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Partial Purification from Hot Dogs of *N*-Nitroso Compound Precursors and Their Mutagenicity after Nitrosation

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Hot dogs contain apparent *N*-nitroso compounds (ANC) and ANC precursors (ANCP). ANCP purification was followed by nitrosation, sulfamic acid treatment, and analysis for ANC. Aqueous hot dog extracts were adsorbed on silica gel, which was eluted with MeCN and MeOH. The MeOH eluate was adsorbed on cation exchange resin (H^+ form) and eluted with NH_4OH. Eluted ANCP traveled at moderate speeds in high-performance liquid chromatography (HPLC) on amino and Pb^{2+} columns. Gas chromatography−mass spectrometry (GC-MS) of trimethylsilyl (TMS) derivatives of crude water extract indicated the presence of glycerol, phosphate, lactic acid, and two monosaccharides. GC-MS of TMS derivatives of Pb^{2+} column HPLC eluates indicated that ANCP included 1-deoxy-*N*-1-glucosyl glycine. The nitrosated NH_4OH eluate showed 4× background mutagenic activity for *Salmonella typhimurium* TA-100. Un-nitrosated fractions showed 2× background activity. Although tryptophan nitrosation gave 88% ANC yield, tryptophan is probably not a major ANCP in hot dogs. Hot dog patties prepared with or without sucrose or glucose showed similar ANC and ANCP levels. We discuss possible implications of these findings for the etiology of colon cancer.

KEYWORDS: Hot dog; nitrite; mutagenicity

INTRODUCTION

N-Nitroso compounds are produced by the reaction of nitrite and nitrogen oxides with secondary amines and *N*-alkylamides. Most *N*-nitroso compounds are carcinogenic in laboratory animals (*1*, *2*), and these compounds are likely risk factors for the induction of several types of human cancer (*3*, *4*). Sodium nitrite is added to certain meat and fish products as a preservative (*5*). Prospective cohort and case-control studies indicated that fresh and especially "processed" (mainly nitrite-preserved) red meats are risk factors in the etiology of colon cancer (*6*−*8*). Most recently, the effect of diet was studied in patients who had had colorectal adenomas removed (*9*). It was reported that processed but not red meat was a significant risk factor (relative risk, 1.75) for the recurrence of advanced colonic adenomas.

In 2001, we described (*10*) modifications of the Walters method (*11*, *12*) for determining total apparent *N*-nitroso compounds (ANC) in food extracts. [We include *apparent* in this term because our method may probably measure a minor proportion of compounds that are not *N*-nitroso compounds (*10*, *13*).] Samples are injected into refluxing EtOAc containing HBr. This reacts with ANC to form NO, which is analyzed by thermal energy analysis (TEA). ANC are determined after extracts are treated with sulfamic acid (SA) to destroy nitrite (*10*). ANC precursors (ANCP) are measured by treatment with 110 mM nitrite, the addition of sulfamic acid, and determination of the resulting ANC (*10*). We examined hot dogs (wieners, frankfurters, and sausages) because they are a widely consumed nitrite-preserved meat product and because of the proposed linkage of such products with colon cancer (*7*). Water extracted most of the ANC and ANCP in hot dogs [mean levels in water extracts, 5.5 μmol ANC/kg and 2700 μmol ANCP/kg (*10*)]. Near maximum ANC yields were obtained with the 110 mM nitrite used to determine ANCP (*10*). Because feeding red meat to humans raised their fecal excretion of ANC, it was proposed that colonic ANC are responsible for the correlation between red meat consumption and colon cancer (*14*, *15*). Fecal ANC outputs rose by factors of 2.0−2.9 in mice fed beef and of 3.7−5.0 in mice fed hot dogs (*16*).

For these reasons, we wished to identify the ANC in hot dogs. Simple volatile nitrosamines constitute 1−2% and nitrosamino acids constitute 15−20% of the total ANC in fried bacon (*13*),

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but the remaining 70−80% of the ANC and their precursors (the ANCP) in this food, as well as the ANC and ANCP in hot dogs, were unidentified when we began this study. Because ANCP are ~500 times more abundant than ANC in hot dogs (*10*), we decided to purify the principal ANCP rather than the ANC in hot dogs. These ANCP might be converted to ANC during storage of the hot dogs or in vivo (*4*). We also examined the nitrosation of tryptophan (Trp) and histidine under our standard conditions because these amino acids react with nitrite to give mutagenic nitrosamines and hence could be significant ANCP in hot dogs. Thus, nitrosation of histidine produced a frameshift mutagen (*17*) and Trp yielded a product nitrosated at the indole nitrogen, which was directly mutagenic for *Salmonella typhimurium* TA-100 (*18*, *19*). Finally, to help test the possible role of sugars in ANCP formation, we determined ANC and ANCP in "hot dog patties" prepared with different types and amounts of sugars.

MATERIALS AND METHODS

Safety. ANC fractions, dimethylnitrosamine, and methylnitronitrosoguanidine were handled with gloves in a chemical hood and with other due precautions, as they are potential or actual carcinogens.

General Procedures. We bought at a supermarket large batches of a brand of hot dogs that had previously (*10*) shown a relatively high ANCP level of 6.9 μmol/g and stored them at −15 °C for <3 months. The hot dog ingredients were listed on the packages in order of decreasing amount as pork, beef, chicken, corn syrup, water, salt, flavoring, hydrolyzed soy protein, Na and K phosphate, sugar, Na erythorbate, and $NaNO_2$. Most chemicals used in this study were purchased from standard suppliers. Solutions were evaporated in a rotary evaporator at ~20 Torr and <40 °C or at room temperature with a stream of N_2 in an unheated Reacti-Vap (Pierce Chemicals, Rockford, IL).

Determination of ANC and ANCP. These were measured by our published methods (*10*, *16*) and were expressed as nmol or μmol of ANC or ANCP based on comparison with a standard injection of 1.0 nmol of *N*-nitrosoproline.

ANC. Aqueous samples (2 mL) were treated with 300 μL of a freshly prepared saturated solution of sulfamic acid in water ("sulfamic acid reagent") and 50 μL of 2 M HCl. The solutions were kept for 15 min at room temperature to allow sulfamic acid to decompose nitrite and for <4 h in ice and then analyzed for ANC.

ANCP. Aqueous samples were analyzed directly or after evaporation to dryness and resolution in 1.5 mL of water. Then, 0.2 mL of 1 M $NaNO_2$ (final level, 110 mM) and 0.1 mL of 2 M HCl were added. The vials were capped and reacted for 1 h at 37 °C, 0.2 mL of sulfamic acid reagent was added, and the solutions were analyzed for ANC. As before (*10*), aqueous test samples (usually 100 μL) were injected into a solution containing EtOAc, HCl, HOAc, and HBr, which was refluxed under reduced pressure at ~28 °C to liberate NO from ANC. A stream of argon passed the NO through seven washbottles and into a Thermal Energy Analyzer (model 502, Thermo-Orion, Beverly, MA) to determine the NO. *N*-Nitrosoproline (0.1 nmol) served as the standard.

Purification of ANCP from Hot Dogs (Figure 1). Samples of all fractions were analyzed for ANCP and then, in most cases, evaporated to determine dry weights. **Figure 1** summarizes the purification and shows the ANCP yield, dry weight, and ANCP concentration for most steps. The total ANC content of each fraction was derived from the formula: ANC content (nmol, μmol, or mmol) = ANC concentration (mM, μM, or nM) × volume in mL/1000.

Step 1: Extraction with Water. An initial extraction with water was adopted because this solvent extracted the largest amount of ANCP from hot dogs (*10*). One package (370 g) of the hot dogs brand described in section 1 of the Materials and Methods was ground with 600 mL of distilled water for 15 min in a Waring blender. The mixture was kept for 4 h at room temperature with occasional stirring and then centrifuged (6600*g*, 4 °C, 20 min). An upper layer of fat was removed. The sediment was blended and extracted twice more with 400 mL of water. The combined extract was stored at −15 °C. This extraction was repeated four times with additional packages of hot dogs. The combined extract (7.0 L) was thawed, filtered while cold through glass wool to remove fat and solids, and evaporated to give 570 g of gummy residue. To this we added 200 mL of water, which dissolved most of the gum.

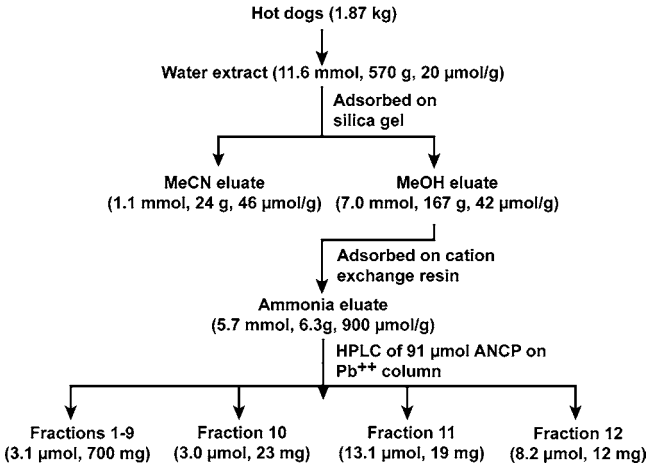

Figure 1. Purification of the ANCP in hot dogs. The amount of ANCP, dry weight, and μmol ANCP/g dry weight are shown for most stages of the purification. The mixtures analyzed by HPLC were derived from workups similar but not identical to that shown in the upper part of the figure.

Step 2: Adsorption on Silica Gel. The aqueous extract from step 1 was mixed with 1.0 L (580 g) of silica gel [Merck, grade 60, 70−230 mesh, Aldrich (Milwaukee, WI)] to give a sticky solid. This was mixed with 1.4 L of acetonitrile (MeCN) and stirred occasionally for 30 min. The supernatant liquid was filtered off. The silica gel was extracted twice more with 1.4 L of MeCN. The combined MeCN extract was evaporated to give 24 g of residue, which was discarded. The silica gel was re-extracted similarly with 3 × 1.5 L of MeOH. The combined MeOH extract was concentrated to 250 mL. A white precipitate (probably silica gel) was removed by filtration through a sintered glass funnel. The resulting filtrate was evaporated to constant weight to give an oily yellow solid (167 g), termed the "MeOH eluate".

Step 3: Adsorption on Cation-Exchange Resin. The residue from the MeOH eluate in step 2 was stirred for 5 min with 330 g of cation exchange resin (50W-8X, H^+ form, BioRad, Hercules, CA). The mixture was stirred for another 5 min with 500 mL of 1 M HCl, and the supernatant was discarded. The resin was stirred similarly twice more, each time with 500 mL of water, which was discarded, and was then stirred three times, each time with 500 mL of 2 M NH_4OH. The combined NH_4OH washes were evaporated to give 6.3 g of residue, which was dissolved in 94 mL of water to give the "NH_3 eluate".

Step 4: High-Performance Liquid Chromatography (HPLC) on a Pb^{2+} Column. A portion of NH_3 eluate containing 91.3 μmol of ANCP was subjected to HPLC in nine runs on a Benson carbohydrate BC-100 Pb^{2+} column (300 mm × 7.8 mm, Alltech, Deerfield, IL) that was kept at 90 °C and was developed with water at 1.2 mL/min. Ten minute fractions were collected. Corresponding fractions from the nine runs were combined and analyzed for ANCP.

Gas Chromatography−Mass Spectrometry (GC-MS) of Trimethylsilyl (TMS) Derivatives of Components of the Initial Water Extract. We evaporated a water extract of hot dogs prepared as in the Materials and Methods, section 3, step 1. To a 2 mL vial containing 20 mg of the residue were added 1 mL of pyridine dried over NaOH, 0.2 mL of hexamethyldisilylazane, and 0.1 mL of TMS chloride. The vial was filled with N_2, stoppered, and heated for 2 h at 50 °C. A sample (5 μL) of the mixture was analyzed on an Agilent model 6890 GC apparatus interfaced with an Agilent 5791A mass selective detector using electron impact MS at 70 eV ionization voltage. A fused silica capillary column (30 m × 0.25 mm internal diameter) of DB-WAX bonded phase (film thickness, 0.25 mm; J & W Scientific, Folsom, CA) was used for the GC. The average velocity of the helium carrier

gas was 30 cm/s. The injector was set at 250 °C. The oven temperature was raised from 50 to 180 °C at 3 °C/min and held there for 40 min. Mass spectra were compared with those from an Agilent MS ChemStation Data system.

GC-MS Analysis of TMS Derivatives of Eluate Fractions from the Pb^{2+} Column: HPLC Fractions Were Evaporated. The entire residue from each fraction or 2.0 mg of glucose was heated for 1 h at 50 °C in pyridine-*N*,*O*-bis(TMS)trifluoracetamide-trimethylchlorosilane (50:49:1). GC-MS was performed on an EC-5 Econocap GC column (30 m × 0.25 mm internal diameter; film thickness, 0.25 mm; Alltech), connected to a fused silica guard column (2 m × 0.53 mm internal diameter). The column was kept for 2 min at 60 °C, raised 4 °C/min to 300 °C, and kept there for 10 min. The splitless GC injector port was at 280 °C. The GC was interfaced with a Finnigan TSQ 7000 mass spectrometer (Thermo-Electron, San Jose, CA) operated under positive ion chemical ionization conditions. The reagent gas in the source was methane. The scan range was 70−1000 atomic mass units.

Nitrosation of Trp, Trp Dipeptides, and Histidine. This was studied by our standard method for determining ANCP (Materials and Methods, section 2), except that 50, 110, and 400 mM nitrite were used instead of 110 mM nitrite.

Determination of Free Amino Acids. Water extracts of three hot dogs brands were prepared (*10*), deproteinized by addition of 30 mg sulfosalicylic acid/mL extract, and centrifuged. Amino acids in 10 μL of extract were derivatized with 6-aminoquinolyl-*N*-hydroxysuccinoyl carbamate. The products were analyzed by HPLC on a C-18 column with fluorescence detection (*20*). We used this method before (*21*). His and Trp peaks coincided with those produced from standards and were absent when the derivatizing agent was omitted.

Study of Hot Dogs "Patties". We followed a published method for preparing "quality deli franks" (*22*), except that the mixture was not stuffed into sausage skins. Thus, we ground 1130 g of refrigerated chuck beef with 290 g of crushed ice in a mincer attached to a food processor. To a stainless steel bowl containing the mixture were added 215 g of crushed ice, 22.5 g of NaCl, 22.5 g of sucrose or glucose, a solution of 169 mg of $NaNO_2$ in 25 mL of water, and a solution of 500 mg of ascorbic acid in 50 mL of water. The mixture was stirred for 5 min every 20−30 min over 2 h (the temperature rose to 17 °C) and poured into an aluminum tray (22 cm × 22 cm × 4 cm) to form a patty 3 cm thick. This was heated in a circulating-air oven for 30 min at 52 °C, for 1 h at 60 °C, for 1 h at 74 °C, and for 10 min at 82 °C. The internal temperature was then 68 °C, and the patty surface became dark brown. Portions were sealed in plastic bags and stored for 1−3 days at 4 °C or for 28 days at 4 or −15 °C. Samples were then analyzed for ANC and ANCP as in the Materials and Methods, section 2, except that the extracts were not evaporated before the analyses.

Mutagenesis Tests. These were performed by a standard method (*23*). A portion of hot dog-derived ANCP (the NH_3 eluate unless specified otherwise) was evaporated. The residue was weighed and redissolved in water. On the day of the mutagenesis test, aliquots of this solution were treated with nitrite and then sulfamic acid as in the assay for ANCP (Materials and Methods, section 2) to give "solution A". This was kept for 15 min at room temperature and then on ice for <2 h. Sterile conditions were used in all subsequent procedures; for example, all solutions were passed through 0.45 μm filters (Millex syringe-driven filter units, Millipore, Bedford, MA) just before the assay. We typically prepared duplicate 1.2 mL vials, each containing 700 μL of 0.2 M Na phosphate buffer (pH 7.4); 100 μL of a suspension, freshly grown for 12 h at 37 °C, of *S. typhimurium* TA-100 kindly supplied by B. Ames (University of California−Berkeley); 0, 50, 100, or 200 μL of solution A, and, respectively, 200, 150, 100, or 0 μL of distilled water. The vials were capped, mixed, and incubated for 30 min at 37 °C with shaking twice/s. The vial contents were mixed with top agar, poured onto hard agar plates, and incubated for 48 h at 37 °C. Mutant colonies were counted twice in a Biotran-III automatic count/area totalizer (model C-112, New Brunswick Scientific, Edison, NJ), and the mean results were recorded. Individual results for each plate generally agreed within 10%. Each experiment included blanks without test material and positive controls with dimethylnitrosamine or 1-methyl-3-nitro-1-nitrosoguanidine. In addition, a solution of 20 mg of 1-deoxy-*N*-1-D-fructosylvaline (**Figure 2**, R = H, from Color Your Enzymes, Kingston, Ontario, Canada) in 100 μL of water was nitrosated and treated with sulfamic acid as described above. Mutagenicity tests were conducted as described above on volumes of the resulting solution corresponding to 0.5, 1.0, and 2.0 mg of fructosyl valine.

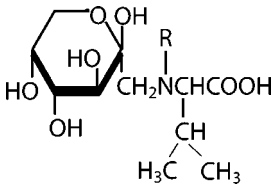

Figure 2. Structure of 1-deoxy-*N*-1-D-fructosyl valine (R = H) and its *N*-nitroso derivative (R = NO).

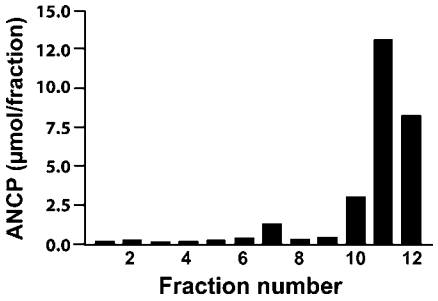

Figure 3. ANCP analysis of fractions obtained by HPLC of the NH_3 eluate on a Pb^{2+} column.

RESULTS

Purification of ANCP in Hot Dogs. In step 1 of the purification (**Figure 1**), the water extract from 1.87 kg of hot dog contained 11.6 mmol of ANCP. In step 2, the water extract from step 1 was adsorbed onto silica gel, which was eluted with MeCN and then MeOH. The MeCN extract was discarded because it contained only 14% of the extracted ANCP. The MeOH extract typically contained 60% of the ANCP applied to the silica gel and 86% of the ANCP extracted from the silica gel and probably contained more hydrophilic ANCP than did the MeCN extract. In step 3, the MeOH fraction from step 2 was adsorbed on the acidic form of a cation exchange resin, which was washed with various solvents and eventually eluted with NH_4OH to give the "NH_3 eluate". This contained 81% of the ANCP applied to the resin and had a specific activity of 900 μmol ANCP/g dry weight. In step 4, a sample of the NH_3 eluate from step 3 was subjected to HPLC on a Pb^{2+} column recommended for the separation of sugars (**Figure 3**) (*24*). Each of the 12 fractions eluted from this column weighed <25 mg except for fraction 8, which weighed 565 mg. Fractions 7 and 10−12 contained the highest amounts of ANCP. As the final eluate (fraction 12) still showed a high ANCP level, some ANCP probably remained on the column. In a run on the Pb^{2+} column where UV absorption at 240 nm was followed, fractions 5 and 6 showed prominent peaks containing 44 and 23% of the total eluted absorption.

GC-MS of TMS Derivatives Prepared from the Initial Water Extract. We decided to identify the major low molecular weight components of the crude water extract of hot dogs, in case there were significant ANCP. A sample of the water extract was converted to TMS derivatives and analyzed by GC with flame ionization detection. Five prominent peaks were observed by this method (results not shown). Analysis of the same mixture by GC−low-resolution electron impact-MS also revealed five prominent peaks (**Figure 4**). These were eluted with similar

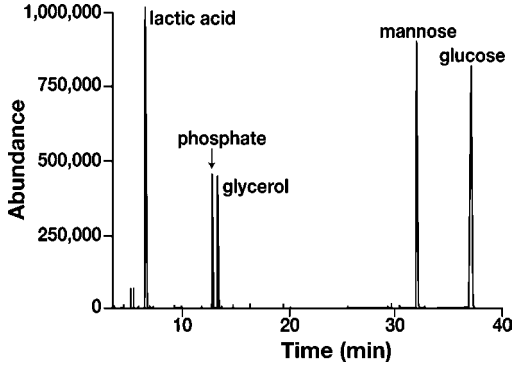

Figure 4. GC-MS profile of a crude water extract of hot dogs after it was dried and converted to TMS derivatives. The total ion current was measured.

Table 1. GC-MS and Tentative Identifications of Five Prominent GC Fractions of TMS Derivatives Prepared from a Crude Water Extract of Hot Dogs

GC retention time (min)	tentative identification	MS [*m*/*z* (% relative intensity in observed and reference MS)]
6.6	lactic acid	219 (4, 7), 191 (13, 14), 147 (94, 68), 117 (81, 68), 73 (100, 100)
12.8	phosphate	314 (17, 21), 299 (100, 100), 211 (11, 5), 133 (14, 7), 75 (49, 43)
13.3	glycerol	218 (17, 5), 205 (48, 21), 147 (70, 33), 133 (18, 7), 117 (34, 20), 103 (28, 20), 73 (100, 100)
32.2	D-mannose	217 (28, 16), 204 (100, 100), 191 (49, 40), 147 (29, 19), 73 (84, 79)
37.3	D-glucose	217 (20, 17), 204 (100, 100), 191 (50, 47), 147 (29, 19), 73 (84, 59)

retention times and were probably identical to the components observed by flame ionization detection. **Table 1** lists the MS of the five peaks and their tentative identification, obtained by comparing their MS profiles with those in a computer library. On this basis, the peaks (listed in order of their retention times) were assigned to lactic acid, inorganic phosphate, glycerol, a monosaccharide (possibly mannose), and glucose. None of these compounds was unexpected for a muscle extract except mannose (if it was present). Phosphate and the sugars may have been present because, according to the package label, the hot dogs contained added phosphate and sugar, presumably sucrose.

GC-MS of TMS Derivatives of Glucose and of Fractions Obtained by HPLC of the NH_3 Fraction on the Pb^{2+} Column. GC-MS was performed on the TMS derivatives of glucose and of fractions 7 and 10−12 (which contained the largest amounts of ANCP) from the Pb^{2+} HPLC column (**Figure 3**). Glucose yielded a prominent GC peak at 35.2 min and a small peak at 37.2 min, which showed similar mass spectra (**Table 2**). HPLC fraction 7 showed prominent GC peaks at 12.8, 18.3, and 32.4 min, the MS of which could not be interpreted. HPLC fraction 10 produced large GC peaks at 35.0 and 37.3 min, a small GC peak at 44.2 min, and large GC peaks at 51.8, 52.6, and 53.5 min (**Figure 5**). **Table 2** shows the main MS fragments for these peaks. The largest MS peak of the GC peaks at 44.2, 51.9, and 52.6 min showed *m*/*z* 671.4, 903.6, and 903.6, respectively. Fractions 11 and 12 from the Pb^{2+} column did not produce GC peaks at retention times exceeding 27 min that were >20% of the maximum response.

ANC Production from Histidine, Trp, and Trp Dipeptides: Their Formation and Stability as Compared to the Stability of Hot Dog ANCP. Nitrosation of histidine gave <2% yields of ANC under our standard conditions and was not further investigated. Nitrosation of Trp gave up to an 88% yield of ANC (**Table 3**, exp 2). After the addition of sulfamic acid in exp 1 of **Table 3**, the ANC yield at 0−1 h dropped from 84 to 11% as the nitrite concentration was raised from 50 to 400 mM. In this experiment, the pH before the incubation dropped from 2.0 when 50 mM nitrite was used to 1.0 for 400 mM nitrite, because more HCl was added with the higher nitrite levels. In all four experiments on Trp, the ANC level dropped steadily when the nitrosation mixtures were stored at 17−18 °C after sulfamic acid was added. The decrease in ANC yield from 0−1 to 2−3 h was 32 ± 11%/h (mean ± SD for six results in exps 2 and 3). After nitrosation at pH 2.0, tryptophanyl alanine and alanyl Trp produced ANC yields of up to 31 and 5% (**Table 3**, exps 5 and 6). The ANC derived from tryptophanyl alanine disappeared on storage at about the same rate as the ANC produced from Trp. In contrast to these results, when aqueous hot dog extracts were treated with nitrite and sulfamic acid and

Table 2. GC-MS of TMS Derivatives of Glucose and of Fraction 10 from the Pb^{++} Column[a]

material analyzed	GC peak [retention time in min (% intensity)]	MS fragments [*m*/*z* (% intensity)]
glucose	35.2 (100)	525.1 (22),[b] 435.1 (84), 361.1 (100)[c]
glucose	37.2 (10)	525.2 (14),[b] 435.1 (100), 361.1 (90)[c]
fraction 10	35.0 (98)	525.2 (38),[b] 435.2 (74), 361.2 (100)[c]
fraction 10	37.3 (100)	525.3 (34),[b] 435.2 (100), 361.2 (97)[c]
fraction 10	44.2 (22)	671.4 (20),[d] 581.4 (10),[e] 539.3 (9),[f] 507.3 (20),[g] 452.0 (8),[h] 435.3 (8),[i] 361.2 (100)[c]
fraction 10	51.9 (70)	903.6 (4),[j] 814.6 (7), 723.4 (5), 525.3 (8), 451.2 (8)
	52.6	435.2 (7), 361.2 (100)[c]

[a] Footnotes *b*–*j* show the tentative identifications of some MS fragments. M_1, M_2, and M_3 are the molecular ions of the TMS derivatives of glucose, Glu-Gly, and a disaccharide, as defined in footnotes *b*, *d*, and *j*, respectively. All assignments agreed with the assigned formulas to within 0.5 mass units. [b] *m*/*z* 525.2: M_1 ($C_{21}H_{52}O_6Si_5$ = 541-16), where M_1 is glucose with five TMS groups and 16 may be CH_3 + H. [c] *m*/*z* 361.1: $C_6H_6O_4(SiMe_3)_3$ = 361.7. [d] *m*/*z* 671.4: [M_2 ($C_{26}H_{63}NO_7Si_6$ = 670.3)] + H. [e] *m*/*z* 581.4: M_2 − $OSiMe_3$. [f] *m*/*z* 539.3: M_2 − $CH_2CO_2SiMe_3$. [g] *m*/*z* 507.4: M_2 − $HOSiMe_3$ − $SiMe_3$. [h] *m*/*z* 452.0: M_2 - $NSiMe_3CH_2COOSiNMe_3$. [i] *m*/*z* 435.3: M_2 − CH_3OSiMe_3 − $CH_2COOSiMe_3$. [j] *m*/*z* 903.6: M_3 ($C_{36}H_8O_{11}Si_8$ = 920-16), where M_3 is a disaccharide with eight TMS groups and 16 may be CH_3 + H. The peak at 52.6 min showed MS fragments similar to those for the peak at 51.6 min.

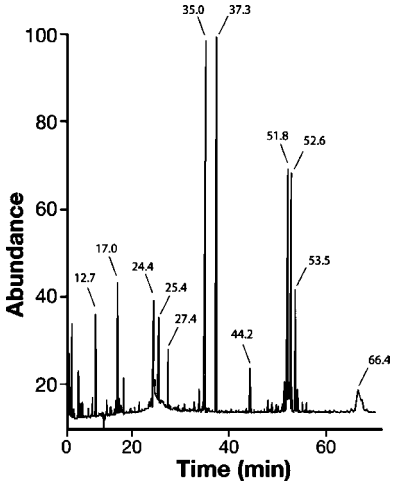

Figure 5. GC-MS profile of TMS derivatives of fraction 10 from the Pb^{2+} column. The total ion current was measured.

Table 3. Nitrosations of Trp as Compared to that of ANCP Derived from Hot Dogs: ANC Yield and Stability on Storage after Adding Sulfamic Acid

exp no.	sodium nitrite (mM)	pH at beginning of nitrosation	pH after adding sulfamic acid	increase in volume on adding sulfamic acid (%)[a]	ANC yield after storage for different times[b] % yield[d,e] 0–1 h	1–2 h	2–3 h	3–4 h	% loss (h)[c]
tests on Trp and Trp dipeptides									
1[e]	50	2.0	1.5	60	84	*e*, *f*			
1[e]	100	1.5	1.0	60	39				
1[e]	400	1.01	0.6	60	11				
2[e]	50	2.0	1.2	12	53	37	27		26
2[e]	110	2.0	1.0	60	88	41	28		34
2[e]	400	2.0	0.7	60	27	8	3		48
3[e]	50	2.0	1.5	60	42	39	28		17
3[e]	110	1.5	1.0	60	45	29	17		31
3[e]	400	1.0	0.6	60	13	4	2		43
4[e]	50	2.5	1.2	30	21			10	17
4[e]	110	2.4	1.0	30	51			29	15
4[e]	400	2.5	0.7	30	46			5	
tests on tryptophanyl alanine[g]									
5	50	2.0	1.1	50	31	14	12		31
5	110	2.0	0.9	50	26	7	6		39
5	400	2.0	0.6	50	10	1	1		45
tests on alanyl Trp[g]									
6	50	2.0	1.0	50	5	1	1		40
6	110	2.0	0.8	50	2	1	1		25
6	400	2.0	0.5	50	3	1	1		34

exp no.	sodium nitrite (mM)	pH at beginning of nitrosation	pH after adding sulfamic acid	increase in volume on adding sulfamic acid (%)[a]	ANC yield after storage for different times[b] ANC level (μmol ANCP/g hot dog) 0–1 h	1–2 h	2–3 h	3–4 h	% loss (h)[c]
tests on ANCP derived from hot dog									
7[h]	110	2.0	1.1	15	310			285	3
8[h]	110	2.0	1.1	15	1510 ± 130[i]			1450 ± 130[i]	1 ± 1[i]

[a] Volume after adding sulfamic acid reagent and water/volume of nitrosation mixture. [b] ANC yields were measured over about 1 h. Hence, times are recorded as 0–1, 1–2 h, etc. [c] Measured after 0–1 h and either 2–3 or, in exp 3a and 3b, 6 and 3–4 h. To calculate loss/h, we assumed that the ANCs were stored for 2 or 3 h in the reactions kept from 0 to 1 h for, respectively, 2–3 and 3–4 h. [d] Percent yield from Trp or Trp dipeptides. [e] In exps 1–4, 500 μM Trp was reacted with 50–400 mM nitrite in a total volume of 2–10 mL. HCl was added in amounts equimolar to those of $NaNO_2$, and the pH at the beginning of the nitrosation was not adjusted, except in exps 2 and 4, where only sufficient HCl was added to reach the indicated pH. After incubation for 1 h at 37 °C, sulfamic acid reagent (147 g/L, 1.52 M) was added in at least a 2-fold molar excess relative to nitrite. The pH was recorded after mixing the reactants (column 3) and after adding sulfamic acid (column 4). Column 5 shows the increase in volume after adding sulfamic acid reagent and, in some cases, water. After the addition of sulfamic acid, the mixtures were stored at room temperature (17–18 °C) for 0–1 to 3–4 h and ANCs were determined as in section 2 of the Materials and Methods. [f] Blank cells, not measured. [g] Tryptophanyl alanine and alanyl Trp were nitrosated, and ANCs were determined as for the Trp nitrosations in exp 2. [h] Ten grams of hot dog was homogenized in 25 mL of water and centrifuged. One milliliter (exp 7) or 0.12 mL (exp 8) of the supernatant was nitrosated in a total volume of 2.0 mL. The product was analyzed for ANCP after storage for different times as described for Trp in exp 2. Exps 7 and 8 used different brands of hot dog. [i] Mean ± SD for three samples from the same package of hot dog.

were then stored at 17−18 °C, the ANC yield dropped by only 1−3%/h (**Table 3**, exps 7 and 8).

Free Amino Acid Levels in Hot Dogs. Analysis of aqueous extracts of four samples, each from a different brand of hot dog, showed concentrations in μmol/g hot dog of 0.4 ± 0.3 for histidine, 0.13 ± 0.11 for Trp, 2.1 ± 1.6 for glycine, and 18 ± 14 for total amino acids (mean ± SD).

ANC and ANCP Levels in Hot Dog Patties. Sucrose is typically included in hot dogs, but other sugars are sometimes used in its place (*22*). For this reason and because of our evidence (see Discussion) that the ANCP in hot dogs include 1-deoxy-*N*-1-D-glucosyl glycine (Glu-Gly), it was possible that the observed variation in ANCP content was due to variation in the nature or amount of the added sugar or in the time for which the hot dogs were kept in cold storage before sale. For commercial hot dogs, the storage time can be several weeks (R. W. Mandigo, Animal Science Department, University of Nebraska−Lincoln, personal communication). Accordingly, we prepared hot dog patties using a standard recipe (*22*) and variations thereof. The patties were analyzed after storage under different conditions. They showed 1.8−6.5 nmol ANC/g and 880−1510 nmol ANCP/g (**Table 4**). Omitting the 2% sucrose from the standard recipe or replacing it with glucose had little effect. In exps 1 and 2 of **Table 4**, the storage of patties for 4 weeks at 4 or −15 °C or for 1 day at 4 °C mostly gave similar results. In exp 3, the combined ANCP results for patties prepared with either sucrose or glucose rose 25% from 970 ± 80 nmol/g (mean ± SD) for four samples stored for 1 day at 4 °C to 1250 ± 170 nmol/g for four samples stored for 28 days at 4 °C. This 25% increase was significant ($P < 0.05$) by the Wilcoxon rank order test.

Mutagenesis Tests. Solutions from three stages of the ANCP purification were nitrosated and treated with sulfamic acid. Different amounts of each solution were analyzed for direct mutagenicity in *S. typhimurium* TA-100. The TA-100 strain was employed because of its extensive use for studying base substitution mutagenesis by *N*-nitroso compounds (*25*). After nitrosation, the initial water extract, the MeOH eluate, and the NH_3 eluate (**Figure 1**) showed maximum activities of 1.2, 2.1, and 2.9 times background (**Figure 6**). We investigated whether

Table 4. ANC and ANCP Contents of Frankfurter Patties Made with or without Sucrose or Glucose and Stored for up to 4 Weeks at 4 or –15 °C

exp. no.	sugar added	storage conditions[a] time (days)	storage conditions[a] temp (°C)	nmol/g[b] ANC	nmol/g[b] ANCP
1	sucrose	28	4	5.8 ± 0.6	1480 ± 90
	sucrose	28	–15	5.6 ± 0.5	1490 ± 80
2	sucrose	1	4	6.2, 6.5	1050, 1140
	sucrose	28	4	4.5, 6.3	1060, 1360
	sucrose	28	–15	5.1, 5.7	1290, 1400
	none	1	4	5.9, 6.1	1010, 1120
	none	28	4	3.9, 6.7	1020, 1370
	none	28	–15	5.6, 6.1	1510, 1560
3	sucrose	1	4	2.9, 3.1	880, 970
	sucrose	28	4	4.8, 5.9	1100, 1130
	glucose	1	4	3.1, 3.2	970, 1080
	glucose	28	4	1.8, 6.0	1320, 1450

[a] Storage conditions after initial storage for 1–2 days at 4 °C. [b] Each row includes results for three samples, listed as means ± SD (exp 1), or for two samples, shown as individual values (exps 2 and 3). The duplicate analyses performed on each sample generally agreed within 20%.

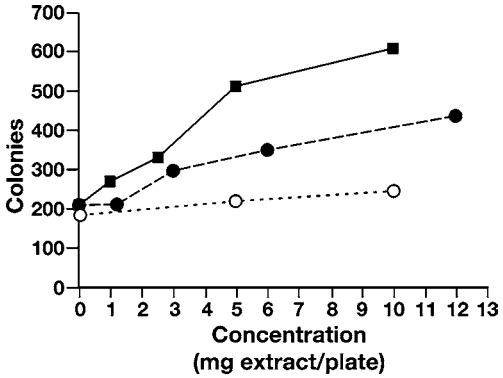

Figure 6. Direct mutagenicity (i.e., without activation) of nitrosated samples from three stages of the purification of the ANCP in hot dogs. Results are shown for a crude water extract of hot dogs (○), the MeOH eluate from silica gel (●), and the NH_3 eluate from cation exchange resin (■), all prepared as in the Materials and Methods (section 3). On the day of the test, samples of each solution with known dry weights and ANCP contents were nitrosated and treated with sulfamic acid. Mutagenesis tests were performed on different volumes (up to 200 μL) of these solutions.

liver S-9 from Aroclor-induced hamsters would increase the mutagenicity of the nitrosated NH_3 eluate (**Table 5**, exp 1). The mutagenicity of DMN increased 22-fold after adding S-9 (**Table 5**, exp 1), confirming that the S-9 contained active cytochrome P450s (*25*). The test of 50, 100, and 200 μL of NH_3 eluate produced 1.05-, 1.08-, and 1.38-fold increases in activity on adding S-9, indicating that most but perhaps not all of the activity did not require metabolic activation, i.e., was due to direct mutagens. Subsequent experiments were performed without adding S-9. In exp 2 of **Table 5**, NH_3 eluates that were or were not nitrosated increased the number of mutants ~4 and ~2 times relative to the blank. On this basis, the ratios of the activities of nitrosated ANCP that were due to (i) the blank without hot dog fractions, (ii) un-nitrosated material, and (iii) nitrosation were ~1:1:2. Hence, one-third of the activity of the nitrosated fraction was present before nitrosation.

The stability of the mutagens in nitrosated NH_3 eluate was tested by assaying this solution just after sulfamic acid was added and after storage for 3 or 24 h at room temperature (**Table 5**, exp 3). The mutagenic activity remained stable after storage under these conditions. For comparison, we determined ANC in the identical solution. We found 50−55 μmol ANC/g dry weight after the same solution was stored at room temperature for 0, 3, and 24 h; that is, the ANC was stable under these conditions. According to the results before storage, 50 μL of NH_3 eluate produced 212 mutants after subtracting the blank.

Table 5. Mutagenesis Tests of Nitrosated NH_3 Eluate from Hot Dogs[a]

experiment	mutants/plate: mean blank	mutants/plate: test results[b]	mean of test-blank
Exp 1: Effect of S-9			
a. tests with S-9[c]			
dimethylnitrosamine[d]	169	594 ± 60	425
50 μL of solution A[e]	169	414 (412, 415)	245
100 μL of solution A	169	547 (532, 562)	378
200 μL of solution	169	587 (565, 608)	418
b. tests without S-9			
dimethylnitrosamine[d]	139	158 ± 13	19
50 μL of solution A	139	282 (273, 290)	143
100 μL of solution A	139	489 (484, 494)	350
200 μL of solution A	139	442 (424, 459)	303
Exp 2: Effect of Nitrosation			
a. 50 μL of NH_3 eluate[f]			
with nitrosation + sulfamic acid	113	483 (467, 498)	370
no nitrosation, no sulfamic acid	113	208 (196, 220)	95
b. 100 μL of NH_3 eluate			
with nitrosation + sulfamic acid	113	637	524
no nitrosation, no sulfamic acid	113	148 (112,183)	35
Exp 3: Stability of Mutagenic Activity on Storage[g]			
a. 50 μL of solution A[h]			
no storage	143	355 (338, 371)	212
storage for 3 h	143	353 (345, 360)	210
storage for 24 h	143	297 (290, 304)	154
b. 200 μL of solution A			
no storage	137	481 (461, 501)	344
storage for 3 h	137	507 (493, 519)	370
storage for 24 h	137	505 (494, 516)	368

[a] On the day of the mutagenesis test, samples of NH_3 eluate of known ANCP content and dry weight were treated with nitrite and then sulfamic acid to give "solution A". Different preparations of solution A were used in each experiment. Mean mutants/plate for the positive controls with 8 μg of methylnitronitrosoguanidine in 12 μL of dimethyl sulfoxide were 960, 796, and 956 for exps 2 and 3 with storage for 0 and 3 h and exp 3 with storage for 24 h. Dimethylnitrosamine was used as the positive control in exp 1. [b] Results are listed as means (individual results) for a single plate (in one case) or for two plates or as means ± SD for three plates. [c] We mixed 1.0 mL of 10% S-9 (Aroclor 1254-induced hamster liver S-9, Bioreliance Toxicology Laboratories, Rockville, MD) with 9 mL of the standard cofactors for S-9 (*23*) and used 700 μL of this mixture instead of the 700 μL phosphate buffer specified in the standard assay. [d] Dimethylnitrosamine (10 μL, 10 mg; final concentration, 135 mM) was added to the 1 mL incubation mixture. [e] Fifty microliters of this solution contained 9.8 μg of dry weight and 0.92 μmol of ANCP before the nitrosation. [f] This contained 2.85 μmol nitrosated ANCP/50 μL before nitrosation. [g] Samples were analyzed immediately after nitrosation and treatment with sulfamic acid or after storage of these solutions for 3 or 24 h at room temperature (17–18 °C). [h] This contained 0.5 μmol nitrosated ANCP/50 μL before nitrosation.

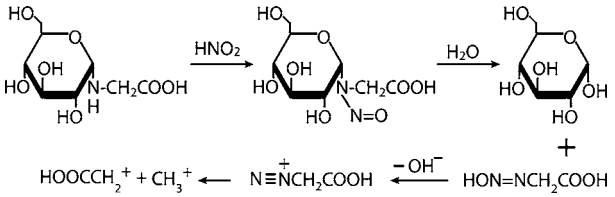

Figure 7. Postulated formation of *N*-nitroso-Glu-Gly from Glu-Gly and its postulated decomposition to give carboxymethyl and methyl carbocations.

As this solution contained 0.39 μmol of ANCP, its specific activity was 544 mutants/μmol nitrosated ANCP.

Finally, we tested 0.5, 1.0, and 2.0 mg of 1-deoxy-*N*-1-D-fructosyl-L-valine ("fructosyl valine", **Figure 2**, R = H) for mutagenicity after nitrosation and addition of sulfamic acid. We performed this test because we wished to ascertain whether the *N*-nitroso derivatives of all glycosyl amino acids were mutagenic and fructosyl valine was the only glycosyl amino acid that we possessed. Fructosyl valine would have been extensively nitrosated, because it gave a 66% yield of ANC under conditions similar to those used here (*10*). Mean results in mutants/plate for 2.0 mg fructosyl valine after nitrosation were as follows. Without S-9: blank, 137; 8 μg of MNNG, 902; and test sample, 159. With S-9: blank, 137; 10 mg of DMN, 473; and test samples, 138. Results for 0.5 and 1.0 mg of fructosyl valine after nitrosation were similar to those for 2.0 mg of fructosyl valine. Clearly, nitrosated fructosyl valine was not mutagenic.

DISCUSSION

The NH_3 eluate from step 3 of the ANCP purification showed the highest specific activity (μmol ANCP/g dry weight) of any fraction. This activity corresponded to 33 wt % of ANCP if the ANCP had a mean molecular weight of 237, the value for Glu-Gly (**Figure 7**). After the NH_3 eluate was subjected to HPLC on a Pb^{2+} column, GC-MS of TMS derivatives was used to characterize those HPLC fractions that showed the highest ANCP contents, especially fraction 10 (**Figure 5** and **Table 2**). The 35.0 and 37.3 min GC peaks for fraction 10 were clearly due to glucose, because the GC retention times and mass spectra were almost identical to those for a glucose standard run at the same time. The 44.2 min peak from fraction 10 was tentatively attributed to the TMS derivative of Glu-Gly, because the largest of its MS peaks occurred at *m*/*z* 671.4, equal to M + H (one mass unit more than the molecular weight) for Glu-Gly and because the smaller MS peaks of the 44.2 min GC peak support the structure. For example, the peaks at *m*/*z* 452.0 and 435.3 could reasonably be due to loss of glycine fragments. Glucose could have arisen in fraction 10 by hydrolysis of Glu-Gly before the TMS derivatives were prepared. The GC peaks from fraction 10 at 51.9 and 52.6 min were attributed to disaccharides because their largest MS fragments showed *m*/*z* 903, attributed to M − CH_3 − H, where M is the mass of the TMS derivative of disaccharides.

This provisional identification of Glu-Gly in hot dog extracts suggests that the ANCP in hot dogs also included other glycosyl amino acids. A high level of free glycine might explain why only a glycine derivative was identified, but glycine was not especially prominent among the free amino acids in hot dogs extracts (Results, section 5). Fractions 11 and 12 from the Pb^{2+} column HPLC showed high ANCP levels (**Figure 3**), but their TMS derivatives did not show prominent GC peaks with retention times greater than 27 min. The ANCP in these fractions could have been glycosyl peptides, which would probably have formed poorly volatile TMS derivatives.

Several other points support the view that the ANCP in hot dogs are mostly *N*-glycosyl amino acids and peptides: (i) Conversion of the ANCP to ANC demonstrates that the ANCP contain NH groups. (ii) The ANCP-derived ANC are stable in alkali (*10*) and hence are not nitrosamides (*26*). (iii) The behavior of the ANCP on cation exchange resin supports the view that the ANCP are secondary amines, which would be absorbed from acid as $R_1R_2NH_2^+$ and eluted under basic conditions as the free bases R_1R_2NH. [Others have used cation exchange resins similarly to isolate fructosyl amino acids from soy sauce (*27*) and liver (*28*).] (iv) On HPLC on a Pb^{2+} column, the ANCP showed moderate retention times similar to those for sugars (*24*). (v) The TMS derivative of one ANCP traveled on GC between the TMS derivatives of glucose and those of disaccharides (**Figure 5**), suggesting that part of the ANCP molecule was a sugar. (vi) Both fructosyl valine and the ANCP in hot dogs were extensively nitrosated under the conditions used to assay ANCP (*10*), indicating facile nitrosation. *N*-1-Glucosyl amino acids are expected to be extremely weak bases, because the NH is linked to a carbon bearing a glycosidic oxygen (**Figure 7**). Confirming this view, *N*-glucosyl valylhistidine shows an acidic dissociation constant (pK_a) of only 5.6 (*29*). Such weak bases are very readily nitrosated (*30*). (vii) Similar to the ANCP-derived ANC, several *N*-nitroso glycosylamines and amino acids were direct mutagens in the Ames test (*31*, *32*) or induced DNA repair synthesis (*33*).

The finding that ANC and ANCP levels in hot dog patties did not vary much when sucrose was omitted or replaced by glucose (**Table 4**) does not support the proposed ANCP structure. However, the rate-limiting factor for ANCP formation in the patties could have been the concentration of free amino acids and peptides, rather than that of sugars.

The ANCP in rat feces (*16*) and human gastric juice (unpublished results from this laboratory) were purified by adsorption−desorption on silica gel and cation exchange resin under conditions similar to those used here for the ANCP in hot dogs. Hence, the ANCP from all three sources may be similar and consist mainly of glycosyl amino acids and peptides.

Nitrosation of Trp gave up to an 88% yield of ANC (**Table 3**, exps 1−4). The lowering of ANC production from Trp as nitrite concentration was raised (**Table 3**, exps 1 and 3) and was probably due to the low reaction pH of 1.0 at the highest nitrite concentration, because nitrosation typically shows maximum rates at pH 3.0−3.4 for amines (*30*) and 2.5 for amino acids (*34*). This view was supported by our finding (**Table 3**, exp 1) that ANC yield increased from 21 to 46% with increasing nitrite concentration when the pH was adjusted to 2.4−2.5 before the nitrosation. The results for the Trp dipeptides (**Table 3**, exps 5 and 6) suggest that *N*-terminal but not *C*-terminal Trp in peptides is readily nitrosated.

The mean ANCP level in hot dogs was 2700 μmol/kg (*10*), whereas the mean free Trp level in hot dogs was only 13.3 μmol/kg (Results, section 5). Therefore, only a small proportion of the ANCP in hot dogs could have been due to free Trp. On storage at ~18 °C, the level of ANC derived from Trp and tryptophanyl alanine decreased by mean values of 29 and 38%/h (**Table 3**, exps 1−5). In contrast, the level of ANC derived from the ANCP in hot dogs decreased by only 1−3%/h under similar conditions (**Table 3**, exps 7 and 8). This difference supports the view that the main ANCP in hot dogs was not Trp. However, peptides with *N*-terminal Trp could have been more significant ANCP than Trp itself.

The view that most of the observed mutagenicity was due to ANCP-derived ANC is supported by findings that (i) mutagenic

activity increased as the ANCP was purified (**Figure 6**), (ii) the activity of the nitrosated NH_3 eluate was twice as high in nitrosated as in un-nitrosated material (**Table 5**, exp 2), and (iii) both mutagenic activity (**Table 5**, exp 3) and ANC content (Results, section 7) of the nitrosated ANCP were stable on storage for 24 h. With respect to point (ii), the ratios of the activity of the blank, un-nitrosated NH_3 eluate and nitrosated NH_3 eluate were about 1:2:3 (**Table 5**). Hence, we ascribe about one-third of the activity of the nitrosated NH_3 eluate to the blank, one-third to the un-nitrosated NH_3 eluate, and one-third to the nitrosation. The mutagenicity of the un-nitrosated material suggests that some nitrosation of ANCP had already occurred before the laboratory nitrosation, presumably due to the nitrite present in hot dogs. [This product is manufactured with up to 120 mg nitrite ion/kg and contains an average of 10 mg nitrite/kg at the time of sale (*35*, *36*).] Alternatively, direct mutagens other than ANC could have been present. If all of the mutagenicity was due to *N*-nitroso compounds, then the maximum mutagenicity (that in the NH_3 eluate) was 345 mutants/μmol *N*-nitroso compound. The activity/mol ANC was only 2.1% of that for the direct mutagen methylnitronitrosoguanidine, which showed a mean activity of 16.7 mutants/nmol (**Table 5**, footnote *a*).

To induce mutations, most nitrosamines must undergo cytochrome P450-catalyzed oxidation to α-hydroxy derivatives, which spontaneously decompose to give monoalkyl nitrosamines. These are converted to diazonium ions ($RCH_2N_2^+$), which can alkylate DNA bases and thereby produce mutations (*25*). In contrast, we postulate here that the ANCP-derived ANC are nitrosamines that are direct mutagens. This would be the case if monoalkyl nitrosamines were produced from *N*-nitroso-1-deoxy-1-glucosyl amino acids by chemical or glucosidase-catalyzed hydrolysis. For example, *N*-nitroso Glu-Gly could decompose to give the diazonium ion $HOOCCH_2N_2^+$ (**Figure 7**). This ion reacts with DNA to give guanine alkylated by carboxymethyl and methyl groups at the 7 and O^6 positions (*37*, *38*). Methylguanine adducts occur in DNA of the human esophagus, stomach, and colon (reviewed in ref *38*). Finally, O^6-carboxymethyl guanine was detected in the DNA of exfoliated colonic cells of humans when they ate a high red meat diet (*39*). This level was significantly higher than that when the subjects ate a vegetarian diet. We suggest that this adduct in colonic DNA arose from *N*-nitroso Glu-Gly in the colon contents.

Methoxymethylmethyl nitrosamine [$CH_3OCH_2N(N{=}O)CH_3$], which, like *N*-nitroso Glu-Gly, contains an alkoxy group α to the nitrosamine function, is stable enough to be distillable (*40*). Nevertheless, hydrolysis of 1-deoxy-1-glucosyl-*N*-nitroso amino acids could occur readily because it was facilitated not only by the glycosidic oxygen but also by the 2-hydroxy group of the glucose moiety. Hydrolysis of 1-deoxy-1-fructosyl-*N*-nitroso amino acids (e.g., see **Figure 2**, R = NO) is unlikely to be facilitated by the relatively distant glycosidic oxygen. This may explain why nitrosated fructosyl valine was not mutagenic (Results, section 7).

Our results revive the suggestion (*31*, *32*, *41*) that nitrosamines derived from glycosyl amino acids are significant carcinogens. The mutagenicity of the purified ANCP after nitrosation could help explain the reported linkage between the consumption of nitrite-preserved meat products and the etiology of colon cancer. This would be the case if (among other conditions) the ANCP-derived ANC reach the colon and are carcinogenic in that organ and if the ANC that occur as such in hot dogs are identical to those formed by laboratory nitrosation of the hot dog ANCP. The possible hazard presented by colonic ANC is not a problem specific to hot dogs because other foods, e.g., sauces and salted dried fish, contain ANCP levels in the same range as that in hot dogs (*10*). The up to 240-fold variation in ANC and 8-fold variation in ANCP contents of different hot dog brands (*10*) suggest that, if necessary, manufacturing methods could be modified to lower the ANC and ANCP levels.

The main achievements reported here are the partial purification of the hot dog ANCP, the demonstration that nitrosated partially purified ANCP were directly mutagenic, and the tentative identification of an ANCP as glucosyl glycine. Much remains to be done. In future studies, we would like to (i) identify additional ANCP using other HPLC systems, GC-MS of TMS derivatives, and HPLC-MS of underivatized ANCP; (ii) synthesize Glu-Gly and compare its properties with that of the apparent Glu-Gly in hot dogs; (iii) investigate the toxicology of ANC derived from the hot dog ANCP; and (iv) determine the mutagenicity of nitrosated extracts of hot dog patties prepared in the absence of individual constituents or after adding nitrosation inhibitors.

ABBREVIATIONS USED

ANCs, apparent *N*-nitroso compounds; ANCP, ANC precursors; exp, experiment; Glu-Gly, 1-deoxy-*N*-1-D-glucosyl glycine; TEA, thermal energy analysis; TMS, trimethylsilyl; Trp, tryptophan.

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