Investigation on the Possible Formation of *N*-Nitroso-*N*-methylurea by Nitrosation of Creatinine in Model Systems and in Cured Meats at Gastric pH

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N-Nitroso-*N*-methylurea (NMU) is a highly potent direct-acting carcinogen that has been shown to induce cancer in a number of animal species. Although previous research has indicated that nitrosation of creatinine (CRN), a common constituent of meats, dried fish, and seafoods, can form traces of NMU, there is uncertainty as to (1) the yield of NMU and (2) whether detectable amounts of NMU can be formed from cured meats following nitrosation under acidic conditions given the low residual levels of nitrite found in cured meats at the present time. Lack of sensitive and specific analytical methods most likely has hindered progress in research in these areas. An HPLC postcolumn denitrosation−thermal energy analyzer technique and a GC-MS confirmation technique were developed for the determination of NMU in cured meats. Both techniques are highly sensitive (0.5 and 0.03 ppb, respectively) and specific. The optimum pH for NMU formation from CRN ranged between pH 1 and pH 3, and the yields of NMU under variable reactant concentrations ranged between 0.00004 and 0.0046%. When 27 samples of various cured meats (10 g aliquots each) were acidified with HCl (final pH values of 0.8−2.5) and incubated at room temperature for 2 h, *without any additional nitrite*, 24 gave results below detectable levels but 3 formed 2−26 ng of NMU/10 g of meat. Incubation of the negative meats with additional nitrite (50−500 μg/g of meat) formed 0.6−176 ng of NMU/10 g of sample. Although the amounts of NMU formed were extremely small, this seems to be the first reported formation of NMU from cured meats with and without additional nitrite.

Keywords: *N-Nitroso-N-methylurea; NMU; creatinine; cured meats; nitrite; determination of NMU in foods*

INTRODUCTION

N-Nitroso-*N*-methylurea (NMU) is a potent direct-acting carcinogen that has been shown to induce cancer of various organs, mainly of the forestomach, brain, and the nervous system, in a wide variety of animal species (Magee et al., 1976; Preussmann and Stewart, 1984). NMU belongs to a class of compounds generally known as *N*-nitrosamides, which are unstable in aqueous solution, especially at pH >5. Therefore, it is highly unlikely that NMU or other nitrosamides would be found in foods or beverages in appreciable concentrations. It is, however, possible that these compounds could be formed in vivo in the human stomach from ingested precursors (amides and nitrite) because the rate of formation of some of the nitrosamides (e.g., NMU) from the corresponding amide precursors (e.g., methylurea) is very high under the acidic conditions existing in the human stomach (Mirvish, 1975).

Previous studies by Mirvish et al. (1982, 1993) have indicated that creatinine (CRN), which is present in fairly high concentrations (30−4100 ppm) in meat, fish, and seafoods (Mirvish et al., 1982), can be nitrosated to form traces of NMU. According to the scheme (Figure 1) proposed by these and other researchers (Greenwald and Levy, 1948; Archer et al., 1971), CRN on nitrosation first produces 5-oxo-CRN-5-oxime (CRNO), which on further nitrosation forms 1-methyl-5-oxohydantoin-5-oxime (MHO). The step from MHO to NMU is, however, not clear; MHO might be nitrosated directly to NMU or indirectly via the intermediate formation of methylurea (MU). The levels of nitrite used in these studies were quite high (0.1−2.6 M), and also there is uncertainty as to the yield of NMU from CRN. The yields (0.02−2.7%) of NMU from CRN as reported by Mirvish et al. (1993) seemed to be much higher than that (0.0003%) observed by Sen and Seaman (1988). Therefore, there is a need to reinvestigate these experiments using up-to-date and more specific analytical methodologies. This seemed to be even more relevant and necessary due to the fact that several epidemiological studies in the United States have suggested a link between consumption of cured meats and the incidence of childhood leukemia and brain tumors (Preston-Martin et al., 1982; Peters et al., 1984; Sarasua and Savitz, 1994). Although there are many inconsistencies in the above studies and the existence of such a link has not been well established, the suggestion deserves further investigation. As far as it is known, no studies have been carried out to determine if detectable amounts of NMU can be formed after nitrosation of cured meats. The lack of suitable analytical methodologies for trace

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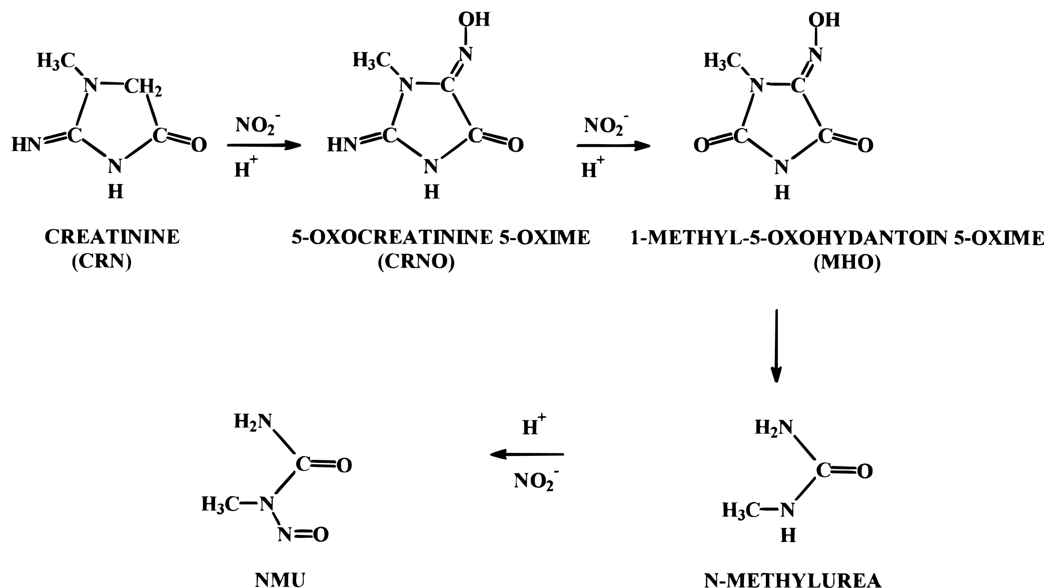

Figure 1. Pathways of NMU formation from nitrosation of CRN according to Mirvish et al. (1982, 1993).

analysis of NMU in cured meats has probably hindered progress in research in these areas.

In this paper, we report two highly sensitive and specific methods, one for rapid screening and the other for mass spectrometric confirmation, for the determination of NMU in cured meats. Some of our latest findings on NMU yields from CRN, CRNO, MHO, as well as from various cured meats, following nitrosation at different pH values and nitrite concentrations are also presented.

MATERIALS AND METHODS

(1) Apparatus. A postcolumn chemical denitrosation system attached to a thermal energy analyzer (TEA) (Thermedics Detection Inc., Chelmsford, MA), as described previously by Sen et al. (1994) and Havery (1990), was used for the determination of NMU. The TEA is a chemiluminescence detector used widely for the sensitive and specific detection of NO and *N*-nitrosamines, which release NO upon pyrolysis (Fine et al., 1976). A 250 mm × 4.6 mm (i.d.) stainless steel column packed with LC-18 (5 μm) (Supelco Canada, Oakville, ON) and 1% acetonitrile in water as the mobile phase (isocratic mode) with a flow rate of 1 mL/min were employed for the HPLC analysis of NMU. The mobile phase was delivered using a Beckman pump (model 110 B, Beckman, San Ramon, CA), and the sample was injected through an Altex Rheodyne injector (model 7125, sample loop = 50 μL).

A VG Analytical 7070EQ tandem mass spectrometer (a hybrid MS/MS with EBQQ configuration) coupled with a Varian 6000 gas chromatograph was used for GC-MS analysis. A 10 m × 0.25 mm (i.d.) DB1701 (J&W Scientific Inc., 0.25 μm film thickness) or an SPB-1701 (Supelco) fused silica column was directly interfaced to the ion source of the mass spectrometer. Helium with a head pressure of 8 psi was used as the carrier gas. The initial column injector temperature was 50 °C with a temperature program to 120 °C at 100 °C/min. The GC column was held at the initial temperature of 30 °C for 1.5 min and then programmed to 60 °C at 2 °C/min (held for 4 min) followed by another ramp of 40 °C/min to 200 °C.

Only the conventional configuration (EB) of the mass spectrometer was used for this work. The conditions were as follows: 70 eV electron-impact ionization; trap current, 500 μA; source temperature, 100 °C; all re-entrant and transfer lines, 100 °C; and resolution 1000 at low resolution for repetitive scanning and 5000 (10% valley definition) at high resolution for selected ion monitoring (SIM) mode. The ion monitored in SIM mode was m/z 60.0324, which is a major peak in the standard spectrum of NMU.

(2) Reagents. NMU, CRN, and L-ascorbic acid were purchased from Sigma (Oakville, ON), whereas sulfamic acid was obtained from Fisher Scientific Ltd. (Nepean, ON). Anhydrous sodium sulfate (granular) and potassium iodide (Analar grade) were procured from BDH Inc. (Toronto, ON). MHO was obtained as a gift from Sidney Mirvish, and CRNO was synthesized as described previously (Archer et al., 1971) using a 1:1.2 molar ratio of CRN and sodium nitrite.

The primary NMU standard (1 mg/10 mL) was prepared in dichloromethane (DCM), which was found to be stable for at least a month if stored in the dark at −20 °C. Aqueous standards, which were used for HPLC-TEA analysis as well as for carrying out recovery studies, were prepared by accurately measuring 10−50 μL aliqouts of DCM standard in a test tube, evaporating off DCM by gently rubbing the test tube between the palms for a few seconds (avoid prolonged warming), and dissolving the residue in water. Alternatively, exactly 8−10 μL of DCM standard was taken into a 15 mL graduated centrifuge tube, and the test tube was rotated gently (without rubbing between the palms) in a tilted position for exactly 3 min, by which time all of the DCM was evaporated off. The residue was dissolved in 1−2 mL of water and used as a standard. Such water standards were prepared fresh daily and, if not used immediately, were kept in an ice bath covered with a black cloth or aluminum foil. Aqueous solutions of CRN, MHO, and CRNO standards were also prepared fresh daily or weighed and directly added to the reaction mixtures.

All organic solvents used were of glass-distilled varieties and, except for DCM, were obtained from EM Science, BDH Inc. DCM was purchased from Burdick and Jackson (Muskegon, MI). Both of the sample preparation cartridges (Sep-Pak C_{18} and Sep-Pak Plus silica cartridges) were obtained from Waters (Milford, MA).

(3) Samples. The cured as well as the raw meat samples were purchased from local retail outlets. These included a wide variety of products such as frankfurters, ham, sausages, pastrami, pepperoni, fried bacon, smoked meat, and bologna. The raw meats included extra lean hamburger meat, pork cutlet, chicken breast, and roast beef. Further details as to the number of samples tested for each type are presented in Table 4. Each raw meat was nitrosated as such and again after cooking to determine the effect of cooking on NMU formation following nitrosation. For this, half portions of each raw meat were cooked on a Teflon-coated frying pan (set at 175 °C) to approximately medium doneness. All samples were cut into small pieces, homogenized using a blender, and stored in screw-cap glass containers at 4 °C until use (usually within 5 days). If longer storage was necessary, they were kept frozen at −20 °C.

Table 1. Effect of pH on NMU Yields from Creatinine and Nitrite[a]

pH	theor yield of NMU,[b] mg	actual yield of NMU,[c] μg, mean ± SD	% theor yield
1.0	49.7	0.986 ± 0.158	0.002
2.0	49.7	0.864 ± 0.090	0.0017
2.5	49.7	0.998	0.002
3.0	49.7	0.956 ± 0.151	0.0019
3.5	49.7	0.122	0.0002
4.0	49.7	0.044 ± 0.008	0.00009

[a] Each experiment was carried out with 100 mg each of creatinine (0.88 mmol) and $NaNO_2$ (1.45 mmol) as described under Materials and Methods (incubation period = 3 h at room temperature). [b] Assumed at least three molecules of $NaNO_2$ were required for each molecule of CRN to form NMU (Mirvish et al., 1993). [c] Average of duplicate or triplicate determinations (SD given for triplicate determinations only).

Table 2. NMU Yields from Creatinine and Sodium Nitrite at Various Reactant Concentrations[a]

CRN used		$NaNO_2$ used					
mg	mmol	mg	mmol	pH	theor yield of NMU,[b] mg	actual yield of NMU, ng	% theor yield
100	0.884	61	0.884	2.5	30.35	471	0.0015
100	0.884	244	3.536	2.5	91.14	4230	0.0046
100	0.884	60[c]	0.869	2.5	29.83	159	0.0005
100	0.884	10	0.145	2.5	4.98	53	0.0011
100	0.884	6	0.087	2.5	2.98	47	0.0016
100	0.884	6[c]	0.087	2.5	2.98	24	0.0008
25	0.221	10	0.145	2.5	4.98	11.5	0.0015
25	0.221	25	0.36	2.5	12.43	21	0.0002
25	0.221	50	0.725	2.25	22.76	64	0.0003
25	0.221	50	0.725	3.0	22.76	9	0.00004
25	0.221	60.7	0.88	2.5	22.76	62	0.0003
10	0.088	10	0.145	2.5	4.98	12	0.00024

[a] All incubations were carried out for 3 h at room temperature as described above. All results are based on single or average of duplicate determinations. [b] Assumed at least three molecules of $NaNO_2$ were required for each molecule of CRN to form NMU (Mirvish et al., 1993). [c] Nitrite was divided into six equal portions and added every 30 min (instead of adding all at once as was done in all other cases).

Table 3. NMU Yields from CRNO and MHO Following Nitrosation under Acidic Conditions[a]

precursor		$NaNO_2$					
mg	mmol	mg	mmol	pH	theor yield, mg[b]	actual yield, ng	% theor yield
CRNO							
20	0.14	19.28	0.28	2.25	14.4	79	0.0005
10	0.07	19.28	0.28	2.25	7.2	52	0.0007
10	0.07	9.64	0.14	2.25	7.2	24	0.0003
5	0.035	4.82	0.07	2.25	3.6	8	0.0002
MHO							
20	0.14	9.64	0.14	2.0	14.4	1730	0.012
20	0.14	38.56	0.56	2.0	14.4	1960	0.014
5	0.035	2.41	0.035	2.25	3.6	75	0.002
5	0.035	10	0.14	1.5	3.6	98	0.0027
5	0.035	10	0.14	2.0	3.6	182	0.005
5	0.035	10	0.14	2.5	3.6	89	0.0025
5	0.035	10	0.14	3.0	3.6	26	0.0007
5	0.035	10	0.14	3.5	3.6	8	0.0002

[a] All incubations were carried out for 3 h at room temperature as described above. All results are based on single or average of duplicate determinations. [b] Assumed at least two molecules of nitrite were required for one molecule of CRNO to form NMU, whereas only one may be sufficient for conversion of MHO to NMU (Mirvish et al., 1993).

Caution. Necessary precautions should be taken when one is handling and working with NMU standards. Old unused standards should be destroyed properly before disposal.

(4) Determination of NMU in Cured Meats. The details are as follows.

Table 4. NMU Formation in Uncured and Cured Meats under in Vitro Acidic Incubation Conditions without Any Additional Nitrite[a]

type of meat or sample used	residual nitrite level (as $NaNO_2$), ppm	pH of incubation mixture	NMU detected, ng (uncorrected for recoveries)
reagent blanks	NA[b]	1−2.5	nil[c]
uncured raw meats[d]	NM[e]	1.1	nil
cooked	NM	1.1	nil
turkey salami	51	2	3
smoked eye round pastrami	214	1.1	26
		1.3	7
		2.5	2.5
		0.8	2
ham and bacon baked meat loaf	48	0.8	4.6
		1.3	6
		2.5	1.6
other cured meat products[d]	0.8−45	0.8−2.5	all negative

[a] Aliquots (10 g) of meats were mixed with water and dilute HCl to the desired pH, and the mixture was incubated for 2 h at room temperature in the dark. The samples were processed and analyzed as described in the text. [b] Not applicable. [c] Lower detection limit was 0.3 ng/10 g of meat (all samples were analyzed by GC-MS). [d] Included 1 each of extra lean hamburger meat, pork cutlet, chicken breast, and roast beef. [e] Not measured. [f] The samples included a wide variety of cured meats such as frankfurters (4), ham (1), sausages (6), pastrami (1), salami (1), pepperoni (2), knackwurst (2), fried bacon (4), smoked meat (2), and bologna (1). Only some of these samples were analyzed for residual nitrite.

(a) Extraction. A 10 g sample of homogenized meat was taken in a Sorval Omni mixer (DuPont Instruments, Newton, CT), mixed with 10−15 mL of water, 4 mL of 3 M HCl, and 100 mg each of ascorbic acid and sulfamic acid (to prevent artifactual formation), and the mixture was allowed to sit for 10−15 min at room temperature. About 100 mL of ethyl acetate and 50 g of anhydrous sodium sulfate (granular) were added, and the sample was blended for 2 min at one-third speed setting. The supernatant was carefully decanted and filtered through a Whatman filter paper (No. 1) that already contained ∼20 g of anhydrous sodium sulfate. The extraction and filtration steps above were repeated twice, each time with 100 mL of ethyl acetate. The combined filtrate was collected into a 500 mL round-bottom flask and the extract evaporated to ∼5 mL using a rotary evaporator (heat turned off in the water bath). Exactly 5 mL of water was added to the concentrated extract, and the mixture was concentrated again to ∼5 mL as above to evaporate off most of the ethyl acetate. The residue was quantitatively transferred (Pasteur pipet) into a 60 mL separatory funnel using 8 mL each of methanol and an acidic buffer (0.2 M KCl−HCl, pH 2) for rinsing. The solution was extracted with two 25 mL portions of *n*-hexane, and the hexane was discarded (to remove fats and lipids). The aqueous layer containing NMU, if any, was saturated with NaCl (2 g), the solution extracted with three 20 mL portions of dichloromethane (DCM) (separatory funnel), and the combined DCM extract concentrated to ∼5 mL using a rotary evaporator (no heat). Exactly 1 mL of water was added to the concentrated extract, and the mixture was re-evaporated as above until all DCM was gone (avoid excessive evaporation of the aqueous residue).

(b) Cleanup on C_{18} Sep-Pak Cartridge. A 10 mL glass hypodermic syringe was attached, through its luer-lock fitting, to two such cartridges, which were connected in series with a small piece of Tygon tubing. Before use, the cartridges were conditioned by passing (gravity flow) a mixture of 4 mL of 50% acetonitrile in water and 0.2 mL of 0.1 M HCl followed by washing with 5 mL of water. If the flow rate was unacceptably slow, gentle pressure was applied by introducing the syringe plunger at the top and forcing it down gently until a desired flow rate of ∼1 drop/s was obtained. Before the plunger was dismantled, the vacuum inside the reservoir was released by unscrewing the luer-lock at the bottom and screwing it back

after removal of the plunger. The aqueous residue in the evaporating flask from above was added (Pasteur pipet) into the syringe reservoir and allowed to pass through the two cartridges as above. The flask was rinsed with 2 mL of water and the rinse passed through the cartridges. Finally, NMU from the cartridges was eluted by passing 4 mL of 20% methanol in water and collection into a 30 mL separatory funnel.

Approximately 10 mL of NaCl-saturated water was added to the above eluate, the mixture extracted with three 10 mL portions of DCM, and the combined extract dried by passing through ~1 g of anhydrous sodium sulfate (granular) placed on a small sintered glass (coarse) filtered funnel. The filtrate was then evaporated to ~5 mL using a rotary evaporator (no heat). At this stage, one can continue to step c below or proceed directly to step d, bypassing preliminary rapid screening by HPLC-TEA.

During the workup, an attempt was made to complete the whole process as quickly as possible without unnecessary delays. If an extract had to be stored for >1 h, it was always kept in DCM medium (NMU is unstable in aqueous medium) and stored at −20 °C in a freezer.

(c) Determination by HPLC-TEA. Exactly 1 mL of water was added to the concentrated DCM extract, and the mixture was evaporated as above until all DCM was gone (avoid excessive evaporation). Suitable aliquots (10−50 μL) of the aqueous residue were analyzed by LC-TEA for NMU as described above.

(d) Cleanup on Silica Sep-Pak Cartridge. If proceeding directly from step b, the dried DCM extract was evaporated to ~2 mL instead of 5 mL (excessive evaporation must be avoided), and the residue was mixed with 3 mL of anhydrous *n*-pentane. Just before use, the silica Sep-Pak cartridge was washed with 5 mL of *n*-pentane, and the above mixture was passed through the cartridge. Following this, the cartridge was successively washed with 2 mL of *n*-pentane, 4 mL of DCM, and 4 mL of a mixture of DCM and methanol (1:1). The last fraction (containing the eluted NMU) was collected into a small separatory funnel.

About 10 mL of NaCl-saturated water was added to the above eluate, and the mixture was extracted with three 10 mL portions of DCM. The combined DCM layer was dried by passing through 5 g of anhydrous sodium sulfate placed in a small sintered glass (coarse) filter funnel and then evaporated to ~2 mL (excessive evaporation must be avoided) using a rotary evaporator (no heat) as described above. The DCM concentrate was quantitatively transferred into a graduated glass stoppered test tube (rinsed with 0.5 mL of DCM), and the solution was evaporated to 1 mL by blowing down with a gentle stream (through a Pasteur pipet) of nitrogen while the test tube containing the solution was kept immersed in cold water. A 2−4 μL aliquot was analyzed by GC-MS as described under Apparatus.

On the other hand, continuing from step c, the aqueous solution left behind following HPLC-TEA determination can be mixed with 10 mL of NaCl-saturated water, the mixture re-extracted with three 10 mL portions of DCM, and the extract processed exactly as described above at the beginning of step d, that is, taken completely through the silica Sep-Pak cartridge cleanup. Because NMU is unstable in aqueous solution, this should be done as soon as HPLC-TEA analyses are completed.

(5) Recovery Studies. Recovery studies of NMU added to both model systems and cured meats were carried out to determine the efficiency of the analytical method. Appropriate amounts of aqueous NMU standards were added to various incubation mixtures (either model systems or meat), and the samples were taken through all of the analytical steps. It should be pointed out that no nitrite was added to these mixtures and the incubation period was also omitted. The main objective of this exercise was to test the efficiency of the overall analytical steps only (not the loss that might occur during the incubation period). Separate experiments were carried out with standard NMU at different pH values (in model systems without nitrite) to determine the stability of NMU during incubation. Because the model system reaction mixtures did not contain any meats, these were extracted and directly analyzed for NMU as described below.

(6) NMU Formation from Various Precursors in Model System Studies. Appropriate aliquots of freshly prepared aqueous solutions of CRN, CRNO, or MHO were taken into separate 50 mL Erlenmeyer flasks, volumes were made up to ~20 mL with water, and the pH of the solutions was adjusted to the desired value using dilute HCl (0.1, 1, or 3 M). Required amounts of freshly prepared sodium nitrite solutions were added to each flask, and the pH values were readjusted as above. After incubation of the reaction mixtures at room temperature (20−22 °C) or at 37 °C in the dark for the desired periods (30 min to 3 h), the nitrosation reaction in each was stopped by adding 1 mL of 3 M HCl, excess amounts of sulfamic acid (usually twice the molar ratio of nitrite), or, in some cases, 100 mg each of ascorbic and sulfamic acids. The mixtures were swirled gently to dissolve the added sulfamic acid and allowed to sit at room temperature in the dark for an additional 15 min. Each solution was extracted with three 20 mL portions of DCM, the combined extract was dried by passing through a bed (10 g) of anhydrous sodium sulfate (granular) placed in a sintered glass (coarse) filter funnel, and the filtrate was collected into a 250 mL round-bottom evaporation flask. The filtrate was evaporated to ~5 mL using a rotary evaporator (no heat), the residue transferred quantitatively (Pasteur pipet) with rinsing (2 mL of DCM) into a graduated test tube, and the solution concentrated to 1 mL by blowing down gently with a stream of nitrogen (no heat). A 2−4 μL aliquot of each was analyzed by GC-MS as described above.

If HPLC-TEA was used for the end determination of NMU, the rotary evaporation (above) was interrupted briefly when the DCM extract had been concentrated to ~5 mL. Exactly 1 mL of water was added to the concentrated extract, and the rotary evaporation (no heat) was resumed until all DCM was gone (excessive evaporation must be avoided). Suitable aliquots (10−50 μL) of the aqueous residues were analyzed for NMU by HPLC-TEA.

(7) NMU Formation from Cured Meats Following Nitrosation with or without Additional Nitrite. A 10 g aliquot of a well-homogenized meat was taken into a Sorval cup and mixed with 10−15 mL of water and 1−4 mL of 3 M HCl to attain the desired pH. After 2−3 min of mixing with a spatula, the pH was readjusted, and the sample was incubated at room temperature in the dark for 2 h. Occasionally, the pH of the mixture rechecked after 30 min and readjusted, if necessary. If additional nitrite was added, it was done at the very beginning, that is, prior to the addition of HCl and adjustment of the pH. Following the incubation, 100 mg of sulfamic acid or 100 mg each of sulfamic and ascorbic acids were added to the sample and mixed well using a spatula. The pH of the sample was rechecked (~1), and the sample was allowed to sit at room temperature in the dark for an additional 10−15 min. The sample was then extracted with ethyl acetate, and the extract analyzed for NMU as described above under sections 4a−4d.

(8) Determination of Residual Nitrite in Cured Meats. Selected samples of cured meats were analyzed for residual nitrite levels using a highly specific and sensitive HPLC chemiluminescence detection method as reported previously (Sen et al., 1994).

RESULTS AND DISCUSSION

(1) Analytical Method Development for NMU. The GC-TEA or HPLC-TEA techniques commonly used for the determination of *N*-nitrosamines are unsuitable for the analysis of NMU and other *N*-nitrosamides because these compounds produce poor yields of NO in the TEA pyrolyzer (Hansen et al., 1979; Fine et al., 1984). To overcome this drawback, various workers have used hydrogen halide induced chemical denitrosation (Walters et al., 1980; Cox et al., 1982; Sen et al., 1984; Havery, 1990) or photolysis (Conboy and Hotchkiss,

1988) and then measured the liberated NO by TEA. Of these methods, that reported by Havery (1990) seemed to be the most suitable for the present investigation and was, therefore, adopted after minor modifications as reported previously by us in connection with chemiluminescence determination of nitrite (Sen et al., 1994) and *N*-nitrosoglyphosate (Sen and Baddoo, 1996). These modifications included (a) addition of both sulfamic acid (0.5 mL, 20 mg/mL in water) and glacial acetic acid (1 mL) to a 10% KI solution to destroy any nitrite impurity in the reagents, which greatly improved the background noise; (b) addition of special cold traps and trap-emptying devices, which allowed continuous use of the system without the need to dismantle it between runs; and (c) insertion of an extra KOH (pellets) trap between the second dry ice cold trap and the TEA to remove the last traces of HI fumes, which might otherwise corrode the TEA.

The cleanup method used for the analysis of cured meats was essentially based on an earlier one developed for the determination of NMU in fried bacon (Sen et al., 1985). Two extra cleanup steps on Sep-Pak cartridges have been incorporated to ensure adequate purification necessary for GC-MS analysis. Also, both ascorbic and sulfamic acids were added following nitrosation to destroy excess nitrite and prevent artifactual formation. Because NMU is thermolabile, photosensitive, and unstable in aqueous solution, extra precaution must be taken to avoid exposure to strong lights, warm temperature, and excessive delays in processing the sample extracts. If procedures are not followed exactly as described, excessive losses of NMU can occur even during the concentration of DCM extracts to low volumes (<1 mL) or during phase transfer from DCM to water.

Both the HPLC-TEA and GC-MS techniques worked well, the latter being more sensitive. About 0.5−1 ng and 2−5 pg of NMU per injection could be detected by the two techniques, respectively. The minimum detection limits for NMU in cured meats were approximately 5 ng/10 g (0.5 ppb) by HPLC-TEA and 0.3 ng/10 g (0.03 ppb) by GC-MS. The percentage recoveries of NMU added to cured meats or negative meat incubation mixtures (no extra nitrite added and incubation omitted) at spiking levels of 10−25 ppb ranged between 64 and 112 (average = 83.2%). Additional recovery studies with meats at 1−2 ppb spiking levels indicated somewhat lower recoveries (42−68%), but this was not entirely unexpected. In view of the lengthy and complex cleanup involved and the instability of NMU, such recoveries and detection limits are considered to be highly satisfactory and, in fact, seemed to be better than those of any method reported thus far. In fact, these recovery data at such a low level of spiking add credence to the data reported for meats in Tables 4 and 5. This suggests that the low-level data reported here, if not quantitative like those reported previously for the determination of *N*-nitrosodimethylamine in beer (Sen et al., 1982), are at least semiquantitative. In the aforementioned international collaborative study, 12 participating laboratories obtained an average recovery of 101.4% from beer at spiking levels of 0.5−5 ppb of NDMA. This was possible mainly because NDMA is a fairly stable volatile nitrosamine that is amenable to simple and efficient cleanup by distillation from alkaline aqueous media. It would be unreasonable, however, to expect such high accuracies for the determination of NMU in cured meats or any other complex substrates. It should also be emphasized that, in this study, none of the data were corrected for recovery losses. Therefore, the amount of NMU detected in each meat incubation mixture was, in fact, underestimated; the actual amount formed was much higher. Recoveries of NMU from blank reaction mixtures used in model system experiments were much better (85−95%) as these were not carried through extensive cleanups. Similarly, recovery studies from blank reaction mixtures indicated very little loss (>90% recoveries) of NMU during the incubation of NMU at pH 0.8−2 for 2 h at room temperature but considerable loss (∼50%) of NMU when the incubation was carried out at 37.5 °C.

The most prominent fragment ion in the mass spectrum of NMU was observed at m/z 60, which most likely corresponds to $CH_3N{=}N{-}OH$ (Rainey et al., 1976).

Table 5. Formation of NMU Following Incubation of Various Meats with Additional Nitrite under in Vitro Acidic Conditions[a]

type of meat used	pH of incubation mixture	added $NaNO_2$ level,[b] ppm	NMU detected, ng (uncorrected for recoveries)
uncured raw meats[c]	1−1.3	200	nil [d]
uncured cooked meats[e]	1−1.3	200	nil
all-beef hot dog	0.7−0.8	500	21[f]
all-beef frankfurter	0.9	500	36
summer sausage	0.7−0.8	280	12[f]
	0.8−1	500	28[f]
Polish sausage	0.7−0.8	200	6
	0.8−1	500	41
pepperoni	0.6−0.7	50	nil
	0.7−0.8	150	4
	0.7−0.8	100	11
fried bacon, brand A	0.6−0.7	50	3
	0.7−0.8	200	176
	1−2	200	16
fried bacon, brand B	0.9−1	200	10
	2.5	200	4.7
fried bacon, brand C	0.8	200	9
	2.5	200	nil
fried bacon, brand D	0.8	200	19.8
smoked ham	1	200	nil
	1	500	5.3
paté	0.7−0.8	500	33
	2.5	500	1
beef pastrami	1	200	0.3
all-beef wiener	2	200	nil
	1	200	trace
	0.7−0.8	500	26
	2.5	500	0.9
Black Forest ham	1	200	0.3
	2.5	500	0.9
	0.7−0.8	500	3.3
pastrami eye of round	1	200	0.6
	2.5	500	0.6
	0.6−0.7	500	8.7
Hungarian dry-cured salami	1	200	0.4
other cured meats[g]	0.6−1	50−500	all negative

[a] Samples were processed as those in Table 4, except additional nitrite was added prior to adjustment of the pH and incubation. Therefore, these data do not represent typical levels of NMU formation that would be expected from current commercial cured meat products which contain much lower levels of residual nitrite. [b] Levels of $NaNO_2$ used were based on the weight of the meat used. [c] Included one each of chicken breast, pork cutlet, and extra lean ground beef. [d] Lower detection limits were 0.3 ng/10 g of meat by GC-MS and 5 ng/10 g of meat by HPLC-TEA. [e] Included roast beef plus three others mentioned under raw meats. [f] Analyzed by HPLC-TEA; all others were analyzed by GC-MS. [g] Included one each of frankfurter, pepperoni, kielbasa roll, smoked sausage and two of turkey salami.

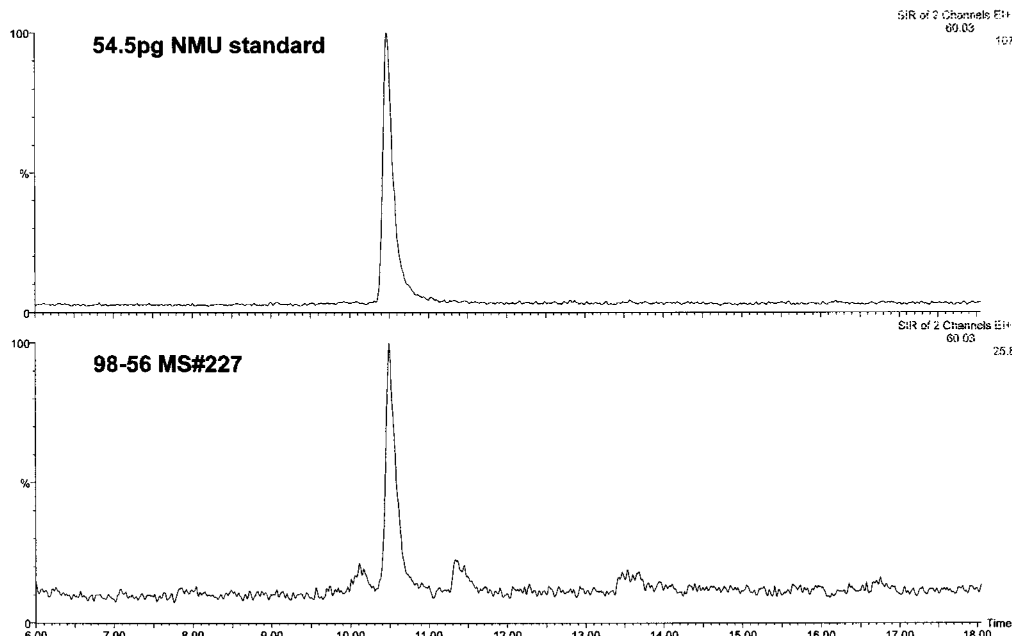

Figure 2. GC-MS-SIM tracings at *m/z* 60.0324: top, 54.5 pg of NMU standard; bottom, 4 μL/1.0 mL cleaned up extract of a fried bacon (Table 5, brand D) incubated with 200 ppm of $NaNO_2$ at pH 0.8.

Unlike *N*-nitrosamines, which usually cleave at the N−NO bond during mass spectrometric fragmentation, most *N*-nitrosamides (e.g., NMU) cleave at the N−CO bond. Further rearrangement follows, and the resulting molecule splits and yields an alkyl diazohydroxide fragment. The molecular ion at *m/z* 103 was very small (∼10% abundance) and, therefore, was not used for routine monitoring. There seemed to be a definite matrix effect on the peak shape of NMU under the GC conditions used. The tracing for NMU at *m/z* 60, when injected in pure DCM solvent, showed slight tailing, but those in the final cleaned up meat extracts or model system extracts, both of which were also prepared using DCM, appeared to be more symmetrical. For this reason, NMU standards for SIM monitoring were prepared using meat extracts (in DCM) that gave negative results for NMU. Also, the peaks were sharp when the GC column was new, but they became somewhat broader after analyses of ∼30−40 samples. However, the peak shape did not deteriorate any further and the column was usable for ∼100 analyses. Typical examples of GC-MS-SIM tracing for NMU standard and a nitrosated fried bacon extract showing formation of NMU are shown in Figure 2.

(2) Model System Studies. Data presented (Table 1) indicate that at relatively high reactant concentrations of both CRN and nitrite, the maximum theoretical yields of NMU at various pH values at room temperature (20−22 °C) were extremely low (0.002%) and remained practically constant within the pH range of 1−3 and then decreased at pH >3. It should be pointed out that these results refer to the overall final yields of NMU and not of the pH optima of the initial step of CRN nitrosation. As discussed by Mirvish et al. (1993), conversion of CRN to NMU involves four steps as outlined in the following sequence of reactions: CRN → CRNO → MHO → MU → NMU. At least three of these (the first two and the last) require nitrosation, and the first step is the slowest and the last one the fastest. Mirvish et al. (1993) have studied the kinetics of CRN and CRNO nitrosation and determined the rate constants of the first two steps. These researchers reported pH maxima of 3 and 2.7−3.0 for nitrosation of CRN and CRNO, respectively. They also observed a fairly high rate of nitrosation for CRN at pH 1. Therefore, our results are not inconsistent with those reported by Mirvish et al. (1993).

Initially, we wanted to study these reactions both at room temperature and at 37.5 °C (body temperature), but NMU was found to be quite unstable at the higher temperature; therefore, all experiments were carried out at room temperature.

Additional experiments were carried out to determine NMU yields at pH 2.5 with various levels of CRN and sodium nitrite. These results (Table 2) suggested that NMU yields decreased with a decrease in reactant concentrations. With both 100 and 25 mg of CRN at pH 2.5, the actual yields (4230 and 11.5 ng) as well as the percentage yields (0.0046−0.0015% of theoretical) of NMU decreased with a decrease in nitrite concentrations. When both CRN and nitrite concentrations were reduced to the lowest level (i.e., 10 mg of each /reaction mixture), the percentage yield of NMU was found to be extremely low (0.00024%). It appears from these data that at low reactant concentrations, NMU yields are unlikely to exceed these values.

NMU yields from CRNO (Table 3) at comparable low reactant concentrations were slightly higher (0.0002−0.0007%), and, as expected, that from MHO was significantly higher (up to 0.014%). Due to limitation of the amounts of CRNO at our disposal, it was nitrosated at only one pH level (pH 2.25). Therefore, the pH optimum of NMU formation from CRNO is yet to be determined, but it lies most likely in the range of 2−3. This assumption is based on findings by Mirvish et al. (1993), which suggested 2.7−3 to be the pH optimum for MHO formation from CRNO (not necessarily that for NMU formation). That for NMU formation from MHO was found to be 2.0 (Table 3). The relatively high rate of conversion of MHO to NMU suggests that the presence of MHO in any food might pose a health risk due to the possibility of intragastric nitrosation in the human stomach (Mirvish et al., 1993). No information,

however, is available on the levels of this compound in foods.

In their two studies, Mirvish et al. (1982, 1993) reacted various amounts of CRN (113−565 mg) and sodium nitrite (1.38−100 g; 0.1−2.6 M) at pH 1 (at 20−25 °C) and observed MU yields of 0.004−3.7%. These values are much higher than those observed by us in this as well as in our previous study (Sen and Seaman, 1988). For example, in our previous study the concentrations of nitrite in the reaction mixtures ranged between 0.036 and 0.36 M. Amounts of NMU formed decreased with a decrease in nitrite concentrations; none were detected at the lowest level, and the yield was only 0.0003% at the highest level. In this study, the concentrations of nitrite used in the low-level experiments (Table 2, rows 4−12) ranged between 0.0043 and 0.0442 M, resulting in NMU yields of 0.00004−0.0016% depending on the pH and how much CRN was used in each experiment. In only one case (row 2 in Table 2), when a high concentration of nitrite was used, did the yield of NMU reach 0.0046%, which was comparable to the lowest yield reported by Mirvish and co-workers as mentioned above. There might be two logical explanations for these differences. First, the above researchers did not measure NMU formation directly; CRN was nitrosated, and the products were denitrosated back to MU, which was measured, whereas we measured NMU directly. Second, as evident from the above, they used much larger amounts as well as higher concentrations of sodium nitrite in their experiments, which may explain the high yields (up to 3.7%) of MU they observed. Because NMU yields drastically decrease with a decrease in reactant concentrations, the extremely low yields observed by us at lower nitrite concentrations are not entirely unexpected. Similar reasons may explain the higher yields of MU Mirvish et al. (1993) observed in experiments in which CRNO (0.02% yield) and MHO (7% yield) were nitrosated.

(3) NMU Formation in Nitrosated Cured Meats. In the first group, various cured meats (Table 4) were acidified to different pH values and incubated for 2 h at room temperature without any additional nitrite. The residual nitrite in meat under acidic conditions provided the nitrosating agent in these experiments; no additional nitrite was added. Of 27 samples tested, only 3 formed detectable levels of NMU and the rest were negative (<0.5 ng/10 g of meat). It seemed that a relatively high (∼>50 ppm) residual $NaNO_2$ level in the meat was needed to form detectable levels of NMU. Because residual nitrite levels in most of the cured meats tested were low (5−40 ppm), the available nitrite was not sufficient to form detectable levels of NMU.

In view of the above, the second phase of the meat study was concentrated on determining the effect of additional nitrite on NMU formation. The main purpose was to artificially create meat−nitrite incubation mixtures with higher nitrite levels. The rationale was that such information would be useful in predicting the extent of intragastric NMU formation that might occur following consumption of cured meats containing high residual nitrite levels. The data would also be useful in predicting if such NMU formation could have occurred in the past when residual nitrite levels in cured meats were quite high and often exceeded 100 ppm levels (Dennis et al., 1990; Sen et al., 1997; White, 1975). These results are presented in Table 5. In total, 29 samples were nitrosated with various levels (50−500 ppm) of additional nitrite, and of these, 16 formed detectable levels of NMU; the rest were negative. It should be emphasized that the level of nitrite added in each case was always based on the weight of the meat used and not on its concentration in the reaction mixture. As can be seen from Table 5, three uncured raw and four uncured cooked meats were also nitrosated and tested as above as controls. None of these formed any NMU even though they were nitrosated with 200 ppm of nitrite.

Three observations can be made from these results. First, as one would expect, NMU formation in most samples increased with an increase in nitrite levels used. Second, the amount of NMU formed with a particular level of nitrite varied widely among samples; some (those in the last row) did not form any even with the addition of 5 mg of $NaNO_2$/10 g of meat (i.e., 500 ppm). Third, lowering the pH of the incubation mixture increased the amount of NMU formed. This suggests that CRN was not the only precursor present in such samples because, in that case, one would have observed approximately the same amounts of NMU formation at pH 2.5 and at pH 1 (see Table 1). As demonstrated previously by others (Mirvish, 1975; Mirvish et al., 1982; Masuda et al., 1978), NMU can be formed from a variety of precursors such as CRN, CRNO, MHO, MU, methyl guanidine (MG), and methylnitrosocyanamide. Except for some limited data on CRN levels in meats and of MG (Kawabata et al., 1978) in certain foods, not much information is available on the occurrence of these compounds in cured meat products. In view of the above findings, it would be advisable to determine which specific precursors are present in cured meats.

Adequate precautions were taken to prevent artifactual formation of NMU during analysis of the samples. Following nitrosation, each incubation mixture was treated with excess of both ascorbic and sulfamic acids, which are well-known nitrite scavengers (Archer, 1984). Moreover, the uncured meats as well as some of the cured meats did not form any detectable levels of NMU. Therefore, it is highly unlikely that NMU detected in these samples was formed as an artifact. Because the levels of NMU formed in the meat samples were extremely low, most meat incubation mixtures were analyzed by the GC-MS technique. However, in a few limited cases, the analyses were carried out by both the HPLC-TEA and GC-MS techniques, and the results were comparable (within ±15%). To our knowledge, this appears to be the first reported formation of NMU in cured meats with or without additional nitrite.

Thus far, we have demonstrated that nitrosation of cured meats under acidic conditions can form traces of NMU. This does not necessarily mean that this occurs in the human stomach. There are many conflicting situations that will influence NMU formation from cured meats due to intragastric nitrosation such as the concentration of nitrite and that of the precursors in the meat, the concentration of nitrite in the saliva, the presence of nitrosation inhibitors (e.g., ascorbate) in the meat, and the pH of the gastric contents following consumption of the meat. The only thing that can be concluded is that this is the first such study that lends direct support to such a possibility. It is not unreasonable for a person to consume ∼100 g of cured meat in a meal, and if it contains >50 ppm of residual nitrite, formation of minute traces of NMU due to intragastric nitrosation cannot be ruled out. Further studies are,

however, needed to determine if this actually occurs in vivo in the human stomach. Current Canadian Food and Drug (Anonymous, 1982) and USDA regulations (*Code of Federal Regulations*, 2000) permit the addition of 100–200 ppm of sodium nitrite in various cured meat products. On the other hand, it should be emphasized that most cured meats at the present time contain on average <50 ppm of residual nitrite (Sen et al., 1997). Therefore, the possibility of in vivo NMU formation in the human stomach following consumption of these cured meats would be extremely small. This was probably not true in the past because earlier surveys indicated a much higher average (up to 65 ppm) as well as higher individual residual nitrite levels (up to 275 ppm) in such products (Sen et al., 1997; Dennis et al., 1990; White, 1975). One way to prevent or minimize such formation will be to make it mandatory to add ascorbate or erythorbate, both of which are excellent nitrosation inhibitors, to all cured meats at ~500 ppm levels. Such a regulation already exists in the United States for bacon (*Federal Register*, 1978), and it might be advisable to extend it to all cured meats.

NOTE ADDED IN PROOF

Referring to the preparation of aqueous NMU standard for HPLC-TEA analysis: A more convenient way to prepare the aqueous NMU working standard would be to dissolve the weighed NMU crystals in either methanol or acetonitrile. NMU is stable in both of these solvents for at least 1 month at −20 °C. Such a standard can then be diluted appropriately with water and be used for HPLC-TEA analysis.

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