N-Nitrosamine Formation in Fried-out Bacon Fat: Evidence for Nitrosation by Lipid-Bound Nitrite

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The capacity of fried-out bacon fat to form N-nitrosamines after removal of the edible portion was investigated by adding 2,6-dimethylmorpholine to the fried-out fat and heating to 170 °C in sealed ampules. Fried-out bacon fats from 17 commercial bacon samples were all capable of forming low mg/kg amounts of N-nitroso-2,6-dimethylmorpholine after 90 min of heating. Fried-out fat rendered from bacon fried for 3 min/side consistently produced more N-nitroso-2,6-dimethylmorpholine than that of bacon samples fried 6 min/side. Lipids that were Soxhlet extracted from raw bacon adipose tissue also contained considerable nitrosation capability after drying and removal of the solvent. The unknown nitrosating agent could not be removed from the fried-out fat or extracted lipids by extraction with NaOH and/or N_2 purging, leading to the conclusion that dissolved oxides of nitrogen are not responsible for these observations.

The precursor(s) to the formation of NPYR (N-nitrosopyrrolidine) when cured bacon is fried, as well as the mechanism of formation, has (have) been the subjects of several reports in recent years (Gray and Randall, 1979; Gray, 1981; Scanlan, 1983). A number of possible amine precursors exist, but most data indicate that proline is the major precursor of NPYR (Coleman, 1978). The order in which proline is nitrosated and decarboxylated (or visa versa) has not been directly proven, but the kinetic data of Lee et al. (1983) indicate that at bacon frying temperatures (80–165 °C), nitrosation precedes decarboxylation. Nakamura et al. (1976) believe the pathway to be temperature dependent; below 175 °C NPRO (N-nitrosoproline) is an intermediate while above 175 °C pyrrolidine is the major intermediate. The work of Bharucha et al. (1979) also supports NPRO as an intermediate based on the fact that NPRO decarboxylates at a lower temperature than proline.

While the amine precursor has been determined with reasonable certainty, the nature of the nitrosating agent is less clear. Added nitrite serves as the source of nitrosating agent and the amount of NPYR formed is related to ingoing nitrite; higher inputs give higher NPYR contents (Sen et al., 1974). The relationship to residual nitrite at the time of frying is less clear. Sen et al. (1974) found no relationship while others have claimed a positive relationship (Sebranek, 1979).

Nitrite, per se, is not a nitrosating agent but must first be converted to a nitrosating species (Mirvish, 1975). While a number of nitrite derivatives can nitrosate amines under certain conditions, most authors have assumed that oxides of nitrogen (NO_x) are responsible for nitrosamine formation during bacon frying (Bharucha et al., 1979). Nitrite is in equilibrium with nitrous acid, which is formed in small amounts in the weakly acidic meat (Cassens et al., 1979). Nitrous acid is in equilibrium with NO_x compounds, which are known to be quite soluble in lipophilic solvents (Mirvish et al., 1978) and could migrate to the adipose tissue. Nitrosation by NO_x is known to occur rapidly in lipophilic solvents by a free radical mechanism (Mirvish et al., 1978). This reasoning could explain why nitrosamines are preferentially formed in the fatty tissue of bacon during frying (Mottram et al., 1977; Coleman, 1978).

We have previously reported that the fat rendered from cured bacon during frying (fried-out bacon fat, FOBF) contains considerable ability to nitrosate added amines after the edible rasher has been removed. This results in the formation of mg/kg amounts of VNA (volatile N-nitrosamines) when noncured foods are fried in FOBF (Hotchkiss and Vecchio, 1984). The objectives of the present work were to expand our observations on nitrosation in FOBF and to determine if dissolved NO_x compounds are responsible for the formation of nitrosamines when amines are added to FOBF.

MATERIALS AND METHODS

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

Reagents. Dichloromethane and water were glass distilled, and each lot was analyzed for positive peaks. N-Nitrosamine standards were made gravimetrically and diluted to 1 ng/μL. NPYR, NDMA (N-nitrosodimethylamine), and NDPA (N-nitrosodipropylamine) were obtained commercially (Sigma Chemical Co.), and NDMM (N-nitroso-2,6-dimethylmorpholine) was synthesized (Lijinsky and Taylor, 1975) from DMM (2,6-dimethylmorpholine), Aldrich Chemical Co.

Analyses. Volatile N-nitrosamines were determined by GC–TEA (gas chromatography–thermal energy analyzer; Fine et al., 1975) after distillation from mineral oil using a modification of the procedure of Owens and Kinast (1980) in which mineral oil containing 0.25 g of α-tocopherol and 6 g of an ammonium sulfamate–2 N H_2SO_4–Celite 545 (1:1:2) mixture was added to each distillation. NDPA (0.25 μg in MeOH) was added as an internal standard (average recovery = 98%). Recovery of NDMA, NPYR, and NDMM from spiked FOBF averaged 72, 94, and 79%, respectively. Addition of DMM (100 μg/g) to the distillation indicated that NDMM was not an artifact. Instrumental conditions were as follows: GC, Aerograph 200; column, 3 m × 0.32 mm i.d. SS, 10% Carbowax 20 M on 80–100 Chromosorb WHP, helium, 25 mL/min, injector 190 °C, column 150 °C; TEA, thermal energy analyzer, Model 543; interface, 175 °C; pyrolyzer, 525 °C; trap, −150 °C; pressure, 2.2 mmHg.

Frying Procedures. All bacons were purchased from local retail outlets. Samples were fried (171 or 190 °C) in a Farberware electric pan (Model 310-B). The pan temperature was calibrated by cementing a thermocouple (Omega Engineering Co., Model CO3-J) to the center surface to allow continuous monitoring (Omega Engineering Co., Model 872). The pan temperature varied ±1 °C across its surface and cycled ±5 °C from the set point.

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Table I. Formation of NDMM in Fried-out Bacon Fat after the Addition of DMM[a]

sample	temp, °C[b]	DMM[c]	NDMM[d]	nitrosation, %
A	190	0	nd[e]	0
B	190	10	1.4	11
C	171	100	3.2	2.6
D	171	100	3.3	2.6
E	171	100	2.8	2.2
F	171	100	3.0	2.4
G	171	100	5.0	4.0
H	171	100	2.3	1.8
I	171	100	3.0	2.4
J	171	100	0.5	0.4
K	190	100	5.0	4.0
L	190	100	2.8	2.2
M	190	100	3.8	3.0
N	190	100	3.8	3.0
O	190	100	4.8	3.8
P	190	100	2.3	1.8
Q	190	100	1.5	1.2
R	190	100	3.4	2.7

[a] Reacted for 90 min at 170 °C. [b] Fried-out fat rendered from bacon cooked 3 min/side at the indicated temperatures. [c] mg of amine/kg of fried-out fat. [d] mg/kg. [e] Not detected.

Most samples were covered during frying. The FOBF was poured off at the end of frying and stored (−5 °C) in sealed jars.

***N*-Nitrosation of DMM.** Aliquots of FOBF (5 g), taken after the particulate had settled out, along with 0.50 or 0.05 mg of DMM in 0.5 mL of dichloromethane were sealed in 10 mL glass ampules. The ampules were heated in a block heater, cooled, opened, and analyzed as given above.

RESULTS AND DISCUSSION

When DMM was added to the FOBF from 17 different commercial bacon samples and heated, NDMM was formed in mg/kg amounts in all samples (Table I). Addition of 100 mg/kg DMM produced an average conversion to the *N*-nitroso derivative of 2.4% (R = 0.4–4.0). There was no apparent correlation between the percent nitrosation of added amine and the NDMA or NPYR content of the fried edible rasher or rendered fat.

These data demonstrate that FOBF has considerable capacity to form more *N*-nitrosamine than is normally found in FOBF. This suggests that during bacon frying, the amount of VNA formed is limited by the concentration of free amine (or amino acid) and not the concentration of nitrosating agent. This is further supported by the data in Figure 1. When FOBF was heated in sealed ampules without the addition of DMM, the concentration of NDMA and NPYR increased by less than 10 and 50 μg/kg, respectively. However, when DMM was present (100 mg/kg), 1–4 mg/kg quantities of NDMM were formed. These data may indirectly support that of Sen et al. (1974), which indicates that factors other than residual nitrite are important in determining the amount of VNA formation during bacon frying.

Frying conditions also influence the capacity of FOBF to further nitrosate added amines. When bacons were randomized into matched groups and fried for 3 or 6 min/side and the rendered fats tested for the ability to nitrosate added DMM, the 3 min/side FOBF consistently produced more NDMM (Table II). As expected, the 6 min/side edible portions had higher NDMA and NPYR levels than the matched bacon samples fried for 3 min/side. It is likely that the longer frying time results in the thermal decomposition of the nitrosating agent in FOBF. The dilution due to increasing the volume of FOBF by frying for 6 rather than 3 min/side is insufficient to account for the decrease in NDMM formed. Volatilization of the nitrosating agent during the longer frying time is also a possibility.

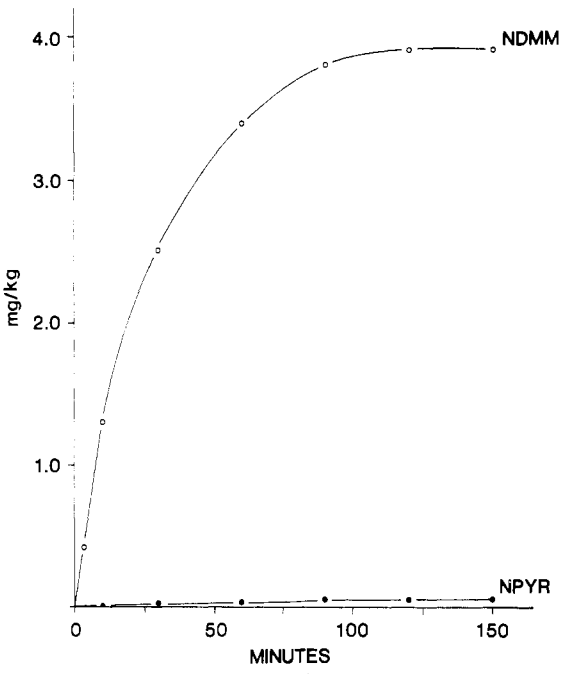

Figure 1. Formation of NPYR and NDMM when fried-out bacon fat containing 100 mg/kg 2,6-dimethylmorpholine was heated to 170 °C in sealed ampules for 0–150 min.

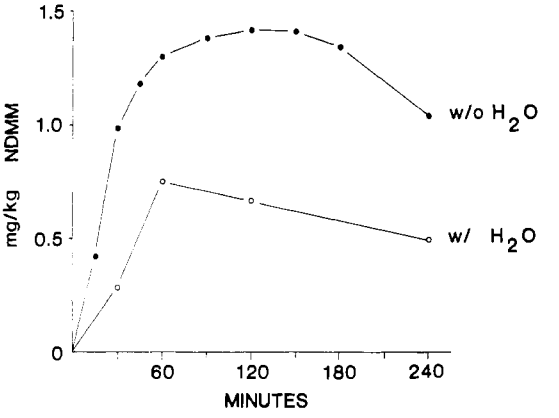

Figure 2. Formation of NDMM when fried-out bacon fat containing 100 mg/kg 2,6-dimethylmorpholine without or with 2% H_2O was heated to 170 °C in sealed ampules for 0–240 min.

Table II. Effect of Frying Time on Nitrosation Capability of Fried-out Bacon Fat

	NDMM[a,b]		
sample	3 min	6 min	ratio (3:6)
A	2.7	2.3	1.2
B	3.8	3.2	1.2
C	4.8	3.9	1.2
D	2.3	1.3	1.8
E	1.6	1.4	1.1
F	3.4	1.9	1.8
G	5.4	5.0	1.1

[a] After addition of 100 mg/kg DMM and heating 90 min at 170 °C. [b] mg/kg.

The highest amounts of NDMM were consistently formed after 90–150 min of heating and decreased slowly with longer heating times (Figure 2). Addition of preformed NDMM to FOBF prior to heating indicated that NDMM is slowly decomposed in FOBF at 170 °C. After heating for 90 min or longer, decomposition exceeds for-

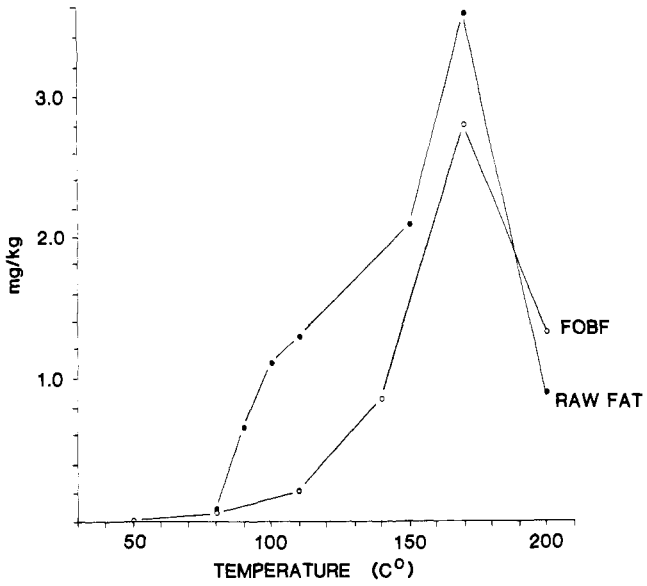

Figure 3. Formation of NDMM when either fried-out bacon fat (○) or Soxhlet-extracted lipids from raw bacon fat (●) each containing 100 mg/kg 2,6-dimethylmorpholine were heated to temperatures of 50–200 °C for 120 min (FOBF) or 90 min (raw fat).

mation. Similar results have been shown for NPYR in model systems (Nakamura et al., 1976). The addition of 2% H_2O to the FOBF inhibited the formation of NDMM by 50–65% (Figure 2). Inhibition by water has also been observed for NPYR formation in model systems designed to simulate the formation of VNA during bacon frying (Coleman, 1978).

Raw bacon fat, which was Soxhlet extracted from uncooked bacon adipose tissue by dichloromethane and the solvent removed under rotary vacuum, contained more nitrosative capacity than a similar fat rendered during frying (Figure 3). Both the uncooked and fried fat samples formed the largest amounts of NDMM at 170 °C after heating for 90 min (raw) or 120 min (FOBF). Only trace amounts of NDMM were formed below 80 °C. If NO_x were the nitrosating agent, NDMM would be formed readily at 25 °C. Mirvish et al. (1978) demonstrated extremely rapid nitrosation rates at 4 °C for NO_x dissolved in dichloromethane.

Other indirect evidence seems to preclude dissolved NO_x as the nitrosating agent. For example, we cleaned up Soxhlet-extracted raw bacon fat by washing the dichloromethane extract 3× with 0.5 N NaOH, removing the solvent by rotary vacuum evaporation, twice redissolving the residue in dichloromethane, and vacuum evaporating off the solvent. Mirvish and Sams (1983) have used a similar but less rigorous procedure to remove dissolved NO_x. When DMM (100 mg/kg) was heated (170 °C, 90 min) in a sealed ampule with 5 g of the lipid residue, 3.5 mg of NDMM/kg of extracted fat was formed. Uncured fresh pork fat that was extracted and similarily treated failed to produce detectable NDMM. We also attempted to N_2 purge the nitrosating agent from hot FOBF (37 or 80 °C) in a gas washing bottle directly into a trap containing Griess reagent (Fan and Tannenbaum, 1971). No nitrite could be detected in the Griess reagent after purging; however, when NO_x was dissolved in dichloromethane by the method of Mirvish et al. (1978) and 0.1 mL added to 100 mL of mineral oil and the mineral oil purged in the same manner, up to 96% of the NO_x was recovered in the Griess trap. When we directly extracted FOBF with 0.1 N NaOH, decolorized the extract, and analyzed by the Griess test for nitrite, less than 45 ng of NO_2^-/g of FOBF was detected.

Freshly fried out bacon fat (25 g) was also cleaned up by hot filtration through Celite 545, followed by extraction (0.1 N NaOH), and 1-h N_2 purging after the addition of 25 mL of dichloromethane. The solvent was evaporated and a 5-g aliquot was tested at each step to determine if any loss in nitrosative capacity occurred. Each aliquot gave the following NDMM concentrations: no cleanup, 3.8 mg/kg; filtered, 3.3 mg/kg; filtered and extracted, 4.4 mg/kg; filtered, extracted, purged, and evaporated, 3.4 mg/kg.

We believe that this indicates that the nitrosating agent is not dissolved NO_x but an unknown reaction product of lipids and NO_x derived from the NO_2^- added during curing. Other authors have presented indirect evidence supporting this hypothesis. Walters et al. (1979) reacted the product(s) of nitric oxide and palmitodiolein with morpholine and found that *N*-nitrosomorpholine was formed. They speculated that unsaturated fatty acids or triglycerides formed pseudonitrosites when reacted with nitric oxide and that these lipid–NO_x products were the source of this nitrosating agent during bacon frying. Others have also demonstrated that nitrite reacts with unsaturated lipids to form covalently bound compounds (Goutefongea et al., 1977). Mirvish and Sams (1983) have investigated the ability of the reaction product(s) of nitrogen dioxide and methyl esters of fatty acids to nitrosate morpholine. Their data indicate that increasing the unsaturation of the fatty acid increases the amount of *N*-nitrosomorpholine formed. The work of Gray et al. (1983) has demonstrated a relationship between the degree of unsaturation in the adipose of unfried bacon and the amount of NPYR and NDMA formed upon frying: more unsaturation produced more VNA in the fried bacon and the FOBF.

In conclusion, we have demonstrated that FOBF has the capacity to form considerably more VNA than is normally present in either the rendered fat or edible portions. Further, these unknown nitrosating agents are not dissolved oxides of nitrogen but more likely a lipid–nitrite reaction product(s) that decomposes during heating to form a nitrosating agent(s) such as NO_x. Components of bacon such as thiols are also capable of forming nitroso derivatives that can transnitrosate (Dennis et al., 1979) and could possibly account for a portion of the nitrosative capability of FOBF. However, it is likely that these nitrosating agents would have been base extracted and the remaining fat would not retain its nitrosative capacity.

Registry No. NDMM, 1456-28-6; nitrite, 14797-65-0.

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Biooxidation of Chrysanthemate Isobutenyl Methyl Groups Examined by Carbon-13 Nuclear Magnetic Resonance Spectroscopy

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[1,3-^{13}C]Acetone (92.6 atom %) is used to label the isobutenyl methyl groups of methyl *trans*-chrysanthemate and *trans*-chrysanthemic acid. ^{13}C NMR chemical shifts are assigned for the analogous allylic carbon atoms of 12 alcohols, aldehydes, and acids prepared by chemical oxidation of methyl *trans*- and *cis*-chrysanthemates. The chemical shifts of these carbon atoms in pH 7.4 phosphate buffer are compared with those of metabolites formed on biooxidation of the ^{13}C-labeled compounds. Direct NMR examination of rat and mouse liver microsomal oxidase systems, the bile of treated rats, and the excreta of orally dosed lepidopterous larvae confirms the previously reported dependence of *E*/*Z* oxidase specificity on the system. Metabolites in rat liver enzyme systems and in the bile of treated rats indicate that the *E*-hydroxymethyl intermediate, but not its *Z* isomer, is rapidly oxidized to the corresponding acid. Studies with ^{14}C compounds establish the importance of isobutenyl methyl oxidation, glucuronide conjugation, and biliary excretion in eliminating the chrysanthemic acid isomers from the body of treated rats.

Chrysanthemate insecticides are detoxified in part by metabolic oxidation at the isobutenyl methyl substitutents (Casida and Ruzo, 1980). Previous studies using [^{14}C]-chrysanthemates and thin-layer chromatography (TLC) revealed some organismal differences in the stereoselectivity of the methyl hydroxylation reactions and in the extent of further oxidation at these sites or conjugation before excretion (Yamamoto and Casida, 1966; Yamamoto et al., 1969; Ueda et al., 1975a,b; Soderlund and Casida, 1977). These findings are not complete or definitive since many of the metabolites were unidentified or underwent some decomposition during workup and analysis. These difficulties might be overcome by in situ examination by ^{13}C nuclear magnetic resonance (NMR) spectroscopy of appropriate ^{13}C-labeled materials and comparison of the ^{13}C chemical shifts of the metabolites with those of suitable synthetic standards.

The present study utilizes ^{13}C NMR to determine the metabolic fate of the isobutenyl methyl groups of methyl (1*RS*,*trans*)-[^{13}C]chrysanthemate in rat and mouse liver enzyme systems and a lepidopterous larva. The in vivo relevance of these findings is further examined by comparing the in vitro metabolites with those present in bile from cannulated rats treated with (1*RS*,*trans*)-chrysanthemic acid bearing a ^{14}C label (for quantitation of urinary, fecal, and biliary metabolites) or a ^{13}C label (for identification of biliary metabolites by direct NMR examination).

MATERIALS AND METHODS

Designations. Figure 1 gives the structure of methyl chrysanthemate and some of its oxidation products along with their designations. Chrysanthemic acid is referred to as CA and its methyl ester as CA-CH_3. Isomers about the cyclcopropane ring are designated as cis or trans and of the side chain (from oxidation of the allylic methyl groups) as *E* or *Z* alcohols (CH_2OH), aldehydes (CHO), or carboxylic acids (COOH).

Chemicals. *trans*-CA and *trans*/*cis*-CA (7:3) were provided by Sumitomo Chemical Co. (Osaka, Japan). Sources of the ^{14}C-labeled cyclopropanecarboxylic acids used were as follows: (1*R*,*trans*)-[2,2-*dimethyl*-^{14}C]-CA (56 mCi/mmol) from Roussel-Uclaf-Procida (Paris, France); (1*R*,*trans*)-[*carbonyl*-^{14}C]-CA and (1*R*,*cis*)-[*carbonyl*-^{14}C]-CA (2.7 mCi/mmol) (Nishizawa and Casida, 1965; Ueda et al., 1974); (1*RS*,*trans*)-[*carbonyl*-^{14}C]-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylic acid (Cl_2CA) (58 mCi/mmol) from hydrolysis (0.5 N NaOH in methanol, 37 °C, 24 h) of (1*RS*,*trans*)-[*carbonyl*-^{14}C]permethrin (Gaughan et al., 1977).

Spectroscopy and Chromatography. NMR spectra were obtained with a Bruker WM-300 wide-bore spectrometer operating at 300.13 MHz (^{1}H) or 75.47 MHz (^{13}C)

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