Hosaka, S.; Hirono, I. *Gann* **1981**, *72*, 327.
Ivie, G. W.; Holt, D. L.; Ivey, M. C. *Science (Washington, D.C.)* **1981**, *213*, 909.
Levin, D. E.; Yamasaki, E.; Ames, B. N. *Mutat. Res.* **1982**, in press.
Lu, S.-H.; Camus, A.-M.; Tomatis, L.; Bartsch, H. *JNCI, J. Natl. Cancer Inst.* **1981**, *66*, 33.
Maruta, A.; Enaka, K.; Umeda, M. *Gann* **1979**, *70*, 273.
McCann, J.; Choi, E.; Yamasaki, E.; Ames, B. N. *Proc. Natl. Acad. Sci. U.S.A.* **1975**, *72*, 5135.
Meltz, M. L.; MacGregor, J. T. *Mutat. Res.* **1981**, *88*, 317.
Miller, J. A.; Miller, E. C. *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **1976**, *35*, 1316.
Morino, K.; Matsukura, N.; Kawachi, T.; Ohgaki, H.; Sugimura, T.; Hirono, I. *Carcinogenesis (N.Y.)* **1982**, *3*, 93.
Nagao, M.; Takahashi, Y.; Yamanaka, H.; Sugimura, T. *Mutat. Res.* **1979**, *68*, 101.
Pamukcu, A. M.; Yalciner, S.; Hatcher, J. F.; Bryan, G. T. *Cancer Res.* **1980**, *40*, 3468.
Saito, D.; Shirai, A.; Matsushima, T.; Sugimura, T.; Hirono, I. *Teratog., Carcinog., Mutagen.* **1980**, *1*, 213.
Stich, H. F.; Rosin, M. P.; Chin. H. W.; Powrie, W. D. *Cancer Lett. (Shannon, Irel.)* **1981**, *12*, 1.
Sugimura, T. In "Naturally occurring carcinogens/mutagens and modulators of carcinogenesis"; Miller, E. C., et al., Eds.; Japanese Science Society Press: Tokyo, 1979.
Sugimura, T.; Kawachi, T.; Nagao, M.; Yahagi, T. In "Nutrition and cancer, etiology and treatment"; Newell, G. R.; Ellison, N. M., Eds.; Raven Press: New York, 1981; pp 59–71.
Sugimura, T.; Nagao, M. *CRC Crit. Rev. Toxicol.* **1979**, *6*, 189.
Takahashi, Y.; Nagao, M.; Fujino, T.; Yamaizumi, Z.; Sugimura, T. *Mutat. Res.* **1979**, *68*, 117.
Tikkanen, L.; Matsushima, T.; Natori, S., paper presented at the 12th annual European Environmental Mutagen Society meeting, Helsinki, Finland, 1982.
Umezawa, U.; Matsushima, T.; Sugimura, T.; Hirakawa, T.; Tanaka, M.; Katok, V.; Takayama, S. *Toxicol. Lett.* **1977**, *1*, 175.
Uyeta, M.; Taue, S.; Mazaki, M. *Mutat. Res.* **1981**, *88*, 233.
van der Hoeven, J. C. M.; Fennis, J., Agricultural University, Wageningen, The Netherlands, unpublished data, 1982.
Visek, W. J.; Clinton, S. K.; Truex, C. R. *Cornell. Vet.* **1978**, *68*, 3.
Wehner, F. C.; Thiel, P. G.; Durand, M. *Appl. Environ. Microbiol.* **1979**, *37*, 658.

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Determination of Nitrite and Volatile Nitrosamines in Animal Diets

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Animal diets (chow, agar, and casein) similar to those used in two nitrite feeding studies that reported an increase in the incidence of lymphomas in rats were analyzed over a 14-day period. Sodium nitrite was added to the chow diet at levels of 0, 1000, and 2000 ppm, to the agar diet at 0, 500, 1000, and 2000 ppm, and to the casein diet at 0 and 1000 ppm. Trace amounts (0.5 to <1.0 ppb) of volatile nitrosamines were found in the agar and casein diets. The levels in the chow diet were significantly higher (5–45 ppb) and increased with the duration of storage as well as with the concentration of added nitrite. The predominant nitrosamine found in the chow diet was *N*-dimethylnitrosamine (NDMA); chow to which no nitrite had been added contained an average of 4 ppb of NDMA. Measurements of the nitrite levels in these diets indicate that the amount of nitrite found was substantially less than the amounts that had been added, possibly because of loss, degradation, or interaction with dietary components.

Two recent studies reported that the addition of sodium nitrite to the diet or drinking water of rats increased the incidence of lymphoreticular tumors (Shank and Newberne, 1976; Newberne, 1979). The object of the experimental approach was to determine whether nitrites per se and not nitrosamines induced cancer in rats. Since direct documentation concerning the presence or absence of nitrosamines in the original experimental diets was not obtainable, similar diets were reconstructed and treated with nitrite to determine whether or not volatile nitrosamines were formed (during mixing or storage) in the diets fed to the animals. We have therefore measured the volatile nitrosamines and nitrite in these diets, stored in the same way as the diets used in the original feeding studies.

EXPERIMENTAL SECTION

Most nitrosamines are potent carcinogens in experimental animals (Magee et al., 1976); extreme care should be exercised in their storage, handling, use, and disposal.

Chemicals. All solvents were distilled in glass grade (Burdick and Jackson Laboratories, Muskegon, MI). Nitrosamine standards were diluted from stock solutions supplied by the Thermo Electron Analytical Services Laboratory (Waltham, MA). These solutions were calibrated by comparison with standards supplied by the Food and Drug Administration (FDA). Other chemicals (reagent grade) were supplied by Chemical Dynamics Corp., South Plainfield, NJ (±-α-tocopherol), Fisher Inc., Fairlawn, NJ (ammonium sulfamate), Aldrich Chemical Co., Milwaukee, WI (sulfanilamide), and J. T. Baker Chemical Co., Phillipsburg, NJ [*N*-(1-naphthyl)ethylenediamine].

Diets. For this 14-day study, diets were prepared at the Massachusetts Institute of Technology, Cambridge, MA. Two kilograms of each of three types of diet was made as described by Wogan and Newberne (1967) and Newberne (1979). These included dry reground pellets of a commercial rodent chow (Agway-Charles River RMH 3000), a dry casein diet, and a wet agar diet prepared in the same way as the casein diet except that agar (1.17%) and water (50%) were added, producing a gel instead of a powder.

Sodium nitrite was added to the diets at the following levels: chow diet, 0, 1000, and 2000 ppm; agar diet, 0, 500, 1000, and 2000 ppm; casein diet, 0 and 1000 ppm. In the

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case of the dry diets, nitrite was dissolved in 10 mL of water and mixed for 10 min in a Hobart paddle mixer. For the agar diet, the nitrite solution was added as the diet was cooling (at approximately 50 °C), and mixing was continued for at least 10 min before the diet was poured into plastic containers and allowed to solidify. The sodium nitrite concentrations are given on a dry weight basis (Newberne, 1979).

The diets were stored at 4 °C in a refrigerator that had never been used for nitrosamine storage.

Analysis Schedule. Day 0 analyses for nitrosamine and nitrite were performed between 4 and 10 h after the nitrite had been added to the diets. Samples were removed for reanalysis on days 3, 7, 11, and 14. In all cases the diets were analyzed in duplicate for nitrosamine and nitrite.

The identity of all nitrosamines found at levels >5 ppb (ng/g) was confirmed by mass spectrometric (MS) analysis of an extracted composite of four 25-g samples of each diet within 2 days.

Nitrite Determination. Nitrite determinations were performed by the modified Griess reagent test described by Fiddler (1977).

Reagents. Sulfanilamide (0.5 g) was dissolved in 150 mL of 15% acetic acid and stored in a brown bottle. *N*-(1-Naphthyl)ethylenediamine dihydrochloride (125 mg) was dissolved in 20 mL of boiling water. The hot solution was added to 150 mL of 15% acetic acid and stored separately in a brown bottle. Sodium nitrite standards were prepared by using a stock solution containing 9.94 mg/L. A standard curve was obtained from at least five dilutions of this stock solution on each day that the dietary nitrite determinations were completed.

Procedure. Two separate 5-g samples of each diet were thoroughly mixed with a 300-mL portion of hot (80 °C) distilled water and heated in a boiling water bath for 2 h; each flask was shaken occasionally. The mixture was then cooled to room temperature. The total volume of each sample was adjusted to 500 mL with distilled water, and the sample was shaken and filtered. A suitable aliquot from each solution was mixed with 2 mL of the modified Griess reagent (add 1 mL of sulfanilamide, wait 5 min, and then add 1 mL of *N*-(naphthyl)ethylenediamine dihydrochloride reagent) and diluted to 50 mL. The absorbance of the solution was measured after 20 min at 540 nm, and the nitrite level in each diet was calculated from the standard curve. The detection limit of the assay was 20 ppm.

Nitrosamine Determination with Gas Chromatography–Thermal Energy Analyzer (GC–TEA). The GC–TEA mineral oil distillation technique (Fine et al., 1975) was adapted to determine volatile nitrosamines in these diets. Samples were run in duplicate, without and with inhibitors.

Without Inhibitors. A 25-g sample of each diet was placed in a two-neck 500-mL distillation flask containing 50 mL of mineral oil. All nine flasks (four agar, three chow, and two casein samples) were connected to separate cold traps immersed in liquid nitrogen. A tenth flask, connected in a similar manner, contained either a chow, agar, or casein control (zero nitrite) diet spiked with 125 ng (5 ppb) each of seven nitrosamines (*N*-nitrosodimethylamine, NDMA; *N*-nitrosodiethylamine; *N*-nitrosodi-*n*-propylamine; *N*-nitrosodi-*n*-butylamine; *N*-nitrosopiperidine; *N*-nitrosopyrrolidine, NPYR; *N*-nitrosomorpholine, NMOR) to determine the recovery of each nitrosamine. The traps were connected to a vacuum manifold and the flasks were slowly heated under a vacuum of 2–3 torr over a 30-min period up to 120 °C and then held at this temperature for 20 min to complete the distillation.

With Inhibitors. The other nine 25-g diet samples were treated identically except that before distillation inhibitors were added to prevent artifactual nitrosamine formation during the analysis. To each flask was added 1 g of (±)-α-tocopherol, 1 g of ammonium sulfamate, 17.5 mL of distilled water, and sufficient 6 N H_2SO_4 to adjust the pH of the mixture to 1.5 (1.5 mL for the casein diets and 3.0 mL for the chow). Again, a tenth (zero nitrite) diet sample spiked with the seven nitrosamines plus the inhibitors was also run as a recovery standard.

Following the distillation procedure, each cold trap was warmed to room temperature and its contents were transferred to a 125-mL separatory funnel with water (10 mL) and methylene chloride (MC, 15 mL) to rinse the trap. The funnel was shaken (1 min) and the MC layer collected. The water fraction was extracted with three 10-mL portions of MC and the combined MC extracts were dried by passing them through a funnel containing 10 g of sodium sulfate prewet with MC. The MC extract (plus an MC rinse of the funnel) was concentrated to 1 mL at 52 °C in a Kuderna-Danish distillation apparatus. A 5-μL aliquot was used in the GC–TEA analysis.

Analyses were performed with a Varian Model 3700 gas chromatograph interfaced to a TEA analyzer as described by Fine et al. (1975). The chromatograph was equipped with a 3.7 m × 3.2 mm stainless steel column packed with 5% Carbowax 20M plus 2% KOH on 100–120-mesh Chromosorb WHP maintained at 175 °C with a nitrogen flow rate of 30 mL/min. Data were recorded on a Hewlett-Packard 3380A integrator/recorder. Each nitrosamine was identified and quantitated on the basis of its retention time and peak area, respectively, relative to that of an authentic standard. Since the recovery of each of the seven nitrosamines was not significantly different among the control diets, they were averaged (Table I) and the level of nitrosamine found in the diet sample was corrected according to this recovery value.

Nitrosamine Determination with GC–MS. When more than 5 ppb of a particular nitrosamine was detected in a diet sample containing the inhibitors, the compound was confirmed by MS within 2 days after the initial analysis. To provide sufficient nitrosamine for MS confirmation, four 25-g samples of each of the diets were subjected to the mineral oil distillation procedure (inhibitors added) as described above. The MC extracts from each of these samples were combined with the remainder of the original extract and concentrated to 1 mL in a Kuderna-Danish apparatus. This concentrate was diluted with 8 mL of *n*-pentane and placed on a column containing 5 g of aluminum oxide II–III prewashed with acetone, MC, and *n*-pentane. After the column was washed with 40 mL of pentane, the nitrosamines were eluted with 20 mL of MC. The eluate was concentrated to 2 mL in a Kuderna-Danish apparatus, cooled in an ice bath, and further concentrated to 0.5 mL under a gentle stream of nitrogen.

For GC–MS confirmation, the nitrosamine, specifically, NDMA, NPYR, and NMOR, was separated and isolated from the MC concentrate by high-performance liquid chromatography (HPLC). Replicate 25-μL aliquots of the concentrate were resolved on a 4.6 × 250 mm Lichrosorb NH_2 10-μm column (equipped with a Varian Model 8500 pump) interfaced to a TEA, and the nitrosamine was eluted with a mobile phase consisting of *n*-pentane–MC (22:3) at a flow rate of 2 mL/min. In this manner at least 300 ng of each of the above nitrosamines was collected according to its retention time compared to that of a standard mixture of NDMA, NPYR, and NMOR. The

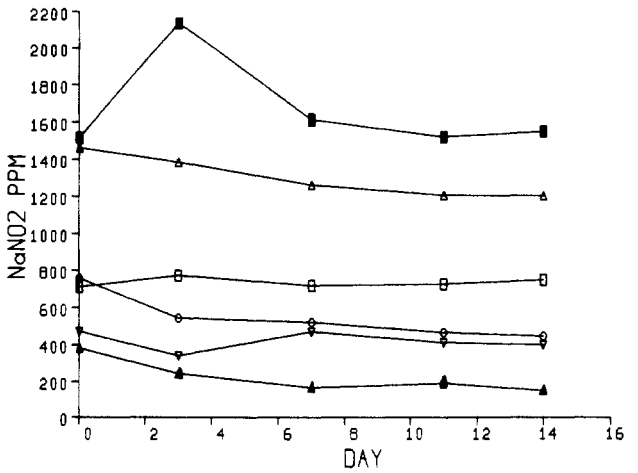

Figure 1. Sodium nitrite content of diets. (▲) Agar, 500 ppm of $NaNO_2$; (○) agar, 1000 ppm of $NaNO_2$; (△) agar, 2000 ppm of $NaNO_2$; (□) casein, 1000 ppm of $NaNO_2$; (▽) chow, 1000 ppm of $NaNO_2$; (■) chow, 2000 ppm of $NaNO_2$.

HPLC collection process was monitored by GC–TEA to verify that only a single peak had been collected.

The collected material was concentrated to 0.6–0.7 mL, placed in the bulb of a small drying tube, and transferred under nitrogen to a connected 3 × 80 mm Tenax column, previously conditioned at 225 °C for 30 min under a stream of nitrogen. The Tenax column served as a liner for the injection port of a Perkin-Elmer 3920B capillary column gas chromatograph interfaced to a Nuclide 1290G mass spectrometer equipped with a Nuclide DA/CA 1.2 data system. The injection port of the chromatograph containing the Tenax tube was heated from room temperature to 200 °C over a 16-min period with a helium flow rate of 2–3 mL/min. By this process, the nitrosamine was transferred onto a 0.2 mm × 25 m glass capillary column coated with Carbowax 20M, cooled at 0 °C. The column was programmed at a rate of 8 °C/min to 200 °C, and the nitrosamine was introduced into the ionization chamber of the mass spectrometer. When the NDMA levels in the diet were 30 ppb and above, a full-scan low-resolution mass spectrum was obtained, sufficient for confirmation. However, to confirm NMOR and NPYR, and for lower levels of NDMA, the selective ion monitoring mode was used to detect the presence of the parent ion, the NO ion, and other characteristic fragment ions (m/z: 42 for NDMA, 41 for NPYR, and 86 for NMOR).

RESULTS AND DISCUSSION

Dietary Nitrite Content. Even at 0 time (actually 4–10 h after nitrite addition) the measured nitrite content (Figure 1) of all the diets was substantially lower than the amount added. [Nitrite was not detected in the control (zero nitrite) diets.] We presume that this loss could be attributed to degradation or interaction of the nitrite with the components of the diet; however, it is more probable that most of the loss occurred during the initial extraction and heating step of the method (preceding the colorimetric determination) where the pH values of the diet solutions were between 4.5 and 5. At this pH there can be conversion of NO_2^- to N_2O_3 (Challis, 1980), which is known to decompose thermally to gaseous nitric oxide and nitrogen dioxide (Bayliss and Watts, 1963).

As shown in Figure 1, the wet agar diets lost considerable nitrite with time, while no such trend was apparent with the dry diets. A similar loss of sodium nitrite with time has been noted in other wet foods (Fiddler, 1977). However, an HPLC method recently developed to simultaneously determine nitrite and nitrate showed no substantial decrease in nitrite within 24 h in a similar agar diet containing 2000 ppm of nitrite (Yang et al., 1981). The nitrite level, however, gradually fell during storage and decreased to 1000 ppm after 40 days.

The variation in nitrite content between duplicates was minimal with the casein and agar diets, but changed considerably with the chow diets. We assume that this variation was due to a nonhomogeneous distribution of nitrite in the chow diet, possibly due to incomplete mixing of the added nitrite solution to the diet.

In summary, in future studies, to determine nitrite in these particular diets, it will be necessary to ensure the uniform distribution of nitrite in the chow diet. This may require a longer mixing period or a different mode of adding nitrite. Equally important is the procedure used to determine nitrite. The Griess colorimetric procedure may not be an accurate quantitative method to determine nitrite in these particular diets because of the potential for nitrite loss during the method. However, although this observation would have to be substantiated, more recent direct methods have been developed that may be more suitable to determine nitrite in these diets, e.g., the HPLC method previously cited and/or the group-selective assay for nitrite, "total nitrosamines", and nitrate (Hart et al., 1982).

Inhibition of Artifactual Nitrosamine Formation. Several preliminary experiments were conducted to determine the optimal conditions needed to prevent artifactual nitrosation of amines during the analytical procedure. In each experiment, 200 μg of morpholine was added to a 25-g diet sample before the mineral oil distillation step. This amine is probably the most easily nitrosated of all the common volatile nitrosamine precursors. We sought to establish conditions that would prevent nitrosation of morpholine, even in the presence of 0.2% $NaNO_2$. After several trials with various amounts of sodium ascorbate, ammonium sulfamate, and tocopherol, it was established that no nitrosation (<0.5 ppb) of morpholine occurred when 1 g of sulfamate and 1 g of α-tocopherol per 25 g of diet were added and with the pH adjusted to 1.5. Thus, these two inhibitors were used throughout the experiments to inhibit artifactual nitrosamine formation. The effect of adding these inhibitors is readily apparent from the results presented in Tables I and II, especially when the chow diets are compared with and without inhibitors. Therefore, it is necessary that these inhibitors be added to prevent artifactual nitrosamine formation when using the TEA procedure, especially if nitrite is a component of the sample.

To further demonstrate the efficacy of inhibition, four replicate samples of each of the 1000 and 2000 ppm of nitrite chow diets containing morpholine and the inhibitors were analyzed on day 2 of the study (Table III). The results of the analysis of the chow diet on days 0, 2, and 3 are given in Table IV, which shows good agreement between the day 0 and day 3 results. Further, the NMOR values reported for day 2 samples are almost identical with the NMOR seen in the day 0 and day 3 samples when one considers that 200 μg of morpholine was added to each of the day 2 diet samples before the mineral oil distillation step. Consequently, these results support the conclusion that the nitrosamines found in the inhibitor-containing diets were not due to artifactual nitrosation during analysis.

Nitrosamines Found in Diets. Neither of the semisynthetic diets (agar and casein) contained appreciable (>5 ppb) levels of any of the volatile nitrosamines when the

Table I. Volatile Nitrosamine Content of Chow Diets Stored at 4 °C for 0-14 Days[a]

$NaNO_2$ added, ppm	nitrosamine[b]	day 0	3	7	11	14	14-day average[c]
0[d]	NDMA	4.1 (3.5)	3.5 (3.6)	3.9 (3.8)	3.5 (4.3)	4.4 (3.3)	3.9 (3.7)
	NDEA			tr[e] (ND)[f]	tr (ND)	ND (tr)	ND (ND)
	NDPA					tr (ND)	ND (ND)
1000	NDMA	10 (56)	9.4 (47)	13 (49)	14 (51)	22 (54)	14 (51)
	NDEA	ND (tr)	ND (tr)	tr (tr)	ND (ND)	ND (ND)	ND (tr)
	NDPA	ND (ND)	ND (ND)	ND (ND)	ND (ND)	ND (tr)	ND (ND)
	NDBA	ND (ND)	ND (ND)	ND (ND)	ND (ND)	ND (tr)	ND (ND)
	NPIP	ND (2.0)	ND (ND)	tr (2.7)	tr (2.6)	1.2 (4.1)	tr (2.3)
	NPYR	ND (16)	ND (9.6)	ND (9.7)	2.5 (13)	5.1 (22)	1.5 (14)
	NMOR	ND (11)	ND (tr)	tr (3.4)	tr (4.0)	tr (4.9)	tr (4.8)
2000	NDMA	15 (372)	19 (299)	27 (216)	31 (292)	45 (220)	27 (280)
	NDEA	ND (9.5)	ND (27)	ND (10)	ND (9)	ND (12)	ND (14)
	NDPA	ND (3.2)	ND (4.6)	ND (2.8)	ND (4.5)	tr (6.9)	ND (4.4)
	NDBA	ND (4.4)	ND (ND)	ND (ND)	ND (5.9)	ND (2.1)	ND (2.5)
	NPIP	ND (18)	ND (16)	tr (14)	1.3 (19)	1.6 (16)	tr (17)
	NPYR	2.7 (73)	2.7 (69)	4.5 (34)	5.8 (56)	7.4 (51)	4.6 (57)
	NMOR	3.0 (27)	tr (31)	4.1 (18)	4.3 (25)	6.6 (24)	3.7 (25)

[a] Values are given as ppb (μg/kg dry weight) corrected for recovery of each nitrosamine from a simultaneously run spiked sample. Samples were analyzed in presence or absence of inhibitors. Values obtained without inhibitors are in parentheses. [b] NDMA = *N*-nitrosodimethylamine; NDEA = *N*-nitrosodiethylamine; NDPA = *N*-nitroso-*n*-propylamine; NDBA = *N*-nitroso-*n*-butylamine; NPIP = *N*-nitrosopiperidine; NPYR = *N*-nitrosopyrrolidine; NMOR = *N*-nitrosomorpholine. [c] The average percent recovery (±standard deviation) of spiked standards in diets ($n = 10$) was as follows: NDMA, 100 ± 8; NDEA, 94 ± 6; NDPA, 91 ± 9; NDBA, 73 ± 13; NPIP, 88 ± 9; NPYR, 74 ± 13; NMOR, 71 ± 7. [d] NDBA, NPIP, NPYR, and NMOR were not detected in this diet on days 0-14. [e] tr = 0.5 to <1.0 ppb. [f] ND = not detected (<0.5 ppb).

Table II. Volatile Nitrosamine Content of Agar and Casein Diets Stored at 4 °C for 0-14 Days[a]

$NaNO_2$ added, ppm	nitrosamine[b]	day 0	3	7	11	14	14-day average
				Agar[c]			
500[d]	NDMA	ND[e](ND)	ND (ND)	tr[f] (tr)	tr (tr)	ND (ND)	ND (ND)
	NDEA	ND (2.2)	ND (2.2)	ND (tr)	ND (tr)	ND (tr)	ND (tr)
1000[d]	NDMA	ND (ND)	ND (1.3)	tr (tr)	tr (tr)	tr (tr)	tr (tr)
	NDEA	ND (2.2)	ND (2.2)	ND (tr)	ND (tr)	ND (tr)	ND (tr)
2000	NDMA	tr (3.8)	ND (5.3)	tr (2.4)	1.0 (3.2)	tr (1.6)	tr (3.3)
	NDEA	tr (4.0)	tr (30)	tr (4.8)	ND (9.6)	ND (2.8)	tr (10)
	NDPA	ND (ND)	ND (5.9)	ND (tr)	ND (3.0)	ND (ND)	ND (1.9)
	NDBA	ND (ND)	ND (ND)	ND (3.6)	ND (ND)	ND (tr)	ND (tr)
	NPIP	ND (ND)	ND (ND)	tr (3.6)	ND (3.0)	ND (tr)	ND (1.4)
	NPYR	ND (ND)	ND (ND)	ND (ND)	ND (ND)	ND (ND)	ND (ND)
	NMOR	ND (ND)	ND (ND)	ND (tr)	ND (ND)	ND (tr)	ND (ND)
				Casein[c]			
1000	NDMA	ND (ND)	ND (ND)	ND (ND)	tr (3.3)	ND (1.3)	ND (tr)
	NDEA	ND (4.8)	ND (3.1)	ND (5.1)	ND (13)	ND (3.2)	ND (5.8)
	NDPA	ND (ND)	ND (ND)	ND (2.4)	ND (3.9)	ND (1.6)	ND (1.6)
	NDBA	ND (ND)	ND (ND)	ND (ND)	ND (4.6)	ND (tr)	ND (1.0)
	NPIP	ND (ND)	ND (ND)	ND (ND)	ND (ND)	ND (tr)	ND (ND)
	NPYR	ND (ND)	ND (ND)	ND (ND)	ND (2.6)	ND (tr)	ND (tr)
	NMOR	ND (ND)	ND (ND)	ND (ND)	ND (ND)	ND (tr)	ND (ND)

[a] Values are given as ppb (μg/kg dry weight) corrected for recovery of each nitrosamine from a simultaneously run spiked sample. Samples were analyzed in presence or absence of inhibitors. Values obtained without inhibitors are in parentheses. [b] See Table I, footnote *b*. [c] None of the seven nitrosamines were detected in the agar and casein diets to which no sodium nitrite had been added. [d] NDPA, NDBA, NPIP, NPYR, and NMOR were not detected in this diet on days 0-14. [e] ND = not detected (<0.5 ppb). [f] tr = 0.5 to <1.0 ppb.

Table III. Analysis[a] of Four Chow Diet Replicates on Day 2 (Inhibitors plus 200 μg of Morpholine Added to Each Diet)

diet	nitrosamine[b]	A	B	C	D	average
chow with 1000 ppm of $NaNO_2$	NDMA	8.3	9.9	13.0	8.9	10.0
chow with 2000 ppm of $NaNO_2$	NDMA	18.7	17.8	17.2	17.9	17.9
	NPYR	2.6	2.4	2.8	2.7	2.6
	NMOR	tr[c]	tr	tr	tr	tr

[a] ppb (ng of nitrosamine/g of diet). [b] See Table I, footnote *b*. [c] tr = 0.5 to <1.0 ppb.

Table IV. Analysis[a] of Chow Diet Containing Added Nitrite on Days 0, 2, and 3

diet	nitrosamine[b]	day 0[c]	2[d]	3[c]
chow with 1000 ppm of $NaNO_2$	NDMA	10.4	10.0	9.4
chow with 2000 ppm of $NaNO_2$	NDMA	14.8	17.9	18.7
	NPYR	2.7	2.6	2.7
	NMOR	3.0	tr[e]	tr

[a] ppb (ng of nitrosamine/g of diet). [b] See Table I, footnote *b*. [c] Single determinations (diet plus inhibitors). [d] Table III results. [e] tr = 0.5 to <1.0 ppb.

analysis was carried out in the presence of the nitrosation inhibitors (Tables I and II). The chow diet with added nitrite contained NDMA and, in some samples, NPYR at levels above 5 ppb. The NDMA and NPYR levels in the

1000 and 2000 ppm of nitrite chow diet increased with time, more than doubling over the 2-week storage period. For example, NDMA levels in the 1000 ppm of nitrite chow diet increased from 10 ppb at day 0 to 22 ppb on day 14 and respectively from 15 to 45 ppb for the 2000 ppm of nitrite chow diet. The former findings parallel values of 17 and 20 ppb for NDMA found (Wasserman, 1979) in a chow diet (NIH-007) containing 1000 ppm of added nitrite analyzed over several weeks by the FDA methanolic potassium hydroxide digestion procedure (Fazio et al., 1971). Prior to digestion, sulfamic acid was added to destroy the nitrite in the diet to prevent artifactual formation of nitrosamine. The low (3–4 ppb) levels of NDMA found in the chow diet with no added nitrite are within the range (<5 ppb) previously reported for such diets (Edwards et al., 1979).

Clearly, omission of the nitrosation inhibitors ammonium sulfamate and tocopherol resulted in artifactual nitrosamine formation during the analytical procedure, which demonstrates the need for careful control where the analysis of nitrosamines is of concern. This was evident in the chow diet where the artifactual nitrosamine formation was the greatest, implying that the possibility of in situ or in vivo nitrosation with chow diets is much greater than with either casein or agar diets.

The influence of these volatile nitrosamines in the cited nitrite feeding studies is unclear. Further, the effect of other nitrosation products potentially capable of being formed in situ or in vivo (e.g., nonvolatile *N*-nitrosoureas, *N*-nitrosamides, etc.) cannot presently be assessed until methods are developed that are capable of detecting and measuring these compounds as well.

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LITERATURE CITED

Bayliss, N. S.; Watts, D. W. *Aust. J. Chem.* **1963**, *16*, 943.
Challis, B. C., Queens College, London, England, personal communication, 1980.
Edwards, G. S.; Fox, J. G.; Policastro, P.; Goff, U.; Wolf, M. H.; Fine, D. H. *Cancer Res.* **1979**, *39*, 1857.
Fazio, T.; Howard, J. W.; White, R. H. "Proceedings of the International Agency for Research on Cancer"; International Agency for Research on Cancer: Heidelberg, West Germany, 1971; pp 16–24.
Fiddler, R. N. *J. Assoc. Off. Anal. Chem.* **1977**, *60*, 594.
Fine, D. H.; Rounbehler, D. P.; Oettinger, P. E. *Anal. Chim. Acta* **1975**, *78*, 383.
Hart, R. J.; Walters, C. L.; Newberne, P. M.; Keefer, L. K., unpublished data, 1982.
Magee, P. N.; Montesano, R.; Preussman, R. In "Chemical Carcinogens"; Searle, C. E., Ed.; American Chemical Society: Washington, DC, 1976; pp 491–625.
Newberne, P. H. *Science (Washington, D.C.)* **1979**, *204*, 1079.
Shank, R. C.; Newberne, P. H. *Food Cosmet. Toxicol.* **1976**, *14*, 1.
Wasserman, A. E., U.S. Department of Agriculture, Philadelphia, PA, personal communication, 1979.
Wogan, G. N.; Newberne, P. H. *Cancer Res.* **1967**, *27*, 2370.
Yang, G. C.; Joshi, A.; Ragelis, E. P. "Abstracts of Papers", 181st National Meeting of the American Chemical Society, Atlanta, GA, March 29–April 3, 1981; American Chemical Society: Washington, DC, 1981; AGFD 26.

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Nitrogen-Containing Heterocyclic Compounds Identified in the Volatile Flavor Constituents of Roasted Beef

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Volatile flavor compounds were isolated from 500 lb of roasted beef. The flavor isolate was subjected to extensive gas chromatographic fractionation, and the pure fractions obtained were identified by GC–mass spectrometry. A total of 44 nitrogen-containing heterocyclic compounds were identified. They included 15 thiazoles, 1 thiazoline, 6 oxazoles, 11 pyrazines, 6 pyrroles, 2 piperidines, and 3 pyridines.

Due to the great sensory and economic value of beef, an enormous amount of research has been conducted on beef flavor. Over 500 compounds have been mentioned in the literature as components identified in the volatiles of cooked beef (MacLeod and Seyyedain-Ardebili, 1981).

Heterocyclic compounds play an important role in roasted flavors and particularly in meat products (Ohloff and Flament, 1978). Some of them have interesting organoleptic properties and very low thresholds. The present paper reports on the identification of nitrogen-containing heterocyclic compounds in the volatile flavor of roasted beef.

EXPERIMENTAL SECTION

Preparation of Roasted Beef. A total of 25 lb of Longissimus beef muscle (cross rib roast), pieces averaging 3–4 lb, was pan-browned with no added fat. The browned

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