rent chemical studies are, therefore, directed to employment of ^{13}C-enriched growth medium for *A. ochraceus* Wilh. in order to study further the biosynthesis of I and related compounds using cmr.

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Formation of *N*-Nitrosopyrrolidine from Pyrrolidine Ring Containing Compounds at Elevated Temperatures

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The formation of *N*-nitrosopyrrolidine from various pyrrolidine ring containing compounds and sodium nitrite at elevated temperatures was investigated. *N*-Nitrosopyrrolidine was formed when dry samples of L-proline, glycyl-L-proline, L-prolylglycine, and pyrrolidine were heated with nitrite at 170° for 2 hr and when proline was heated for 2 hr with sodium nitrite at 170° in a pH 6.2 buffer solution. Buffered collagen samples produced *N*-nitrosopyrrolidine at temperatures of 120° and above. L-Hydroxyproline did not produce *N*-nitrosopyrrolidine. The identity of *N*-nitrosopyrrolidine was confirmed in the heated samples by gas–liquid chromatography coupled with mass spectrometry.

N-Nitrosopyrrolidine (NPY), which has been shown to be carcinogenic in animal feeding trials (Druckrey *et al.,* 1967), has been identified in cooked but not in raw bacon. Fazio *et al.* (1973) reported levels of NPY from eight commercial brands of fried bacon ranged from 10 to 108 ppb. Crosby *et al.* (1972) and Sen *et al.* (1973) detected NPY in 13 of 24 samples and in 7 of 8 samples of fried commercial bacon, respectively.

Lijinsky and Epstein (1970) suggested cooking proteinaceous food might cause pyrolysis of protein and produce amino acids and the nitrosatable secondary amine pyrrolidine (PY). The greatest source of pyrrolidine ring compounds in foods would be the two α-imino acids proline (PR) and hydroxyproline (HP). Proline and HP are abundant amino acid residues in collagen which is the most abundant protein in mammalian muscle tissue (Price and Schweigert, 1971). In a recent report, Bills *et al.* (1973) identified *N*-nitrosoproline, pyrrolidine, spermidine, proline, and putrescine as potential precursors for the formation of NPY.

The purpose of this study was to investigate the heat-induced formation of NPY by reacting sodium nitrite with various pyrrolidine ring containing compounds. Included in this study were PR, HP collagen, and two proline containing dipeptides.

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EXPERIMENTAL PROCEDURES

Chemicals. L-Proline, HP, *N*-nitrosodipropylamine (NDPA), and NPY were purchased from the Eastman Kodak Co., Rochester, N. Y. Glycyl-L-proline (GP), L-prolylglycine (PG), and collagen from bovine Achilles tendon were purchased from the Sigma Chemical Co., St. Louis, Mo. Pyrrolidine was obtained from the Aldrich Chemical Co., Milwaukee, Wis. The GP, PG, and collagen were shown by thin-layer chromatography (Brenner and Niederwieser, 1960) to contain no free proline. Control samples of GP, PG, and collagen spiked with 1% free proline were easily detected by the thin-layer procedure. Since NDPA and NPY are potent carcinogens, caution was taken in handling nitrosamine solutions, spiked samples, heated vials, and concentrates, and work was done in efficient fume hoods whenever possible.

Preparation of Samples. Weighed amounts of compounds under investigation and powdered sodium nitrite were added to 35-ml test tubes. PR and HP samples were heated in the dry state and also in pH 6.2 buffer. For the collagen samples which were heated in the dry state, nitrite was added in solution and the collagen–nitrite mixture was subsequently freeze-dried. For the samples which were heated in solution, 1 ml of buffer was added to the test tube prior to sealing. The test tubes were shaken to completely mix the components, sealed in an oxygen–methane flame, and immediately placed in the oven. After heating, the samples were removed from the oven and allowed to cool. Samples not analyzed immediately were stored at −15° until extraction.

Extraction of Samples. The vials were broken at the tip and the contents were dissolved in 50 ml of distilled water saturated with anhydrous Na_2SO_4. The internal standard (NDPA) was weighed into the mixture and the solution was transferred to a separatory funnel. The aqueous solution was extracted three times with CH_2Cl_2 and the CH_2Cl_2 layer was drained to a flask containing anhydrous Na_2SO_4 to remove any traces of moisture.

A different extraction procedure was employed for the collagen samples. After heating, the vial was broken and the contents were placed in Whatman single strength cellulose thimbles. The broken vial was crushed into small pieces and additional anhydrous Na_2SO_4 was added and stirred with the contents of the thimble. The internal standard was gravimetrically added to the receiving flask and the samples were continuously extracted with CH_2Cl_2 for 18 hr.

Sample Concentration. The CH_2Cl_2 extracts were transferred to the Kuderna-Danish evaporative concentrator and concentrated to approximately 4 ml. Further concentration to approximately 1 ml was accomplished with a slow stream of prepurified nitrogen at room temperature.

Gas Chromatographic Analysis. A Varian gas chromatograph (series 1400) equipped with a flame ionization detector was used for the quantitative analyses of NPY. The column was 5 ft × ⅛ in. o.d. stainless steel packed with 5% Carbowax 20M on 70–80 mesh Chromosorb G, AW. Column temperature was 120° isothermal and the nitrogen flow rate was 30 ml/min at 120°. Injection port and detector temperatures were 210 and 270°, respectively.

A factor for relating the weight and peak area of the internal standard to the weight and peak area of NPY was determined from four runs in which authentic NPY and NDPA were weighed into 50 ml of distilled water saturated with anhydrous Na_2SO_4 and carried through the extraction, concentration, and gas chromatographic (gc) procedures. The addition of the internal standard prior to extraction and concentration provided a means to compensate for losses of NPY that undoubtedly occurred during these steps.

Gas–Liquid Chromatograph/Mass Spectrometry. An Atlas CH 4 rapid-scanning mass spectrometer connected directly to an F&M Model 810 gas chromatograph was used to identify NPY from the heated PR samples. The chromatographic conditions were as described above except helium was used for the carrier gas. The flow rate was 30 ml of helium/min at 120°. The gc was fitted with an effluent splitter which directed 17% of the column effluent to the flame detector and the remainder to the silicone membrane helium separator of the mass spectrometer. A Carle valve in the gc–mass spectral interface enabled the venting of excess solvent to the atmosphere. The mass spectral operating conditions were: filament current, 20 μA; electron voltage, 70 eV; accelerating voltage, 3.0 kV; analyzer pressure, 1.75×10^{-6} mm; and multiplier voltage, 1.6 kV. Spectra were scanned from m/e 25 to 250 in 2.5 sec.

A Finnigan Model 1015C gc–mass spectrometric system which included a Varian Aerograph Model 1400 gc was used to identify NPY in the heated GP, PG, and collagen samples. A 10 ft × ⅛ in. o.d. stainless steel column, packed with 5% Carbowax 20M on 70–80 mesh Chromosorb G, AW, was used to separate components of the samples. The flow rate was 30 ml of helium per min. The injector and column temperatures were 210 and 174°, respectively. The gc–mass spectrometer interface was a Gohlke all-glass, jet orifice separator which allowed optimal amounts of sample components to pass to the ion source. A total ion current monitor provided a chromatographic trace. The operating conditions were: filament current, 400 μA; electron voltage, 70 eV; analyzer pressure, 5×10^{-7} Torr; and multiplier voltage, 2.9 kV. Spectra were scanned from m/e 34 to 300 in 1.0 sec.

Table I. Amounts of *N*-Nitrosopyrrolidine Produced from Dry Samples Heated at 170° for 2 hr[a]

Compound	mg of NPY[b]
L-Proline	3.70
Glycyl-L-proline	0.80
L-Prolylglycine	0.44
Pyrrolidine	0.44
L-Hydroxyproline	N.D.[c]

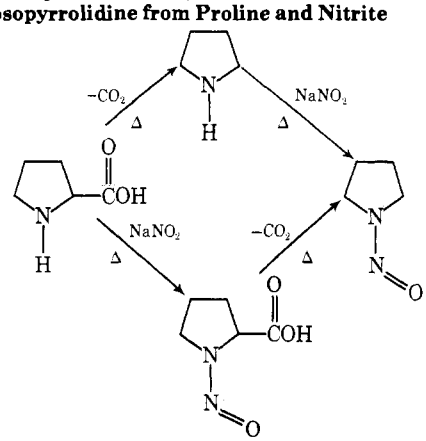
[a] From 1 mmol of compound and 1 mmol of sodium nitrite. [b] Mean from three replicate experiments. Maximum variation of replicates from the mean was approximately ±30%. [c] NPY not detected.

RESULTS AND DISCUSSION

The amounts of NPY produced from the dry samples of PR, GP, PG, PY, and HP are given in Table I. The amounts of NPY produced from PR agree closely with the values reported by Ender and Ceh (1971) when PR was heated under similar conditions. Ender and Ceh (1971) used starch while we employed sealed vials instead of starch to contain the nitrosamines in the heated samples. Our findings confirm the thin-layer Greiss identification of NPY reported by Ender and Ceh (1971).

Concerning the formation of NPY from PR, Ender and Ceh (1971) suggested heat-induced decarboxylation forming PY which reacted with nitrite to produce NPY. Sen *et al.* (1973) pointed out the possibility of nitrosation of PR and subsequent decarboxylation to NPY. A general outline as shown in Scheme I takes both possibilities into account. This dual route scheme would be consistent with the mechanism recently suggested by Sen *et al.* (1973).

Scheme I. Proposed Pathways for the Heat-Induced Formation of *N*-Nitrosopyrrolidine from Proline and Nitrite



The two dipeptides formed NPY when heated with $NaNO_2$ under dry conditions at 170° for 2 hr. It should be pointed out that GP is a common sequence unit in the protein collagen.

When 1 mmol each of PR and sodium nitrite were heated to 170° in pH 6.2 buffer solution, 0.58 mg of NPY was produced (Table II). HP did not produce NPY in either

Table II. Amounts of *N*-Nitrosopyrrolidine Produced from Proline and Hydroxyproline Heated at 170° for 2 hr at pH 6.2[a,b]

Compound	mg of *N*-nitrosopyrrolidine
L-Proline	0.58
L-Hydroxyproline	N.D.[c]

[a] Buffer prepared by adding 50 ml of 0.1 *M* potassium dihydrogen phosphate to 25.9 ml of 0.1 *M* sodium hydroxide; 0.3 ml of buffer was added to each sample and the pH was measured before heating. [b] One millimole of compound was allowed to react with 1 mmol of sodium nitrite. [c] NPY was not detected.

Table III. Amounts of *N*-Nitrosopyrrolidine Formed from Collagen When Heated at Various Temperatures and pH Values, and Dry for 2 hr[a-c]

Temp, °C	mg of *N*-nitrosopyrrolidine			
	pH 4.6[d]	pH 6.2[e]	pH 9.0[f]	Dry
120	N.D.	0.09	N.D.	N.D.
145	0.07	0.13	0.09	N.D.
170	0.38	0.86	1.02	N.D.
195	1.20	0.75	1.30	4.5

[a] All samples contained 0.300 g of collagen and 0.150 g of $NaNO_2$; buffered samples contained 1 ml of buffer. [b] pH was measured before heating. [c] Mean from two replicate experiments. Maximum variation of replicates from the mean was approximately ±30%. [d] Buffer prepared by adding 50 ml of 0.1 *M* potassium hydrogen phthalate to 8.7 ml of 0.1 *M* NaOH. [e] Buffer prepared by adding 50 ml of 0.1 *M* potassium dihydrogen phosphate to 25.9 ml of 0.1 *M* sodium hydroxide. [f] Buffer prepared by adding 50 ml of 0.05 *M* sodium bicarbonate to 10.7 ml of 0.1 *M* sodium hydroxide.

aqueous solution or dry under the conditions used. These results suggest that HP would not be a precursor of NPY. *N*-Nitroso-3-hydroxypyrrolidine may have been produced but it was not detected in this study.

Collagen samples were heated in buffer solutions at pH 4.6, 6.2, and 9.0 and dry using temperatures of 120, 145, 170, and 195° for 2 hr. Results are given in Table III, and three observations were noted: (1) only the pH 6.2 samples produced NPY at 120°, (2) small amounts of NPY were produced in all aqueous samples at 145°, and (3) in the temperature range 145–195° there was an increase in the amounts of NPY produced. The dry collagen samples did not produce NPY in the temperature range from 120 to 170° although it was formed at 195°. The results indicate that the pyrrolidine ring can be cleaved from a polypeptide chain and nitrosated in acidic, neutral, and alkaline buffers and dry at elevated temperatures. The identity of NPY in the heated samples was confirmed by comparing the mass spectrum obtained from the heated samples with a standard spectrum of NPY obtained under the same analytical conditions.

The results of this study suggest that collagen could be a precursor of NPY in cooked bacon. Caution must be observed in extrapolating the results of our work directly to cooked bacon because of the differences between the two systems. Nevertheless, PR makes up about 12% of the total amino acid residues in collagen. Raw bacon contains approximately 8% protein, and as much as 25% of the protein could be collagen (Price and Schweigert, 1971). The presence of nitrite, coupled with high cooking temperatures, could favor the formation of NPY during cooking.

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Analytical Studies of Cis–Trans Isomerization of Diethylstilbestrol Monomethyl Ether

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Solutions of the monomethyl ether of diethylstilbestrol (MME) reach a cis–trans equilibrium mixture in Et_2O very rapidly. Chloroform solutions equilibrate less rapidly, in approximately 15 min, as determined by gas–liquid chromatography (glc) and nuclear magnetic resonance (nmr). The equilibrium mixture concentrations are about 27–30% cis and 70–73% trans. Silylation decreases the rate of equilibration in both solvents and alters the equilibrium mixture if MME is silylated before the addition of $CHCl_3$ at room temperature. Increasing temperature tends to increase the rate of equilibration of silylated MME sample solutions. Mass spectral data helped to confirm that the two peaks observed by glc were each due to MME.

The monomethyl ether of diethylstilbestrol (MME) was the subject of much investigation for several years immediately following the discovery of diethylstilbestrol (DES). Geschickter and Byrnes (1942) and Reid and Wilson (1942) reported the results of studies of its estrogenic activity. Gottlieb (1948) and Kelly and James (1952) reported general assay procedures for synthetic estrogens including MME. The synthesis of MME was reported by Dodds *et al.* (1939), Reid and Wilson (1942), Rubin *et al.* (1945), and Wilds and Biggerstaff (1945).

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The synthesis of DES by Dodds *et al.* (1938), like many subsequent syntheses reported by Solmssen (1945), concludes with the demethylation of the dimethyl ether of DES (DME). Rubin *et al.* (1945) showed that MME can be formed under certain conditions during this demethylation step. The "British Pharmacopoeia" (1968) and The Pharmaceutical Society of Great Britain (1969) proposed thin-layer chromatography as a method of detection of MME in DES. More recently, Gainer and Chiasson (1974) reported the determination of MME by a gas–liquid chromatography (glc) assay.

The cis–trans isomerization of other stilbenes has been studied by Gegiou *et al.* (1968) and Saltiel and Megarity (1969). The cis–trans isomerization of DES has been investigated by Derkosch and Friedrich (1953), Rutherford