Creatinine: A Food Component That Is Nitrosated-Denitrosated To Yield Methylurea

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In a previous study, nitrosation-denitrosation of a dried fish product and of fried bacon yielded 25 mg/kg methylurea (MU). The MU precursor in the fish product was studied by using production of ureas as an assay. The precursor was isolated by three chromatographic systems and identified as creatinine (CRN). Nitrosation-denitrosation of CRN gave a 2.7% yield of MU, but similar treatment of creatine yielded <0.2% of total ureas. Under milder conditions, CRN yielded 0.05% of total ureas, and CRN 5-oxime and 1-methylhydantoin 5-oxime, known products of CRN nitrosation, yielded 0.04% and 7.0% MU, respectively. The fish product contained 4.1 g/kg CRN and fried bacon, 3.3 g/kg. It appears that creatine in meat and fish is dehydrated to CRN under anhydrous conditions. The findings are related to the hypothesis that gastric cancer is due to intragastric production of nitrosoureas.

Carcinogenic nitrosamides, including nitrosoureas, might be produced under the acidic conditions of the stomach and might be a factor in the causation of gastric cancer in man (Mirvish, 1971, 1977; Correa et al., 1975; Haenszel and Correa, 1975; Weisburger, 1979; National Academy of Sciences, 1981). This view appears reasonable because nitrosamide formation from nitrite and amides is an acid-catalyzed reaction (Mirvish, 1971), nitrosamides are direct-acting carcinogens that might act in the stomach if produced there, and in rodents nitrosamides [including methylnitrosourea (MNU)] and especially the related compound 1-methyl-3-nitro-1-nitrosoguanidine have induced glandular stomach tumors resembling human gastric cancer (Druckrey et al., 1971; Sugimura and Kawachi, 1973). In addition, gastric cancer incidences in different countries are positively correlated with exposure to nitrate (which is partly converted in vivo into gastric nitrite) and negatively correlated with the consumption of fresh fruits and vegetables containing ascorbate, which inhibits nitrosation by reducing nitrite (Haenszel and Correa, 1975; Mirvish, 1981; National Academy of Sciences, 1981).

Dried, salted fish products and (much more tentatively) fried bacon have been associated with gastric cancer (Haenszel and Correa, 1975) and hence might contain amides or related nitrosamide precursors. These and other foods do not contain alkylureas that could yield nitrosoureas directly (Mirvish et al., 1980; Kawabata et al., 1980). However, when these foods were treated with a very large excess of nitrite at pH 1 to produce nitrosoureas, and then with strong acid to convert nitrosoureas to ureas, they yielded about 25 mg of methylurea (MU)/kg of food (Mirvish et al., 1980). The process was termed "nitrosation-denitrosation" and the results indicated that these foods contain MU precursors. Nitrosation of the fish product without denitrosation produced MNU.

We report here on the identification of creatinine (CRN) as an MU precursor in the fish product, on some analyses of foods for CRN and creatine (CRE), and on data related to intermediates involved in CRN conversion to MU. An abstract of some of these results has been published (Mirvish and Cairnes, 1981).

EXPERIMENTAL SECTION

Most procedures followed those used by Mirvish et al. (1980). Mass spectra (MS) were measured with a modified AEI MS-902 mass spectrometer (by using a direct probe at 220 °C), linked to computer analysis of each peak. Cited compositions agreed with the measured masses to within 3 mmu. $A \pm B$ indicates mean ± SD.

(A) Materials. CRN (mp 254 °C) and CRE monohydrate (mp 292 °C) were obtained from Eastman Kodak Co., Rochester, NY. To prepare CRN 5-oxime, CRN (6.1 g, 54 mmol) was nitrosated as by Archer et al. (1971). The temperature of the nitrosation mixture reached 70 °C. After the reaction, 55 mL of water was added to prevent crystallization of the more water soluble 1-methylhydantoin 5-oxime, which was also produced, and the solid that separated was triturated with 3 × 55 mL of water and recrystallized from hot water, to give 1.5 g (19%) of product. The mp was 255 °C dec (cf. 259 °C reported by Archer et al.). 1-Methylhydantoin 5-oxime was synthesized as described by Archer et al. (1971) and showed mp 180 °C (cf. reported mp of 181 °C).

The UV, IR, and ^{1}H NMR spectra (the last in NaOD-D_2O) of the two oximes agreed with those reported by Archer et al. The ^{1}H NMR spectra in dimethyl sulfoxide were as follows: for CRN, δ 2.89 (CH_3), 3.29 (CONH), 3.65 (CH_2), and 7.45 (C=NH); for CRN 5-oxime, δ 3.31 (CONH), 3.49 (CH_3), and 8.2 (C=NH); for 1-methylhydantoin 5-oxime, δ 2.92 (CONH) and 3.36 (CH_3). The ^{1}H NMR spectra proved the most useful for checking purity.

The MS of the two oximes agreed with those given by Archer et al. (1971), who did not record the ion formulas. We found the following m/z values, relative intensities, and probable ion formulas (in that order): for CRN 5-oxime, 142 (67, M^+, $C_4H_6N_4O_2$), 126 [57, $(M-O)^+$], 125 [100, $(M-OH)^+$], 112 [31, $(M-NO)^+$], 99 [45, $(M-HNCO)+$], 72 (39, $C_2H_4N_2O^+$), 69 (45, $C_2HN_2O^+$), and 57 (24, $C_2H_4N_2^+$); for 1-methylhydantoin 5-oxime, 143 (55, M^+), 127 [27, $(M-O)^+$], 126 [100, $(M-OH)^+$], 113 [11, $(M-NO)^+$], 84 (17, $C_3H_4N_2O^+$), 83 (14, $C_3H_3N_2O^+$), 72 (52, $C_2H_4N_2O^+$), and 70 (39, $C_2NO_2^+$).

The foods used were shaved, dried bonito fish product imported from Japan ("hana katsuo"), denoted "the fish product", bacon (three brands), beef hamburger meat, and beef jerky (one brand), all purchased locally.

(B) Purification and Identification of an MU Precursor in the Fish Product. This is summarized in Figure 1.

Detection of Precursors. The purification was monitored by subjecting aliquots (usually 10% of the sample) to nitrosation-denitrosation, as follows. To an aliquot in 15 mL of water was added 1 g of $NaNO_2$ (in six equal lots) over 3 h at room temperature. After each addition, the pH was adjusted to 1 by using pH paper. The solution was

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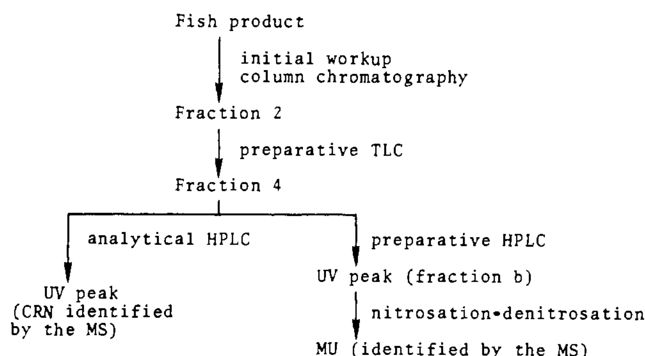

Figure 1. Summary of the purification of the MU precursor and its identification as CRN.

left for 18 h at 0 °C, reacted with 0.45 g of ammonium sulfamate, brought to pH 0 with HCl, stored at 6 °C for 2 days, evaporated, and dissolved in 1 mL of methanol. Aliquots (e.g., 0.2 mL) were analyzed colorimetrically for ureas, as in Mirvish et al. (1980). The presence of salts made these results only semiquantitative.

Initial Workup. A 250-g sample of the fish product was homogenized in a Waring blender with 1.6 L of 95% ethanol. The extract was evaporated under vacuum with a rotary evaporator to remove any volatile nitrosamines. The residue was dissolved in 50 mL of water, adjusted to pH 1 with $HClO_4$, and left overnight to precipitate proteins. These were filtered off, and the filtrate was basified with NaOH and extracted with 3 × 250 mL of ether. The aqueous phase was adjusted to pH 7 with NaOH and extracted with 4 × 200 mL of 1-butanol. The extract was evaporated at <60 °C to give 7.7 g of residue.

Column Chromatography. The residue was dissolved in 150 mL of ethanol, 10 g of silica gel 60 (70–230 mesh, J. T. Baker and Co., Phillipsburg, NJ) was added, and the solvent was evaporated. The resulting powder was added to a column (53 × 3 cm), previously packed with 80 g of silica gel as a slurry in $CHCl_3$–CH_3OH (1:1). The column was eluted with 250 mL each of 2.5, 5, 7.5, 10, 20, and 50% concentrated (58%) NH_4OH in $CHCl_3$–CH_3OH (1:1). Fractions of 250 mL were collected, evaporated, and dissolved in 15 mL of CH_3OH, and 1–2-mL aliquots were analyzed for the precursor.

Thin-Layer Chromatography (TLC). A methanolic solution of the urea-yielding fraction (no. 2) from the previous step was applied as streaks to eight preparative TLC plates (200 × 2 mm, silica gel GF-254, Brinkmann Instruments Co., Westbury, NY). The plates were developed twice with $CHCl_3$–CH_3OH–58% NH_4OH (49:49:2). Bands were detected by their absorption of long- or short-wavelength UV light, and scraped off. Corresponding bands from each plate were combined and eluted with 100 mL of $CHCl_3$–CH_3OH–58% NH_4OH (5:5:1). The eluates were evaporated and dissolved in CH_3OH.

High-Performance Liquid Chromatography (HPLC). Analytical HPLC was performed on a LiChrosorb SI-60 (5-μm) column (250 × 3.2 mm, Altex Scientific, Inc., Berkely, CA), with development by cyclopentane–CH_3OH–ether (87:11:2) at 1.5 mL/min. Preparative HPLC was performed on a 1 × 25 cm column of LiChrosorb SI-60 (10 μm), which was eluted with cyclopentane–CH_3OH–ether (78:20:2) at 6 mL/min and gave fractions a, b, and c (see Results). Detection was by UV absorption at 206 nm.

(C) Identification of the Urea Obtained by Nitrosation–Denitrosation of Preparative HPLC Fractions. Each fraction from the preparative HPLC performed as described under section B was evaporated and dissolved in 50 mL of water. The entire solution was subjected to nitrosation–denitrosation as described under section B, Detection of Precursors (but with 10 g of $NaNO_2$ and 4.5 g of ammonium sulfamate), and evaporated. The residue was dissolved in 50 mL of water and extracted with 3 × 25 mL of ether and then 3 × 25 mL of 1-butanol. The butanol extract was evaporated at <60 °C. The residue was heated at 80 °C and 2 torr for 10 min to remove interfering substances and dissolved in 25 mL of water. One milliliter was analyzed for ureas.

Ureas in the remaining 24 mL of the solution derived from HPLC fraction b were purified as before (Mirvish et al., 1980) by using the urea test to follow the purification. To do this, the solution was adjusted to pH 1 and applied to a column of Bio-Rad AG-50W-X8 cation-exchange resin (100–200 mesh, 2.3-cm diameter, 25-cm height, H^+ form, Bio-Rad Laboratories, Richmond, CA), which was eluted with 500 mL of water and 1 L 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 were determined. The urea-containing eluates (which had pH 2–5) were combined and evaporated.

The urea-containing fraction was further purified by descending paper chromatography. Half the residue (dissolved in methanol) was applied as a strip 10 cm from the end of a Whatman 3 MM paper (15 × 56 cm, previously washed with the developing solvent), and this was developed with 1-propanol–58% NH_4OH (9:1 v/v) for 18 h (some solvent ran off the paper). Urea traveled 12 cm and MU traveled 22 cm in this system. The paper was dried and cut into 3-cm strips, each of which were eluted with 15 mL of methanol. Samples of each fraction were analyzed for ureas. The urea-positive fractions that appeared from their migration rate to contain MU were combined, evaporated, and dissolved in 400 μL of methanol.

A portion (100 μL) of the MU fraction was subjected to HPLC on a silica gel column (25 cm × 3.2 mm i.d., 5-μm LiChrosorb 60 from E. Merck and Co., Rahway, NJ) with cyclopentane–methanol–ether (90.5:7.5:2.0) as the solvent. The flow rate was 2 mL/min and 1-min fractions were collected. Analysis of aliquots showed a urea-containing fraction eluting at 14–16 min. This was subjected to mass spectrometry, which identified it as MU.

(D) MU Production from CRN and Related Compounds. CRN, CRE, CRN 5-oxime, and 1-methylhydantoin 5-oxime were nitrosated as described under section B, Detection of Precursors, by using the amounts and volumes listed under Results. The solutions were denitrosated as described under section B by using an amount of ammonium sulfamate equal to half the weight of the $NaNO_2$. The resulting MU was purified as described under section C, except that, during evaporation of the butanol extract, salts that precipitated were filtered off.

(E) CRN and CRE Determination in Foods. Bacon strips and hamburger patties were fried until well done in an electric frying pan set at 160 °C, without added fat. Some bacon was fried to a similar appearance in a microwave oven. Ten grams of fish product, fried bacon, fried hamburger, or beef jerky was homogenized in 100 mL of water in a Waring blender for 3 min, cooled, reblended after adding 25 mL of saturated picric acid solution, and filtered through Celite. Total CRN plus CRE was determined by the Jaffe reaction after the solution containing picric acid was boiled for 40 min. CRN alone was determined similarly but without boiling. In both cases we used the method for urine (without evaporation of the sample) in Snell and Snell (1970, p 14); this involved making the

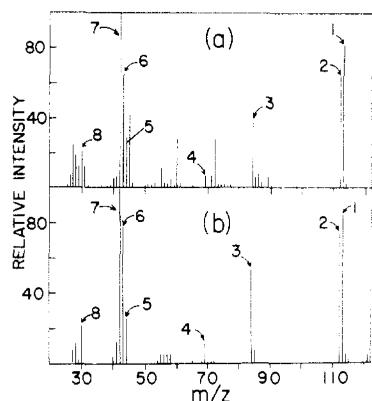

Figure 2. MS of (a) the chief UV-absorbing peak obtained by HPLC of the fish product extract and (b) authentic CRN. The numbers in (a), m/z values, and probably ion formulas of peaks attributed to CRN were as follows (in that order): 1, 113, $C_4H_7N_3O^+$, M^+; 2, 112, $(M-H)^+$; 3, 84, $C_3H_6N_3^+$; 4, 69, $C_2HN_2O^+$; 5, 44, $C_2H_6N^+$; 6, 43, $C_2H_5N^+$ and $CH_3N_2^+$; 7, 42, $C_2H_4N^+$; 8, 30, CH_4N^+. Peaks at m/z 72, 60, and 45 were not derived from CRN. (b) shows the MS of authentic CRN, in which numbered peaks had the same m/z values and probable ion formulas as the corresponding peaks in (a).

picric acid solutions alkaline and determining absorbance at 540 nm. Raw bacon or raw hamburger meat (25 g) was homogenized in 250 mL of 95% ethanol and filtered through Celite. The filtrate was evaporated at 30 °C and partitioned between 100 mL of water and 250 mL of *n*-hexane, and the aqueous phase was analyzed as before after adding picric acid.

The recovery of CRN from 10–20 g of fish product or fried bacon, spiked with 100–200 mg of CRN, was 105 ± 10% for seven analyses. Addition of CRN did not increase the results for CRE and vice versa for addition of CRE. Six picric acid containing filtrates (from one fried fish, one fried bacon, and four fried hamburger samples) were also analyzed for CRN by HPLC, with the system described under section B. (Picric acid was eluted at 3.5–7.0 min.) These analyses gave CRN values that were 91 ± 10% of those measured colorimetrically.

RESULTS

Purification and Identification of an MU Precursor in the Fish Product (See Experimental Section B and Figure 1). The fish product (250 g) was extracted and subjected to column chromatography on silica gel. Fraction 2 (dry weight 2.2 g), which was eluted with 5% ammonia in a 1:1 chloroform–methanol mixture, was the principal fraction to produce ureas (total yield 3.8 μmol). This fraction was subjected to preparative TLC. Seven UV-absorbing bands were observed and their eluates were analyzed. The fourth and sixth bands from the origin (fraction 4, R_f 0.5, dry weight 0.34 g; fraction 6, R_f 0.8) produced ureas. Fraction 6 was not worked on further.

Five percent of TLC fraction 4 was subjected to analytical HPLC. About 75% of the UV absorbance appeared as a single peak (retention time, 29 min). Figure 2 shows the MS of the corresponding fraction, which identified it as CRN. HPLC of a mixture of authentic CRN and TLC fraction 4 showed an increase in the main UV-absorbing peak, with no new peaks.

To determine whether the main UV-absorbing fraction obtained on HPLC contained the urea precursor, we subjected one-third of TLC fraction 4 to preparative HPLC. As before, a single principal UV-absorbing fraction was obtained (fraction b, 70 mL; retention time, 12–23 min). Fraction a (70 mL) refers to the eluate collected before and fraction c (130 mL) to the eluate collected after

Table I. MU Yields from the Nitrosation–Denitrosation of CRN and Related Compounds[a]

expt no.	compound	MU yield, %
1	CRN[b]	1.6,[c] 3.7[c]
2	CRE[d]	0.08, 0.19
3	CRN[e]	0.004, 0.04
4	CRN 5-oxime[e,f]	0.04,[c] 0.04[c]
5	1-methylhydantoin 5-oxime[e]	5.9,[c] 8.2[c]

[a] Experimental Section D describes the methods used. Each result refers to a separate experiment. [b] A solution of CRN (463 mg, 4.1 mmol) in 700 mL of water was nitrosated with 100 g (1.45 mol) of $NaNO_2$ and then denitrosated. [c] Identity was confirmed by the MS, which gave spectra typical of MU. [d] A solution of CRE hydrate (611 mg, 4.1 mmol) in 700 mL of water was nitrosated-denitrosated as in footnote *b*. Total urea yields of 0.08% and 0.19% were measured after the desalting ion-exchange column, and the workup was stopped at this point. [e] A solution or suspension of 5 mmol of compound in 200 mL of water was nitrosated with 1.38 g (20 mmol) of $NaNO_2$ and then denitrosated. [f] Urea was also separated by the paper chromatography and identified by its MS. Its yield was 70% of that for MU.

the UV-absorbing fraction. The peak area of fraction b corresponded to 400 mg of CRN/kg of fish product (from a comparison with HPLC of authentic CRN).

Identification of Ureas Obtained from Preparative HPLC Fractions (See Experimental Section C). Each fraction of the preparative HPLC was subjected to nitrosation–denitrosation, and the ureas were determined on 1-mL samples (out of 25 mL). On the basis of these analyses, nitrosation–denitrosation of the entire 25 mL derived from each of fractions a, b, and c yielded 0.17, 0.73, and 0 μmol of ureas, respectively. Hence, most of the ureas were derived from the CRN peak. The ureas in the remainder of the nitrosated–denitrosated fraction b were purified. Paper chromatography separated a urea-compound fraction, which from its mobility was probably urea, and one that traveled 22 cm, as expected for MU. The latter fraction was further purified by HPLC and its MS was measured. The m/z value, relative intensity, and probable ion formula of the principal peaks (in that order) were 74, 91, and $C_2H_6N_2O^+$, 58, 24, and $C_2H_4NO^+$, 44, 52, and CH_2NO^+, 31, 54, and CH_5N^+, and 30, 100, and CH_4N^+, similar to the MS of MU reported by Mirvish et al. (1980). Hence, the CRN fraction yielded MU and probably also urea. The amounts of the ureas determined after paper chromatography (which are usually accurate) corresponded to yields from CRN of 1.3% for MU and 0.9% for urea.

Nitrosation–Denitrosation of CRN and Other Compounds (See Experimental Section D). Nitrosation–denitrosation of authentic CRN under the conditions used for treating foods (which involved the use of 2.7 M nitrite) gave an average MU yield of 2.7% (Table I, experiment 1). The identity of the MU was confirmed by its MS. Similar treatment of CRE gave <0.2% yield of total ureas, and MU was not shown to be present (Table I, experiment 2).

CRN is known to be nitrosated to give CRN 5-oxime and 1-methylhydantoin 5-oxime (Figure 3) (Greenwald and Levy, 1948; Archer et al., 1971), and these could be intermediates in the production of MU. Accordingly, we nitrosated samples of these oximes and also of CRN, this time using only 100 mM nitrite. The MU yield followed the order methylhydantoin oxime > CRN oxime ≈ CRN (Table I, experiments 3–5). CRN oxime was also shown to yield urea.

Determination of CRN and CRE in Foods (See Experimental Section E). Our results showed that CRN

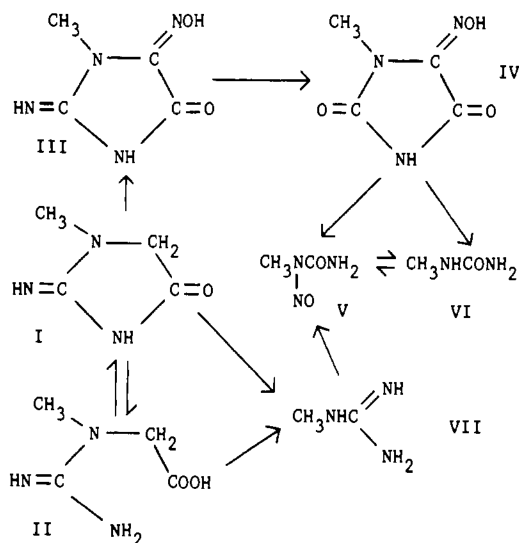

Figure 3. Possible routes whereby nitrosation-denitrosation of CRN could yield MU. I, CRN; II, CRE; III, CRN 5-oxime; IV, 1-methylhydantoin 5-oxime; V, MNU; VI, MU; VII, methylguanidine.

Table II. Analysis of Foods for CRN and CRE[a]

food product	no. of analyses	CRN, g/kg	CRE, g/kg
bonito fish product	2	4.1 ± 0	2.0 ± 0.3
fried bacon			
brand A	2	3.3 ± 0.1	0.3 ± 0.4
brand B	5	3.2 ± 0.4	0.6 ± 0
bacon fried in microwave oven[b]	2	3.2 ± 1.0	0.6 ± 0.1
raw bacon			
brand A	3	0.09 ± 0.01	1.1 ± 0.1
brand B	3	0.09 ± 0.09	1.0 ± 0.4
fried hamburger	7	0.54 ± 0.32	2.3 ± 0.5
raw hamburger meat	1	0.03	1.9
beef jerky	4	2.2 ± 0.1	0.8 ± 0.2

[a] Results are given as mean ± SD. [b] One sample each of brands A and B was used.

is a precursor of MU in the fish product and this may also apply to the MU obtained from fried bacon (Mirvish et al., 1980). Accordingly, we determined CRN and CRE in the dried fish product, raw and fried bacon, raw and fried hamburger, and beef jerky. The CRN concentration followed the order fish product > fried bacon > beef jerky > fried hamburger > raw bacon and hamburger meat (Table II).

DISCUSSION

Our results demonstrated that CRN was a major precursor of the MU produced from a dried fish product, when it was nitrosated and then denitrosated. Living vertebrate muscle contains 3–5 g/kg CRE, some of it as CRE phosphate. CRE is dehydrated to CRN in the body and excreted as such in the urine, but the blood CRN level in man is only 0.01–0.02 g/L (Hawk et al., 1954). Our results on the CRN and CRE content of foods (Table II) indicate that CRE is dehydrated to CRN when bacon and, to a lesser extent, beef hamburgers are fried and (presumably) when the dried fish product and beef jerky are manufactured from raw fish or beef. The conversion of CRE to CRN was previously reported to occur during the manufacture of smoke-dried skipjack fish (Kawabata et al., 1980), during storage at 30 °C of nitrite-treated meat (Davidek et al., 1976), and in boiled soup (Carisano et al., 1969). Hence, the main source of dietary CRN may be meat and fish products that have been stored or heated, especially under dehydrating conditions.

Although direct analysis of the fish product showed a CRN content of 4.1 g/kg, we obtained only 400 mg/kg CRN when the MU precursor was purified. This difference probably arose because CRN was hydrolyzed to CRE during the purification. This probably also explains why the precursor disappeared rapidly during early attempts by us to isolate it using purely aqueous systems.

The most likely route for CRN conversion into MU involves the known reaction with nitrite to give CRN 5-oxime and 1-methylhydantoin 5-oxime (Figure 3), since the MU yield was greatest for methylhydantoin oxime (Table I). If the presumed final step, i.e., conversion of methylhydantoin oxime into MU (or MNU), involves a nitrosation, then the entire process entails three successive reactions with nitrite. CRN 5-oxime is probably not itself hazardous, since it was not carcinogenic when injected into newborn mice (Wogan et al., 1975). Another possibility is that methylguanidine is produced by oxidation of CRN during the nitrosation (Endo et al., 1976; Kawabata et al., 1980) and is then nitrosated (via conversion into methylnitrosoguanidine and methylnitrosocyanamide) to give MNU (Mirvish, 1971; Mirvish et al., 1973). However, methylguanidine is also produced by the oxidation of CRE (Endo et al., 1976; Kawabata et al., 1980), which yielded little or no MU (Table I).

We showed by cation-exchange chromatography of a fish product extract that methylguanidine itself was not a precursor of the MU (Mirvish et al., 1980). This was true even though nitrosation of methylguanidine produces MNU and the fish product contained 26 mg/kg methylguanidine [unpublished analysis by K. Karlowski and S. Mirvish, using the method of Fujinaka et al. (1976)]. The latter result was similar to that reported by Fujinaka et al. (1976) for a similar product. The fact that the molar concentration of CRN in the fish product (Table II) was about 100 times that of methylguanidine explains why CRN and not methylguanidine was a principal source of MU.

The production of MU from nitrosated-denitrosated dried fish and fried bacon offers some support for the hypothesis that intragastric nitrosamide production is involved in the etiology of stomach cancer, since MU was probably produced by denitrosation of the carcinogenic MNU formed by nitrosation of CRN. Two points militate against this view: (a) The MU yield from CRN was only 0.022% at a nitrite concentration of 100 mM (Table I, experiment 3), whereas the nitrite level in gastric juice reached 54 μM in subjects from an area with a high gastric cancer incidence (Tannenbaum et al., 1979). (b) The conversion is complex, since three successive reactions (Figure 2) would probably have to occur in the stomach for MNU to be produced there. In favor of a role for CRN is its very high concentration in foods, up to 4.1 g/kg (Table II). This is 6 times higher by weight than the highest reported occurrence in foods of secondary amines, which are precursors of nitrosamines (Singer and Lijinsky, 1976). It is also possible that CRN oxime and/or methylhydantoin oxime are produced from CRN in certain stored foods and further nitrosated to MNU in the stomach, despite the failure by Davidek et al. (1976), using a polarographic method of analysis, to detect CRN conversion into either of the oximes in nitrite-treated meat stored at 30 °C.

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LITERATURE CITED

Archer, M. C.; Clark, S. D.; Thilly, J. E.; Tannenbaum, S. R. *Science (Washington, D.C.)* **1971**, *174*, 1341–1343.
Carisano, A.; Bonecchi, A.; Riva, M. *J. Chromatogr.* **1969**, *45*, 264–268.
Correa, P.; Haenszel, W.; Tannenbaum, S.; Archer, M. *Lancet* **1975**, *2*, 58–60.
Davidek, J.; Velisek, J.; Klein, S.; Janicek, G. *Fleischwirtschaft* **1976**, *56*, 99–100.
Druckrey, H.; Landschütz, C.; Preussman, R.; Ivankovic, S. *Z. Krebsforsch.* **1971**, *75*, 229–239.
Endo, H.; Minoru, I.; Endo, T. In "*In Vitro* Metabolic Activation in Mutagenesis Testing"; DeSerre, F. D., et al., Eds.; Elsevier: Amsterdam, 1976; pp 217–221.
Fujinaka, N.; Masuda, Y., Kuratsune, M. *Gann* **1976**, *67*, 679–683.
Greenwald, I.; Levy, I. *J. Org. Chem.* **1948**, *13*, 554–559.
Haenszel, W.; Correa, P. *Cancer Res.* **1975**, *35*, 3452–3459.
Hawk, P. B.; Oser, B. L.; Summerson, W. H. "Practical Physiological Chemistry", 13th ed.; McGraw-Hill: New York, 1954.
Kawabata, T.; Ino, M.; Ohshima, H. *Nippon Suisan Gakkaishi* **1980**, *45*, 971–975.
Mirvish, S. S. *J. Natl. Cancer Inst. (U.S.)* **1971**, *46*, 1183–1193.
Mirvish, S. S. *J. Toxicol. Environ. Health* **1977**, *2*, 1267–1277.
Mirvish, S. S. In "Cancer 1980: Achievements, Challenges, Prospects for the 1980's"; Burchenal, J. H.; Oettgen, H. F., Eds.; Grune and Stratton: New York, 1981; pp 557–587.
Mirvish, S. S.; Cairnes, D. A. *Proc. Am. Assoc. Cancer Res.* **1981**, *22*, 140.
Mirvish, S. S.; Karlowski, K.; Cairnes, D. A.; Sams, J. P.; Abraham, R.; Nielsen, J. *J. Agric. Food Chem.* **1980**, *28*, 1175–1182.
Mirvish, S. S.; Nagel, D. L.; Sams, J. *J. Org. Chem.* **1973**, *38*, 1325–1329.
National Academy of Sciences "The Health Effects of Nitrate, Nitrite, and *N*-Nitroso Compounds"; National Academy of Sciences: Washington, DC, 1981; Part 1.
Singer, G. M.; Lijinsky, W. *J. Agric. Food Chem.* **1976**, *24*, 550–553.
Snell, D.; Snell, C. T. "Colorimetric Methods of Analysis", 3rd ed.; Van Nostrand: Princeton, NJ, 1970; Vol. IV-AA.
Sugimura, T.; Kawachi, T. *Methods Cancer Res.* **1973**, *8*, 245–308.
Tannenbaum, S. R.; Moran, D.; Rand, W.; Cuello, C.; Correa, P. *JNCI, J. Natl. Cancer Inst.* **1979**, *62*, 9–12.
Weisburger, J. H. *Cancer (J. Am. Cancer Soc.)* **1979**, *43*, 1987–1995.
Wogan, G. N.; Paglialunga, S.; Archer, M. C.; Tannenbaum, S. R. *Cancer Res.* **1975**, *35*, 1981–1984.

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Formation of Pseudoglycinins and Their Gel Hardness

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Native subunit proteins of glycinin, the acidic and basic subunits designated as ASI, ASII, ASIII, and ASIV and BS, respectively, were isolated by DEAE-Sephadex column chromatography. Pseudoglycinins were reconstituted from the combinations between BS and each acidic subunits except ASIV. The pseudoglycinins thus formed were similar to native glycinin; they all consisted of reconstituted intermediary subunits that were composed of acidic and basic subunits linked together by disulfide bridges and had molecular weights that were about 6 times that of the intermediary subunit. The hardness of the heat-induced gels from pseudoglycinins was different from those derived from native glycinin, depending on the acidic subunit composition. ASIII appeared to cause a significant increase in the hardness of the gel.

Not only are soybeans used for various kinds of traditional Japanese foods but also their protein products are used commercially as ingredients in foods. The gel-forming ability of soybean protein is of significance with respect to their usage in food systems. It has been reported that the quality of tofu-gel (one of the Japanese traditional foods made from soybeans) differs according to the cultivars used (Smith et al., 1960) and that the proportion of 7S and 11S globulins is responsible for the differences in the physical properties of tofu-gel among soybean cultivars (Saio et al., 1969). The differences in functional properties of 7S and 11S globulins were reviewed extensively by Saio and Watanabe (1978). On the other hand, it has previously been demonstrated that the subunit compositions of 11S globulins of soybeans are different among the cultivars (Mori et al., 1981). Thus, it seems likely that the subunit composition of 11S globulin is related to the physical properties of foods made from soybeans or their isolated proteins. However, very little information is available with respect to the correlation between the physical properties and the protein structure at the subunit level.

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11S globulin (referred to as glycinin), one of the major components of the soybean storage protein, has been shown to be composed of at least three kinds each of acidic and basic subunits (Catsimpoolas, 1969; Kitamura and Shibasaki, 1975). Glycinin has been shown to have intermediary subunits in which the acidic (α) and basic (β) subunits are linked by disulfide bridges in 1:1 ratio and to be composed of $(\alpha\beta)_6$ (Badley et al., 1975; Kitamura et al., 1976; Mori et al., 1979). In a previous paper we investigated the reconstitution of intermediary subunits from native acidic and basic subunits proteins of glycinin and the hybrid intermediary subunits from combinations of native subunit proteins of glycinin and sesame 13S globulin (Mori et al., 1979). Further, preliminary results were obtained for the formation of artificially reconstituted 11S globulins that have different subunit compositions from native 11S globulins, i.e., pseudo and hybrid 11S globulins from combinations of native subunit proteins of glycinin and broad bean legumin (Utsumi et al., 1980a).

In the present study, in order to elucidate how the subunits of glycinin contribute to the physical properties of its gel, we investigated the reconstitution of various pseudoglycinins from its native subunit proteins and the textural properties of the gel made from the pseudoglycinins.

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