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Nitrosating Agents from the Reaction Between Methyl Oleate and Dinitrogen Trioxide: Identification and Mutagenicity

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ABSTRACT

Dinitrogen trioxide (N_2O_3) was reacted with methyl oleate and the residual N_2O_3 removed. The resulting products were capable of nitrosating 2,6-dimethylmorpholine above 80°C with a maximum yield of nitrosamine at 130°C. N-*nitroso-2,6-dimethylmorpholine formation was first order in nitrosating agent. When the reaction products were added to ground uncured pork and fried,* N-*nitrosodimethylamine and* N-*nitrosopyrrolidine were formed in amounts similar to those formed in fried bacon. 2,3-Dimethyl-2-nitro-3-nitrosobutane also nitrosated amines in the presence of pork lipids. Individual products were separated by HPLC and identified by IR and MS as nitronitroso-, dinitro-, nitronitrate-, and nitro oxime additions to methyl oleate. The nitronitroso derivative, as previously predicted, occurred in the fraction with the greatest capacity to nitrosate the amine. The products of the reaction between oleic acid and N_2O_3 were mutagenic in* Salmonella typhimurium *strain TA1535 without S-9 activation showing a 5·5- to 66-fold increase in revertants at doses of 90 to 200 μg/plate. These findings support, but do not prove, the theory that nitrite reacts with unsaturated lipids in bacon to form compounds which are responsible for nitrosamine formation during frying.*

INTRODUCTION

The formation of *N*-nitrosamines in fried bacon has been the subject of considerable research (Gray & Randall, 1979). Sodium nitrite added to

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cured bacon correlates with increasing *N*-nitrosopyrrolidine formation during frying (Sen *et al.*, 1974). However, free detectable nitrite is reduced to less than 50% of that added shortly after the curing process (Cassens *et al.*, 1974). Cassens *et al.* (1977) concluded from several studies that nitrite reacts with many components in bacon, including the lipid portion.

Mottram *et al.* (1977) demonstrated that nitrosamine formation occurs primarily in the lipid phase when frying bacon. Two- to five-fold more *N*-nitrosopyrrolidine is detected in the fat than in the lean (Bharucha *et al.*, 1979). The possible role of unsaturated fatty acids in nitrosation was first suggested by Goutefongea *et al.* (1977). Gray *et al.* (1983) demonstrated a correlation between increasing amount of unsaturation in the uncured pork with increasing amount of nitrosamine formed during bacon frying. Hotchkiss *et al.* (1985) demonstrated that a lipid-bound nitrite compound exists and is responsible for nitrosating amines in fried out bacon fat and extracted raw bacon fat. The nitrosating agent could not be extracted or purged from the fat.

The reaction between oxides of nitrogen (NO_x) and olefins has been known for several years. Shechter (1964) has reviewed the reaction and has shown that at least eight different products are formed depending on the particular nitrogen oxide, olefin and solvent used. Walters *et al.* (1979) speculated that nitrite reacts with unsaturated fatty acids in triglycerides in cured meats to form nitronitroso (pseudonitrosites) products which could be responsible for nitrosamine formation during bacon frying. They reacted palmitodiolein with nitrogen dioxide (NO_2) and showed that the crude product was capable of nitrosating amines. Mirvish *et al.* (1983) reacted NO_2 with methyl esters of unsaturated fatty acids in a model system and demonstrated that the crude product was capable of nitrosation. Nitrosation capacity generally increased with increasing unsaturation. Oxides of nitrogen have been shown to react *in vivo* with skin and lung lipids (Pryor *et al.*, 1982) to form unidentified compounds which could, in turn, nitrosate amines *in vitro* (Mirvish *et al.*, 1983). Zubillaga *et al.* (1984) reacted methyl oleate with NO_x while studying the nature of the antioxidant properties of nitrite and concluded that products were formed but no attempt at identification was made. Kurechi & Kikugawa (1979) concluded that, in aqueous systems, lipids could inhibit nitrosation by competing with the amine for nitrite. Separation by HPLC indicated that at least two unidentified methyl linoleate-nitrite products formed.

The above studies lead to the conclusion that NO_x compounds react with unsaturated lipids in model systems to form compounds which are capable of nitrosating amines. However, there is no direct evidence as to the structure of the compound(s) formed or their potential biological activity. The objectives of our work were to study the nature of the products formed

in the reaction between dinitrogen trioxide (N_2O_3) and methyl oleate, to determine which products were capable of nitrosation, and to determine if the reaction between oleic acid and N_2O_3 formed bacterial mutagens.

MATERIALS AND METHODS

Safety

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

Reagents

Dichloromethane (DCM) and water were glass distilled and analyzed for artefacts. Methyl oleate, oleic acid, methyl linoleate, methyl linolenate, *N*-nitrosodimethylamine (NDMA) and *N*-nitrosopyrrolidine (NPYR) were purchased from Sigma Chemical Co., St. Louis, MO; 2,6-dimethylmorpholine (DMM) and 2,3-dimethylbut-2-ene were purchased from Aldrich Chemical Co., Milwaukee, WI. *N*-nitroso-2,6-dimethylmorpholine (NDMM) was synthesized according to the procedure of Lijinsky & Taylor (1975). 2,3-Dimethyl-2-nitro-3-nitrosobutane was synthesized by the procedure of Park & Williams (1972). After recrystallization from ether, the structure was confirmed by the IR spectrum with bands at 1637 cm^{-1} (lit. 1640 cm^{-1}) and 1545, 1379 and 850 cm^{-1} which agree with the structure assigned by Pfab (1977). Purity of all reagents was determined by gas chromatography or HPLC. All fatty acid methyl esters gave only one peak by HPLC.

Analysis

Volatile *N*-nitrosamines were determined by GC–TEA (gas chromatography–Thermal Energy Analyzer; Fine *et al.*, 1975). Instrumental conditions were as follows: GC, Hewlett Packard 5890A, column, 10 m × 530 μm glass capillary, Carbowax 20 M, helium, 8·0 ml/min, injector 190°C, column 85°C; TEA, Thermal Energy Analyzer, Model 543; interface, 175°C, pyrolyzer, 550°C, trap, −160°C, pressure 1·1 mm Hg. Injection volumes of 2 to 8 μl were used.

Reaction products were separated by HPLC on a Beckman 110A solvent metering pump, Model 210 injection valve with a 20 μl injection loop, and Model 160 detector (214 nm); column, Zorbax C_{18}ODS; mobile phase, 85% acetonitrile, 15% water; flow rate, 1 ml/min.

Infrared spectra (IR) were obtained on a Perkin-Elmer grating Model 281. Samples were prepared as a thin film on sodium chloride cells and scanned from 600 to 4000 cm^{-1}.

Mass spectra (MS) were obtained by a Hewlett Packard 5995A, direct probe. MS conditions were: 70 eV electron energy; source, 184°C; analyzer, 180°C; electron multiplier, 1400 V; probe 30°C for 1 min, increase 60°C/min to 200°C. Samples were scanned from 29 to 50 m/z.

Reaction conditions

N_2O_3 was synthesized and reacted with the methyl esters of fatty acids under conditions similar to those used by Park & Williams (1972) to study the products of N_2O_3 and simpler olefins. Perchloric acid (1·2 N, 10 ml) was purged with N_2 and then mixed with $NaNO_2$ (3·6 mmol or a tenfold excess above olefin concentration) in a closed glass apparatus. The N_2O_3 formed was purged with nitrogen through anhydrous calcium sulfate and trapped as a light blue solid in a glass vapor trap immersed in liquid N_2. The olefin (1·6 to 0·34 mmole) in 1·0 ml DCM was added to the solid N_2O_3 and allowed to react at −77°C for 12 h. The liquid was rotary vacuum-evaporated, 10 ml DCM added and evaporated twice to remove traces of NO_x. The products were washed with 40 ml water three times, dried, filtered, and again vacuum evaporated to dryness. The aqueous extracts were tested by Griess reaction (Sen & Donaldson, 1978) to ensure the absence of free NO_x. The products were used as a mixture for nitrosation kinetics and mutagenicity assay and fractionated by HPLC for identification and testing for nitrosating capacity.

N-Nitrosation of DMM

Aliquots of the reaction products (180 μg) or fractions separated by HPLC, 500 μg DMM and 0·60 μl heptane were sealed in a 2·0 ml glass ampoule. The ampoules were heated in a block heater, cooled, brought to 1·0 ml with DCM and analyzed for volatile nitrosamines as described above. Kinetic parameters were determined using procedures similar to those of Mirvish (1975). A 10-fold molar excess of amine was treated with the products of the N_2O_3-methyl oleate reaction at five temperatures between 100 and 170°C in sealed ampoules as above except that the reaction was stopped at 2 min and quantitatively assayed for NDMM. Energy of activation was calculated as the slope of 1/K versus $\ln k$. The reaction order was determined by several doublings of the amount of products added to excess DMM.

Mutagenicity

Strain TA 1535 of *Salmonella typhimurium* was donated by Dr Bruce N. Ames, Berkeley, CA, and characterized by the method of Maron & Ames (1983). Six geometrically spaced doses of the products of the reaction between N_2O_3 and oleic acid (40 to 300 μg in DMSO) were tested with and without S-9 activation. Each dose was tested in triplicate by the plate incorporation assay of Maron & Ames (1983) with the modifications of Batzinger *et al.* (1978). Sodium azide (0·625 μg/plate) and solvent-only samples all served as positive and negative controls. Ampicillin and crystal violet disks were used to check the genetic markers of TA1535.

RESULTS AND DISCUSSION

The yellow products formed when N_2O_3 was reacted with methyl oleate were capable of nitrosating DMM only when heated (Fig. 1). Nitrosation did not occur appreciably below 80°C and maximized at 130°C. Previous experiments using either lipids extracted from raw cured bacon fat or fried-out bacon fat as a source of nitrosating agent produced similar curves

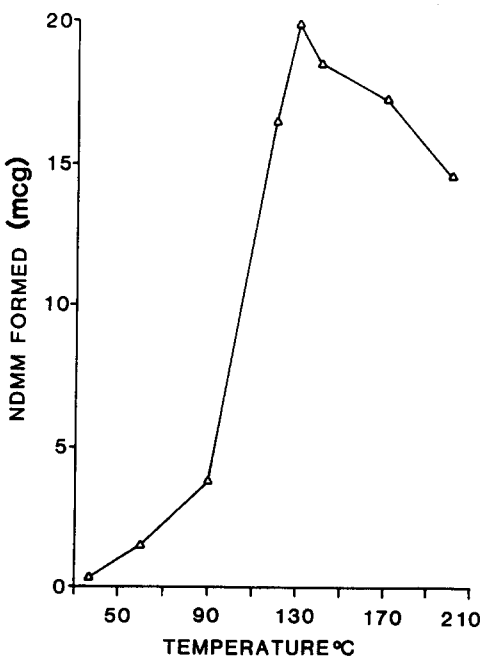

Fig. 1. Effect of temperature on the formation of *N*-nitroso dimethylmorpholine from 2,6-dimethylmorpholine and the products of the reaction between methyl oleate and N_2O_3 (single observations).

(Hotchkiss *et al.*, 1985). In those experiments, nitrosation did not occur below 80°C and maximized at 170°C.

The initial rate of NDMM formation increased linearly with increasing amounts of the reaction products when DMM was in excess. This indicated a first order dependence on nitrosating agent and not the second order dependence that would be expected if nitrite were involved (Mirvish, 1975). The fact that appreciable nitrosation did not occur below 80°C indicates that residual oxides of nitrogen are not the nitrosating agents. Challis & Kyrtopoulos (1979) found very rapid nitrosation by NO_x at 25°C. A rate constant was calculated by assuming that each mole of methyl oleate (MW 296) reacted stoichiometrically with N_2O_3 (MW = 76) to form a compound with a molecular weight of 372. This underestimates k by as much as 10- to 100-fold because, as is shown below, not all of the products are capable of nitrosation and the actual amount of nitrosating compound was considerably less than 100%. At 130°C, k was $6{\cdot}8 \times 10^{-4}\,s^{-1}$ which is much lower than the $0{\cdot}42\,mol^{-1}\,s^{-1}$ reported for aqueous nitrosation of morpholine (Mirvish, 1975) or the $9{\cdot}6 \times 10^{7}\,mol^{-1}\,s^{-1}$ reported for non-aqueous nitrosation by N_2O_3 (Challis & Krytopoulos, 1979). Transnitrosation has, however, been reported to have a k of $3{\cdot}1 \times 10^{-5}\,s^{-1}$ at 50°C (Singer *et al.*, 1980). We have estimated the energy of activation for the nitrosation of DMM to be 11·8 kcal/mole, which is similar to the values reported for model systems designed to simulate frying (Lee *et al.*, 1983).

When 39 mg of the reaction products were thoroughly mixed with 100 g of ground fresh pork and fried at 170°C for 10 min, 4·3 and 13 μg of NDMA and NPYR, respectively, were found in the condensed steam above the skillet. This represents the majority of nitrosamine formed during frying (Hotchkiss & Vecchio, 1985). No other nitrosamines were detected. The amounts of these two nitrosamines formed were higher than those found in previous studies where cured meats were fried under similar conditions (Hotchkiss & Vecchio, 1985). These data indicate that the products of the reaction between N_2O_3 and methyl oleate were capable of nitrosating amines endogenous to uncured pork under frying conditions. These findings support the suggestion of Walters *et al.* (1979) that oxides of nitrogen might react with lipids to form intermediates in the formation of nitrosamines during frying. They treated palmitodiolein with nitric oxide in solvent for 6 h. The resulting product was then refluxed with 1% morpholine in ethyl acetate for six more hours. *N*-nitrosomorpholine was formed in 13% yield based on an assumption that the nitric oxide formed only the nitronitroso derivative. They did not present any chromatographic or spectroscopic data in order to determine the nature of the product(s) formed, nor did they add the product to uncured meat to determine if nitrosamines would form during frying.

When 2,3-dimethyl-2-nitro-3-nitrosobutane (DNNB) and excess **DMM** were heated in a sealed vial containing 5·0 g of lipids extracted from raw pork fat, the yield of NDMM was 15% of theoretical based on the amount of DNNB. Walters *et al.* (1979) reported a 15% yield of *N*-nitrosomorpholine when 'pseudonitrosites' of cyclohexane were refluxed with 1% morpholine. Our yields in hexane were consistently lower than in the extracted lipids.

Methyl esters of steric, linoleic and linolenic acids were similarly treated with N_2O_3 and the products reacted with DMM. Methyl stearate was unreactive with N_2O_3 and did not nitrosate DMM (Table 1). Both methyl linoleate and methyl linolenate reacted with N_2O_3 and the products were capable of nitrosating DMM. NDMM was produced in the greatest amount by the methyl linolenate products. Methyl linoleate products gave somewhat less NDMM than did methyl oleate. These results differ from those of Mirvish & Sams (1983) who reported that the nitrosation capacity of NO_2-treated methyl linoleate increased more than 4-fold over methyl oleate and that methyl stearate had a nitrosation capacity of over one-half that of methyl oleate. The reasons for these differences are unknown but could be related to differences in reaction conditions, solvents, and the fact that NO_2, rather than N_2O_3, was used. In our hands, the procedures used by Mirvish & Sams (1983) resulted in residual NO_x, as determined by Griess reaction, remaining after solvent removal.

Separation of the products of the reaction between N_2O_3 and methyl esters of unsaturated fatty acids by HPLC showed that the spectrum of products was more complicated than earlier thought (Walters *et al.*, 1979). Methyl stearate did not show any peaks in addition to unreacted ester under the HPLC conditions described. All other esters gave at least ten major peaks, all with shorter retention times than the parent methyl ester, indicating a greater polarity for the products (Fig. 2). Unreacted ester could not be detected. Several HPLC fractions were collected and their capacity

TABLE 1

Formation of *N*-nitroso-2,6-dimethylmorpholine from Various Fatty Acid Methyl Esters after Reaction with N_2O_3

Methyl ester-N_2O_3	*μg NDMM formed/ mg product*
Methyl stearate (18:0)	—
Methyl oleate (18:1)	99·8
Methyl linoleate (18:2)	49·9
Methyl linolenate (18:3)	169·0

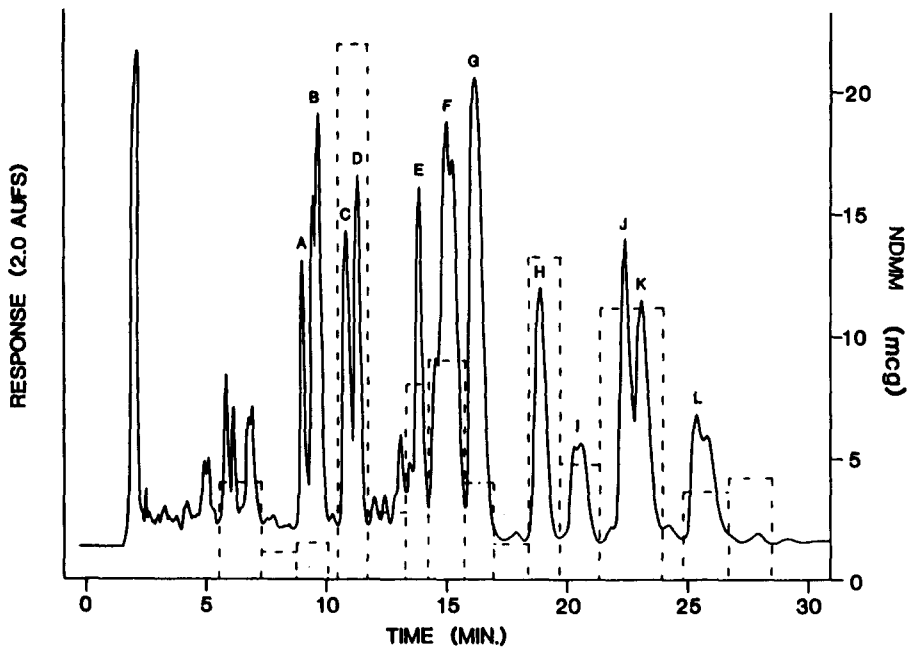

Fig. 2. HPLC chromatogram of the products of the reaction between N_2O_3 and methyl oleate. Fractions were isolated and reacted with 500 μg 2,6-dimethylmorpholine at 130°C and the formation of *N*-nitroso-2,6-dimethylmorpholine quantitated (broken lines).

to nitrosate DMM determined (Fig. 2). The total amount of NDMM formed from 1 mg of the crude mixture was approximately 110 μg while the sum of the NDMM formed from all fractions in Fig. 2 (corrected to 1 mg injected) was 100 μg. This indicated that the peaks shown represent a large majority of the nitrosation capacity. The fraction containing peaks C and D had the greatest nitrosative capacity followed by fractions containing peak H and peaks J and K. Analysis of several fractions by gas chromatography–mass spectrometry (GC–MS) gave peaks with the same retention time and nearly identical spectra to unreacted methyl oleate. Chemical ionization HPLC–MS also gave equivocal data which was probably due to the exposure of the compounds to temperatures above their decomposition points. This has been observed for other similar compounds analyzed by MS (Ahmad *et al.*, 1985).

Methyl oleate was reacted with $^{15}N_2$-N_2O_3 made from NO_2^- containing the ^{15}N stable isotope of nitrogen. The direct probe MS spectra from m/z 29 to 50 of isolated peaks (Fig. 2) was compared to ester treated with $^{14}N_2$-N_2O_3 (Table 2). Comparison of m/z 30, 31 and 46, 47 ions (^{14}N-NO$^+$, ^{15}N-NO$^+$ and ^{14}N-NO_2^+, ^{15}N-NO_2^+ respectively) confirmed the presence of the nitro group in peaks C, D and E and supported the presence of the nitroso group in compound D. Ions at m/z 30 are characteristically more abundant

TABLE 2

Mass Spectra of the Products of the Reaction between Methyl Oleate and both ^{14}N- and ^{15}N-N_2O_3

Compound	*C*		*D*		*E*		*G*		*H*		*J*	
m/z[a]	^{14}N	^{15}N	^{14}N	^{15}N	^{14}N	^{15}N	^{14}N	^{15}N	^{14}N	^{15}N	^{14}N	^{15}N
30	7·6	0·8	16·4	—	6·4	—	15·9	—	27·6	2·6	11·0	—
31	1·4	7·4	1·2	8·3	—	8·3	—	9·5	—	37·0	—	4·9
39	14·3	12·3	15·1	15·7	16·0	11·0	14·7	14·4	14·3	1·0	16·0	15·1
41	85·2	87·2	100·0	100·0	100·0	74·3	95·8	91·4	91·2	16·7	100·0	98·2
42	—	19·9	—	—	21·4	—	19·5	21·8	—	—	49·1	—
43	100·0	100·0	92·4	74·0	90·8	100·0	100·0	100·0	100·0	100·0	76·3	100·0
44	5·5	—	5·4	3·6	4·4	4·9	9·6	9·6	5·1	12·0	5·8	5·2
45	4·3	9·6	3·0	6·7	1·7	1·2	2·9	5·9	—	—	4·7	5·5
46	1·4	—	1·3	0·3	3·6	—	27·4	—	42·6	4·2	5·0	—
47	—	1·0	0·7	0·6	0·1	7·0	0·3	35·2	—	10·9	0·2	2·4

[a] Fractions were scanned from m/z 29 to 50.

TABLE 3
Additional IR Absorption Bands Formed After Treating Methyl Oleate with N_2O_3[a]

Compound	*Wavenumber*	*Assignment*	
C[b]	1 552·5 (s)	NO_2 asym.	
	1 520·4 (m)	NO_2 asym.	dinitro
	1 375·7 (m)	NO_2 sym.	
	958·2 (m)	C—N	
D	*1 562 (s)	N=O	
	1 527·9 (s)	NO_2 asym.	nitroso nitro
	1 490·0 (w)	—	
	1 375·9 (m)	NO_2 sym.	
	960·0 (w)	C—N	
E	*3 381·0 (w)	OH oxime	
	*1 638·6 (w)	C—N oxime	
	1 597·9 (w)	—	nitro oxime
	1 520·9 (vs)	NO_2 asym.	
	1 488·8 (w)	—	
	1 376·5 (m)	NO_2 sym.	
	* 958·5 (m)	N—O oxime	
	834·8 (w)	C—N	
G	1 609·4 (vs)	NO_2 asym. (NO_3)	
	1 523·2 (s)	NO_2 asym.	nitronitrate
	1 375·4 (m)	NO_2 sym.	
	1 262·8 (s)	NO_2 sym. (NO_3)	
	829·5 (m)	C—N	
H	1 664·4 (s)	—	
	1 584·0 (m)	—	
	1 562·9 (s)	—	
	1 524·9 (m)	NO_2 asym.	
	1 375·0 (m)	NO_2 sym.	
	1 275·0 (m)	NO_2 sym. NO_3	
	965·0 (w)	—	
	886·5 (mw)	—	
	821·0 (m)	—	
J	1 638·3 (m)	—	
	*1 558·5 (m)	N=O	
	1 519·9 (s)	NO_2 asym.	
	1 489·0 (m)	—	
	1 375·0 (m)	NO_2 sym.	
	1 335·2 (m)	—	

[a] Thin film of compounds on NaCl cells.
[b] See Fig. 2.
* Indicates peaks which disappeared with heating at 140°C for 15 min.

than m/z 46 ions in nitro compounds (Silverstein *et al.*, 1981) but the m/z 30, 31 ions in compound D are larger than would be expected without the presence of the nitroso group. Aliphatic nitrites do not have m/z 46 ions. Peaks G and H had abundant m/z 46, 47 ions which would be expected of alkyl nitrates.

P-NMR studies (Cl_3CD) of peaks C and D were inconclusive. Pfab (1977) has reported that P-NMR cannot unambiguously discriminate structures of alkanes with two NO_x functional groups.

IR analyses of individual peaks also indicated that the methyl oleate remained intact but with additional absorption bands. Table 3 lists these bands and gives assignments based on those reported by Brown (1955), Feuer (1969) and Lambert *et al.* (1976). Some bands disappeared after heating to 140°C. For example, compound D lost the band at 1563 cm^{-1} which was assigned to N=O (Fig. 3; Brown, 1955). This supported the experimental evidence that this compound nitrosated DMM in the same temperature range. Bands at 1528 cm^{-1}, 1376 cm^{-1} and 960 cm^{-1} indicated the presence of a nitro group. These bands are similar to those seen in authentic nitroso-nitro-dimethylbutane. The IR spectrum of peak C indicated a dinitro function which was stable at 140°C. Absorptions at 1639 and 3381 cm^{-1} in peak E were evidence of the oxime. Nitro bands at 1521 and 1377 cm^{-1} were also present.

Our spectral data are consistent with the structures assigned in Table 3. Peaks C and D were identified as the dinitro and nitronitroso derivatives, respectively. This supports the speculation of Walters *et al.* (1979) that

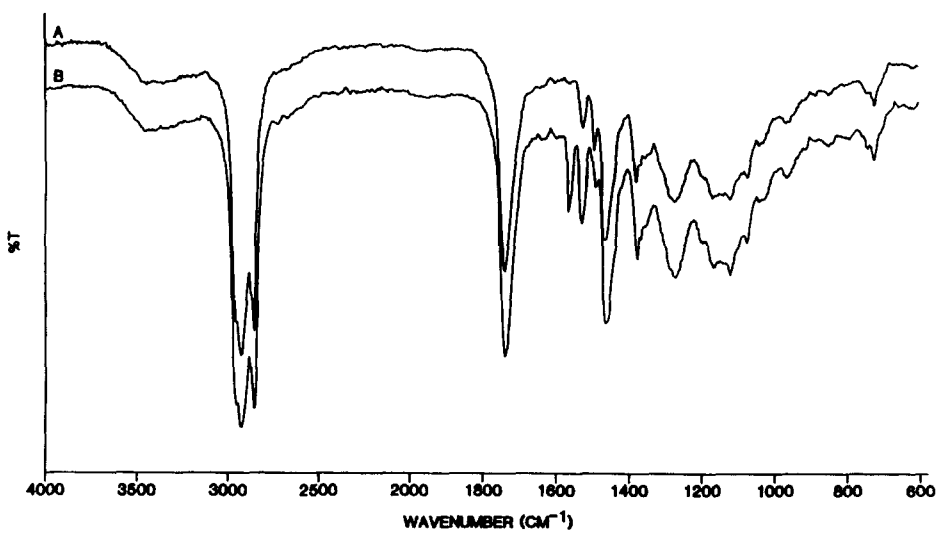

Fig. 3. IR spectra of compound D before (spectra B) and after (spectra A) heating at 140°C for 15 min. Band at 1563 cm^{-1} was lost.

nitronitroso compounds form from triglycerides containing unsaturated fatty acids. Mirvish *et al.* (1983) favored the nitronitrite ester structure. That structure is not consistent with any major peak in our work, particularly the absence of an IR absorption in the 1650–1680 and 617–691 cm^{-1} regions which are characteristic of nitrite esters. It is possible that, upon heating, the nitro group rearranges to the nitrite ester as such rearrangements are known (Chapman *et al.*, 1966). Peaks E and G were identified as nitro-oxime and nitronitrate addition products, respectively. The four products identified here are consistent with compounds identified as products of the reaction between oxides of nitrogen and simpler olefins (Shechter, 1964; Park & Williams, 1972; Pfab, 1977).

The products of the reaction of N_2O_3 and oleic acid as a mixture (after cleanup) demonstrated a dose-related increase in mutants (Fig. 4). Regression analysis of the square root of revertants per plate (minus spontaneous) versus log of the dose demonstrated a slope equal to 57·8 revertants/log dose with an $R^2 = 0{\cdot}899$. The dose estimated to give a statistically significant increase in revertants was 63·7 μg/plate (Table 4). Potency was 369 revertants/100 μg oleic acid-N_2O_3 product. These data are consistent with other data for C—NO_x compounds as direct base pair mutagens (Namiki *et al.*, 1981). These mutagenic compounds may not occur

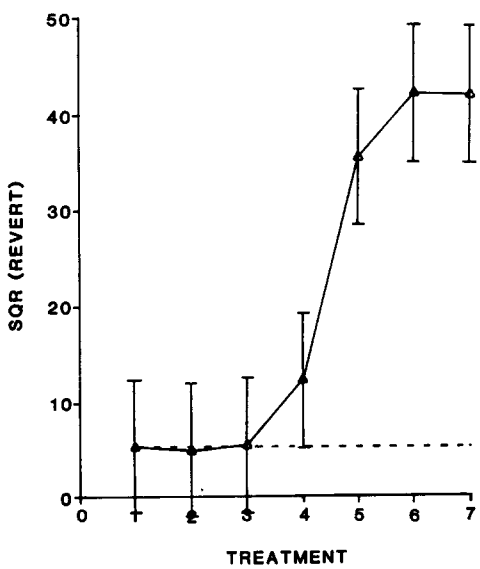

Fig. 4. Dose–response curve for mutagenicity (TA 1535) of the products of the reaction between N_2O_3 and oleic acid. Treatment 1 was the control; treatments 2 to 7 represented 40 to 300 μg of products per plate, geometrically spaced.

TABLE 4
Mutagenicity of the Crude Products of the Reaction between Oleic Acid and N_2O_3

Oleic acid–N_2O_3 products	*His$^+$ revertants/plate*[a]
0 μg/plate	27 (27–33)
40 μg/plate	24 (23–24)
60 μg/plate	29 (28–30)
90 μg/plate	150 (35–324)
135 μg/plate	1 256 (971–1 771)
200 μg/plate	1 777 (1 474–1 896)
300 μg/plate	1 763 (1 569–2 087)
DMSO 50 μl	28 (27–29)
NaN_3 0·625 μg	306 (263–351)
Oleic acid 131 μg	21 (18–25)

[a] Incubated without S-9 mix for 72 h at 37°C.

in bacon in sufficient amounts to demonstrate mutagenicity. Miller & Buchanan (1983) tested raw and fried bacon with *Salmonella typhimurium* TA 98 and found raw bacon not to be mutagenic while fried bacon was mutagenic with S-9 activation.

SUMMARY AND CONCLUSION

Our work confirmed previous work which indicated that certain oxides of nitrogen react with unsaturated fatty acid esters to form compounds capable of nitrosating amines under certain conditions. We further showed that these compounds will nitrosate amines endogenous to raw pork under frying conditions to form the same nitrosamines most common to fried bacon. The reaction is capable of producing a wide range of compounds even under the mild conditions of our work, rather than the single compound suggested by previous reports. Structural identification of the more abundant products agreed with the previous literature for simple olefins. Additionally, these compounds may have undesirable biological activity. We agree with previously published speculation that it is likely that lipid-NO_x compounds such as those demonstrated here are formed in nitrite-treated meats and are the nitrosating agents responsible for the heat-dependent nitrosamine formation during frying. We are currently attempting to isolate similar products from cured pork in order to establish their presence in cured meats.

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REFERENCES

Ahmad, M. U., Libbey, L. M., Barbour, J. F. & Scanlan, R. A. (1985). Isolation and characterization of the products from the nitrosation of the alkaloid gramine. *Food Chem. Toxicol.*, **23**, 841–7.

Batzinger, R. P., Ou, S-Y.L. & Bueding, E. (1978). Antimutagenic effects of 2(3)-*tert*-butyl-4-hydroxyanisole and of antimicrobial agents. *Cancer Res.*, **38**, 4478–85.

Bharucha, K. R., Cross, C. K. & Rubin, L. J. (1979). Mechanism of *N*-nitrosopyrrolidine formation in bacon. *J. Agric. Food Chem.*, **27**(1), 63–9.

Brown, J. F. (1955). Infrared spectra of nitro and oxidized nitrogen compounds. *J. Am. Chem. Soc.*, **77**, 6341–51.

Cassens, R. G., Sebranek, J. G., Kubberod, G. & Woolford, G. (1974). Where does the nitrite go? *Food Prod. Dev.*, **8**(10), 50–6.

Cassens, R. G., Woolford, G., Lee, S. H. & Goutefongea, R. (1977). Fate of nitrite in meat. In: *Proceedings of the Second International Symposium on Nitrite in Meat Products.* (Tinbergen, B. J. & Krol, B. (Eds)), PUDOC, Wageningen, The Netherlands, 95–100.

Challis, B. C. & Kyrtopoulos, S. A. (1979). The chemistry of nitroso compounds. Part 11. Nitrosation of amines by the two-phase interaction of amines in solution with gaseous oxides of nitrogen. *J. Chem. Soc. Perkin*, **I**, 299–304.

Chapman, O. L., Cleveland, P. G. & Hoganson, E. D. (1966). A photochemical rearrangement of nitro-olefins. *Chem. Commun.*, 101.

Feuer, H. (Ed.) (1969). *The chemistry of the nitro and nitroso groups. Part 1.* New York, Interscience Publishers.

Fine, D. H., Rufeh, F., Lieb, D. & Rounbehler, D. P. (1975). Description of the Thermal Energy Analyzer (TEA) for trace determination of volatile and nonvolatile *N*-nitroso compounds. *Anal. Chem.*, **47**(7), 1188–91.

Goutefongea, R., Cassens, R. G. & Woolford, G. (1977). Distribution of sodium nitrite in adipose tissue during curing. *J. Food Sci.*, **42**(6), 1637–41.

Gray, J. I. & Randall, C. J. (1979). The nitrite/*N*-nitrosamine problem in meats: An update. *J. Food Protec.*, **42**(2), 168–79.

Gray, J. I., Skrypec, D. J., Mandagere, A. K., Booren, A. M. & Pearson, A. M. (1983). Further factors influencing *N*-nitrosamine formation in bacon. In: *N-nitroso compounds: Occurrence, biological effects and relevance to human cancer.* (O'Neill, I. K., Von Borstel, R. C., Miller, C. T., Long, J. & Bartsch, H. (Eds)), IARC, Scientific Publication No. 57, 301–9.

Hotchkiss, J. H. & Vecchio, A. J. (1985). Nitrosamines in fried-out bacon fat and its use as a cooking oil. *Food Technol.*, **39**(1), 67–73.

Hotchkiss, J. H., Vecchio, A. J. & Ross, H. D. (1985). *N*-nitrosamine formation in fried-out bacon fat: Evidence for nitrosation by lipid-bound nitrite. *J. Agric. Food Chem.*, **33**, 5–8.

Kurechi, T. & Kikugawa, K. (1979). Nitrite-lipid reaction in aqueous system: Inhibitory effects on *N*-nitrosamine formation. *J. Food Sci.*, **44**, 1263–71.

Lambert, J. B., Shurvell, H. F., Vervit, L., Cooks, R. G. & Stout, G. H. (1976). *Organic structural analysis.* New York, Macmillan Pub. Co., Inc.

Lee, M. L., Gray, J. I., Pearson, A. M. & Kakuda, Y. (1983). Formation of *N*-nitrosopyrrolidine in fried bacon: Model system studies. *J. Food Sci.*, **48**, 820–4.

Lijinsky, W. & Taylor, H. W. (1975). Increased carcinogenicity of 2,6-dimethylmorpholine compared with nitrosomorpholine in rats. *Cancer Res.*, **35**, 2123–5.

Maron, D. M. & Ames, B. N. (1983). Revised methods for the *Salmonella* mutagenicity test. *Mut. Res.*, **113**, 173–215.

Miller, A. J. & Buchanan, R. L. (1983). Detection of genotoxicity in fried bacon by the *Salmonella*/mammalian microsome mutagenicity assay. *Food Chem. Toxicol.*, **21**(3), 319–23.

Mirvish, S. S. (1975). Formation of *N*-nitroso compounds: Chemistry, kinetics, and *in vivo* occurrence. *Toxicol. Appl. Pharm.*, **31**, 325–51.

Mirvish, S. S. & Sams, J. P. (1983). A nitrosating agent from the reaction of atmospheric nitrogen dioxide (NO_2) with methyl linoleate: Comparison with a product from the skins of NO_2-exposed mice. In: N-*Nitroso compounds: Occurrence, biological effects and relevance to human cancer.* (O'Neill, I. K., Von Borstel, R. C., Miller, C. T., Long, J. & Bartsch, H. (Eds)), IARC Scientific Publication No. 57, 283–9.

Mirvish, S. S., Sams, J. P. & Issenberg, P. (1983). The nitrosating agent in mice exposed to nitrogen dioxide: Improved extraction method and localization in the skin. *Cancer Res.*, **43**, 2550–4.

Mottram, D. S., Patterson, R. L. S., Edwards, R. A. & Gough, T. A. (1977). The preferential formation of volatile *N*-nitrosamines in the fat of fried bacon. *J. Sci. Food Agric.*, **28**, 1025–9.

Namiki, M., Osawa, T., Ishiashi, H., Namiki, K. & Tsuji, K. (1981). Chemical aspects of mutagen formation by sorbic acid–sodium nitrite reaction. *J. Agric. Food Chem.*, **29**, 407–11.

Park, J. R. & Williams, D. L. H. (1972). Kinetics of the reactions of nitrous acid with olefins. Kinetic identification of probable nitrosating agents. *J. Chem. Soc. Perkin*, **II**, 2158–63.

Pfab, J. (1977). Orientation of dinitrogen trioxide addition to 2-methylpropene and structure of the addition product. *Chem. Soc. (London) Chem. Comm.*, 766–7.

Pryor, W., Lightsey, J. & Church, D. (1982). Reaction of nitrogen dioxide with alkenes and polyunsaturated fatty acids: Addition and hydrogen abstraction mechanisms. *J. Am. Chem. Soc.*, **104**, 6685–92.

Sen, N. P. & Donaldson, B. (1978). Improved colorimetric method for determining nitrate and nitrite in foods. *J. Assoc. Offic. Anal. Chem.*, **61**(6), 1389–94.

Sen, N. P., Iyengar, J. R., Donaldson, B. A. & Panalaks, T. (1974). Effect of sodium nitrite concentration on the formation of nitrosopyrrolidine and nitroso-dimethylamine in fried bacon. *J. Agric. Food Chem.*, **22**, 540–1.

Shechter, H. (1964). The chemistry and mechanisms of reactions of oxides of nitrogen and olefins. *Rec. Chem. Prog.*, **25**, 54–76.

Silverstein, R. M., Bassler, G. C. & Morrill, T. C. (1981). *Spectrometric identification of organic compounds.* (4th edn), New York, John Wiley and Sons.

Singer, S. S., Singer, G. M. & Cole, B. B. (1980). Alicyclic nitrosamines and nitrosamino acids as transnitrosating agents. *J. Org. Chem.*, **45**, 4931–5.

Walters, C. L., Hart, R. J. & Perse, S. (1979). The possible role of lipid pseudonitrosites in nitrosamine formation in fried bacon. *Z. Lebensm.-Unters. Forsch.*, **168**, 177–80.

Zubillaga, M. P., Maerker, G. & Foglia, T. A. (1984). Antioxidant activity of sodium nitrite. *J. Amer. Oil Chem. Soc.*, **61**, 772–6.