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

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Several primary amines were treated with nitrite in a high temperature–low moisture system and in buffered solution. Gas chromatography and mass spectrometry were used to analyze for nitrosamines. Analysis of nitrosamino acids was facilitated by making the methyl ester derivatives. When treated in a system with 5% H_2O at 160°, nitrosamines in yields of 1–22% were produced from amines as follows: putrescine yielded nitrosopyrrolidine, ornithine yielded nitrosopyrrolidine and nitrosoproline, cadaverine yielded nitrosopiperidine, and lysine yielded nitrosopiperidine and nitrosopipecolic acid. The same products were identified from reactions in buffer solution, except ornithine did not produce nitrosopyrrolidine and lysine did not yield nitrosopiperidine. Butylamine, when allowed to react with nitrite, was found to produce low levels of dibutylnitrosamine. The pH optimum for the formation of nitrosopyrrolidine from putrescine was 3.8, and the pH optimum for the formation of nitrosopipecolic acid from lysine was 3.4. Reaction kinetics appeared to be second order with respect to nitrite concentration.

The formation and occurrence of carcinogenic nitrosamines in foods have become a matter of recent concern. Dimethylnitrosamine has been sporadically detected in cured meat and fish products (Crosby et al., 1972; Fazio et al., 1971; Sen, 1972; Wasserman et al., 1972) while nitrosopyrrolidine (NPYR) has been detected in a number of cooked bacon samples (Crosby et al., 1972; Fazio et al., 1973; Sen et al., 1973).

Nitrosamines result from the action of nitrite on secondary amines but tertiary amines and quarternary ammonium compounds have also been shown to yield nitrosamines when allowed to react with nitrite (Ender et al., 1967; Fiddler et al., 1972; Lijinsky et al., 1972; Malins et al., 1970; Scanlan et al., 1974, Smith and Loeppky, 1967).

In general, primary amines are not considered obvious precursors of nitrosamines because reaction with nitrite leads to diazotization and deamination resulting in olefins, alcohols, or substituted products (Ridd, 1961). Several investigations have presented evidence, however, that some primary amines may produce nitrosamines when allowed to react with nitrite under certain conditions. Early studies by von Linnemann (1872) demonstrated that dipropylnitrosamine (DPNA) was a product of the propylamine–nitrite reaction and Meyer et al. (1877) determined that dibutylnitrosamine (DBNA) was a product of the butylamine–nitrite reaction. Adamson and Kenner (1934) also found dialkylnitrosamines resulted from the action of nitrite on various primary aliphatic amines. More recently, Ender and Ceh (1971) reported low yields of dimethylnitrosamine and diethylnitrosamine from the reaction of nitrite with methylamine and ethylamine, respectively. The authors also reported that several primary amino acids, when heated with nitrite in a starch mixture, resulted in dialkylnitrosamines. Lijinsky and Epstein (1970) suggested that the primary amines putrescine and cadaverine may cyclize to secondary amines at the cooking temperatures of food and react with nitrite to form carcinogenic nitrosamines. Putrescine and spermidine were shown to produce NPYR when treated with nitrite in an oil and water system heated to 170° (Bills et al., 1973).

Primary amines are common in foods and the formation of nitrosamines upon reaction with nitrite is an important consideration. The purpose of this study was to investigate the reaction of several common primary amines and nitrite with regard to the formation of nitrosamines.

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EXPERIMENTAL PROCEDURES

Amine Purity. Nitrosamine yields from primary amines were generally low, so it was necessary to establish the purity of each amine with respect to certain secondary amines. Gas chromatography (GC) was used to determine if the primary amines used in this study contained secondary amines as impurities which would lead to the same nitrosamines as those believed to be products of the primary amines. A Varian 1400 gas chromatograph equipped with a flame ionization detector and a glass column (10 ft × 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 and column temperatures, 100°; detector temperature, 280°; nitrogen carrier gas flow, 30 ml/min. For the analysis of butylamine, the injector and column temperatures were 150°.

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&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 limit of 0.02%. Pipecolic acid and proline were determined by ninhydrin after elution with pH 3.25 citrate–HCl buffer from a column containing Beckman type 50A resin according to the procedure of Ivey (1974).

High Temperature–Low Moisture Reactions. Various

amine hydrochloride salts were mixed with reagent grade sodium nitrite and quantities representing 0.5 mmol of amine and 1.0 mmol of nitrite were weighed into an 18 × 150 mm culture tube (Kimax). Five percent distilled water was added and mixed with the salts. After sealing the open end of the tube in an oxygen–methane flame, the material was placed in a 160° oven for 2 hr. After cooling, the container was broken open at the tip and the material was rinsed into a separatory funnel with approximately 30 ml of distilled water saturated with sodium sulfate. The contents of the separatory funnel were acidified to a pH of approximately 1 with H_2SO_4 prior to extraction. Amines which reacted with nitrite 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 was added to adjust the solutions to the desired pH as determined by a pH meter. The amine and nitrite buffer solutions were then combined in a 25 × 150 mm culture tube with a Teflon-lined screw cap, mixed, and immediately incubated in a water bath at the desired temperature. The amine concentration in each reaction was 0.025 *M* and the sodium nitrite concentration was usually 0.050 *M*. The reaction was terminated by addition of ammonium sulfamate in a 5 molar excess of the initial nitrite concentration followed by H_2SO_4 to acidify the solution to a pH of approximately 1. The reaction material was then saturated with sodium sulfate and transferred to a separatory funnel for extraction. All reactions were performed in duplicate. Amines which reacted with nitrite in buffer solution were putrescine, cadaverine, ornithine hydrochloride, and lysine hydrochloride. Butylamine was allowed to react in a similar manner, except the amine concentration was 0.50 *M* and the sodium nitrite concentration was 1.0 *M*. The reaction was carried out in a nonbuffered solution adjusted to pH 3.4 with H_2SO_4.

Extraction and Concentration. Redistilled reagent grade dichloromethane was used to extract NPYR, nitrosopiperidine (NPIP), and DBNA from acidified aqueous solution. Aqueous fractions from the high temperature–low moisture reactions were extracted in a separatory funnel three times with equal volumes of dichloromethane, while aqueous fractions from the buffered reactions were extracted twice with equal volumes of 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°. When the extract was concentrated to approximately 4 ml, the apparatus was removed from the water bath. Further concentration was accomplished by fitting the concentrator tube with a micro-Snyder column prewetted with dichloromethane and evaporating the solvent with a slow stream of nitrogen to a final volume of 0.3–0.5 ml.

Esterification of Nitrosamino Acids. Reagent grade ethyl acetate was used to extract nitrosoproline (NPRO) and nitrosopipecolic acid (NPCA) from aqueous reaction solutions following the same procedures outlined for the dichloromethane extraction of the volatile nitrosamines. After drying over sodium sulfate, the ethyl acetate fraction was transferred to a round-bottomed 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 milliliters of anhydrous methanol containing 2% concentrated H_2SO_4 was added to the flask and the sample was incubated at room temperature for 1 hr. Samples containing NPCA were treated with acidic methanol for 18–24 hr. At the completion of the methanol treatment, 10 ml of distilled water saturated with sodium sulfate was added to the flask and the contents were transferred to a separatory funnel and 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.

Gas Chromatography of Nitrosamines. A Varian 1400 gas chromatograph equipped with a flame ionization detector was used for the nitrosamine analyses. A 12 ft × 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 × 0.13 in. i.d. stainless steel column packed with 1% Carbowax 20M on Chromosorb G was employed. Operating conditions for the instrument were as follows: injector temperature, 190°; column temperature, 170°; detector temperature, 280°; 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 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 3373B) 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. For the GC estimation of NPRO and NPCA, known amounts of the nitrosamino acids were added to acidified aqueous solutions and carried through the extraction, concentration, methylation, and analysis procedure in the same manner as the reaction material. The peak areas of MeNPRO and MeNPCA were compared to an internal standard, methylacetylproline, which was added at the completion of the methylation step. Nitrosamine yields were calculated as percent of theoretical yield based on the amine concentration.

Gas Chromatography–Mass Spectrometry. A Finnigan Model 1015C quadrapole mass spectrometer (MS) in combination with a Varian 1400 gas chromatograph was used to confirm the identity of nitrosamines found as products of the various reactions. Samples were introduced into the MS through the GC operated under the conditions described for GC analysis but with helium as the carrier gas. Operating conditions for the MS were as follows: filament current, 400 μA; electron voltage, 70 eV; analyzer pressure, 5×10^{-7} Torr; scan time, 1 sec. Identification was confirmed by comparing the spectra to those of nitrosamine standards.

Safety Precautions. Many nitrosamines have been demonstrated to be potent carcinogens and safety precautions should be observed in the laboratory regarding the handling and storage of nitrosamine solutions. In this investigation, measures were taken to avoid inhalation and skin contact.

RESULTS AND DISCUSSION

Various heterocyclic nitrosamines were identified as reaction products when the hydrochloride salts of putrescine, cadaverine, ornithine, and lysine were allowed to react with sodium nitrite at 160° for 2 hr. The yields of nitrosamines from duplicate samples are given in Table I. Yields of het-

Table I. Amounts of Nitrosamines Formed when Various Diamine Hydrochlorides Were Treated with Sodium Nitrite for 2 hr at 160°

Amine	Nitrosamine formed[a]	% theor. yield[b] A	B
Putrescine · diHCl	NPYR	23.5	21.1
Ornithine · HCl	NPYR	1.6	0.8
	NPRO[c]	3.9	2.7
Cadaverine · diHCl	NPIP	22.0	21.0
Lysine · HCl	NPIP	1.3	1.0
	NPCA[c]	2.7	2.2

[a] NPYR (nitrosopyrrolidine), NPRO (nitrosoproline), NPIP (nitrosopiperidine), and NPCA (nitrosopipecolic acid). [b] Based on amount of amine in system. [c] NPRO and NPCA determinations were from different reacted samples than the respective NPYR and NPIP determinations.

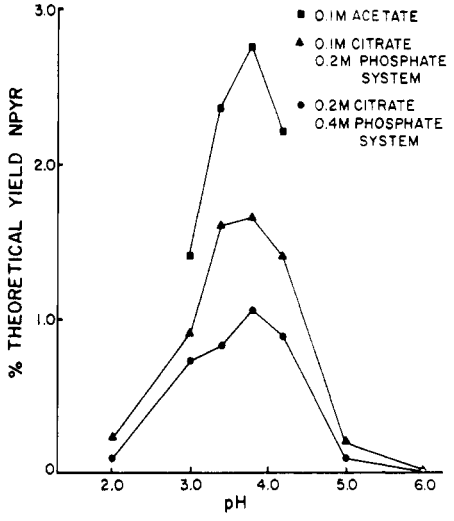

Figure 1. Effect of buffer type and pH on the formation of NPYR from putrescine and sodium nitrite. Reaction conditions: 0.025 *M* putrescine, 0.050 *M* sodium nitrite, 100° for 60 min.

erocyclic nitrosamines from straight chain diamines were considerably higher than from the corresponding amino acids. The presence of the carboxyl group may have inhibited nitrosamine formation or the nitrosamino acids may have been less stable at the reaction conditions employed. Fan and Tannenbaum (1972) demonstrated that nitrosamines with a carboxyl group adjacent to the amino nitrogen may be less stable at elevated temperatures than nitrosamines without the carboxyl group.

When putrescine was allowed to react 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 are shown in Figure 1. The pH values shown are initial pH and do not reflect changes occurring during the reaction. When the pH was measured at the completion of the reaction, differences from initial pH were not greater than 0.1 pH unit with the exception of reactions at pH 3.0 and 2.0 where decreases of 0.3–0.5 pH unit were noted. Yields of NPYR ranged from 2.8% at pH 3.8 to less than 0.005% at pH 6.0. Although the type and strength of buffer influenced the amount of NPYR formed, maximum yield was obtained at about pH 3.8 in all three buffer systems.

An estimate of experimental variation was obtained by treating 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°, for 30 min. The yield of NPYR for the 12 samples was 745.5 ± 45.6 μg (mean ± standard deviation). The standard deviation in this case represented 6.1% of the mean.

Table II. Nitrosamines Produced from Various Diamines Treated with Sodium Nitrite at 100° for 1 hr at pH 3.8[a]

Amine	Nitrosamine	% theor. yield[b]
Putrescine	NPYR	2.76
Ornithine	NPRO	7.24
Cadaverine	NPIP	0.02
Lysine	NPCA	0.45

[a] Amine (0.025 *M*) and sodium nitrite (0.050 *M*) in 0.1 *M* acetate buffer. [b] Based on amount of amine in system.

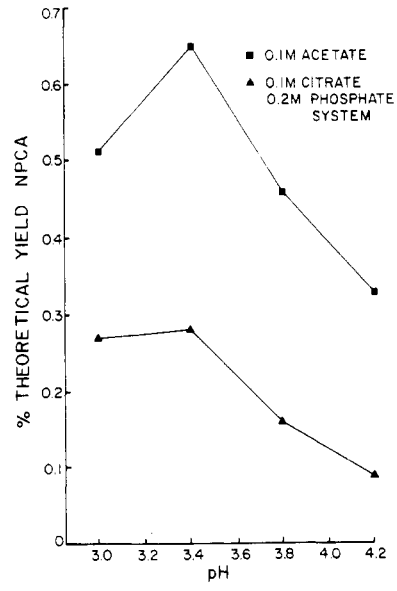

Figure 2. Effect of buffer type and pH on the formation of NPCA from lysine and sodium nitrite. Reaction conditions: 0.025 *M* lysine, 0.050 *M* sodium nitrite, 100° for 60 min.

When lysine was allowed to react 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 2. The pH optimum for the formation of NPCA from lysine appeared to be about 3.4. Cadaverine which reacted with nitrite in buffered solution was found to give rise to NPIP and ornithine resulted in NPRO.

Table II shows the yields of the various heterocyclic nitrosamines obtained from the four amines allowed to react with nitrite under identical conditions. Higher yields of the five-membered ring compounds were obtained compared to the six-membered ring compounds, and the amino acids produced more nitrosamines than did the corresponding straight-chain compounds. Nitrite is known to react with nonionized amine functions (Ridd, 1961). Since the carboxyl group lowers the basicity of the α-amino group, at pH 3.8 the amino groups of the amino acids would be more reactive to nitrite than amino groups of the straight-chain diamines, and this may be why higher yields of heterocyclic nitrosamines were obtained from the amino acids. The formation of NPYR and NPIP from ornithine and lysine, respectively, involves decarboxylation in addition to cyclization and nitrosation. Decarboxylation occurred at 160° but decarboxylated nitrosamines were not obtained from the amino acid reactions in buffer at temperatures up to 100°.

Table III. Effect of Nitrite Concentration on the Formation of NPYR from Putrescine[a]

Concn $NaNO_2$, mM	% theor. yield NPYR[b]	$k \times 10^6$ [c]
12.5	0.01	
25.0	0.09	
50.0	0.75	12.0
75.0	2.23	15.9
100.0	3.47	13.9
125.0	4.71	12.1
250.0	8.55	5.5

[a] Reaction conditions: 25 mM putrescine (pH 3.8), 0.065 M citrate–0.07 M phosphate buffer, 100°, 15 min. [b] Based on amount of putrescine in system. [c] k is given in millimoles^{-2} minutes^{-1} liter^2 and is from the equation: rate = k[amine][nitrite]^2.

Table IV. Nitrosamines Produced from Putrescine and Lysine Treated with Sodium Nitrite at 22° for 6 Days

Amine[a]	$NaNO_2$ concn, M	Nitrosamine	% theor. yield[b]
Putrescine[c]	0.050	NPYR	0.39
	0.250		9.22
Lysine[d]	0.050	NPCA	0.03
	0.250		0.28

[a] Amine concentration 0.025 M. [b] Based on amount of amine in system. [c] pH 3.4, 0.065 M citrate–0.07 M phosphate buffer. [d] pH 3.8, 0.1 M acetate buffer.

Table V. DBNA Produced from the Reaction of Butylamine and Sodium Nitrite

Amine	Reaction conditions	Amine, mmol	Na-NO_2, mmol	DBNA, mmol	% theor. yield of DBNA[a]
Butylamine	Solution, pH 3.4, 100° for 1 hr[b]	10.0	20.0	0.0004	0.01
Butylamine · HCl	Dry, 160° for 1 hr	0.5	1.0	0.0026	1.04

[a] Assumes DBNA forms from two molecules of butylamine, based on amount of amine in system. [b] Reaction volume 20 ml, pH adjusted with H_2SO_4.

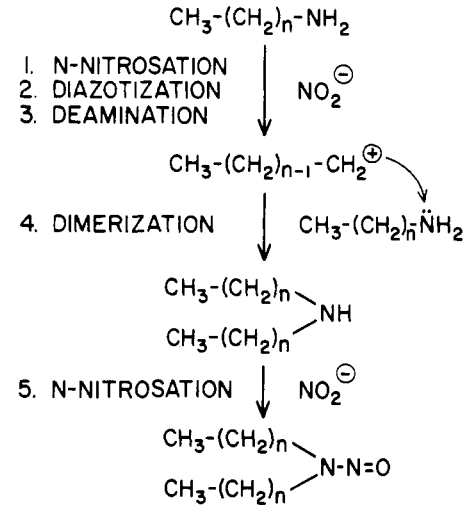

Figure 3. Proposed pathway for dialkylnitrosamine formation from a primary monoamine.

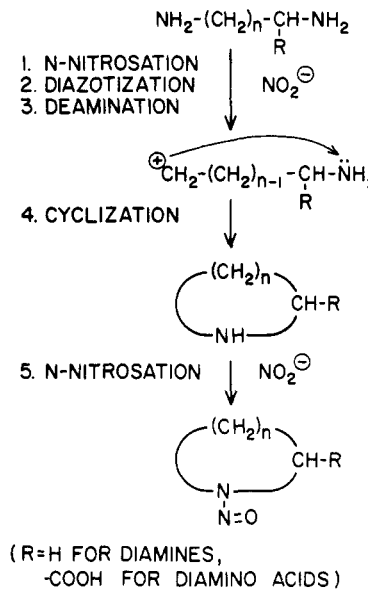

Figure 4. Proposed pathway for heterocyclic nitrosamine formation from a primary diamine.

The production of NPYR from the reaction of putrescine and nitrite was used as a system to determine the effect of nitrite concentration on the yield of a heterocyclic nitrosamine from a primary diamine. As shown in Table III, the nitrite concentration has a marked influence on the yields of NPYR. The highest yield, 8.55%, was obtained with a tenfold excess of nitrite over amine concentration. Values for the pH-dependent rate constant (Mirvish, 1970; Ridd, 1961) are similar for nitrite concentrations of 50–125 mM, and in that range, the reaction kinetics appear to be second order with respect to nitrite concentration.

When incubated with nitrite for 6 days, heterocyclic nitrosamines were produced from diamines at 22°. The yields of nitrosamines produced from putrescine and lysine are shown in Table IV. The production of heterocyclic nitrosamines from primary amines at 22° indicates that elevated temperatures are not necessary for the reaction to occur and suggests that formation could also take place at storage temperatures of food.

Butylamine, a primary monoamine, was allowed to react with nitrite and DBNA was obtained as a product. As shown in Table V, yields in solution at 100° were extremely low, but in the low moisture system at 160°, 1% of the nitrosamine was obtained. The identification of DBNA as a reaction product confirms the finding of Meyer et al. (1877) who reported DBNA, in addition to butylene and butanol, as reaction products of butylamine and nitrite. The mechanism of DBNA formation involves dimerization rather than cyclization and represents nitrosamine formation from a primary monoamine. A second amine molecule would have to be in close proximity during the reaction for dimerization to occur, and the amine concentration would have an important influence on nitrosamine formation. Because of the extremely low yield of DBNA obtained in solution, it appears that dimerization of primary amines and eventual nitrosamine formation would not occur readily in food systems.

The mechanism of heterocyclic nitrosamine formation from a primary diamine involves cyclization. Nitrosamine formation was found to take place at elevated temperatures and also at 22°. Cyclization of the hydrochloride salts of primary diamines is known to occur at elevated temperatures (Norman, 1968). At lower temperatures in buffered

solution, the amine–nitrite reaction may be involved in the cyclization mechanism. Austin (1960) suggested that the carbonium ion formed from the reaction of a primary amine and nitrite could react with a second primary amino group to form a secondary amine. In the case of a primary diamine, a cyclic secondary amine could result. Figure 3 shows a proposed pathway for the formation of dialkylnitrosamines from the reaction of nitrite with a primary monoamine involving the mechanism proposed by Austin (1960). A proposed pathway for the formation of a heterocyclic nitrosamine from a primary diamine–nitrite reaction is shown in Figure 4. Steps in Figures 3 and 4 include (A) nitrosation, diazotization, and deaminiation (Ridd, 1961); (B) reaction of carbonium ion with amino group; (C) nitrosation of secondary amine.

Studies have shown that NPRO and NPCA may not be carcinogenic (Greenblatt and Lijinsky, 1972; Nagasawa et al., 1973). These nitrosamino acids may be potentially hazardous if present in foods, however, as they have been shown to decarboxylate to carcinogenic nitrosamines under conditions of high temperature (Bills et al., 1973; Pensabene et al., 1974) or dilute alkali (Lijinsky et al., 1970).

The reaction of nitrite with diamines to form heterocyclic nitrosamines may be a potentially important reaction with regard to nitrosamine formation in foods. Putrescine has been previously demonstrated to be a possible precursor of NPYR (Bills et al., 1973). Ornithine, cadaverine, and lysine are structurally similar amines that have been reported to occur along with putrescine in fresh and cured meat (Dierick et al., 1974; Lakritz et al., 1973; Piotrowski et al., 1970; Wasserman and Spinelli, 1970). The addition of nitrite to foods containing these primary amines could result in the formation of carcinogenic nitrosamines under conditions of storage or heat treatment.

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