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An Investigation into the Potential Formation of N-Substituted Amides and Their Nitrosated Derivatives during the Frying of Bacon

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The formation of N-substituted amides was investigated by using both model systems and bacon. Fatty acids were shown to react readily with selected α-amino acids in model systems at 200 °C to give N-substituted amides. However, formation of primary amines via the decarboxylation of α-amino acids appears to be unlikely at temperatures normally encountered in pan frying of bacon due to insufficient energy for the decarboxylation step. Under these conditions, only amines would react readily with fatty acids to yield secondary amides. N-substituted amides were shown to be nitrosated readily under acid conditions in a model system. It was also demonstrated that *N*-nitrosamides are very unstable under conditions commonly encountered in cooking bacon and thus are unlikely to be present in heat-processed foods.

N-Nitrosamides which arise from the reaction of secondary amides with nitrite (Mirvish, 1977) usually have one alkyl residue and an acyl residue. They are chemically reactive compounds and are relatively easily hydrolyzed to alkylating diazoalkanes (Preussmann, 1974). They are considered to exert both local and systemic activity in the carcinogenesis of experimental animals (Preussmann, 1974). Although their powerful carcinogenic responses are well-known, there has been only a limited number of studies on the occurrence of nonvolatile *N*-nitroso compounds in food systems, due in part to their instability under neutral and alkaline conditions (Mirvish, 1971). However, the precursors of *N*-nitrosamides have been reported in certain foods. Such compounds include uridine and ureas which have been isolated from fish by Mirvish (1975). High concentrations of agmatine, a decarboxylation product of arginine, have been reported in fresh abalone (Kawabata et al., 1978), and citrulline has been reported by Wada (1930) in watermelon.

Recently, Sims and Fioriti (1975) reported that heating fatty acids (or esters) and triglycerides with α-amino acids at temperatures above 150 °C resulted in the formation of N-substituted amides. These results were confirmed by Kakuda and Gray (1980a) using a model system containing amino acids or free amines and fatty acids. They reported that the presence of a secondary amino group in these compounds makes them susceptible to nitrosation. These compounds may thus represent another source of nitrosatable species available for reaction with nitrite.

The present study was undertaken to establish whether N-substituted amides can be formed under conditions encountered in the processing and cooking of bacon and thus be potential precursors of *N*-nitrosamides. Specific objectives of this study were (1) to investigate the formation of N-substituted amides from reactions between fatty acids and/or triglycerides with α-amino acids and amines in both model and bacon systems, (2) to investigate the nitrosation of N-substituted amides in both model and bacon systems, and (3) to study the thermal stability of *N*-nitrosamides during the frying process.

MATERIALS AND METHODS

Note: Because of the extremely hazardous nature of N-nitroso compounds, all work was carried out in efficient fume cupboards whenever possible, and extreme caution was exercised in handling these components.

Reagents. All chemicals and solvents employed were of analytical grade and were used without further purification. Fatty acids and their methyl esters were purchased from Fisher Scientific Co., Fair Lawn, NJ. Pentylamine, norleucine, valine, and methionine were purchased from Eastman Kodak Co., Rochester, NY. Column packing materials were obtained from Supelco, Inc., Bellefonte, PA. Pork bellies were purchased from a local supplier soon after slaughter and stored in a cooler at 2 °C until used.

Preparation of N-Substituted Amides in Model Systems. A series of N-substituted amides were prepared by reacting norleucine with lauric, myristic, palmitic, stearic, oleic, and linoleic acids at 200 °C for 1 h. The unreacted amino acid was removed by suction filtration after the addition of warm (30 °C) diethyl ether to the reaction flask. After removal of the solvent by evaporation, the residue was redissolved in a minimum of warm (40 °C) petroleum ether and then left at room temperature to crystallize. Further purification of the prepared amides was carried out as previously described by Kakuda and Gray (1980a).

Analysis and Identification of N-Substituted Amides. The purity of the synthesized *N*-pentyllauramide, *N*-pentylmyristamide, *N*-pentylpalmitamide, *N*-pentyl-

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stearamide, N-pentyloleamide, and N-pentyllinoleamide was determined by using a Hewlett-Packard 5840A gas chromatograph equipped with a Hewlett-Packard 18850A GC terminal. A glass column (2 m × 2 mm i.d.) packed with 3% OV-101 on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA) was employed in the analyses. The chromatograph was temperature programmed from 240 to 260 °C at a rate of 5 °C/min. Injection port and flame ionization detector temperatures were 250 and 350 °C, respectively, while the carrier gas (nitrogen) flow rate was 30 mL/min.

Mass spectra were obtained by using a Hewlett-Packard 5985A gas chromatograpoh–mass spectrometer. The column was similar to that used for the purity analyses, while helium was the carrier gas with a flow rate of 25 mL/min. The analyses were carried out by using a temperature program from 240 to 260 °C. The ion source and analyzer temperatures were maintained at 290 °C; the electron voltage was 2000 V and the ionizing potential was 70 eV.

Nitrosation of N-Substituted Amides. Nitrosation of the N-substituted amides prepared above was carried out by using essentially the method of White (1955a).

Analysis and Identification of Amides and N-Nitrosamides. The purity of the N-nitrosamides was determined by GC analysis, while their identities were confirmed by mass spectrometry as previously described by Kakuda and Gray (1980b).

Formation of N-Substituted Amides in Pork Belly Slices. *Processing of Pork Bellies.* A fresh pork belly weighing approximately 4 kg was sliced to $^1/_8$ in. in thickness. The slices were divided into two groups. One of the groups was sprayed on the surface with pentylamine, while the other group of slices was freeze-dried and then rehydrated with water containing pentylamine, followed by equilibration for 48 h.

Two pork bellies approximately the same size were selected and stitch pumped with water containing pentylamine and/or norleucine. All treated bellies were smoked for 4 h at 58 °C (dry bulb) and 3 h at 52 °C (dry bulb) at ambient relative humidity in a laboratory smokehouse (Drying System Inc., Chicago, IL). Smoke was applied throughout cooking with a midget-size Mepaco Smoke generator (Meat Packers Equipment Co., Oakland, CA) by utilizing mixed hardwood sawdust. The smoked bellies were transferred to a tempering cooler (2 °C) where they were held overnight prior to slicing.

Frying of Bacon Slices. The treated slices were fried in a Sunbeam (Sears and Roebuck, Chicago, IL) electric frying pan. Each group of slices was fried such that half of them were held at 175 °C for 4 min on each side and the other half for an additional 4 min on each side. Cook-out fat was taken for analysis after each cooking interval.

Analysis of N-Substituted Amides in Fried Pork Belly Slices and Cook-Out Fat. Twenty grams of fried pork belly slices was homogenized with 10 mL of distilled water in a Waring blender and extracted 3 times with 50 mL of methylene chloride. The extract and tissue residue were then transferred to a medium-grade sintered glass funnel and filtered under vacuum. The homogenizer and the residue in the funnel were washed with an additional volume of methylene chloride and filtered.

The filtrate was quantitatively transferred to a 500-mL separating funnel, and distilled water (10% by volume) was added and throughly mixed. The mixture was allowed to separate into two phases until the interface was clear. The upper phase was transferred to a 500-mL volumetric flask and evaporated to dryness in a vacuum Rotavapor-R (Buchi, Switzerland) at 40 °C. The crude product was purified by column chromatography (Supelcosil-ATF 061, Supelco, Inc.). The crude dried extract was dissolved in 5 mL of petroleum ether and applied on the column. The sample was eluted with 200 mL of petroleum ether, followed by 250 mL of petroleum ether–diethyl ether (90:10 v/v). The first fraction was discarded, and the second fraction was collected and evaporated to dryness. The purified amides were dissolved in 5 mL of diethyl ether for GC analysis.

Formation of N-Nitrosamides in Pork Belly Slices. A solution of N-pentylpalmitamide in diethyl ether (100 mg in 3 mL) and sodium nitrite in water (0.5 g in 3 mL) was injected into 50 g of pork belly slices with a Hamilton syringe and stored 24 h at 4 °C. The slices were fried as previously described. The fried pork belly slices and cook-out fat were extracted with chloroform. The pooled chloroform extracts were washed with water and evaporated to dryness. The crude product was partially purified by precipitating the unreacted amide in cold petroleum ether (4 °C) and vacuum filtering. The filtrate was placed on a Supelcosil ATF-061 column and eluted with 100 mL of petroleum ether. The petroleum ether was evaporated and the purified compound was dissolved in 5 mL of diethyl ether for GC analysis.

Thermal Stability of N-Nitrosamides under Frying Conditions. One hundred milligrams of N-nitroso-N-pentylpalmitamide was dissolved in 3 mL of diethyl ether and injected into 100 g of pork belly slices with a Hamilton microsyringe. Fifty grams of pork belly slices was fried as before. The fried pork belly slices and cook-out fat were extracted and analyzed for N-nitroso-N-pentylpalmitamide. The remaining 50 g of raw pork belly were extracted and analyzed in a similar manner and used as the control.

Determination of Fatty Acid Composition of Pork Belly Adipose Tissue. The fatty acid composition of pork belly adipose tissue was determined by GC analysis of the fatty acid methyl esters according to the procedure of Morrison and Smith (1964). The column used was a 2 m × 2 mm i.d. glass column packed with 15% DEGS (diethylene glycol succinate) on Chromosorb W (80–100-mesh). The chromatograph was operated isothermally at 190 °C.

RESULTS AND DISCUSSION

Preparation and Analysis of N-Substituted Amides. Gram quantities of long-chain N-substituted amides were prepared and purified by repeated crystallization in petroleum ether or by column chromatography. These purified compounds were used as standards for GC analysis. The formation of these long-chain amides was confirmed by GC–MS analysis as described by Kakuda and Gray (1980a). The mass spectrum of N-pentylpalmitamide exhibited the major ions m/e 129, 296, 325 (M^+), 239, 268, and 30. The mass spectra of the other amides confirmed their formation from norleucine and fatty acids (lauric, myristic, palmitic, stearic, oleic, and linoleic) at high temperatures. These purified amides were used as standards for the identification of N-substituted amides formed during the cooking of pork belly slices.

Nitrosation of N-Substituted Amides. Gram quantities of N-nitrosamides were prepared by reacting the purified amides with sodium nitrite as previously described. The purity of the N-nitrosamides (N-nitroso-N-pentyllauramide, N-nitroso-N-pentylmyristamide, N-nitroso-N-pentylpalmitamide, N-nitroso-N-pentylstearamide, N-nitroso-N-pentyloleamide, and N-nitroso-N-pentyllinoleamide) was determined by GC, and their

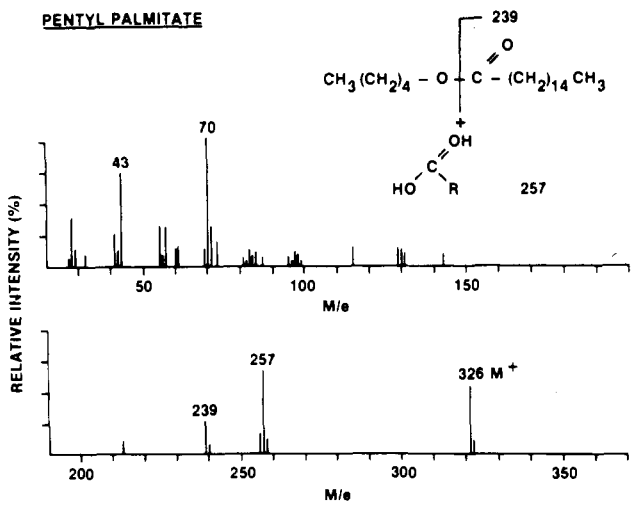
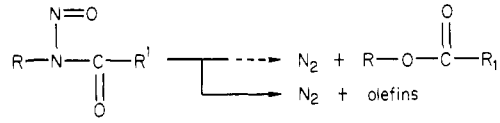


Figure 1. Mass spectrum of pentyl palmitate, a breakdown product of *N*-nitrosopentylpalmitamide.

Table I. Fatty Acid Composition of Pork Bellies and N-Substituted Amides Which Formed upon Heating Pork Bellies Containing Added Pentylamine

fatty acid	%	% amide	N-substituted amide
C14	1.3	0.4	pentylmyristamide
C16	16.5	16.3	pentylpalmitamide
C18	11.1	14.0	pentylstearamide
C18:1	49.8	43.0	pentyloleamide
C18:2	15.4	15.5	pentyllinoleamide

identity was confirmed by GC–MS analysis (Kakuda and Gray, 1980b). The mass spectra of these *N*-nitrosamides detected only the corresponding ester produced by thermal elimination of nitrogen. White (1955b) reported that the formation of esters and olefins occurs according to the scheme



The compound detected by GC–MS analysis of *N*-nitroso-*N*-pentylpalmitamide corresponded to pentyl palmitate (Figure 1), the major ions present being *m/e* 70, 43, 257, 326, (m^+), and 239. Similar fragmentation patterns were recorded for the other *N*-nitrosamides. A standard mixture of these *N*-nitrosamides was prepared and used as standards for the identification of possible *N*-nitrosamides in bacon.

Formation of N-Substituted Amides in Pork Belly Slices Treated with Norleucine and/or Pentylamine. Freeze-dried pork belly slices were rehydrated in water containing 1000 mg/kg pentylamine. After equilibration for 24 h, the treated slices were fried for 4 min on each side at 175 °C and analyzed for amide formation. The analysis revealed that amide formation did not occur either in cook-out or in the fried bacon during cooking. After the fried bacon and cook-out fat were heated for an additional 8 min, five N-substituted amides were identified in the cook-out fat and cooked residue (Figure 2), indicating that the time of frying is a limiting factor in their formation. It was noted that the relative percentages of the various amides formed closely approximated the fatty acid composition of pork belly adipose tissue as indicated in Table I.

Similar results were obtained when pentylamine was sprayed on the surface of pork belly slices, followed by frying. N-Substituted amides were formed from the reaction of added pentylamine and the fatty acids naturally present in the pork belly slices. These results are in agreement with the findings of Kakuda and Gray (1980a), who reported that free amines react readily upon heating with fatty acids and fatty esters. They reported that a temperature of 100 °C for 15 min was sufficient for amide formation from the reaction of pentylamine with tripalmitin or palmitic acid.

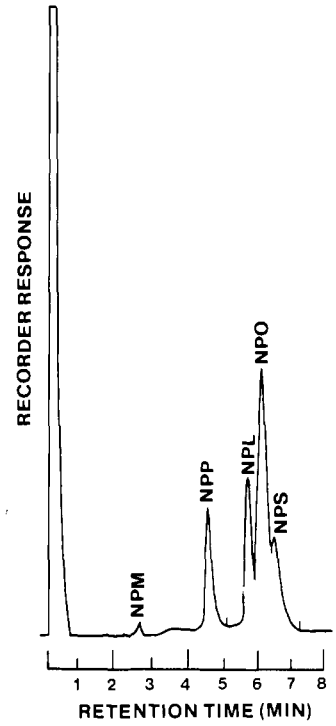


Figure 2. Gas chromatogram of N-substituted amides (*N*-pentylmyristamide, NPM; *N*-pentylpalmitamide, NPP, *N*-pentyllinoleamide, NPL; *N*-pentyloleamide, NPO; *N*-pentylstearamide, NPS) present in cook-out fat from rehydrated freeze-dried pork belly slices containing pentylamine, heated for 16 min at 175 °C.

When the study was repeated with norleucine, amide formation did not take place in the cook-out fat or fried bacon, even after the additional heating period of 8 min at 175 °C. However, when norleucine and pork belly adipose tissue were heated for 1 h at 200 °C, five N-substituted amides (*N*-pentylmyristamide, *N*-pentylpalmitamide, *N*-pentylstearamide, *N*-pentyloleamide, and *N*-pentyllinoleamide) were identified. These results indicate that the normal time and temperature of frying were not sufficient for decarboxylation of norleucine. Sims and Fioriti (1975) reported that decarboxylation of an amino acid in the presence of a fatty acid ester is zero order and is much slower than the aminolysis reaction.

When pork bellies were stitch pumped with pentylamine (or norleucine) and trilaurin, followed by smoking, slicing, and frying, amide formation was not evident in either the smoked belly, the smoked and fried belly, or the cook-out fat. This was true even though previous experiments had confirmed the formation of amides in pork belly slices treated with pentylamine under identical frying conditions. Patterson and Mottram (1974) reported that the concentration of volatile amines in pork carcass meat decreased during curing and processing. Therefore, these results suggest that low temperatures and long periods of smoking resulted in volatilization and loss of pentylamine. The smoking conditions and frying temperature were not sufficient to decarboxylate the norleucine.

These results indicate that formation of primary amines via the decarboxylation of amino acids appears to be un-

likely under normal cooking conditions. There does not appear to be sufficient energy for the decarboxylation reaction. Kakuda and Gray (1980a) reported high temperatures (minimum 150 °C for 45 min) were required for the decarboxylation of norleucine. However, the amount of free amines in foods is not limited to those formed by thermal decarboxylation. Many enzymatic and bacterial decarboxylation reactions are known to occur in many foodstuffs (Maga, 1978), and these reactions may serve as sources of free amines. The presence of amines, fatty acids, and high temperatures during cooking and processing may lead to formation of secondary amides in foods.

Formation of *N*-Nitrosamides in Bacon. In order to determine whether nitrosation of amides can occur in bacon, solutions of *N*-pentylpalmitamide in diethyl ether and sodium nitrite in water were injected separately into pork belly slices that were then stored 24 h at 4 °C. The bacon was then analyzed for the presence of *N*-nitrosopentylpalmitamide before and after frying. Results showed that *N*-nitrosamide formation did not occur in the raw or cooked bacon or in the cook-out fat. This is not surprising since the main nitrosating agent for amides is the nitrous acidium ion (Mirvish, 1975). Thus, nitrosation of secondary amides requires low pH. The extent of nitrosation of secondary amides decreases as the pH of the environment increases, and little reaction occurs above pH 3 (Kakuda and Gray, 1980b). Thus, the pH of foods would militate against the occurrence of nitrosamides, even if their precursors are present. However, in vivo nitrosation of alkylamides has been reported by Mirvish (1971).

Thermal Decomposition of *N*-Nitrosamides. The stability of *N*-nitroso-*N*-pentylpalmitamide under frying conditions similar to those used in the cooking of bacon was also studied. An aliquot of *N*-nitrosamide solution in corn oil (1000 mg/kg) was injected into pork belly slices and stored overnight at 4 °C and then fried. A 95% recovery of the *N*-nitrosamide was obtained from the raw pork belly slices. Analysis revealed that only 8 and 5% of the original *N*-nitrosamide was found in the cook-out fat and fried bacon, respectively. This indicates that 87% of the original amount of the *N*-nitrosamide was decomposed during heating. These results support the findings of KKuda et al. (1980), who reported *N*-nitrosamides are much less stable than the volatile *N*-nitrosamines. In their thermal decomposition studies on *N*-nitrosamides utilizing heating conditions commonly encountered in the pan frying of bacon or in the oven roasting of pork, Kakuda et al. (1980) found that *N*-nitroso-*N*-pentylpalmitamide was degraded to the extent of 74–97% compared to 3–14% for *N*-nitrosopyrrolidine and *N*-nitrosodimethylamine. Chow (1979) reported that at temperatures ranging from ambient to 100 °C, *N*-nitrosamides undergo irreversible thermal rearrangements to form diazo esters. Diazo esters, in turn, decompose rapidly to give carboxylic esters or acids and olefins. The instability of *N*-nitrosamides under alkaline and neutral pH and their rapid thermal decomposition lead to the conclusion that the occurrence of *N*-nitrosamides in food systems is unlikely. The major contribution of N-substituted amides, if present in foods, may be as precursors of *N*-nitroso compounds formed by in vivo nitrosation reactions.

CONCLUSIONS

Previous studies have indicated that amino acids or amines can react with fatty acid esters under appropriate conditions to form N-substituted amides (Sims and Fioriti, 1975; Kakuda and Gray, 1980a). Similarly, studies have also established the conditions under which these amides can react with nitrite to form the corresponding *N*-nitroso derivatives (Kakuda and Gray, 1980b). However, there are few data available regarding their possible formation in food systems containing added nitrite.

This study has shown that the formation of N-substituted amides from amino acids and fatty acids or triglycerides is unlikely during the processing and cooking of foods. This supports the conclusions from the previous investigation (Kakuda and Gray, 1980a) when it was demonstrated that high temperatures (150 °C for 45 min) are necessary to effect the decarboxylation of amino acids. Bacon was chosen as the food substrate in this study as bacon adipose tissue reaches a final temperature of approximately 165 °C when fried at 171 °C for 6 min, 3 min/side (Lee, 1981).

It has also been further demonstrated that the high temperatures obtained during the frying process will bring about substantial decomposition of *N*-nitrosamides in bacon, if present. The observation that *N*-nitroso-*N*-pentylpalmitamide decomposes by approximately 87% in the frying process supports the model system data of Kakuda et al. (1980), who reported a decomposition range of 74–94% for the same *N*-nitrosamide. Thus, it can be concluded that it is very unlikely that *N*-nitrosamides will be present in thermally processed food systems.

Registry No. Norleucine, 327-57-1; *N*-pentyllauramide, 84649-64-9; *N*-pentylmyristamide, 74420-90-9; *N*-pentylpalmitamide, 73392-18-4; *N*-pentylstearamide, 74420-94-3; *N*-pentyloleamide, 74420-97-6; *N*-pentyllinoleamide, 74420-98-7; *N*-nitroso-*N*-pentylpalmitamide, 73392-17-3; *N*-nitroso-*N*-pentyllauramide, 84649-65-0; *N*-nitroso-*N*-pentylmyristamide, 74420-91-0; *N*-nitroso-*N*-pentylstearamide, 74420-95-4; *N*-nitroso-*N*-pentyloleamide, 84649-66-1; *N*-nitroso-*N*-pentyllinoleamide, 84649-67-2.

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