AN ABSTRACT OF THE THESIS OF

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Title: FORMATION OF HETEROCYCLIC N-NITROSAMINES FROM THE REACTION OF NITRITE AND SELECTED PRIMARY DIAMINES AND AMINO ACIDS

Abstract approved: D. D. Bills

The reaction of several primary amines with nitrite was investigated with regard to the formation of N-nitrosamines. When reacted in a low moisture system at 160°C with a 1:2 molar ratio of amine to sodium nitrite, various primary amine hydrochloride salts were found to give rise to heterocyclic nitrosamines. The amines and yields of nitrosamines were as follows: putrescine, 22% nitrosopyrrolidine; cadaverine, 21.5% nitrosopiperidine; ornithine, 1% nitrosopyrrolidine and 3% nitrosoproline; lysine, 1% nitrosopiperidine and 2.5% nitrosopipecolic acid. Nitrosamine analysis was by gas chromatography and identification was confirmed by mass spectrometry. The analysis of the nitrosamino acids was facilitated by making the methyl ester derivatives.

When the reactions were carried out in buffered solution at temperatures up to 100°C, the same products were identified except

ornithine did not produce nitrosopyrrolidine and lysine did not produce nitrosopiperidine. The pH optimum for the formation of nitrosopyrrolidine was 3.8 and the pH optimum for the production of nitrosopipecolic acid from lysine was 3.4. Substantial differences in nitrosamine yield were noted when various types of buffer were used. The effects of time and temperature were investigated with nitrosamines being produced at temperatures as low as 22°C. The concentration of nitrite influenced nitrosamine yield dramatically and the reaction rate appeared to be second order with respect to nitrite concentration. Theoretical yields of heterocyclic nitrosamines from primary diamines reacted with nitrite in buffer were as high as nine percent. On a comparative basis, yields of five-membered heterocyclic nitrosamines were higher than the six-membered ring compounds, and the amino acids gave higher yields of the respective nitrosamines than did the simple diamines.

When butylamine was reacted with nitrite, dibutylnitrosamine was identified as a product. Yields were extremely low in solution, but as a high temperature-low moisture reaction, one percent of the nitrosamine was formed.

A system similar to frying bacon was used to determine if putrescine could contribute to the formation of nitrosopyrrolidine in cooked bacon. Ground pork belly containing 200 ppm sodium nitrite was cooked to 177°C and the cooked meat, cooked-out fat, and

cooking distillate were combined and analyzed for nitrosopyrrolidine. An average of 109 ppb nitrosopyrrolidine was detected without added putrescine. When 0.40% putrescine was added to the pork prior to cooking, an average of 321 ppb nitrosopyrrolidine was formed. In another set of cooking experiments, only the distillate was analyzed. Increased amounts of nitrosopyrrolidine were detected when the pork contained added putrescine or proline.

Formation of Heterocyclic N-Nitrosamines From the Reaction of Nitrite and Selected Primary Diamines and Amino Acids

by

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INTRODUCTION

Many nitrosamines have been shown to be carcinogenic when fed to test animals and the occurrence of these compounds in food may represent a human health hazard. Several investigations have demonstrated the presence of low levels of nitrosamines in certain foods. Dimethylnitrosamine (DMNA) has been sporadically detected in several cured meat products and nitrosopyrrolidine (NPYR) has been reported in cooked bacon samples.

Nitrosamines form from the reaction of nitrite and amines. Information regarding nitrosamine precursors and the conditions of nitrosamine formation may lead to a better understanding of the occurrence of nitrosamines in foods. By knowing which compounds can give rise to nitrosamines and the reaction conditions involved, preventive measures may be taken to reduce nitrosamine occurrence in foods.

Secondary, tertiary, and quanternary amines have been shown to react with nitrite to produce nitrosamines. Primary amines are common in foods, but few studies have been made regarding the possibilities of these compounds acting as nitrosamine precursors.

The purpose of this study was to investigate the formation of nitrosamines from some primary amine-nitrite reactions. Several primary diamines found in foods were reacted with nitrite to determine if these compounds could act as nitrosamine precursors and under what conditions these reactions might take place.

REVIEW OF LITERATURE

The occurrence of N-nitrosamines in the human environment has recently become a matter of concern. Formed from the reaction of certain amines and nitrite, many of these compounds have been shown to be potent carcinogens. Exposure to nitrosamines could represent a cause of human cancer and the presence of nitrosamines in food may constitute a public health hazard.

Magee and Barnes (1956) first aroused the concern of the scientific community when they showed that DMNA produced malignant liver tumors when fed to rats. Studies by Druckrey et al. (1967) revealed that a large number of N-nitroso compounds were carcinogenic to rats and that nitrosamines with varied structures gave rise to tumors in different organs. Magee and Barnes (1967) further reported on the teratogenic, mutagenic, carcinogenic, and acute toxic effects of many nitroso compounds. By 1972 it was determined that 75 out of 100 nitrosamines tested were carcinogenic (Wolff and Wasserman, 1972) and a year later it was claimed that there were over 100 known carcinogenic nitroso compounds (Magee, 1973).

Some evidence regarding the possible effects of nitrosamines on humans has been presented. Investigations into a high incidence of human esophageal cancer in localized areas of Africa have shown

an association with the occurrence of DMNA in a certain edible fruit (Duplessis et al., 1969) and in a local alcoholic drink (McGlashan et al., 1968). Of the animal species tested, all are susceptible to tumor induction by nitrosamines, and this implies that humans are probably also adversely affected (Magee, 1973).

Nitrosamines in Food

The detection of nitrosamines in food has been an active area of investigation. Sebranek and Cassens (1973) have recently reviewed and summarized much of the work regarding the potential health hazards of nitrosamines. The possibility of nitrosamines occurring in food was first realized in 1964 when herring meal that had been preserved with sodium nitrite was found to be hepatotoxic when fed to sheep. The causative agent was determined to be DMNA that had formed during processing (Ender et al., 1964).

Early reports of nitrosamines in food were based primarily on identification using thin-layer chromatography (tlc). It was claimed that wheat flour contained diethylnitrosamine (DENA), especially after heating (Marquardt and Hedler, 1966). Evidence for the occurrence of DENA in other wheat products, as well as milk and cheese, was also presented by the same investigators (Hedler and Marquardt, 1968). Ender and Ceh (1968) reported nitrosamines in samples of meat, fish, and mushrooms, and McGlashan et al. (1968) suggested

that African alcoholic spirits contained DMNA. Cheese and cured meats were said to contain DMNA and DENA, respectively, at levels of 40-120 ppb (Freimuth and Glaser, 1970).

It has been pointed out that these early reports of nitrosamine occurrence employed detection methods that lacked specificity and the findings may be erroneous (Thewlis, 1968; Pensabene et al., 1972; Wolff and Wasserman, 1972). Further analysis of wheat products failed to detect any DENA (Thewlis, 1967; Sen et al., 1969b) and it has been shown that other compounds in food could interfere with the tlc detection of nitrosamines (Thewlis, 1968; Wolff and Wasserman, 1972).

Other investigations employing more reliable detection procedures have indicated the presence of nitrosamines in a variety of foods. In most cases several different methods were used to detect nitrosamines and identification was confirmed by mass spectrometry (ms). Duplessis et al. (1969) found DMNA in an edible African fruit and Fong and Walsh (1971) have reported DMNA and DENA in Cantonese dried fish. The occurrence of 4-46 ppb DMNA was confirmed in smoke-processed sable, salmon, and shad by Fazio et al. (1971a). Low levels of DMNA have also been found in various cured meat products such as salami and dry sausage (Sen, 1972b), ham (Fazio et al., 1972), and frankfurters (Wasserman et al., 1972). Panalaks et al. (1973) also reported evidence for 2-12 ppb DMNA in

a variety of cured meats. Alliston et al. (1972) reported, but did not confirm, the occurrence of DMNA, DENA, and NPYR in food products such as cheese, liver, and cod. The presence of DMNA and DENA at levels of less than 10 ppb was confirmed in several food products by Crosby et al. (1972).

In general, the findings of nitrosamines in food products have been sporadic and inconsistent. The nitrosamines detected have been at low levels and usually only a few of the samples analyzed have been shown to be positive. An exception to this is the detection of NPYR in cooked bacon. Several investigations have revealed the presence of NPYR in a number of cooked commercial bacon samples at levels up to 108 ppb (Crosby et al., 1972; Fazio et al., 1973; Sen et al., 1973). These studies also showed that the cooking process, frying, was an important step because NPYR could not be detected in samples of the raw bacon. Further work has shown that the method of cooking and temperature of frying influence the amount of NPYR produced (Pensabene et al., 1974). The cooked-out fat recovered from frying operations has been found to contain even higher levels of NPYR than the cooked bacon (Fazio et al., 1973; Sen et al., 1973; Pensabene et al., 1974).

Analytical Procedures for Nitrosamines

A number of procedures employing various detection methods

have been used for nitrosamine analysis. Several reviews have been devoted to surveying the approaches to analyzing nitrosamines (Walters, 1971; Wasserman, 1972; Duplessis and Nunn, 1973).

Investigations involving nitrosamines in model systems have employed ultra-violet absorption (Friedman, 1972; Mirvish et al., 1973), colorimetric procedures (Fan and Tannenbaum, 1971), and radiochemical activity in extracted fractions (Mirvish, 1970) to estimate the amounts of nitrosamines formed. Quantitative techniques such as these are useful in rate studies where the concentration of nitrosamines is high and the level of interfering compounds is low.

Procedures for nitrosamine analysis in food can be divided into two main steps; isolation from the food product and nitrosamine detection. Isolation techniques include digestion, distillation, and extraction followed by clean-up procedures involving further extractions and column chromatography (Wasserman, 1972).

One method of nitrosamine detection involves tlc in conjunction with ultra-violet light and specific spray reagents. Procedures employing tlc have been outlined for the analysis of several food products including alcoholic beverages (Sen et al., 1969b; Sen and Dalpe, 1972). Ultra-violet light is used to cleave nitrite from the amine, and spray reagents then react with the nitrite or amine to give a colored complex. Spray reagents that have been used

include diphenylamine with palladium chloride (Preussmann et al., 1964), the Griess reagent, and ninhydrin (Sen et al., 1969b).

Colorimetric procedures have been developed for estimating the total concentration of nitrosamines in solution. These methods include the use of the Griess reagent (Daiber and Pruessman, 1964) a modified Griess reagent of sulfanilic acid and N-1-naphthylethylenediamine (Fan and Tannenbaum, 1971), and conversion of nitrosamines to hydrazines (Ender and Ceh, 1971).

Devik (1967), McGlashan et al. (1968), and Walters et al. (1970), have used polarography to detect nitrosamines. It has been pointed out, however, that compounds other than nitrosamines can interfere with polarographic procedures (McGlashan et al., 1970; Heyns and Koch, 1970).

Gas chromatography (gc) methods have been used by a number of workers to detect volatile nitrosamines (Howard et al., 1970; Foreman et al., 1970; Telling et al., 1971; Fiddler et al., 1972). Specialized detectors have sometimes been used to aid in the gc analysis of nitrosamines. These include an alkali flame ionization detector (Howard et al., 1970) and a Coulson electrolytic conductivity detector (Rhoades and Johnson, 1970; Essigmann and Issenberg, 1972).

In some cases derivatives of the nitrosamines have been made before gc to allow the use of a more sensitive electron capture

detector. Nitrosamines treated in this manner were oxidized to nitramines (Sen, 1970) or electrochemically reduced to polyfluorinated amides (Alliston et al., 1972).

Methods for the detection of several volatile nitrosamines in food based on gc analysis have been proposed (Fazio et al., 1972; Sen, 1972a; Telling et al., 1971; Crosby et al., 1972; Essigmann and Issenberg, 1972).

Confirmation of nitrosamine identity has become an important part of nitrosamine research. Becuase of the possibility of other compounds interfering with nitrosamine analysis and because of the serious implications of the occurrence of nitrosamines in food, it has been suggested that all findings be confirmed using several identification techniques. The most definitive of these is considered to be mass spectrometry (ms) (Pensabene et al., 1972). A combination gc-ms analysis is a common detection step in most procedures. Several reports have been made detailing gc-ms confirmation techniques (Bryce and Telling, 1972; Essigmann and Issenberg, 1972; Pensabene et al., 1972; Gough and Webb, 1973). The technique of gc-ms analysis is limited to volatile nitrosamines but in certain cases, nonvolatile nitrosamines have been analyzed directly by ms (Lijinsky et al., 1970) or by conversion to a volatile derivative (Ivey, 1974).

The Formation of Nitrosamines

Nitrosamines result from the interaction of nitrite and certain amines. Secondary amines are the most obvious amine form that can give rise to nitrosamines. Studies have shown, however, that nitrosamines may also result from the action of nitrite on tertiary amines (Ender et al., 1967; Smith and Loeppky, 1967; Malins et al., 1970; Lijinsky et al., 1972; Scanlan et al., 1974) and even in somewhat lower yields from quaternary amines (Fiddler, et al., 1972).

The occurrence of nitrosamines in foods is often associated with products to which nitrite has been added. Nitrite salts are food additives that may be added to various meat and fish products under prescribed limitations (Code of Federal Regulations). Sodium or potassium nitrite has several functions in cured meat and fish products. Sodium nitrite has been shown to inhibit the outgrowth of Clostridium botulinum spores, the organism responsible for botulism poisoning (Emodi and Lechowich, 1969). This inhibitory effect has recently been demonstrated in bacon (Greenberg, 1973; Herring, 1973), ham (Greenberg, 1972; Christiansen et al., 1973), and frankfurters (Bard, 1973). Nitrite has also been shown to contribute to the flavor of cured meat (Cho and Bratzler, 1970; Wasserman and Talley, 1972; Simon et al., 1973). The characteristic pink color associated with cured products is due, in part,

to the addition of nitrite (Wolff and Wasserman, 1972). Aside from use as an intentional food additive, nitrite may also occur naturally at low levels in some foods or result from nitrate through the reductive action of bacteria (Wolff and Wasserman, 1972).

The formation of nitrosamines from nitrite-amine reactions has been studied kinetically by numerous investigators (Taylor and Price, 1929; Mirvish, 1970; Friedman, 1972; Schweinsberg and Sander, 1972; Fan and Tannenbaum, 1973). Nitrosation takes place most readily under acidic conditions. The optimum pH for nitrosamine formation from secondary and tertiary amines has been reported to be in the range of 2.5-3.4, depending on the basicity of the amine being nitrosated. Under most conditions, the reaction rate has been shown to be related to the amine concentration and the square of the nitrite concentration. It is believed that the reaction takes place between the nonionized amine group and a dimer of nitrite, dinitrogen trioxide.

Investigations have also identified several inhibitors and accelerators of the nitrosation reaction. Inhibitors include neutral salts (Taylor and Price, 1929; Mirvish, 1970) and ascorbate (Mirvish et al., 1972). Compounds determined to be accelerators of nitrosation include thiocyanate, which can occur in saliva (Boyland et al., 1971), and formaldehyde which is a constituent of smoke used in food processing (Keefer and Roller, 1973).

The possibility that nitrosamines may form in vivo, especially during the simultaneous ingestion of nitrite and secondary amines, has been a concern of several investigations. Sen et al. (1969a) demonstrated that DENA could form when diethylamine and sodium nitrite were incubated in human and animal gastric juices. Other researchers have indicated, however, that the formation of nitrosamines from most secondary amines and nitrite under stomach conditions is not likely because of the strong basicity of the amine. Weakly basic amines are more likely to result in nitroso compounds (Sander et al., 1968).

Primary Amines as Nitrosamine Precursors

The action of nitrite on primary amines has been studied for many years, and much of the available information on these reactions has been reviewed by Adamson and Kenner (1934), Streitwieser (1957), and Ridd (1961). In general, a form of the nitrite ion attacks the nonionized primary amine to yield a diazonium ion. It is thought that the diazonium ion then decomposes to a carbonium ion with loss of the amine function and release of nitrogen gas. Through elimination, substitution, or rearrangements, the final products resulting from the carbonium ion include olefins, alcohols, or other substituted compounds. In view of this reaction scheme, primary amines have not been considered obvious precursors of nitrosamines.

Investigations have shown, however, that it is possible to form nitrosamines from primary amines.

Austin (1960) has suggested that a variety of products may result because of the nucleophiles that can be present during the reaction of a primary amine and nitrite. The carbonium ion intermediate may react with another primary amine molecule to yield a secondary amine. It was speculated that dimethylamine may be a product of the reaction of methylamine and nitrous acid.

Several early investigations support the contention that the action of nitrous acid on a primary amine can result in a secondary amine. This product normally undergoes further reaction with nitrite to yield a nitrosamine. Dipropylnitrosamine has been reported as a product of propylamine and nitrous acid (Linnemann, 1872). Meyer et al., (1877) found that products of the butylamine-nitrite reaction included butyl alcohols, butylene, and dibutylnitrosamine (DBNA). A later investigation also suggested that DBNA was a product of this reaction (Whitmore and Langlois, 1932). Similar findings were reported by Adamson and Kenner (1934) who reacted a homologous series of aliphatic amines with nitrite. In addition to alcoholic and olefinic products, the respective nitrosamines, dipentyl- through didecyl-, resulted from the primary amine series pentylamine through decylamine.

If the aliphatic amine in question contains two amine functions,

it is possible that a secondary amine could form by cyclization. Demjanow (1892) reported that tetramethylenediamine (putrescine) when reacted with nitrite, gave in addition to alcohols and olefins, NPYR.

Primary amines may, however, be converted to secondary amines without interaction with nitrite. Certain diamine hydrochlorides have been shown to cyclize to secondary amines at high temperature. Putrescine dihydrochloride, when subjected to high temperature, was found to produce pyrrolidine (Ladenburg, 1887) and cadaverine dihydrochloride, under these conditions, cyclized to piperidine (Ladenburg, 1885). Recently Lijinsky and Epstein (1970) have called attention to the possibilities of putrescine and cadaverine forming cyclic secondary amines during the cooking of meat and fish. It is conceivable that if these secondary amines were formed, they could further react with any residual nitrite to produce carcinogenic nitrosamines.

Since nitrosamines have become a food safety concern, several investigators have studied some of the primary amines found in food as possible nitrosamine precursors. Devik (1967), using polarography as a detection method, claimed DMNA and possibly other nitrosamines were products when several primary amino acids were heated with glucose. Because of the serious implications of nitrosamines resulting from such a common reaction, several

research groups attempted to repeat these findings. It was concluded that amine-sugar reactions did not result in nitrosamines and that the DMNA was likely a misidentification due to the presence of pyrazines as reaction products (Heyns and Koch, 1970; Havre and Ender, 1971; Scanlan and Libbey, 1971). Another report has implicated the same type of reaction as contributing to the formation of nitrosamines, but in this case, sodium nitrite was also part of the reaction system. When lysine was heated with glucose and then with nitrite, nitrosopiperidine (NPIP) was identified as a product (Heyns and Roper, 1973).

A simple primary amine, methylamine, reportedly gave rise to DMNA when reacted with nitrite under conditions of low moisture and high temperature (Ender et al., 1967). Other common primary amino acids such as glycine, valine, and alanine were thought to produce DMNA and other dialkyl nitrosamines when heated with nitrite in a starch mixture (Ender and Ceh, 1971). The findings were not, however, confirmed by ms. The authors also indicated that DMNA and DENA, in extremely low yields, were formed from the action of nitrite on methylamine and ethylamine, respectively, in solution at 100°C.

Working with a high temperature oil and water system designed to simulate frying conditions, Bills et al. (1973) confirmed NPYR as a product from the reaction of nitrite with several amines. The

primary amines, putrescine and spermidine, were two compounds that were found to cyclize and yield NPYR under these conditions.

The formation of carcinogenic nitrosamines from primary amines and nitrite is potentially an important reaction in terms of food safety. Primary amines in the form of free amino acids and decarboxylated analogs are common in foods, especially those to which nitrite is added. Several primary amines have been implicated as nitrosamine precursors but little definitive work has been done to establish the influence of reaction parameters on nitrosamine formation. The possibility that other diamines found in food may undergo cyclization to secondary amines and eventually nitrosamines has not been explored. More information regarding primary amine-nitrite interactions as related to nitrosamine formation seems necessary.

EXPERIMENTAL PROCEDURE

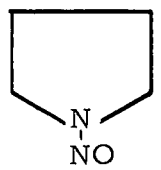
The chemical structures of nitrosamines discussed in this study are shown in Figure 1.

Safety Precautions

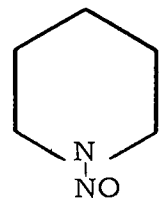
Many nitrosamines have been determined to be potent carcinogens in test animals and it is likely that these compounds are also hazardous to humans. Safety precautions should be observed in the laboratory regarding the storage and handling of nitrosamine solutions. In this investigation, measures were taken to avoid skin contact, inhalation, and accidental spills. Experiments with nitrosamines were conducted in a fume hood. A full length laboratory coat and impervious gloves were worn when handling nitrosamines. The gas chromatograph was operated with the detector vented into a fume hood. Concentrated nitrosamine solutions and glassware in contact with such solutions were treated with a solution of five percent hydrobromic acid in acetic acid following use.

Amine Purity

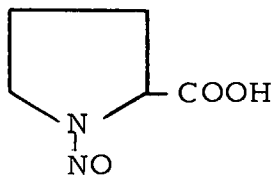
Nitrosamine yields from primary amines were, in most cases, low so it was necessary to establish the purity of each amine with respect to certain secondary amines. Secondary amines could,



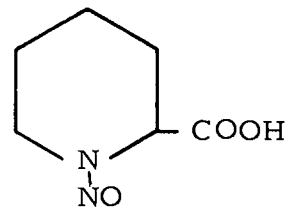
Nitrosopyrrolidine (NPYR)



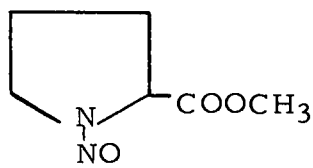
Nitrosopiperidine (NPIP)



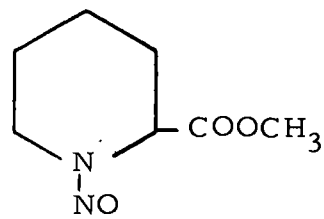
Nitrosoproline (NPRO)



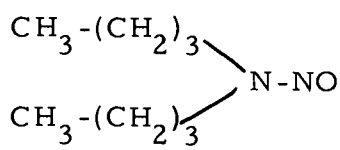
Nitrosopipecolic Acid (NPCA)



Methyl Nitrosoproline (MeNPRO)



Methyl Nitrosopipecolate (MeNPCA)



Dibutylnitrosamine (DBNA)

Figure 1. Structures of nitrosamines discussed in this study.

presumably, form nitrosamines more readily than primary amines under the experimental conditions employed. If present in high levels, specific secondary amines could form nitrosamines and result in erroneous conclusions regarding the production of nitrosamines from primary amines.

Gas chromatography (gc) was used to determine if the primary amines used in this study contained certain secondary amines as impurities which would lead to the same nitrosamine as that found to be a product of the primary amine. A Varian 1400 gas chromatograph equipped with a flame ionization detector and a glass column (10 ft X 0.13 in i.d.) packed with 28% Pennwalt 223 plus 4% KOH on 80/100 Gas-Chrom R (Applied Science Laboratories, Inc.) was used for the amine analysis. Operating conditions were as follows: injector temperature, 100°C; column temperature, 100°C; detector temperature, 280°C; nitrogen carrier gas flow, 30 ml/min. For the analysis of butylamine, the injector and column temperatures were 150°C.

Amines existing as free bases were injected directly onto the column. Amines existing as hydrochloride salts were dissolved in 1.0 N methanolic NaOH to facilitate gc analysis. Compounds tested in this manner were putrescine (Pfaltz and Bauer), putrescine dihydrochloride (K and K Laboratories, Inc.), cadaverine (Aldrich Chemical Co.), cadaverine dihydrochloride (Calbiochem), ornithine

hydrochloride (Aldrich Chemical Co.), lysine hydrochloride (Eastman Chemical Co.), and butylamine (Aldrich Chemical Co.). Secondary amines used as standards were pyrrolidine, piperidine hydrochloride, and dibutylamine (all from Aldrich Chemical Co.).

On the basis of gc retention time, pyrrolidine could not be detected in putrescine, putrescine hydrochloride, or ornithine hydrochloride with a detection limit of 0.01%. Cadaverine dihydrochloride and lysine hydrochloride contained no piperidine at a detection limit of 0.01%. Cadaverine, as a free base, contained less than 0.07% piperidine.

The amount of contaminating dibutylamine in butylamine was reduced to less than 0.002% by fractional distillation. Butylamine hydrochloride was made by bubbling hydrogen chloride gas through butylamine.

Lysine hydrochloride and ornithine hydrochloride were found to be free of pipecolic acid and proline, respectively, at a detection level of 0.02%. Analysis for pipecolic acid and proline was performed using an amino acid column chromatography procedure. Amino acids were determined by ninhydrin after elution from a Beckman type 50A resin with pH 3.25 citrate-HCl buffer according to the procedure of Ivey (1974).

High Temperature-Low Moisture Reactions

Various amine hydrochloride salts were mixed with reagent grade sodium nitrite (Mallinckrodt Chemical Works) in a 1:2 molar ratio and ground with a mortar and pestle. Quantities representing 0.5 m moles of the amine and 1.0 m mole of nitrite were weighed in duplicate into 18 X 150 mm Kimax culture tubes. Five percent water was added and mixed with the salts. The open end of the tubes was sealed in an oxygen-methane flame and the tubes were placed in a 160°C oven for two hr. After cooling, the containers were broken open at the tip and the contents were rinsed into separatory funnels with approximately 30 ml of distilled water saturated with reagent grade sodium sulfate (Mallinckrodt Chemical Works). The contents of the separatory funnel were then acidified to pH < 1 with H_2SO_4.

Amines reacted under conditions of high temperature and low moisture were putrescine dihydrochloride, cadaverine dihydrochloride, ornithine hydrochloride, lysine hydrochloride, and butylamine hydrochloride.

Reactions in Buffer

Buffers were 0.1 M acetate and a citrate-phosphate system made by mixing varying proportions of 0.1 M citrate and 0.2 M disodium phosphate or varying proportions of 0.2 M citrate and 0.4

M disodium phosphate (McIlvaine, 1921). Amines and sodium nitrite were added separately to the buffer, and H_2SO_4 or NaOH were added to adjust the solutions to the desired pH as determined by a Beckman Expandomatic pH meter. The amine and nitrite buffer solutions were then combined in a 25 X 150 mm culture tube with a Teflon-lined screw cap, mixed, and immediately incubated at the desired temperature. The amount of amine used in each reaction was usually 0.5 m moles and the sodium nitrite was usually 1.0 m mole with the total reaction volume being 20 ml.

When the incubation time was less than one hr, the reaction was terminated by addition of ammonium sulfamate (Matheson, Coleman, and Bell) in a five molar excess of the nitrite followed by concentrated H_2SO_4 to acidify the solution of pH < 1. Reaction mixtures were then saturated with sodium sulfate, acidified if not acidified previously, and transferred to separatory funnels for extraction. All reactions were performed in duplicate, except one experiment in which each set of conditions was carried out in triplicate. Amines reacted with nitrite in buffer solution were putrescine, cadaverine, lysine hydrochloride, and ornithine hydrochloride. Butylamine was reacted in a similar manner, except 10 m moles of amine and 20 m moles of sodium nitrite were used. The reaction was carried out in nonbuffered solution adjusted to the desired pH with H_2SO_4.

Extraction and Concentration

Redistilled dichloromethane (Mallinckrodt Chemical Works) was used to extract the volatile nitrosamines, NPYR, NPIP, and DBNA, from the acidified aqueous solutions. Aqueous fractions from buffered reactions were extracted in a separatory funnel twice with equal volumes of dichloromethane. Because particulate matter was present in aqueous fractions from the high temperature-low moisture reactions these samples were extracted three times with dichloromethane.

The dichloromethane extracts were dried over sodium sulfate and transferred to Kuderna-Danish evaporative concentrators (Kontes Glass Co.). A small boiling chip was added and the lower part of the apparatus was immersed in a water bath at 60-65°C. When the extract was concentrated to approximately 4 ml, the apparatus was removed from the water bath. Further concentration was accomplished by fitting the concentration tube with a micro-Snyder column and evaporating the solvent at 20°C with a slow stream of prepurified nitrogen to a final volume of 0.3-0.5 ml.

Extraction and Esterification of Nitrosamino Acids

Reagent grade ethyl acetate (Mallinckrodt Chemical Works) was used to extract the acidic nonvolatile nitrosamines,

nitrosoproline (NPRO) and nitrosopipecolic acid (NPCA), from acidified aqueous solutions following the same procedures as outlined for the dichloromethane extraction of the volatile nitrosamines. After drying over sodium sulfate, the ethyl acetate fraction was transferred to a round bottom boiling flask and evaporated to near dryness on a Buchi Rotavapor.

The methyl esterification of NPRO was according to the procedure of Ivey (1974). Ten ml of anhydrous methanol containing two percent concentrated H_2SO_4 was added to the flask and the sample was held at room temperature for one hr. In applying this method to NPCA, it was observed that the time necessary to get maximum esterification under these conditions was somewhat longer. Samples containing NPCA were treated with the acidic methanol solution for 18-24 hr at room temperature in the absence of light.

At the completion of the methanol treatment, ten ml of distilled water saturated with sodium sulfate was added to the flask and the contents were transferred to a separatory funnel. The material was extracted three times with equal volumes of dichloromethane. The dichloromethane extract containing the methyl ester was dried over sodium sulfate and concentrated in a Kuderna-Danish evaporator as previously described.

Thin-Layer Chromatography (tlc)

Tlc was used for preliminary identification of certain nitrosamines. Silica gel G plates, 0.25 mm in thickness (Brinkman Instrument Co.), were used in the procedure. Dichloromethane or ethyl acetate extracts were spotted, and after development by the solvent, the plates were sprayed with a modified Griess reagent (Fan and Tannenbaum, 1971), and exposed to an ultra-violet lamp for 15-20 min. The developing solvent used for NPYR, NPIP, and DBNA was 5:7:10, hexane: diethyl ether: dichloromethane. The solvent system used for the analysis of NPRO and NPCA was 4:1:1, butanol: acetic acid: water. The spray reagent was two percent sulfanilic acid in 30% aqueous acetic acid and 0.2% N-1-naphthylethylenediamine dihydrochloride in 30% acetic acid mixed in equal volumes prior to use. Nitrosamines appeared as pink spots after ultra-violet irradiation.

Gas Chromatographic (gc) Analysis

A Varian 1400 gas chromatograph equipped with a flame ionization detector was used in all analyses. A 12 ft X 0.13 in i.d. stainless steel column packed with 7% Carbowax 20M on Chromosorb G was used for the analysis of NPYR, NPIP, and DBNA. For the analysis of the methyl esters of NPRO and NPCA (MeNPRO and

MeNPCA, respectively), a 10 ft X 0.13 in i.d. stainless steel column packed with one percent Carbowax 20M on Chromosorb G was employed. Operating conditions for the instrument were as follows: injector temperature, 190°C; column temperature, 170°C; detector temperature, 280°C; flow rate of nitrogen carrier gas, 30 ml/min.

Tentative identification of nitrosamines was made by observing peaks on the chromatogram at the same retention time as the nitrosamine standards. NPYR, NPIP, DBNA, and dipropylnitrosamine (DPNA) were obtained from Eastman Chemical Co. NPRO and NPCA were synthesized from proline and pipecolic acid (both from Aldrich Chemical Co.) according to the method of Lijinsky et al. (1970) and esterified to MeNPRO and MeNPCA as described.

The amount of the various nitrosamines formed from each reaction was estimated by determining the peak area and relating this to the peak area of an internal standard. Peak area was estimated with a digital integrator (Hewlett Packard model 3373 B) or by multiplying the peak height by the retention time. DPNA was used as an internal standard for the quantitation of NPYR and NPIP. NPYR was added as the internal standard for DBNA quantitation. To establish a response and recovery factor, f, a known amount of standard nitrosamine and internal standard were carried through the analytical procedure. The factor f was calculated from the following formula:

$$\frac{\text{nitrosamine peak area}}{\text{internal standard peak area}} = f \frac{\text{mass nitrosamine}}{\text{mass internal standard}}$$

For the gc estimation of MeNPRO and MeNPCA, methyl acetyl proline was used as the internal standard. It was added at the completion of the methylation step and was related to NPRO and NPCA standards which were added to an acidified aqueous solution and carried through the entire esterification and analysis procedure.

Gas Chromatography-Mass Spectrometry (gc-ms)

A Finnigan Model 1015C quadrapole mass spectrometer in combination with a Varian 1400 gas chromatograph was used to confirm the identity of nitrosamines. Samples were introduced into the ms through the gc operated under the conditions described for gc analysis but with helium as the carrier gas. The gc-ms interface was a glass jet orifice separator. Operating conditions were filament current, 400 μA; electron voltage, 70 eV; analyzer pressure, 5×10^{-7} Torr; scan time, one sec. Identification was confirmed by comparing the spectra to those of nitrosamine standards.

Meat Cooking

Two matched pork bellies were obtained fresh from a commercial processor. To insure a heterogeneous sample for cooking experiments, the pork was ground, mixed, and reground with a

Hobart meat grinder fitted with a 3/8 in. plate. The ground pork was stored at -23°C until used in the experiments.

In preparation for cooking, sodium nitrite was stirred into a pork sample at a level of 200 ppm (0.02%). Putrescine was added to the meat in the same manner at levels of 0.10 and 0.40% (added as putrescine dihydrochloride but calculated as putrescine). Proline was mixed into the meat at a level of 0.10%. After the desired compounds had been stirred into the sample, the material was reground at least three times with a Universal hand grinder to insure adequate mixing of the added compounds.

One hundred g of the material was placed in a 500-ml boiling flask which was fitted with a distillation head, condenser, and receiver. Thermocouple leads were placed through the distillation head into the ground meat. Heat was applied to the flask with a prewarmed heating mantle which was removed when the temperature of the meat reached 177°C. The heating process normally took about 12 min. During the cooking process, 30-35 ml of distillate was collected. For each set of experimental conditions, two replicate samples were prepared and cooked. Two samples of commercial bacon were also cooked in this manner.

Analysis for NPYR was carried out in two ways. In one set of experiments, the distillate, cooked meat, and cooked-out fat were combined and analyzed, while in a second set of experiments,

only the distillate collected from the cooking operation was analyzed.

Analysis of NPYR in Cooked Product

The cooking apparatus was disassembled after cooling and the contents of the cooking flask were transferred to a Waring Blendor jar with 100 ml of pH 7.0 citrate-phosphate buffer. Two g of ammonium sulfamate was added and the contents were blended for approximately 15 sec. The blended material was transferred with another 100 ml of buffer to a one-liter boiling flask. The distillation head and condenser were rinsed with buffer and this rinse was placed in the boiling flask along with the cooking distillate. Approximately ten ml of dichloromethane was used to rinse the cooking flask and this also was added to the boiling flask.

The material in the boiling flask was steam distilled until 200 ml of distillate was collected. The distillate was saturated with sodium sulfate, acidified to 0.1 N with HCl, and extracted twice with equal volumes of dichloromethane. The extract was dried over sodium sulfate and concentrated, using the Kuderna-Danish and nitrogen gas procedure, to a volume of two ml. One ml of nanograde hexane was added and the concentration was continued to a volume of 0.2-0.3 ml. The sample was analyzed by gc and the NPYR quantitated with the aid of an internal standard, ethyl undecanoate, added prior to the concentration of the dichloromethane extract.

When necessary, the sample was further concentrated to 0.05 ml for ms analysis.

Percent recovery of NPYR was determined by adding 100 μg of NPYR to a sample of cooked pork that contained no added putrescine or sodium nitrite. This was carried through the same procedure as the other samples. The analysis of raw pork was carried out by blending 100 g of meat containing 0.02% nitrite and 0.40% putrescine with two g of ammonium sulfamate and 200 ml of buffer. The blended material was then steam distilled and analyzed in the same manner as the cooked samples.

Analysis of NPYR in Cooking Distillate

After the pork sample was cooked, the distillation head and condenser were rinsed with distilled water and this was combined with the cooking distillate. After acidifying with HCl and saturating with sodium sulfate, the cooking distillate was extracted twice with equal volumes of dichloromethane. The extract was dried and concentrated to one ml.

Extracts of the distillate were subjected to a column chromatography clean-up procedure similar to that described by Pensabene *et al.* (1974). Five g of 80-200 mesh adsorptive alumina (Fisher Scientific Co.) was placed in a 10 X 200 mm glass column plugged with glass wool. The alumina was washed with 30 ml of hexane and

one ml of the dichloromethane extract was then placed on the column. Five ml of hexane was used to rinse the concentrator tube and wash the sample onto the column. One hundred ml of hexane containing ten percent dichloromethane was passed over the alumina and discarded and NPYR was eluted with 100 ml of dichloromethane. This eluant was concentrated to 0.2-0.3 ml as previously described and analyzed by gc. The internal standard, ethyl undecanoate, was added after the column chromatography step. Recovery of NPYR was estimated by carrying 50 μg of NPYR through the analytical procedure.

RESULTS AND DISCUSSION

Identification of Nitrosamines

The identification of nitrosamine products from the reaction of primary amines and nitrite was based on ms analysis, with results obtained from tlc and gc used as supporting evidence. Nitrosamines present in samples representing the various general reaction conditions were subjected to verification by ms. No nitrosamines, other than those reported, were found as reaction products.

The identification and quantitation of the nonvolatile nitrosamino acids, NPRO and NPCA, was facilitated by making methyl ester derivitives. The mass sprectum of MeNPCA has not been previously reported and is shown in Figure 2. The parent ion is m/e 172 and represents about one percent of the base peak. The ion at m/e 142 indicates the loss of NO, which is characteristic of most nitrosamines. The ion at m/e 113 indicates the loss of $COOCH_3$ from the parent molecule and the base peak at m/e 83 is likely due to the loss of both NO and $COOCH_3$. Other prominent ions are at m/e 55, 28, and 15. Mass spectral analysis of the nonvolatile NPCA was performed by Lijinsky et al. (1970). Ions were reported at m/e 158, 128, 113 and 83 which correspond to the fragmentation pattern observed for the methyl ester in this study.

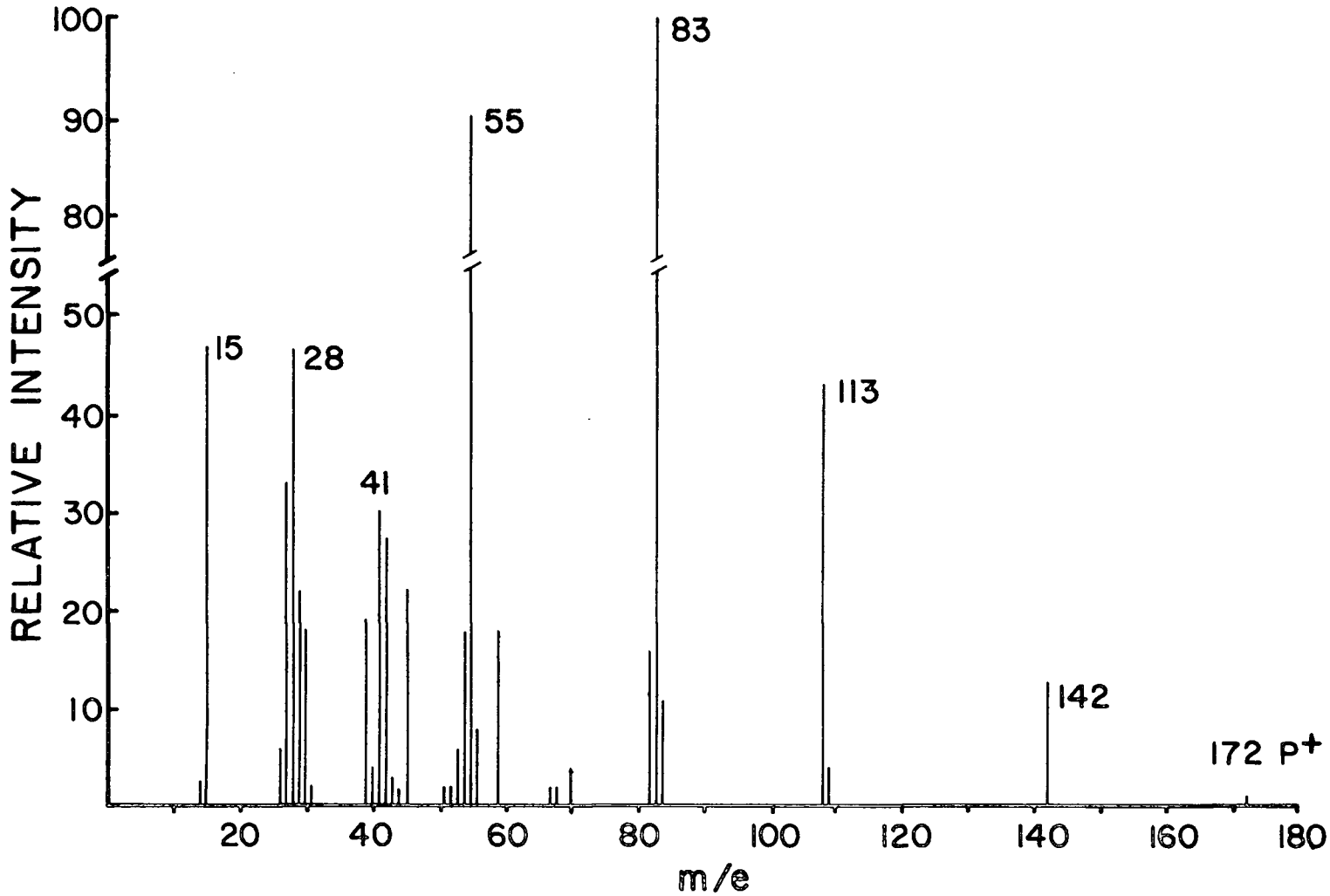


Figure 2. Mass spectrum of MeNPCA.

The identification of nitrosamines by tlc was based on comparison of R_f values with standards. NPRO and NPCA were not compatible with the solvent system proposed by Sen et al. (1969b) for the analysis of a number of nitrosamines. A solvent system of 4: 1: 1 butanol: acetic acid: water was found to give acceptable separation of the nitrosamino acids. The R_f values for NPRO and NPCA were 0.55 and 0.66, respectively.

High Temperature-Low Moisture Reactions

When the hydrochloride salts of two straight chain diamines and two amino acids were reacted with sodium nitrite at 160°C for two hr, various heterocyclic nitrosamines were identified as reaction products. The amount of nitrosamines formed in samples reacted in duplicate are given in Table 1. Putrescine yielded over 20% NPYR and cadaverine produced about the same amount of NPIP. The reaction of nitrite with the amino acids resulted in the formation of two nitrosamines from each amino acid. Ornithine produced about one percent NPYR and three percent NPCA while lysine gave approximate yields of one percent NPIP and two and one-half percent NPCA.

The quantity of heterocyclic nitrosamines produced from the straight chain diamines (putresine and cadaverine) was about five times greater than the combined nitrosamine products from each amino acid. The presence of the carboxyl group seemed to inhibit

Table 1. Amounts of nitrosamines formed when various diamine hydrochlorides were reacted with sodium nitrite for two hr at 160°C.[a]

Amine	Nitrosamine formed	mg		Percent[b] theoretical yield	
		A	B	A	B
Putrescine·di HCl	NPYR	11.8	10.5	23.5	21.1
Ornithine·HCl	NPYR	0.8	0.4	1.6	0.8
	NPRO[c]	2.8	2.0	3.9	2.7
Cadaverine·di HCl	NPIP	12.5	12.0	22.0	21.0
Lysine·HCl	NPIP	0.7	0.6	1.3	1.0
	NPCA[c]	2.2	1.7	2.7	2.2

[a] 0.5 m moles amine was reacted with 1.0 m mole sodium nitrite.

[b] calculated on basis of amine

[c] NPRO and NPCA determinations were from different reacted samples than the respective NPYR and NPIP determinations.

the cyclization or favor other types of reactions. The nitrosamino acids may also be less stable than NPYR and NPIP and could possibly break down to compounds other than NPYR and NPIP resulting in a lower total yield of heterocyclic nitrosamines from the amino acids.

The reaction conditions used in this model system were undoubtedly more rigorous than the temperatures associated with the cooking of food. Rather severe heating conditions were employed for the purposes of product identification and yield comparisons. Five percent water was added to the salt mixture because preliminary studies indicated this enhanced nitrosamine formation, and the added water also served to keep the contents in one area of the vial during the reaction.

The formation of a heterocyclic nitrosamine from a primary diamine, heated in the presence of sodium nitrite, was reported by Demjanow (1892) when NPYR was found to result from putrescine. Bills et al. (1973) obtained a 0.04% yield of NPYR when putrescine and sodium nitrite were reacted in a 1:1 molar ratio at 170°C for about ten min in a water-oil system.

Cadaverine, ornithine, and lysine are compounds similar to putrescine, but the formation of heterocyclic nitrosamines from the reaction of these amines with nitrite has not been previously demonstrated. It has been recently shown, however, that lysine,

heated with glucose and subsequently with nitrite, resulted in NPIP (Heynes and Roper, 1973). These authors inferred that glucose was involved in the formation of NPIP. In light of the findings in the present investigation, glucose is not an essential element of NPIP formation.

In order to form NPYR and NPIP from ornithine and lysine, respectively, decarboxylation, in addition to cyclization and nitrosation, must occur. Other nitrosamine investigations have shown that the high temperature conditions in this experiment were conducive to decarboxylation. Ender and Ceh (1971) and Huxel (1973) demonstrated that proline, reacted in the dry state with sodium nitrite at temperatures of 130-170°C, underwent nitrosation and decarboxylation to produce NPYR. It has also been demonstrated that NPRO will decarboxylate to NPYR at high temperatures (Bills et al, (1973). An optimum temperature of 185°C for the decarboxylation of NPRO to NPYR has been reported by Pensabene et al. (1974).

Possible routes for the formation of heterocyclic nitrosamines from cadaverine and lysine are shown in Figure 3. The processes involving cyclization, decarboxylation, and nitrosation may occur at several points in the scheme. Approximately twice as much NPCA as NPIP was obtained from lysine and about 20 times more NPIP was formed from cadaverine than from lysine.

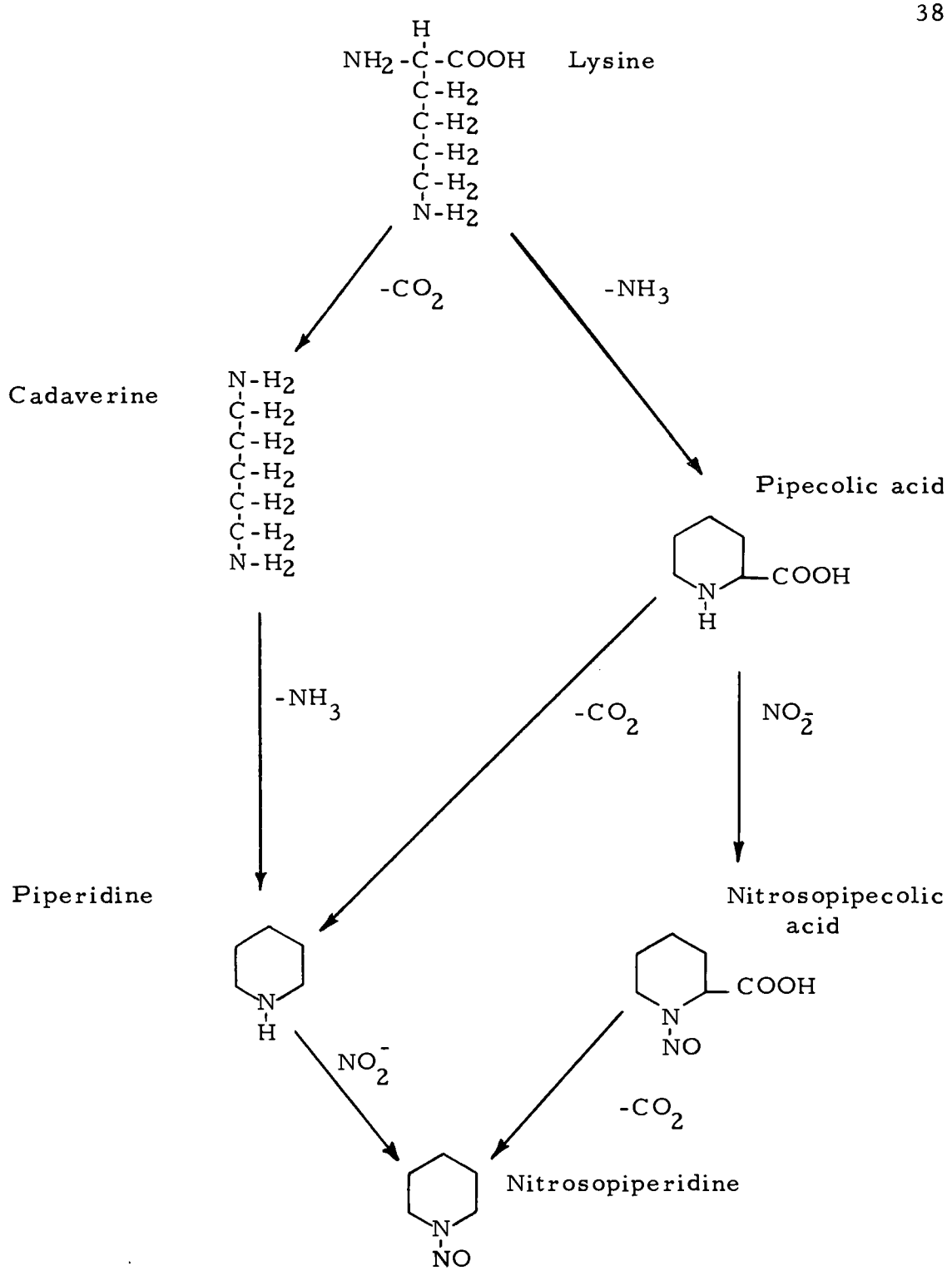


Figure 3. Possible routes for the formation of heterocyclic nitrosamines from lysine and cadaverine.

Influence of Buffer and pH

When putrescine was reacted with sodium nitrite in buffered solution, NPYR was found to be a product. The effects of buffer type and pH on the yield of NPYR can be seen in Figure 4. The pH values shown are initial pH and do not reflect changes occuring during the reaction. When the pH was measured at the completion of the reaction, differences from the initial pH were not greater than 0.1 pH units except for reactions at pH 3.0 and 2.0 where decreases of 0.3-0.5 pH units were noted. Theoretical yields of NPYR ranged from 2.8% at pH 3.8 to less than 0.005% at pH 6.0.

The NPYR yields in the 0.2 M citrate and 0.4 M phosphate buffer system were determined with triplicate samples. Values obtained from reactions performed at pH 3.0-4.2 were subjected to an analysis of variance. Mean values through this pH range were 365-531 μg. The standard error was 19 μg and the coefficient of variability was 5.3%.

An estimate of the variance between different experiments was obtained by reacting the same sample in duplicate in six different experiments. Reaction conditions were as follows: 0.025 M putrescine, 0.050 M sodium nitrite, pH 3.8, 0.065 M citrate-0.07 M phosphate buffer, 100°C for 30 min. The yield of NPYR for the 12 samples was 745.5 $\pm$ 45.6 μg (mean $\pm$ standard deviation). The

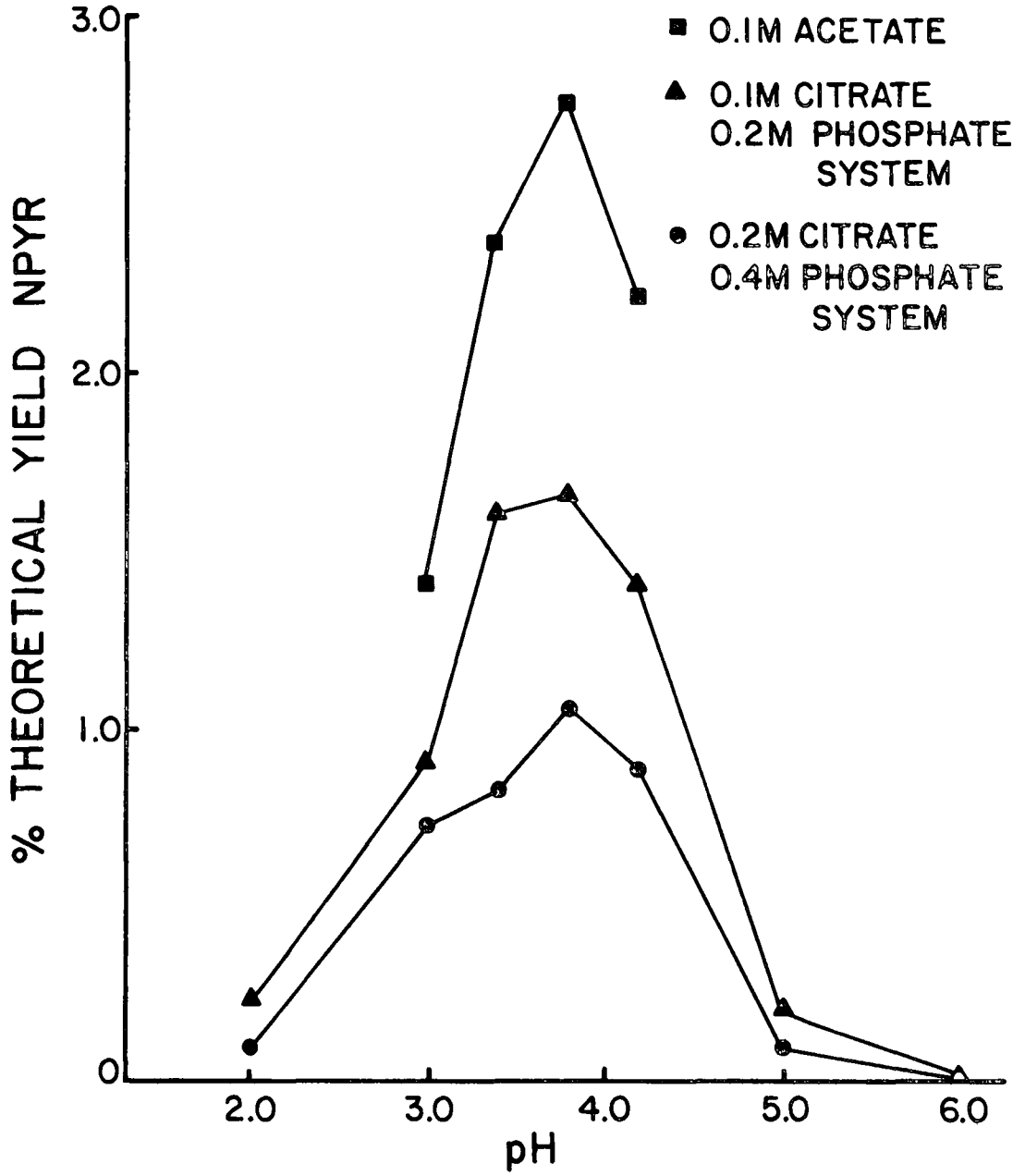


Figure 4. Effect of buffer type and pH on the formation of NPYR from putrescine and sodium nitrite. Reaction conditions: 0.025 M putrescine, 0.050 M sodium nitrite, 100°C for 60 min. Citrate-phosphate buffer systems made by mixing varying proportions of 0.1 M citrate and 0.2 M disodium phosphate or varying proportions of 0.2 M citrate and 0.4 M disodium phosphate (McIlvaine, 1921).

standard deviation in this case represented 6.1% of the mean.

Whether reacted in the 0.2 M citrate-0.4 M phosphate or 0.1 M citrate-0.2 M phosphate buffer systems or in 0.1 M acetate buffer, the maximum NPYR accumulation was at pH 3.8. This pH optimum is slightly higher than those described for the nitrosation of other amines as follows: dimethylamine, 3.4 (Mirvish 1970); morpholine, 3.4 (Fan and Tannenbaum, 1973); triethylamine, 3.3 (Schweinsberg and Sander, 1972); and 2.5 for proline, hydroxyproline, and sarcosine (Mirvish et al., 1973). The reactions reported by other investigators, however, involved the formation of a nitrosamine from a secondary amine at room temperature, except for triethylamine which is a tertiary amine and was reacted at 100°C.

As shown in Figure 4, the type of buffer system employed had a significant influence on the yield of NPYR. Doubling the strength of the citrate-phosphate buffer system decreased the NPYR formed 18-65%, while use of acetate buffer resulted in 47-67% more NPYR than the 0.1 M citrate-0.2 M phosphate system. The effect of the buffer type was also noted in a separate experiment where the amount of NPYR formed in each buffer after 30 min was compared to the amount formed when only H_2SO_4 was used to adjust the pH. The results, summarized in Table 2, show the same trend of inhibition including reduced NPYR in acetate buffer compared to no buffer.

Increases in buffer concentration appear to result in decreased amounts of nitrosamine formed.

Table 2. Effect of buffer on NPYR formed from putrescine at pH 3.8.[a]

Buffer	NPYR μ moles	Relative yield
No buffer[b]	12.97	100
0.1 M acetate	10.66	82
0.065 M citrate 0.07 M phosphate	7.73	60
0.13 M citrate 0.14 M phosphate	5.60	43

a reaction conditions: 0.025 M putrescine, 0.050 M sodium nitrite, 100°C, 30 min.

b pH adjusted with H_2SO_4.

Other investigations have noted that nitrosation is decreased by additional ions in solution. Taylor and Ridd (1929) showed inhibition of the formation of DMNA from dimethylamine when potassium chloride was added to the system. Mirvish (1970) found that the production of DMNA from dimethylamine and nitrite in buffered solution was inhibited 8-9% by 0.05 M sodium chloride. The author attributed this inhibition to increased ionic activity.

When lysine was reacted with sodium nitrite in buffered solution, NPCA was the only nitrosamine found as a reaction product. The effects of buffer and pH on the amount of NPCA formed are

shown in Figure 5. The buffer inhibition is similar to that observed for the formation of NPYR from putrescine. Yields of NPCA in acetate buffer were two to four times greater than in the citrate-phosphate system.

Two important differences can be noted in comparing Figure 5 to Figure 4. Although reaction conditions were comparable, the yields of NPCA are considerably lower than the yields of NPYR. A second difference is that the pH optimum in Figure 5 appears to be about 3.4 as compared to 3.8 in Figure 4.

Comparisons between the formation of nitrosamines from primary and secondary amines are difficult because of differing reaction mechanisms. It has been pointed out, however, that the nitrosation of a heterocyclic amine, such as piperidine, has a higher pH optimum than does the nitrosation of an amino acid such as proline (Mirvish, 1972). This coincides with the observation in this investigation where the formation of NPYR from putrescine seems to have a higher pH optimum than does the formation of NPCA from the amino acid lysine.

Effect of Time

The effect of time on the formation of NPYR from putrescine and sodium nitrite is shown in Figure 6. The relationship appears to be linear up to a reaction time of 30 min. The amount of NPYR

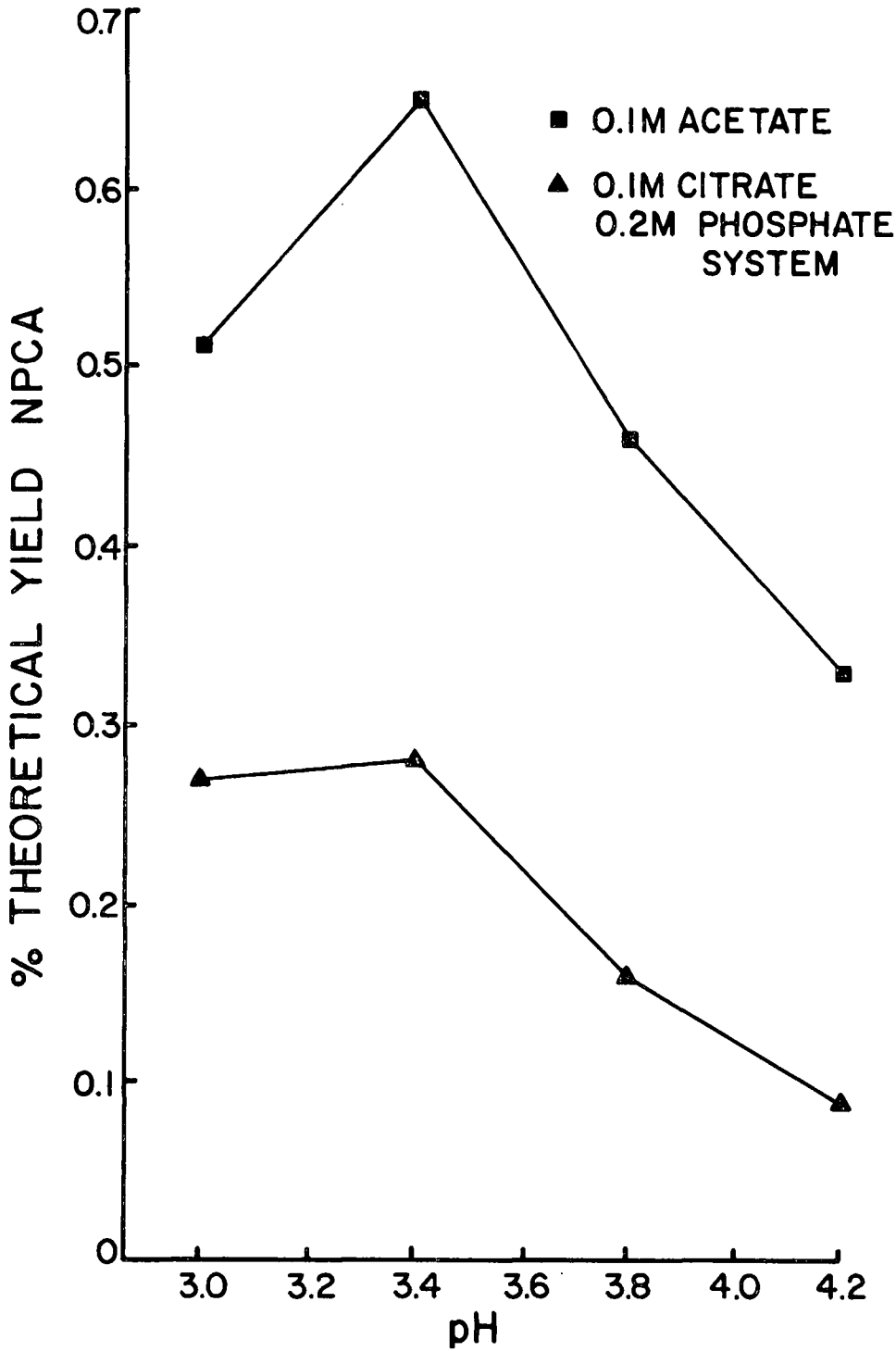


Figure 5. Effect of buffer type and pH on the formation of NPCA from lysine and sodium nitrite. Reaction conditions: 0.025 M lysine, 0.050 M sodium nitrite, 100° C for 60 min. Citrate-phosphate buffer was made by mixing varying proportions of 0.1 M citrate and 0.2 M disodium phosphate (McIlvaine, 1921).

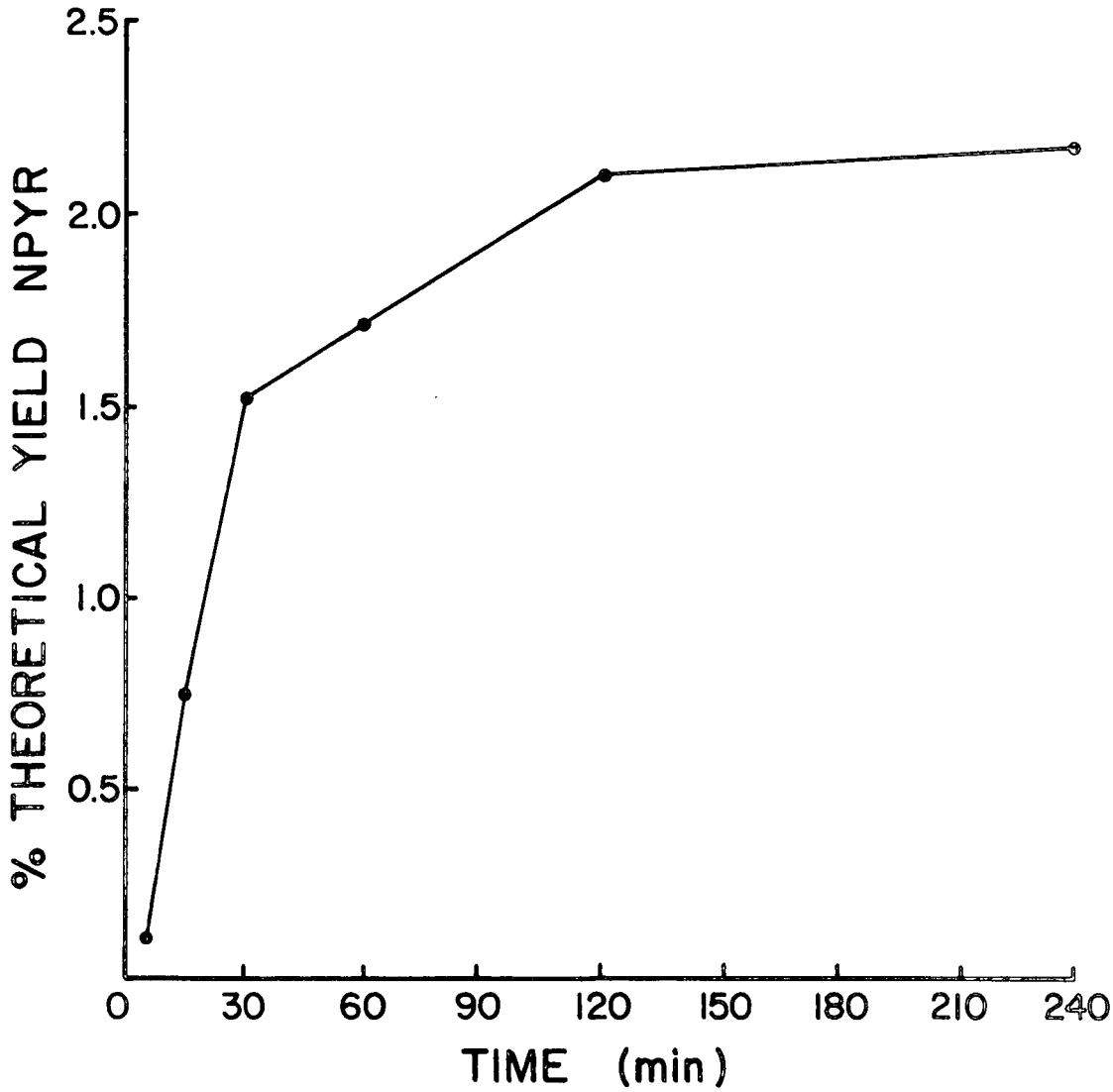


Figure 6. Effect of time on the formation of NPYR from putrescine and sodium nitrite. Reaction conditions: 0.025 M putrescine, 0.050 M sodium nitrite, pH 3.8, 0.065 M citrate - 0.07 M phosphate buffer, 100°C.

formed appears to level off after 120 min. The highest rate of NPYR formation was 0.25 μ moles/min found after 15 and 30 min. The samples were incubated at 100°C and the time was measured from the point the reaction tubes were placed in the water bath. A slower rate of 0.10 μ moles/min after 5 min was probably due to a lower temperature of the reaction mixture when placed in the bath and the time necessary for the sample to reach 100°C. It has been suggested by Fan and Tannenbaum (1973) that kinetic information regarding the rate of nitrosamine formation should be gathered using the initial reaction rate. For later studies involving the effect of nitrite concentration on the reaction rate, a 15 min reaction time was used.

Cadaverine reacted with sodium nitrite in buffered solution was found to give rise to NPIP, but in lower yields than the amount of NPYR formed from putrescine. The effect of time on the formation of NPIP from cadaverine is shown in Figure 7. It should be noted that the concentration of sodium nitrite was 0.125 M because yields from 0.050 M sodium nitrite were extremely low. The reaction rate for the formation of NPIP from cadaverine and nitrite, under these reaction conditions was lower than the rate of NPYR formation from putrescine and nitrite.

Effect of Nitrite Concentration

The production of NPYR from the reaction of putrescine and nitrite was used as a system to test the effect of nitrite concentration

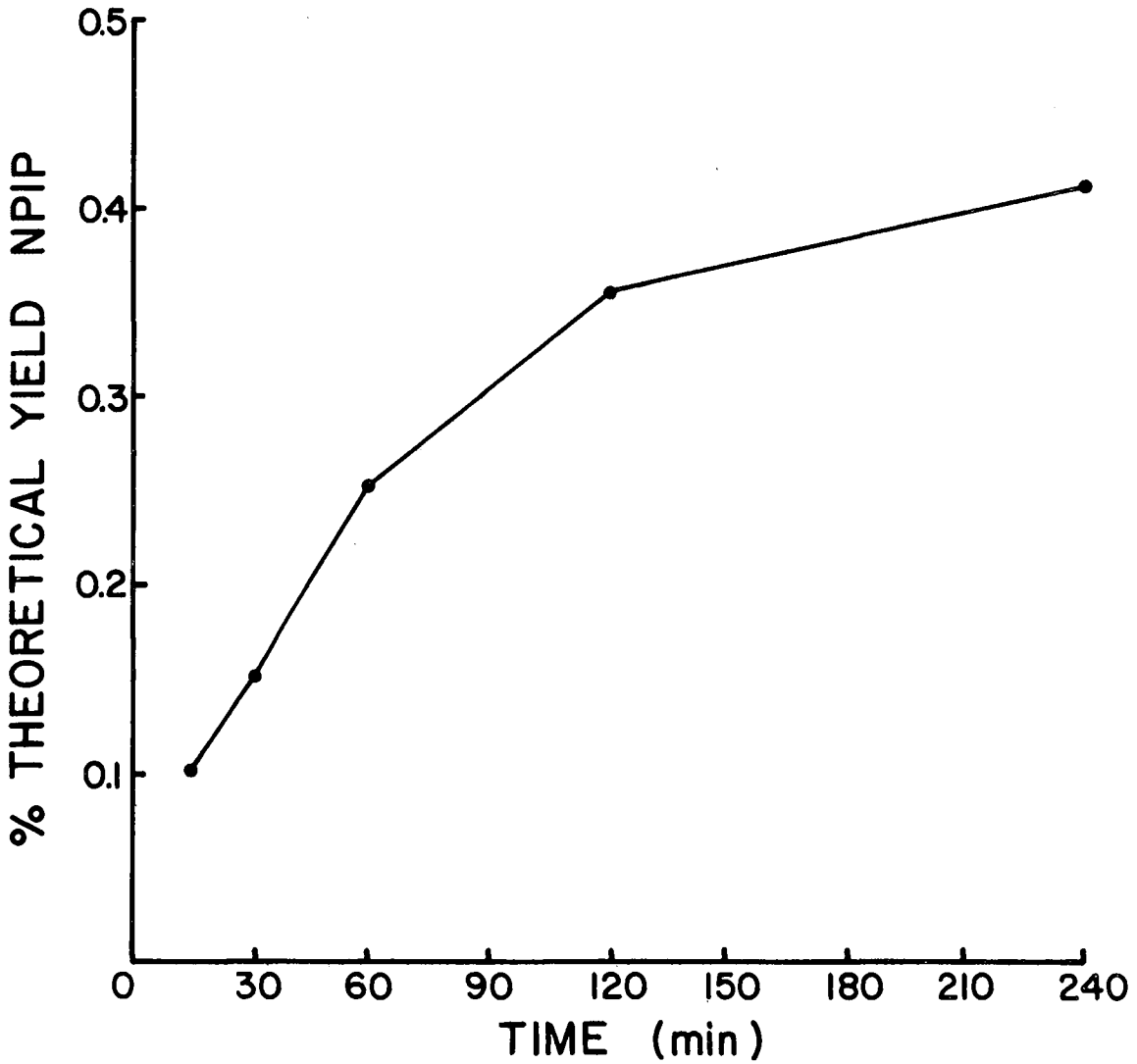


Figure 7. Effect of time on the formation of NPIP from cadaverine and sodium nitrite. Reaction conditons: 0.025 M cadaverine, 0.125 M sodium nitrite, pH 3.8, 0.1 M acetate buffer, 100°C.

on the yield of heterocyclic nitrosamine from a primary diamine. The nitrite concentration had a marked influence on the amount of NPYR formed as shown in Table 3. Increasing the nitrite concentration by a factor of two had more than a doubling effect on the yield of NPYR. The highest yield, 8.55% of theoretical, was obtained with a tenfold excess of nitrite over amine concentration.

The rate of nitrosation reactions and nitrosamine formation has been shown to be dependent on the square of the nitrite concentration (Taylor and Price, 1929; Ridd, 1961; Mirvish, 1970; Fan and Tannenbaum, 1973). Second order dependence is probably why dramatic changes in NPYR yield were observed when the nitrite concentration was altered. A pH dependent rate constant, k, can be calculated from the equation: rate = k [amine] [nitrite]^2 (Mirvish, 1970). The values for k, shown in Table 3, are relatively constant for the nitrite concentrations of 50-125 mM. On the basis of k values in this range, it appears the rate of NPYR formation is dependent on the square of the nitrite concentration. This implies that the formation of heterocyclic nitrosamines from primary diamines is kinetically the same order as the formation of nitrosamines from secondary amines.

Table 3. Effect of nitrite concentration on the formation of NPYR from putrescine. [a]

Concentration $NaNO_2$ (mM)	NPYR formed (mg)	Percent yield	k X 10^6 [b]
12.5	0.004	0.01	--
25.0	0.047	0.09	--
50.0	0.375	0.75	12.0
75.0	1.117	2.23	15.9
100.0	1.736	3.47	13.9
125.0	2.355	4.71	12.1
250.0	4.275	8.55	5.5

[a] reaction conditions: 25 mM putrescine, pH 3.8, 0.065 M citrate -0.07 M phosphate buffer, 100°C, 15 min.

[b] k is given in m moles^{-2} min^{-1} liter^{2} and is from the equation: rate = k [amine] [nitrite]^2.

Effect of Temperature

The influence of reaction temperature on the production of heterocyclic nitrosamines from primary diamines was noted from the results obtained from the high temperature-low moisture studies. Yields of NPYR in excess of 20% were obtained after two hr at 160°C while no NPYR was detected after holding the reaction material at room temperature for several days. The effect of temperature on the formation of NPYR in buffered solution is shown in Figure 8. NPYR was not detected at 22 or 40°C, but a yield of 0.06% was noted at 60°C. The yield increased tenfold at 80°C and 25-fold when the reaction was carried out at 100°C.

When a longer reaction time was used, nitrosamines were formed from primary diamines at a temperature of 22°C. Putrescine and lysine were reacted in buffer with sodium nitrite in the absence of light for six days. The yields of NPYR and NPCA are shown in Table 4. In the case of NPYR, a yield above 9% was found when a ten molar excess of nitrite was used. Approximately equal amounts of NPYR were obtained by incubating the same reaction mixture at 22°C for six days or 100°C for 10-15 min. An important conclusion from this experiment is, with the reaction conditions employed, relatively high levels of heterocyclic nitrosamines were formed at room temperature. It seems likely that heterocyclic nitrosamine

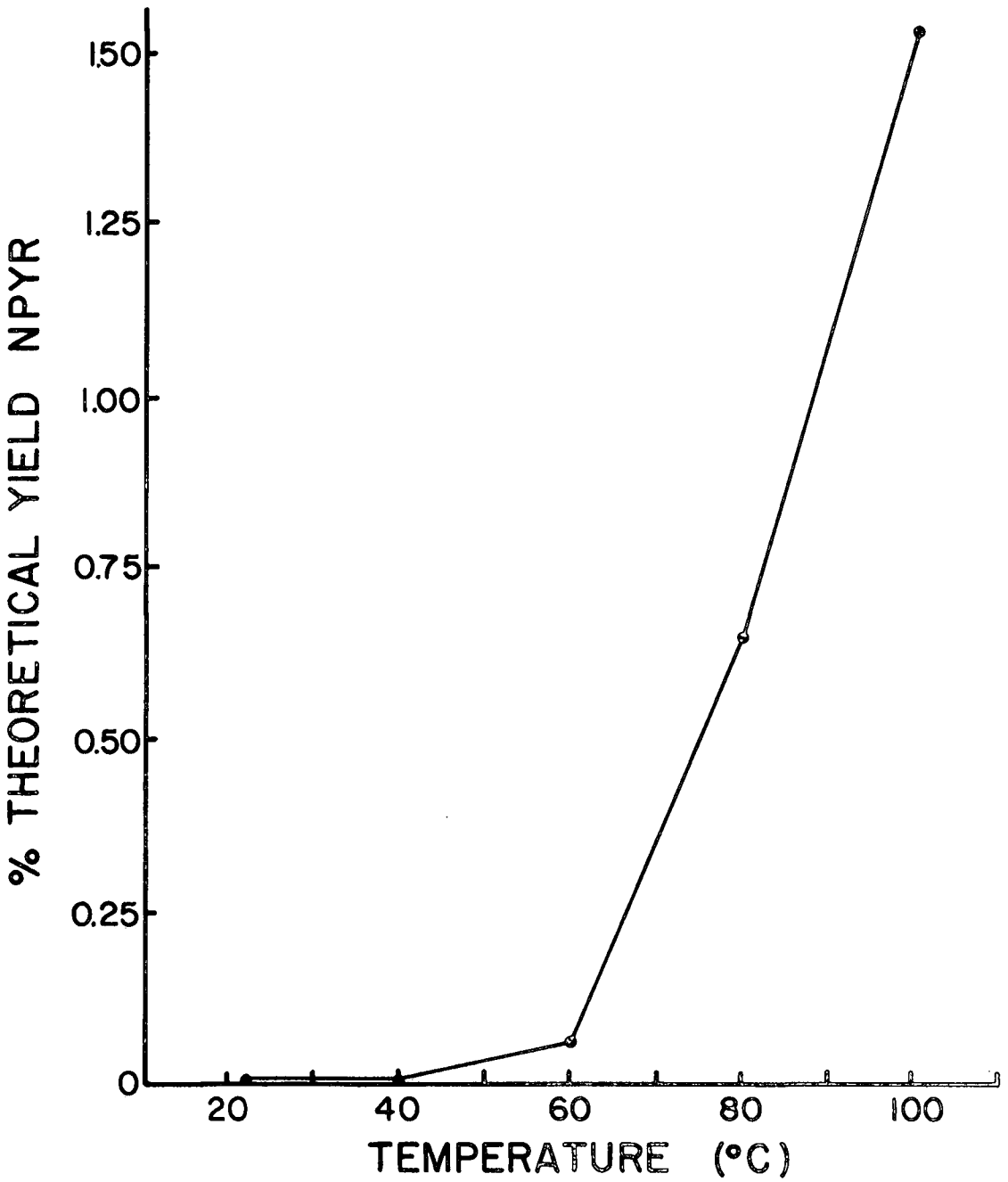


Figure 8. Effect of temperature on the formation of NPYR from putrescine and sodium nitrite. Reaction conditions: 0.025 M putrescine, 0.050 M sodium nitrite, pH 3.8, 0.065 M citrate - 0.07 M phosphate buffer, 30 min.

formation could also take place at the refrigeration temperatures of food.

Table 4. Nitrosamines produced from putrescine and lysine reacted with sodium nitrite at 22 ± 2°C for six days.

Amine [a]	$NaNO_2$ concentration (M)	Nitrosamine	Percent yield
Putrescine [b]	0.050	NPYR	0.39
	0.250		9.22
Lysine [c]	0.050	NPCA	0.03
	0.250		0.28

[a] amine concentration 0.025 M

[b] pH 3.4, 0.065 M citrate-0.07 M phosphate buffer

[c] pH 3.8, 0.1 M acetate buffer.

Comparison of Nitrosamines Formed from Various Primary Diamine Precursors

Four different heterocyclic nitrosamines were formed from the four diamines reacted with nitrite in buffer. The amounts of nitrosamines formed using the same reaction conditions are shown in Table 5. It should be noted that the amines were reacted in pH 3.8 buffer which is the optimum pH for NPYR formation from putrescine. This may not, however, be the optimum pH for the other reactions.

Table 5. Nitrosamines produced from various diamines reacted with sodium nitrite at 100°C for one hr at pH 3.8.[a]

Amine	Nitrosamine	Percent yield
Putrescine	NPYR	2.76
Ornithine	NPRO	7.24
Cadaverine	NPIP	0.02
Lysine	NPCA	0.45

[a] 0.025 M amine and 0.050 M sodium nitrite in 0.1 M acetate buffer.

The amino acids gave rise only to nitrosamino acids and not the decarboxylated compounds. Ornithine did not result in NPYR and lysine did not produce NPIP, as was found in the high temperature-low moisture reactions. Although the buffer reactions were performed at 100°C, these conditions apparently did not promote decarboxylation.

Interesting comparisons can be made regarding the relative amounts of heterocyclic nitrosamines formed. The five-membered rings, NPYR and NPRO, were formed more readily than the six membered rings, NPIP and NPCA. This observation is consistent with the information reported by Salomon (1936) regarding the formation of heterocyclic rings in solution. When comparing the formation of pyrrolidine and piperidine from the corresponding straight chain bromamines, it was observed that the five-membered

ring formed more rapidly than the six-membered ring. Differences in spacial arrangements of the straight chain compounds in solution was offered as an explanation. The observations concerning ring size in Table 5 can be contrasted to the high temperature-low moisture reactions, summarized in Table 1, where little difference could be noted in the amount of five versus six-membered ring products.

From Table 5, a difference can also be noted in the formation of heterocyclic nitrosamines from the simple diamines compared to the amino acids. Approximately three times as much NPRO as NPYR was found and over 20 times as much NPCA as NPIP was formed. This suggests that the carboxyl group functions to make cyclization take place more readily. These observations are at variance with those of the high temperature salt reactions where it was found that the amino acids gave less heterocyclic nitrosamines than did putrescine and cadaverine. The main difference in these two reaction conditions, other than temperature, was that one was a solution reaction while the other was a low moisture salt reaction. Nitrosation reactions in solution are affected by the basicity of the amine and because the carboxyl group serves to lower the basicity of the α-amino group, this may be the reason why higher yields were obtained from the amino acids. The pKa values for the amino groups of cadaverine are 9.7 and 11.0 (Rometsch et al., 1951) compared to

9.0 and 10.5 for lysine (Weast, 1973). Since nitrous acid is known to react with the nonionized amine (Ridd, 1961), the lower basicity of the amine group would increase the effective concentration of the amine in the nitrosation reaction.

Mechanism of Heterocyclic Nitrosamine Formation

At least two separate steps are involved in the formation of heterocyclic nitrosamines from straight chain primary diamines. With cadaverine as an example, presumably the first step would be cyclization to piperidine and the second step would be the nitrosation of the cyclic amine to a nitrosamine. The latter mechanism involves a form of the nitrite ion attacking the secondary amine to yield a nitrosamine. The initial cyclization mechanism may be more complicated and could be influenced by the reaction conditions.

Cyclic secondary amines can result when straight chain diamine hydrochloride salts are subjected to high temperatures (Norman, 1968). A second mechanism of cyclization could involve the reaction of the diamine with nitrite. As a result of interaction with nitrite, a primary amine undergoes diazotization and carbonium ion formation. A cyclic secondary amine could be produced if this carbonium ion reacted with the amine group at the opposite end of the molecule. This mechanism would be similar to that suggested by Austin (1960), whereby the carbonium ion resulting from a primary

monoamine could react with another amine molecule to give a dimer.

In applying the cyclization mechanisms to the present study, the heat-induced cyclization of the diamine hydrochloride seems likely for the low moisture reactions at 160°C. In solution, however, interaction with nitrite may play a role in cyclization, especially where significant yields of heterocyclic nitrosamines were obtained at room temperature.

DBNA Formation from Butylamine

When butylamine was reacted with sodium nitrite both in buffer and at the high temperature-low moisture conditions, DBNA was found to be a product as shown in Table 6. In solution at pH 3.4, the yield of DBNA was extremely low, while the high temperature dry salt reaction produced one percent DBNA.

Table 6. DBNA produced from the reaction of butylamine and sodium nitrite.

Amine	Reaction conditions	Amine (m moles)	$NaNO_2$ (m moles)	DBNA (mg)	Percent yield [a]
Butylamine	solution, pH 3.4, 100°C for 1 hr [b]	10.0	20.0	0.062	0.01
Butylamine·HCl	dry, 160°C for 1 hr	0.5	1.0	0.404	1.02

[a] assumes DBNA forms from two molecules of butylamine.

[b] reaction volume 20 ml, pH adjusted with H_2SO_4.

DBNA as a product of the butylamine-nitrite reaction was first reported by Meyer et al. (1877). Similar primary monoamines, methylamine and ethylamine, have more recently been implicated as precursors of DMNA and DENA, respectively (Ender and Ceh, 1971). The results of the present investigation paralleled the observations of Ender et al. (1967) where very low yields of DMNA were obtained from methylamine and nitrite in solution. When methylamine hydrochloride and sodium nitrite were reacted in the dry state at 135°C, however, DMNA yields reportedly increased to 9%.

The formation of a dialkylnitrosamine from a primary monoamine involves a dimerization step followed by nitrosation. This represents a second reaction scheme whereby amines may act as nitrosamine precursors. Contrasted to the cyclization process, however, dimerization would be dependent on amine concentration. The dimerization process requires a second amine molecule in close proximity during the reaction, whereas the cyclization of a diamine could take place independent of any surrounding amine molecules. This difference can be observed by comparing the NPYR yields of 2-3% from putrescine via cyclization to the 0.01% DBNA yield in an even more concentrated butylamine solution. The increased yield of DBNA in the high temperature reaction system is probably due, in part, to a much more concentrated amine situation.

Although these results demonstrate that primary amines when reacted with nitrite can form nitrosamines through dimerization, this type of reaction is unlikely to occur to a great extent in food systems unless there is a high concentration of the primary amine.

Occurrence of Diamine Precursors

The amines shown to result in heterocyclic nitrosamines in this study have been found to occur in foods, especially those products to which nitrite is added. Ornithine is a nonprotein amino acid while lysine commonly occurs in protein and as a free amino acid. The reaction of nitrite with lysine, as it exists in a protein, is not likely to result in NPIP or NPCA because of the covalent bonding of the α-amino group. Knowles et al. (1974) reacted nitrite with bovine serum albumin and suggested that the lysine in the protein was deaminated and converted to a hydroxy derivative, 6-hydroxynorleucine. Free lysine and ornithine have been reported in cured ham (Piotrowski et al., 1970) and beef (Wasserman and Spinelli, 1970). The presence of putrescine has been demonstrated in many plant materials (Smith, 1970) and, along with cadaverine, in animal tissue (Tabor and Tabor, 1964). Putrescine and cadaverine have been reported at levels of 189 and 48 mg/100 g tissue, respectively, in fresh pork, and 50 and 63 mg/100 g tissue, respectively in cured pork (Lakritz et al., 1973). Dierick et al. (1974) reported much

lower levels of putrescine and cadaverine in dry sausage along with low levels of lysine and ornithine. It was also demonstrated that the levels of putrescine and free lysine increased as the sausage was allowed to ripen.

Carcinogenicity of Heterocyclic Nitrosamines

The heterocyclic nitrosamines found as products of primary diamines have been tested for carcinogenicity. The volatile nitrosamines, NPYR and NPIP, have been demonstrated to be potent carcinogens in rats (Druckrey et al., 1967; Greenblatt and Lijinsky, 1972a). The nonvolatile nitrosamino acids appear to be less hazardous. Nagasawa et al. (1973) determined the LD50 for NPRO and NPCA but these compounds were not shown to be carcinogenic in rats. Greenblatt and Lijinsky (1972b) also concluded that NPRO was not carcinogenic in mice. NPRO and NPCA may be potentially harmful, however, in that they may be converted to carcinogenic compounds by decarboxylation. High temperature or exposure to dilute alkaline conditions have been shown to promote the decarboxylation of nitrosamino acids (Bills et al., 1973; Pensabene, et al., 1974; Lijinsky et al., 1970).

NPYR Formation during Cooking

NPYR was detected in samples of ground pork that had been cooked with added sodium nitrite and putrescine. Table 7 shows the amount of NPYR detected by analyzing the combined cooked meat, cooked-out fat, and cooking distillate. The samples were not analyzed for other nitrosamines. NPYR was not detected in uncooked pork to which nitrite and putrescine had been added. The average amount of NPYR detected increased by a factor of three with a 0.40% addition of putrescine.

Table 7. NPYR in the combined meat, fat, and distillate of pork cooked to 177°C.

Treatment	μg/100 g pork				Corrected[a] ppb			
	A	B	C	Ave.	A	B	C	Ave.
0.02% $NaNO_2$	4.9[b]	3.2[b]	1.7	3.3	163	107	57	109
0.02% $NaNO_2$ plus 0.40% putrescine	14.7[c]	7.7[c]	6.5	9.8	490	257	216	321

[a] corrected for 30% recovery.

[b] tentative ms confirmation.

[c] confirmed by ms.

The gc-ms analysis of the combined cooked products further substantiated the quantitative differences of NPYR in samples to which putrescine had been added. The presence of NPYR in samples

cooked without putrescine could only be tentatively confirmed by ms. This was accomplished by observing increases in ions at m/e 100, 68, 42, and 30, all characteristic ions of the NPYR spectrum, at the retention time of NPYR. A more complete NPYR spectrum was obtained from samples to which putrescine had been added. The ms analysis also showed that another compound, 2-tridecanone, was present in the samples and eluted at approximately the same retention time as NPYR. The gc quantitation procedure was corrected for the contribution of this compound to the peak area of NPYR.

Replicate cooked samples showed considerable variation in the amount of NPYR detected. Wide variations have also been noted in fried commercial bacon samples, even when the same sample of bacon was cooked in the same manner several different times (Pensabene et al., 1974). The level of NPYR in the samples cooked without putrescine averaged 109 ppb. This can be compared to the results reported by Fazio et al. (1973) who found NPYR levels totaling 75-313 ppb in the cooked meat and cooked-out fat from several commercial bacon samples.

Recovery of NPYR in the steam distillation and extraction steps averaged 30%. Any losses of NPYR in the concentration step were compensated by the use of an internal standard. Other investigators, using steam distillation as part of the NPYR analysis procedure, reported recoveries of 21-41% (Telling et al., 1971) and below 30%

(Crosby et al., 1972). Added ammonium sulfamate and pH 7.0 buffer served to inhibit any NPYR formation from nitrite and putrescine during the analysis procedure.

The objective of the cooking experiments was to determine if putrescine could serve as a precursor of NPYR in a meat system subjected to frying conditions. The quantitative results indicate that putrescine can contribute to the amount of NPYR produced. The heating system was designed to approximate those conditions found in cooking bacon because the occurrence of NPYR in cooked commercial bacon is currently a major food safety problem. Pork belly was used because it is the cut of meat normally processed into bacon. The 0.02% sodium nitrite is the maximum legal level allowed in bacon. The 0.40% putrescine, however, is higher than the levels reported by Lakritz et al. (1973) in pork. Heating conditions were similar to frying in that water was allowed to escape while the hot fat remained with the meat. The pork was heated to 177°C which is the recommended temperature for cooking bacon (Pensabene et al., 1974).

In a second set of cooking experiments, only the cooking distillate was analyzed for NPYR. Table 8 shows the NPYR detected in the distillate of various cooked samples. NPYR estimated to be in the distillate of samples cooked with nitrite but without added amine was low and could not be confirmed by ms. Increased amounts

of NPYR were observed with the addition of putrescine and proline. No peaks were observed at the retention time of NPYR in the gc analysis of samples cooked without nitrite. All samples were subjected to a column chromatography clean-up procedure prior to analysis and 2-tridecanone was completely removed from the dichloromethane extracts by this step. Recoveries of NPYR added to the distillate, extracted, and carried through the column chromatography procedure averaged 81%. This was much higher than when cooked meat and fat were combined with the distillate for analysis. The increased recovery is likely due to the elimination of the steam distillation step.

Table 8. NPYR in the distillate of pork cooked to 177°C.

Percent $NaNO_2$	Amine added	μg/100 g pork			Corrected[a] ppb		
		A	B	Ave.	A	B	Ave.
0	-	0	0	0	0	0	0
0.02	-	1.9	1.2	1.6	23	15	19
0.02	0.10% putrescine	7.2[b]	2.5	4.9	89	31	60
0.02	0.40% putrescine	12.2[c]	10.3	11.3	151	127	139
0.02	0.10% proline	43.4[c]	26.7	35.1	536	330	433

[a] corrected for 81% recovery.

[b] tentative confirmation by ms.

[c] confirmed by ms.

Analysis of only the distillate proved to be a satisfactory procedure for testing the influence of added amine on the level of NPYR produced during cooking. The 0.10% added putrescine is within the range of putrescine levels reported in pork (Lakritz et al., 1973). Proline has been previously shown to be a precursor of NPYR (Bills et al., 1973; Huxel, 1973), and formed more NPYR than did putrescine. The amount of NPYR in the distillate can be compared to average levels found in the entire sample given in Table 7. For samples cooked with 0.02% sodium nitrite and 0.02% sodium nitrite plus 0.40% putrescine, approximately 17 and 43%, respectively, of the NPYR appears to have been in the distillate. Although NPYR was not detected in the distillate of two commercial bacon samples cooked in this manner, it is possible that NPYR could be lost from bacon during normal frying operations. The presence of NPYR in the cooking volatiles, as shown in this study, may represent a possible health hazard associated with frying nitrite-containing foods.

SUMMARY AND CONCLUSIONS

The reaction of nitrite with several primary amines was investigated with regard to the formation of nitrosamines. The nitrosamines were analyzed by gc and identification was confirmed using gc-ms. When reacted with sodium nitrite at 160°C, several primary diamines were found to give heterocyclic nitrosamines. Putrescine dihydrochloride and cadaverine dihydrochloride yielded over 20% NPYR and NPIP, respectively. Ornithine hydrochloride resulted in lower amounts of NPYR and NPRO while lysine produced low levels of NPIP and NPCA.

When reacted in buffered solution at 100°C, the optimum pH for the formation of NPYR from putrescine was 3.8 and the pH optimum for the formation of NPCA from lysine was about 3.4. Nitrosamine yield was affected by the type of buffer used, indicating that the ionic environment of the reaction system is an important factor in the formation of heterocyclic nitrosamines from primary diamines.

Nitrite concentration had an important influence on the rate of NPYR formation from putrescine. The reaction kinetics appear to be second order with respect to the nitrite concentration. Although yields of heterocyclic nitrosamines increased at elevated temperatures,

substantial yields were also noted when the reaction mixture was incubated at room temperature for six days.

When the yields of heterocyclic nitrosamines from the various precursors reacted in buffer were compared, the five-member ring compounds formed more readily than the six-membered ring products. Also, yields of the carboxylated nitrosamines, NPRO and NPCA, from ornithine and lysine, respectively, were higher than yields of NPYR and NPIP from the straight chain diamines.

DBNA was identified as a product of the reaction of butylamine with nitrite. In solution, yields were extremely low, but as a high temperature-low moisture reaction, one percent DBNA was produced. The mechanism of DMNA formation involved dimerization, rather than cyclization, and it appears that this type of reaction is unlikely to occur in food systems unless there is a high concentration of the amine.

When ground pork containing 200 ppm sodium nitrite was heated, NPYR was produced in the cooked product. The amount of NPYR increased when putrescine was added to the pork prior to cooking, indicating that putrescine can be a precursor of NPYR in cooked bacon. When only the cooking distillate was analyzed, increased amounts of NPYR were detected when putrescine and proline had been added to the nitrite-containing pork.

This study has shown that several commonly occurring diamines can react with nitrite, under certain conditions, to produce carcinogenic heterocyclic nitrosamines. Although yields are relatively low, these primaryamines may serve as nitrosamine precursors in foods.

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