Identification of Alkylureas after Nitrosation–Denitrosation of a Bonito Fish Product, Crab, Lobster, and Bacon

Sidney S. Mirvish,* Kazimierz Karlowski,[1] David A. Cairnes, James P. Sams, Rose Abraham, and James Nielsen

The in vivo production of nitrosoureas from food constituents and nitrite might contribute to the etiology of gastric cancer. Food extracts were treated with nitrite at pH 1 and left a pH 0 to effect denitrosation. The resulting alkylureas (which may have arisen from intermediate alkylnitrosoureas) were purified by cation-exchange, paper, and high-pressure liquid chromatography. Identity of most alkylurea samples was confirmed by high-resolution mass spectrometry. A smoked, dried bonito fish product from Japan yielded an average of 340 μmol (25 mg) of methylurea (MU)/kg of fish; an intermediate product was shown to be methylnitrosourea. We obtained *n*-propylurea, 3-butenylurea, and [(4?)-hydroxybutyl]urea from dungeness crab and (mainly) 3-butenylurea from Alaskan king crab and slipper lobster tail. Fried bacon yielded an average of 350 μmol of MU/kg of food. The precursors of the alkylureas remain unidentified. The very large excess of nitrite used for the nitrosation makes the results less likely to be significant.

A factor in the causation of human gastric cancer could be exposure to nitrosoureas, which might occur in foodstuffs or be produced under the acidic conditions of the stomach (Mirvish, 1971, 1977; Correa et al., 1975; Weisburger and Raineri, 1975; Weisburger, 1979). This hypothesis appears reasonable because simple and substituted nitrosoureas, when administered orally, have induced gastric adenocarcinomas in experimental animals (Druckrey et al., 1968, 1970, 1971; Druckrey and Landschütz, 1971). Since nitrosoureas are chemically unstable, direct-acting carcinogens, they might act in the human stomach, if they were produced there by in vivo nitrosation. Despite intensive efforts to determine volatile nitrosamines in foods, the occurrence therein of nitrosoureas has not been much studied, mainly because of a lack of suitable analytical methods.

Our main objective was to search for food components that might react with nitrite under acidic conditions to yield alkylnitrosoureas. Since alkylureas are the most obvious such components, a subsidiary objective was to discover whether these compounds occur in foods. Our approach was to treat food extracts with a large excess of nitrite at pH 1, in order to produce alkylnitrosoureas, and to subject the resulting solutions to prolonged acid treatment at pH 0, in order to convert the alkylnitrosoureas to alkylureas (Preussmann and Schaper-Druckrey, 1972; Mirvish et al., 1973). The ureas were then isolated and identified. An advantage of this approach was that alkylureas were easier to work with than the unstable nitrosoureas. A disadvantage was that we did not establish that nitrosoureas were produced by the nitrosation, with one important exception. The nitrite treatment destroyed most of the urea (which might have masked the alkylureas) by conversion into nitrogen and CO_2. Hence, the results for urea did not measure its content in unnitrosated foods.

The high incidence of gastric cancer in Japan is associated with exposure to salted, pickled fish and vegetables (Sato et al., 1959; Haenszel and Correa, 1975). Gastric cancer could be associated with a high consumption of fish and other seafoods in other countries too, including Norway, Iceland, and Chile (Haenszel and Correa, 1975). Accordingly, we studied a salted bonito fish product from Japan and then surveyed selected seafoods and certain common foods.

In a retrospective study on gastric cancer patients in Holland, Meinsma (1964) reported that gastric cancer incidence was correlated positively with bacon consumption and negatively with citrus consumption. In a similar study in Kansas City, Higginson (1966) found positive correlations with the use of animal fats, cooked fats, fried foods, and bacon and negative correlations with the use of dairy products. If these studies were confirmed, bacon consumption could merely indicate a life style conducive to the development of gastric cancer or could itself be an etiologic factor. Consideration of the second alternative should take into account that (a) fried bacon is unlikely to be associated with gastric cancer in high-incidence countries such as Japan and Chile and (b) in the United States, bacon consumption is not falling, whereas the gastric cancer incidence has fallen dramatically over the past 30 years (Haenszel and Correa, 1975). The occurrence of *N*-nitrosopyrrolidine and dimethylnitrosamine in fried bacon (Sen et al., 1979) may be irrelevant, since these nitrosamines are not gastric carcinogens in experimental animals. Hence, we also studied fried bacon. Preliminary reports on this study have been published (Mirvish, 1977; Mirvish and Sams, 1977; Mirvish et al., 1978, 1980).

EXPERIMENTAL SECTION

Analytical-grade solvents were used throughout. Methylurea (MU) and isopropylurea were obtained from Eastman Organic Chemicals and ethylurea, *n*-propylurea, and *n*-butylurea from Aldrich Chemical Co. *n*-Hexylurea was synthesized (Lee et al., 1977). All foods were purchased locally. Proton magnetic resonance (^{1}H NMR) spectra were measured with a Varian CFT-20 instrument. The nitrite treatment could yield carcinogenic nitrosamines from amines present in the foodstuffs (in addition to carcinogenic nitrosoureas) and appropriate precautions were taken. Unless mentioned otherwise, pH's were adjusted with concentrated HCl or saturated NaOH solution. Solutions were evaporated, generally under a water-aspirator vacuum, with a rotary evaporator at <60 °C. Ureas were estimated by a colorimetric method specific for ureas, termed here the "urea test" (Hunninglake and Grisolia, 1966; Mirvish et al., 1979), which was considered positive

Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, Nebraska 68105.

[1]Present address: Department of Food Research, National Institute of Hygiene, 00-791 Warsaw, Poland.

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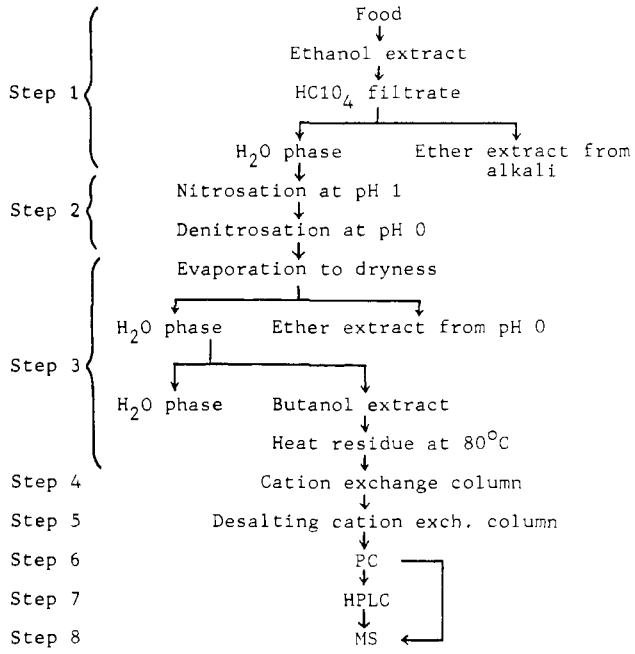

Figure 1. Scheme showing principal operations in the standard procedure for obtaining ureas from foodstuffs. Step numbers are those listed under Experimental Section.

only if it gave a purple color. Standards with 0.1 μmol of MU in a final volume of 3.5 mL showed an absorptivity of 0.50–0.75 at 545 nm.

Standard Procedure for Obtaining Ureas from Foodstuffs (See Figure 1). *Step 1: Sample Preparation.* Each analysis was performed on a separate 250-g food sample. This was extracted with 1.6 L of 95% ethanol in a Waring blender 3 times for 2 min each, with 15-min intervals to cool the sample. The mixture was filtered and the filtrate was evaporated. The residue was dissolved in 500 mL of water, adjusted to pH 1 with $HClO_4$, and left at 6 °C overnight. The protein precipitate was filtered through 100 g of Celite. The filtrate was made alkaline, extracted with ether (3 times with half the filtrate volume) to remove part of the amines that could yield nitrosamines, and readjusted to pH 1.

Step 2: Nitrosation and Denitrosation. The aqueous solution was placed in a 2-L conical flask and 100 g of $NaNO_2$ (in six equal lots) was added over 3 h, without external cooling. Each lot was added over 2 min with slow stirring, the pH was adjusted to 1 by using pH paper, and the stirring was stopped to minimize HNO_2 decomposition. A pH of 1 was used because alkylureas are nitrosated rapidly at this pH (Mirvish, 1971). If a pH meter was used here, the glass electrode was damaged, perhaps due to formation of nitrogen oxide bubbles on the electrode surface. The flask was stoppered loosely and left overnight in ice in the hood. While the solution was cold, 45 g of ammonium sulfamate was added over 20 min to destroy excess HNO_2. The pH was brought to 0 (pH meter), and the solution was left at 6 °C for 2 days.

Step 3: Butanol Extraction. The solution was brought to pH 2.5 (pH paper) and evaporated, to remove part of any volatile nitrosamines present. A solution of the residue in 500 mL water was adjusted to pH 0.0–0.3 and extracted 3 times with 250 mL of ether and 3 times with 250 mL of 1-butanol, with thorough shaking. The butanol extract was evaporated by using, finally, an oil pump (with exhaust gases led into a chemical hood). The residue was heated at 80 °C and 2 torr for 10 min (to remove substances derived from the nitrite that interfered with the urea test) and dissolved in 250 mL of water. The pH was adjusted to 1.0.

To check the solvent partitions, we adjusted 170 g of NaCl (corresponding to NaCl derived from $NaNO_2$ and HCl) and 90 g of ammonium sulfamate in 1 L of water to pH 0.3. In duplicate tests, 20 mL of this solution containing 20 mg of MU, *n*-butylurea, or *n*-hexylurea was extracted 3 times with 10 mL of ether and then 3 times with 10 mL of 1-butanol. The volume of each phase was measured and aliquots were analyzed for ureas; 78% of the MU, 85% of the *n*-butylurea, and 46% of the *n*-hexylurea were extracted into the butanol. With *n*-hexylurea, 50% was extracted into the ether. Hence, the extraction was satisfactory, except for *n*-hexylurea.

To check the heating step, we prepared an unnitrosated food extract by subjecting 500 g of the bonito fish product to step 1 and step 3 until after the heating. This gave 46 g of oil, which was mixed with 40 mL of methanol. A solution of 1 mL of this mixture and 34 μmol of MU, *n*-butylurea, or *n*-hexylurea in 10 mL methanol was analyzed for ureas, evaporated in a 50-mL flask, and heated (80 °C; 2 torr; 10 min). The residue was dissolved in methanol to 9 mL and reanalyzed. Solutions of fish extract alone and alkylurea alone were also examined. The loss was 5% for MU, 0% for *n*-butylurea, and 25% for *n*-hexylurea; i.e., the loss was appreciable only for *n*-hexylurea. The results for the alkylurea solutions with and without fish extract showed that the extract enhanced the color reaction of MU by 76%, of *n*-butylurea by 67%, and of *n*-hexylurea by 57%. Fish extract alone gave little color.

Step 4: First Column. The principle here was that MU (and presumably other alkylureas) shows an acidic dissociation constant (pK_a) of 0.7 (Perrin, 1965) and should be partly retained on a cation-exchange column [as $(RNH_2{\cdot}CONH_2)^+$] at pH 1 but eluted as the nonprotonated urea at pH 3–4. A column of Bio-Rad AG-50W-X8 resin (100–200 mesh; 2.3-cm diameter × 25-cm height) was regenerated with 2 N NaOH (500 mL), water (1 L), 1 N HCl (500 mL), and 0.1 N HCl (until the eluate reached pH 1). The step 3 extract was applied to the column, which was eluted successively with ~1.5 L of pH 2.75 buffer (1 N formic acid with added NaOH, applied until the eluant pH was 2.5), 500 mL of pH 5.75 buffer (1 N acetic acid with added NaOH, applied until the eluant pH was 5.75), and 500 mL of 2 N NH_4OH. The flow rate was 2.5–3.0 mL/min and 20-min fractions were collected. The pH and urea content of every third fraction were measured. The urea-containing fractions (eluted at pH 2.0–3.5) were combined and stored at 6 °C over chloroform. The analysis was stopped if it showed <40 μmol of the ureas/kg of food.

Step 5: Desalting Column. To remove the buffers, we used a cation-exchange column identical with that in step 4, equilibrated to pH 1 as before. The step 4 urea fraction was adjusted to pH 1 and applied to the column, which was eluted with water (500 mL) and 2 N NH_4OH (1 L, until the eluate pH was >10). The pH and urea content of every third fraction were determined. Urea-containing fractions (eluted at pH 2–5) were combined and evaporated. If the residue weighed >100 mg, it was passed through a second desalting column.

Step 6: PC. The residue was dissolved in 5 mL of methanol and half was applied as a 13-cm strip to Whatman 3 MM paper (15 × 56 cm, previously washed with the PC solvent system). Descending PC was performed with 1-propanol–concentrated NH_4OH (9:1 v/v, "solvent 1") for 18 h. The paper was dried and cut into 3-cm strips, which were eluted with 15 mL of methanol. The fractions were analyzed for ureas, combined as ap-

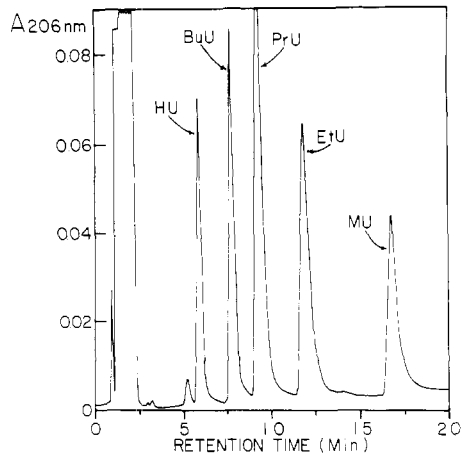

Figure 2. Separation of alkylureas by high-pressure LC. Eight liters of methanol solution containing 1 mg/mL of each alkylurea was subjected to high-pressure LC as described under Experimental Section. Monitoring was by UV absorption at 207 nm (where MU showed an ϵ of 70). Peak identity was checked by comparison with the high-pressure LC of individual ureas. HU, *n*-hexylurea; BuU, *n*-butylurea; PrU, *n*-propylurea; EtU, ethylurea.

propriate, and subjected to descending PC with 1-butanol–acetic acid–water (3:1:1 v/v/v, "solvent 2") and/or with CCl_4–CH_2Cl_2–ethyl acetate–formic acid [70:50:15:10 by volume, modified from Knappe and Rohdewald (1966), "solvent 3"].

In trials with pure ureas, they were visualized by spraying with 1% *p*-(dimethylamino)benzaldehyde in ethanol, followed by exposure to HCl vapor (Ehrlich's reagent). The R_f values for urea, MU, and ethylurea were (in order) 0.20, 0.53, and 0.66 on PC with solvent 1, 0.60, 0.75, and 0.75 with solvent 2, and 0.10, 0.20, and 0.50 with solvent 3. *n*-Butylurea showed R_f 0.76 with solvent 3. The ureido amino acid citrulline showed R_f 0 with solvent 1. Hence, PC with solvent 1 served to separate urea and citrulline from the alkylureas. Subsequent PC with solvents 2 and 3 separated the individual alkylureas. Solvent 3 was used for the higher alkylureas.

Step 7: High-Performance LC. Methanol solutions of PC fractions were subjected to high-performance LC on a silica gel column (25 cm × 3.2 mm i.d., 5 μm Lichrosorb 60 from E. Merck and Co.). The column was eluted with cyclopentane–methanol–anhydrous ether (93:5:2 v/v/v) and flushed with methanol. The flow rate was 2 mL/min. One-milliliter eluate fractions were collected and 25–100-μL aliquots were analyzed for ureas. Alkylureas ranging from methylurea to *n*-hexylurea were separated, when pure solutions were used (Figure 2). Most food extracts were applied to the column in 0.5 or 1.0 mL of methanol; this large volume interfered with the separations, but the high-performance LC removed many impurities and yielded cleaner MS.

Step 8: Mass Spectrometry. In most analyses, the MS (see end of Results) served to confirm the identity of the ureas. We used an AEI MS-902 spectrometer with an AEI DS30 data system, modified to operate with VG System 2040 programs. Computer analysis giving compatible elemental compositions was performed. All cited compositions agreed with the measured masses to within 3 millimass units. Spectra were scanned from m/z 700 to m/z 28 at 8 s/decade. The MS of methanolic solutions of PC or high-performance LC fractions were determined by using a direct probe (ion block temperature, 210 °C).

Quantitation of Results. Total ureas were estimated from the urea method applied to PC by solvent 1 and may be fairly reliable. No corrections were made for the recovery efficiency. Individual ureas were determined from the total urea results and the ratio between the amount of each urea, as determined by PC. If the MS showed several ureas in one fraction, this is reported, generally without attempting to estimate relative amounts.

Further Checks of the Method. We studied the effect of NaCl, included with 7.4 μg of MU in the urea test (final volume, 3.5 mL); 10, 25, 50, and 100 mg of NaCl inhibited color development by 8, 75, 82, and 100%, respectively. This and the results discussed earlier showed that the urea test may give erroneous results, when applied to crude extracts.

For determination of MNU stability during the nitrosation, 700 mg of MU in 700 mL of water was nitrosated as in step 2. MNU was monitored by its UV absorption at 392 nm (ϵ, 93; Mirvish, 1971). The MNU yield was 84% at 5 min, 67% at 1 h, 37% at 3 h, and only 10% at 18 h. MNU decomposition was attributed to HNO_2 attack on the terminal amino group.

For determination of MU recovery after nitrosation–denitrosation, 150 mg of MU in 700 mL of water was subjected to step 2 and step 3 until after the heating. the residue was dissolved in water and analyzed by the urea test. MU recovery was 11 ± 3% (mean ± SE) in four experiments. Methylguanidine is nitrosated to give MNU (Mirvish, 1971; Mirvish et al., 1973) and occurs in some foods (Kawabata et al., 1978, 1979). Accordingly, MU could arise from methylguanidine. To test whether this could occur, we subjected 500 mg of methylguanidine sulfate in 700 mL of water to steps 2 and 3 and analyzed the product by the urea test. MU yield from methylguanidine was 13 and 15% in two experiments.

Isolation of Methylnitrosourea (MNU) from Bonito Fish Product. The fish product (500 g) was subjected to step 1 of the standard procedure. The resulting solution was adjusted to pH 7, extracted 4 times with 250 mL of 1-butanol, and evaporated. The residue (85 g) was dissolved in 500 mL of water and nitrosated as in step 2. Ammonium sulfamate was added as in step 2 but in an ice bath (which was used in all subsequent steps except the evaporations). Materials from all subsequent steps were stored at −15 °C, in view of the instability of MNU. The pH was raised to 4 with solid $NaHCO_3$ over 20 min. The solution was extracted 4 times with 200 mL of ether. The extract was dried over Na_2SO_4, filtered, and evaporated at 20 °C. The residue was redissolved in 50 mL of ether, dried, and evaporated. The residue (206 mg) was dissolved in 2 mL of methanol and applied as strips to two 20 × 20 × 0.025 cm silica gel thin-layer chromatography (TLC) plates (Sil gel G-25 without gypsum, Brinkmann Instruments). The plates were developed with CH_2Cl_2–ethyl acetate (7:3 v/v) and 2-cm bands were eluted with 20 mL of methanol. Eluate samples with R_f 0.6–0.7 reacted positively in the urea test, which determines MNU (Mirvish et al., 1979). According to this test, these eluate fractions (which were evaporated) contained 1.1 mg of MNU.

On reanalysis after storage at −15 °C for 15 days, the product contained 210 μg of MNU. The material in acetonitrile solution was subjected to high-performance LC on the same silica gel column as used in step 7, with elution by CH_2Cl_2–CH_3CN (9:1 v/v). The flow rate was 0.5 mL/min and detection was by UV absorption at 230 nm. Authentic MNU showed a retention time of 8.0 min. High-performance LC of the fish extract showed several peaks, with the largest eluted at 8.0 min. The fraction corresponding to this peak was collected and evaporated.

Table I. Ureas from Bonito Fish Product

no.	conditions	urea, μmol/kg	MU, μmol/kg
1	standard procedure[a]	48,[b] 42,[b] 15	220,[b] 390,[b] 420[c]
2	no nitrosation (steps 2 and 3 omitted)[d]	1180, 1480	0, 0
3	no nitrosation ($NaNO_2$ omitted)[e]	100[b]	0
4	spiked with 8110 μmol/kg of MU[f]	20, 0	1050, 720
5	spiked with 36 500 μmol/kg of methylguanidine·HCl[f]	48	4860
	subdivided after cation-exchange chromatography and then nitrosated:[g]		
6	fraction A (pH 1-7)	33	75
7	fraction B (pH 7-13.5)	20	4
8	fraction C (pH 14)	17[h]	

[a] This involved a single desalting column and successive PC with solvents 1 and 2. [b] Identity was confirmed by the MS. [c] Urea and MU results for the same analysis are listed in corresponding positions. [d] Step 1 of the standard procedure was performed until the $HClO_4$ precipitation. The filtrate was subjected directly to column chromatography (step 4), followed by steps 5-7. [e] The complete standard procedure was performed, but $NaNO_2$ was not added in step 2. [f] The standard procedure was performed up to the first PC (in step 6). [g] A fish product extract was prepared as in step 1 and subjected directly to chromatography as in step 4 but with final elution by 1.5 N NaOH. Each of the three eluate fractions was subjected to the standard procedure from step 2 to PC with solvent 1 (in step 6), and the urea and MU fractions were determined. [h] This represents total ureas (urea plus MU) eluted from the first column but not worked on further.

RESULTS

Analysis of a Bonito Fish Product. We first examined a smoked, dried, and shaved bonito fish (probably *Katsuiconus vagnas*) product, imported from Japan and bought in the United States (Table I). The Japanese name was "hana katsuo". We analyzed three samples by the standard procedure (Table I, no. 1); these gave a mean yield of 340 μmol (25 mg) of MU and 35 μmol (1.8 mg) of urea per kg of fish. Identification of MU and urea in two samples was confirmed by the MS.

The ^1H NMR spectrum in $CDCl_3$ of an MU sample (from the analysis giving 220 μmol of MU/kg) showed a peak attributed to CH_3 at 2.77 ppm (J = 4 Hz, splitting caused by the adjacent NH) and a broad peak at 4.38 ppm attributed to NH. Authentic MU showed identical peaks.

We demonstrated that an intermediate produced by nitrosation of the fish was MNU. An extract of the fish product was nitrosated, but not denitrosated, and the resulting MNU was purified (see Experimental Section) and identified by its ^1H NMR spectrum in $CDCl_3$, where it showed a single peak at 3.23 ppm attributed to CH_3 (Figure 3). Authentic MNU showed a peak at 3.22 ppm.

The fish product was analyzed without nitrosation. The nitrosation–denitrosation step was either completely omitted (Table I, no. 2) or performed without adding nitrite, i.e., the extract was treated only with acid (Table I, no. 3). MU was not detected in either experiment, indicating that it was produced by the nitrite treatment.

We determined the recovery of MU added to the fish product (Table I, no. 4). A 250-g fish sample was mixed with 150 mg of MU (8.11 mmol/kg of fish) and analyzed by the standard procedure. We obtained 1050 μmol of MU/kg of fish. Since this included ~340 μmol of MU/kg that would have been determined in the absence of added MU, recovery of added MU was ~710 μmol/kg, i.e., 9%. The results in a second experiment corresponded to 5% recovery of the added MU. These figures are similar to those for MU recovery after nitrosation–denitrosation in the absence of foodstuff (see Further Checks of the Method, paragraph 3).

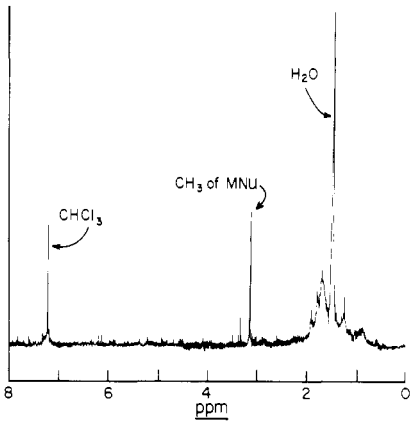

Figure 3. ^1H NMR spectrum in $CDCl_3$ of MNU derived from the bonito fish product. The entire high-pressure LC fraction corresponding to ~200 μg of MNU (see Experimental Section) was dissolved in 40 μL of $CDCl_3$ and subjected to Fourier transform ^1H NMR spectrometry, with 400 transients.

The MU yield was determined after mixing 250 g of fish product with 1.0 g of methylguanidine hydrochloride (Table I, no. 5). After MU arising from the fish was allowed for, the results coresponded to a 12% conversion of methylguanidine into MU, similar to results for the nitrosation–denitrosation of methylguanidine in the absence of foodstuff (see Further Checks of the Method, paragraph 3).

We established in which pH region the MU precursor was eluted from the cation-exchange column (Table I, no. 6-8). After cation-exchange chromatography of an unnitrosated fish product extract, the eluate was combined into fractions eluted at pH 1-7, 7-13.5, and 14. Each fraction was separately nitrosated–denitrosated and analyzed for MU. The results indicate that most MU precursor was eluted at pH 1-7. Under similar column conditions, methylguanidine, as detected by the Sakaguchi reaction, was eluted only by 1.5 N NaOH. Hence, the MU precursor was not methylguanidine.

Analysis of Various Foods, Including Crustaceans (See Table II). The MS were not examined in several of these products because of the low yield of ureas. The common mushroom *Agaricus bisporus* was analyzed without nitrosation; i.e., it was subjected to step 1 without the ether extraction, followed directly by the first column (step 4). We observed urea in the mushroom [confirming an observation by Altamura et al. (1967)] but no alkylureas. The other analyses involved nitrosation–denitrosation and the standard procedure, as for the fish product. Only small amounts of ureas were obtained from red wine, Swiss cheese, salted Schmaltz herring (family Clupeidae), cod fillets (genus *Gadus*), silver smelt fish (genus *Osmerus*), and oysters (probably *Crassotrea virginica*).

After positive results were obtained with the bonito fish product, we thought urea precursors might occur in any high-protein foods preserved and stored at room temperature. Hence, we also examined two meat products, summer sausage and beef jerky, that fulfilled this condition. In both, small amounts of MU were obtained, but the MS were not examined. Both products also yielded "urea X", with an R_f of 0.1 on PC with solvent 1; this could have been citrulline. Shrimp (family Penaeidae) also appeared to

Table II. Ureas from Various Foods

food	procedure used: basis of analysis[a]	procedure used: final step[b]	urea compd: name	urea compd: μmol/kg	MS confirmation[c]
mushroom	PC 1[d]	PC 1	urea	2280	–
swiss cheese	PC 1	PC 1	urea	16	–
red wine[e]	column 1[f]	column 1	total[g]	3	–
schmaltz salted herring	column 1	column 1	total	9	–
cod fillets[h]	column 1	column 1	total	7	–
silver smelt fish[h]	column 1	column 1	total	41	–
oysters[h]	column 1	column 1	total	22	–
summer sausage	PC 1	PC 2	urea	52	–
			MU	14	–
			X[i]	22	–
beef jerky	PC 1	PC 1	urea	15	–
			MU	80	–
			X[i]	96	–
shrimp[h]	PC 1	PC 1	urea	56	–
			MU	24	–
dungeness crab no. 1[h]	PC 3	PC 3	*n*-propylurea plus MU plus 3-butenylurea	90[j]	+
dungeness crab no. 2[h]	PC 3	PC 3	MU plus *n*-propylurea plus 3-butenylurea plus [(4?)-hydroxybutyl]urea	350[k]	+
Alaskan king crab[h]	PC 3	HPLC	3-butenylurea	460	+
slipper lobster tail[h]	PC 2	HPLC	3-butenylurea plus *n*-propylurea	340[l]	+

[a] Step of the procedure on which the determination was based. [b] Final step of the analysis. [c] (+) Confirmed by the MS; (–) MS not examined. [d] PC 1, PC 2, and PC 3 indicate PC with solvents 1, 2, and 3, respectively (see step 6 of the procedure). [e] This was evaporated to dryness before preparing the ethanol extract. [f] First chromatography column (step 4 of the procedure). [g] Total ureas. [h] Purchased as the frozen food. [i] This unidentified urea showed R_f 0.1 on PC with solvent 1. [j] Relative M^+ intensities indicated that this was mainly *n*-propylurea. [k] Results were combined from several PC fractions. [l] Ratio of M^+ intensities indicated only a small proportion of *n*-propylurea.

yield small amounts of MU (MS not examined). For this and other crustaceans, only the edible portions were analyzed.

Dungeness crab (*Cancer magister*) was purchased as a cooked product. Sample 1 was analyzed by the standard method. The urea fraction was subjected to PC with solvents 1 and 2. It traveled at a higher R_f than MU with solvent 2 and was subjected to PC with solvent 3. This separated a fraction with R_f 0.1, containing 88% of the ureas, and a fraction, R_f 0.4, that contained 12% of the ureas and did not give a clear MS. The fraction with R_f 0.1 was identified by its MS as a mixture of *n*-propylurea, MU, and 3-butenylurea.

A second dungeness crab (sample 2) was stored at room temperature for 4 days to induce spoilage and analyzed as for the first crab. The ureas were subjected to PC by solvents 1 and 3. Several fractions were obtained. The MS showed that most fractions were mixtures containing varying proportions of MU, *n*-propylurea, 3-butenylurea, and [(4?)-hydroxybutyl]urea.

Alaskan king crab (*Paralithodes cumtschatica*) was analyzed by the method used for the other crabs, with successive PC by solvents 1 and 3. A fraction with R_f 0.1 in solvent 3 was subjected to high-performance LC; its MS showed 3-butenylurea. A fraction with R_f 0.35 in solvent 3 was not analyzed. Slipper lobster tail, imported from Japan, was also examined. According to the U.S. importer, this was probably spiny lobster (*Ibaccus cilatus*). A sample was treated by the method used for the crabs and subjected to PC by solvents 1 and 2 and to high-performance LC. The MS of fractions with R_f 0.3 in solvent 1 and R_f 0.50–0.63 in solvent 2 revealed mainly 3-butenylurea, with a small proportion of *n*-propylurea.

In summary, of the crustaceans analyzed, shrimp appeared to yield small amounts of MU, but the MS was not examined. Dungeness crab yielded *n*-propylurea, 3-butenylurea, [(4?)-hydroxybutyl]urea, and MU. Alaskan king crab and slipper lobster tail yielded mainly 3-butenylurea. The total amount of ureas obtained from the crabs and lobster (90–460 μmol/kg) was in the same range as that for MU obtained from the bonito fish product.

Table III. Analysis of Bacon

sample no.	brand	fried[a]	nitrosated[a]	MU, μmol/kg	urea, μmol/kg
1	A	+	+	67[b,c]	
2	A	+	+	320[b]	14
3	A	+	+	720[b]	63
4	B	+	+	280[b]	30
5	B	+	+	380[b]	32
6	A	–	+	9[b]	12
7	B	–	+	0	4
8	B	+	–	0	50[b]
9	A	+	–	0	210[b]
10	B	+	–	0	110[b]

[a] (+) Operation was performed; (–) operation was not performed. [b] Identity was confirmed by the MS. [c] The MS indicated a small proportion of urea.

Analysis of Bacon. We analyzed two brands of bacon (Table III). For samples 1–5, ~900 g of bacon was fried in its own fat in a stainless steel electric frying pan set at 165 °C, until the bacon was crisp but not overdone. The fried bacon was blotted and it then weighed ~200 g. Each of these samples were subjected to the standard procedure, involving nitrosation–denitrosation, a single desalting column, PC by solvent 1, and high-performance LC. All five samples yielded MU [mean, 350 μmol (26 mg) per kg of fried weight] and urea. Identification of all MU samples and one urea sample was confirmed by the MS.

In samples 6 and 7, 900 g of bacon was analyzed without frying, by the method employed for the fried bacon. One sample yielded 9.3 μmol of MU/kg (with the identity confirmed by the MS), corresponding to ~42 μmol of MU/kg if the bacon had been fried and MU had remained in the meat. The second sample did not yield MU. These results suggested that most of the MU precursor was produced during frying.

In samples 8–10, fried bacon was analyzed without the nitrite treatment. In sample 8, nitrosation–denitrosation (step 2) was entirely omitted. In samples 9 and 10, the

Table IV. Mass Spectra of Some Simple Alkylureas[a]

ion	ions, shown as m/z (rel intensity)					
	urea	methylurea	ethylurea	*n*-propylurea	isopropylurea	*n*-butylurea
M^+	60 (100)	74 (89)	88 (60)	102 (28)	102 (11)	116 (7)
$(M - CH_3)^+$	ND[b]	ND	73 (58)	87 (25)	87 (55)	101 (2)
$(M - NH_2)^+$	*c*	58 (16)	72 (2)	ND	ND	ND
$(M - NH_3)^+$	*d*	57 (7)	71 (6)	85 (0.3)	85 (1)	ND
$(M - C_2H_5)^+$	ND	ND	ND	73 (40)	73 (2)	87 (10)
$(M - C_3H_7)^+$	ND	ND	ND	ND	ND	73 (33)
$(M - CH_3 - NH_3)^+$	ND	42 (3)	56 (11)	ND	ND	ND
$(M - CONH_2)^+$	16 (14)	*e*	44 (43)	58 (5)	58 (12)	72 (1)
$(M - CH_3 - CONH)^+$	ND	16 (5)	*e*	44 (13)	44 (100)	ND
$(M - CH_3 - CONH_2)^+$	ND	15 (16)	29 (4)	43 (2)	43 (5)	ND
$(M - NHCONH_2)^+$	ND	ND	29 (12)	43 (3)	43 (9)	57 (2)
$(H_2NCO)^+$	44 (72)	44 (34)	44 (28)	44 (11)	44 (8)	44 (9)
$(HNCO)^+$	43 (26)	43 (15)	43 (13)	43 (5)	43 (4)	43 (5)
$(CH_3NH)^+$	ND	30 (100)	30 (100)	39 (100)	30 (2)	30 (100)

[a] Measured as described under Experimental Section. [b] ND, not detected. [c] See $(H_2NCO)^+$. [d] See $(HNCO)^+$. [e] See $(CH_3NH)^+$.

entire standard procedure was performed, but $NaNO_2$ was not added in step 2. MU was not detected in any of these samples.

Identification of Alkylureas by Their MS. MS peaks are listed as m/z values, with the relative abundance and assumed structure in parentheses.

(1) *MS of Authentic Alkylureas.* Table IV compares the MS of five purchased or synthesized ureas. All showed prominent molecular ion (M^+) peaks. Fragmentation occurred by loss of methyl, ethyl, etc., loss of $CONH_2$ to give RNH^+, and loss of NH_2 and NH_3 (probably to give the cyanate, $RNCO^+$). The cyanate peak was minor but useful, since it indicated whether unknown ureas were substituted on one nitrogen only.

(2) *MS of Ureas Isolated from Foods.* (a) *MS of Urea.* The MS of urea, obtained from the bonito fish sample with 16 mg of MU and 2.1 mg of urea/kg (Table I, no. 1), showed peaks at m/z 60 (100, M^+), 44 (82, NH_2CO^+), and 43 (24, $NHCO^+$), as for the MS of pure urea (Table IV).

(b) *MS of MU.* The MS of MU isolated from the bonito fish product sample with 16 mg of MU/kg (Table I, no. 1) showed peaks at m/z 74 (59, M^+), 58 (14, CH_3NHCO^+), 44 (35, $CONH_2^+$), 43 (9, $HNCO^+$), and 30 (100, CH_3NH^+). This spectrum was almost identical with that of authentic MU, shown in Table IV. The MS of all MU samples obtained from the fried bacon (Table III) were also almost identical with that of authentic MU. For example, the MS of MU obtained from sample no. 3 (Table III) showed peaks at m/z 74 (73), 58 (26), 44 (59), 43 (10), and 30 (100).

(c) *MS of n-Propylurea.* This MS was most clearly observed in a sample obtained from dungeness crab no. 2 after PC with solvent 1 (R_f 0.75) and solvent 2 (R_f 0.70). The MS also showed 3-butenylurea and MU, with an M^+ intensity ratio for propylurea, butenylurea, and MU of 100:6:33, respectively. Peaks attributed to *n*-propylurea occurred at m/z 102 (14, M^+), 87 [12, $(M - CH_3)^+$], 85 (1.3, $C_3H_7NCO^+$), 44 (12, NH_2CO^+), 43 (12, $C_2H_5N^+$), 43 (16, $HNCO^+$), and 30 (100, CH_3NH^+). Prominent peaks not attributed to ureas also occurred at m/z 71 (27, $C_5H_{11}^+$ and $C_4H_7O^+$), 70 (22), and 57 (21, $C_4H_9^+$). The position and intensity of the *n*-propylurea peaks closely resembled those of authentic *n*-propylurea and were different from those of isopropylurea (see Table IV).

(d) *MS of 3-Butenylurea.* The cleanest MS of this urea was obtained from the sample of slipper lobster tail after PC with solvent 1 (R_f 0.30), PC with solvent 2 (R_f 0.63), and high-performance LC. The MS showed peaks at m/z 114 (8.9, M^+), 113 [21, $(M - H)^+$], 112 [9.1, $(M - 2H)^+$], 86 [8.6, $(M - C_2H_4)^+$], 73 (3.0, $NH_2CONHCH_2^+$), 70 [100, $CH_2{=}CH(CH_2)_2NH^+$], 69 [20, $CH_2{=}CH(CH_2)_2N^+$], 68 (20, $C_4H_6N^+$), 44 (7.0, NH_2CO^+), 43 (64, $HNCO^+$), 41 (20, $CH_2{=}CHCH_2^+$), 30 (8.2, CH_3NH^+), and 28 (21, CH_2N^+). A urea group was indicated by the positive response in the urea test. The molecular formula, $C_5H_{10}N_2O$, suggested the presence of a C=C double bond. The absence of peaks due to loss of CH_3 indicated that the double bond was at the terminal position and that there were no NCH_3 groups (e.g., the compound was not a propenylmethylurea). The loss of C_2H_4 (to give a peak at m/z 86) is characteristic of terminal ethylenic groups (Budzikiewicz et al., 1967). The base peak at m/z 70 indicated that a butenyl group was attached to nitrogen.

(e) *MS of [(4?)-Hydroxybutyl]urea.* This was detected in a fraction derived from dungeness crab no. 2. The MS of this fraction also showed *n*-propylurea and MU. The intensity ratio of the $(M - 1)^+$ peak for the (hydroxybutyl)urea to the M^+ peaks of *n*-propylurea and MU was (in order) 7:10:4. Peaks due to HCl were also present. Peaks attributed to [(4?)-hydroxybutyl]urea occurred at m/z 132 (1.0, M^+), 131 [9.4, $(M - 1)^+$, $NH_2CONH(CH_2)_3CH{=}OH^+$], and 115 [32, $HOCH_2(CH_2)_3NCO^+$]. The prominent $(M - 1)^+$ peak is characteristic of alcohols (Budzikiewicz et al., 1967). These fragments supported the structure as a (hydroxybutyl)urea. The absence of a $(M - CH_3)^+$ peak suggested that the OH was at the terminal carbon.

DISCUSSION

Our results should be regarded as of comparative value only and not as a quantitative analysis, since we do not know the efficiency of conversion of the unidentified precursors into alkylureas. The conversion efficiency might differ from the recovery of MU added to food, which was 5–9% in the case of the bonito fish product (Table I, no. 4). The recovery of alkylureas in two critical steps after denitrosation was shown to be nearly quantitative.

After nitrosation–denitrosation of food extracts, we obtained MU (mainly from a bonito fish product and fried bacon), *n*-propylurea (mainly from dungeness crab), 3-butenylurea (from dungeness crab, king crab, and slipper lobster tail), and [(4?)-hydroxybutyl]urea (from dungeness crab). For the bonito fish product, MNU was shown to be an intermediate product. Our negative results with silver smelt fish, cod fillet, and salted herring indicated that MU was not produced from all fish species. In this connection, a Japanese product prepared from the mackerel fish "sanma hiraki" was nitrosated to yield a direct-acting mutagen, which was not MNU (Marquardt et al.,

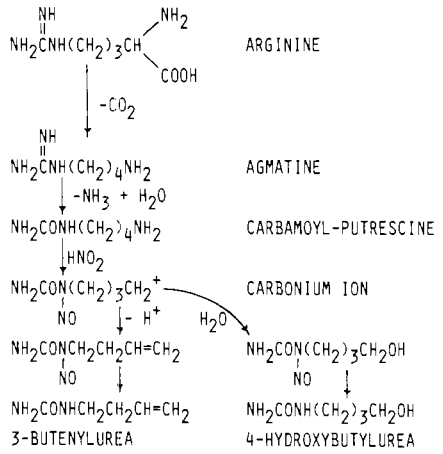

Figure 4. Postulated origin of 3-butenylurea and (4-hydroxybutyl)urea by the nitrosation of *N*-carbamoylputrescine.

1977a,b; Weisburger et al., 1980). When an extract of the nitrosated fish was fed to rats, it produced tumors of the glandular stomach, resembling human gastric cancer (Weisburger et al., 1980).

The origin of *n*-propylurea is of particular interest, since *n*-propyl groups have rarely, to our knowledge, been identified in natural products. 3-Butenylurea and (4-hydroxybutyl)urea could have arisen by the nitrosation of *N*-carbamoylputrescine, which can be produced from arginine or agmatine by certain plants and bacteria (Moller, 1955; Smith and Richards, 1962; Smith and Garraway, 1964; Kleczkowski and Wielgat, 1968) (Figure 4). Agmatine is found in some invertebrates but has not been reported to occur in crustaceans (Kawabata et al., 1978).

MU was not obtained from bonito fish product or fried bacon when treatment with nitrite and acid was omitted or when the extracts were treated with acid alone (Table I and III). This indicates that the unnitrosated foods did not contain MU and that MU was not produced merely by the acid treatment. The results for bacon also indicate that any residual nitrite in fried bacon was insufficient to produce detectable MU during the acid treatment. The absence of MU in these experiments agrees with the finding by Kawabata et al. (1980) that simple alkylureas did not occur in any of 57 samples of Japanese foods. [Their method of analysis involved successive column chromatography on Dowex 50 (H^+ form), Dowex IX (OH^- form), and basic alumina, followed by gas chromatography of the (dimethylamino)methylene derivatives of the ureas.] The urea compounds known to occur naturally possess other functional groups, e.g., citrulline, carbamoylputrescine, and 5,6-dihydrouracil (Figure 4; Mirvish, 1972).

Foodstuffs, such as the bonito fish product and fried bacon, might be involved in the etiology of human gastric cancer, as discussed in the introduction. Such foodstuffs could react with nitrite to yield nitrosoureas either during food storage or in the stomach. The first possibility may not be relevant to carcinogenesis, because nitrosoureas may be unstable during food storage. The second possibility should be more seriously considered. A complex scheme involving two reactions with nitrite is postulated for the formation of 3-butenylnitrosourea and (4-hydroxybutyl)-nitrosourea (Figure 4), if these are indeed the immediate precursors of the corresponding ureas. This complexity considerably lessens the chance that these reactions are environmentally significant. Even if MNU and *n*-propylnitrosourea are the immediate precursors of the corresponding ureas in all the foods analyzed (as seems probable), the formation of these nitrosoureas is less likely to be significant, if a similar complex series of reactions is required for their production. This may apply especially because we used a very large excess of nitrite for the nitrosation.

Further research is needed before the relevance of our findings can be evaluated. We should answer the following questions: What are the alkylurea precursors? How many foodstuffs yield alkylureas after nitrosation–denitrosation? Does nitrosation–denitrosation of all fried foods yield MU? Are alkylnitrosoureas the intermediate nitrosation products in all cases?

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Fatty Acid Analysis on Short Glass Capillary Columns

Elaine Lanza,*[1] John Zyren,[2] and Hal T. Slover[1]

Analyses of fatty acid methyl esters (FAME) were compared on 100-, 10-, and 2-m glass capillary columns coated with SP2340. The accuracy and precision for the analysis of FAME standards were comparable for all three columns. When actual food samples were chromatographed, the 100-m column gave superior resolution of the many positional and geometric isomers in hydrogenated vegetable oils and ruminant animal fats; however, analysis times were 90–135 min/sample. The 10-m column was adequate for quantitation of major fatty acids, but some minor acids were not detected. Analysis time ranged from 5 to 30 min depending on the sample and the chromatographic conditions. Low resolution made the 2-m column undesirable, even though the major fatty acids could be separated in less than 3.5 min. Quantitative data from the analysis of peanut oil, rapeseed oil, shortening, cod liver oil, pork, beef, and beef liver samples on 100- and 10-m columns are compared, and the characteristics of the three columns are discussed.

In order to evaluate current dietary habits, plan and execute nutritional research, and implement current recommendations on dietary fats, nutritionists need detailed data on the amounts and kinds of fatty acids in foods consumed in the United States. Collection of such data would require quantitative analysis of a vast number of foods. The use of packed column gas chromatography (GC) for the quantitative analysis of fatty acids in foods presents problems. (1) Choice of an appropriate internal standard is difficult because complex foods, such as cheeseburgers, contain so many fatty acids. In the absence of an internal standard, fatty acids can be quantitated only as normalized weight percent rather than as the more useful absolute amounts. (2) Resolution of positional isomers of unsaturated fatty acids is inadequate. (3) Separation of the cis and trans isomers is incomplete so the percent trans fatty acids cannot be estimated. The high efficiency of long (60 and 100 m) glass capillary columns coated with SP2340 solved these problems (Slover & Lanza, 1979): (1) separation between peaks was adequate for insertion of the internal standard, methyl heneicosanoate; (2) many positional isomers, particularly the octadecenoate isomers, can be resolved; (3) separation of the geometric isomers was adequate for estimation of the amount of trans fatty acids. However, the analysis took 90–135 min, depending on the sample. Recently, Ettre & March (1974), Johansen (1977), and Rooney et al. (1978) described significant reductions in analysis time with short glass capillary columns. Unfortunately, these authors explored mainly the qualitative aspects of this technique. The purpose of our investigation was to study the use of short capillary columns for the quantitative analysis of fatty acids in foods and the compromises which have to be made between analysis speed and resolution.

MATERIALS AND METHODS

Gas Chromatography. A Hewlett-Packard Model 5840 gas chromatograph equipped with a flame ionization detector, a Hewlett-Packard Model 7671A automatic liquid sampler, and a J & W Scientific, Inc. (Orangevale, CA) glass capillary column splitter were used. All SP2340 glass capillary columns were purchased from Quadrex Corp. (New Haven, CT). SP2340 is a cyanosilicone phase which has chromatographic properties similar to those of polar polyesters. Helium was used as the carrier gas for all analyses. Chromatographic conditions appear in Table II.

Sample Preparation. *Extraction*: All foods were extracted with chloroform–methanol as described by Floch et al. (1957). *Methylation*: Fatty acids were methylated by a modification of the procedure of Metcalfe et al. (1966). For quantification, an internal standard, methyl heneicosanoate, was added to each sample.

Nomenclature. In this paper the designation used for fatty acid identity is the following: the number before the colon gives the number of carbon atoms in the chain; the

U.S. Department of Agriculture, Science and Education Administration, Human Nutrition, Beltsville Human Nutrition Research Center, Nutrient Composition Laboratory, Beltsville, Maryland 20705.
[1]Nutrient Composition Laboratory, Nutrition Institute, Human Nutrition Center, SEA, USDA, Beltsville, MD 20705.
[2]National Food Processors Association, 1133 20th Street, N.W. Washington, DC 20036.