3-Hydroxy-*N*-nitrosopyrrolidine. Isolation from Heated 4-Hydroxy-*N*-nitrosoproline

Formation of 3-hydroxy-*N*-nitrosopyrrolidine from 4-hydroxy-*N*-nitrosoproline was demonstrated in a model system simulating conditions under which bacon is fried at a pan temperature of 170 °C. Identification of the 3-hydroxy-*N*-nitrosopyrrolidine was based on coincidence of gas chromatographic retention times and comparison of mass spectral fragmentation patterns with those of the authentic compound synthesized by a separate route.

Many *N*-nitrosamines have been demonstrated to cause cancer in animals. Recently, much attention has been focused on the occurrence of nitrosamines in cured meat products. Fazio et al. (1973) isolated *N*-nitrosopyrrolidine from pan-fried bacon but not from raw bacon. Lijinsky and Epstein (1970) speculated that *N*-nitrosopyrrolidine may arise either through the formation of *N*-nitrosoproline from proline and sodium nitrite with subsequent decarboxylation to *N*-nitrosopyrrolidine or by the decarboxylation of proline followed by nitrosation of pyrrolidine with nitrite during cooking.

In a model system designed to simulate bacon frying conditions at a pan temperature of 170 °C, Bills et al. (1973) demonstrated that *N*-nitrosoproline, proline plus nitrite, or pyrrolidine plus nitrite yielded *N*-nitrosopyrrolidine when heated. Kushnir et al. (1975) isolated *N*-nitrosoproline from raw bacon. Huxel et al. (1974) and Gray and Dugan (1975) reported that collagen, a protein containing significant amounts of proline, can also yield *N*-nitrosopyrrolidine when heated with nitrite. Crevasse et al. (1969) reported values of approximately 15 and 13% for proline and 4-hydroxyproline in acid-soluble collagen from the epimysium of pork muscle. Since collagen is a predominate source of these two amino acids in animal tissue, and since their concentrations are comparable in pork collagen, it would be reasonable to expect that 4-hydroxyproline might undergo decarboxylation and nitrosation (not necessarily in that order) in a manner analogous to proline to yield 3-hydroxy-*N*-nitrosopyrrolidine in fried bacon (see Figure 1).

The purpose of this investigation was to determine whether 4-hydroxy-*N*-nitrosoproline could be converted to 3-hydroxy-*N*-nitrosopyrrolidine when heated under bacon frying conditions.

EXPERIMENTAL PROCEDURES

Safety Precautions. Many *N*-nitroso compounds are potent carcinogens and care should be exercised in their handling. As a matter of good laboratory practice, all *N*-nitroso compounds, whether previously demonstrated to be carcinogenic or not, should be handled with the respect that cancer-causing agents deserve. Guidelines for laboratory procedures for handling carcinogens are available from the National Cancer Institute, Bethesda, Md.

Model Heating System. The system consisted of a 200-ml balloon flask containing 50 ml of steam-stripped vegetable oil, 0.2 g of 4-hydroxy-*N*-nitrosoproline dissolved in 2.0 ml of H_2O, and a boiling chip. The temperature of the contents of the flask was brought to 170 °C in about 10 min and held at 170 °C for an additional 10 min under reflux (Bills et al., 1973).

In some of the heating trials, the aqueous phase was neutralized with NaOH prior to heating in order to bring the pH of the system to that of bacon. Without neutralization, the aqueous phase had a pH of approximately 2 since 4-hydroxy-*N*-nitrosoproline is a strong organic acid.

Extraction. Following the heating process, the contents of the balloon flask were transferred to a 500-ml separatory funnel and extracted with 50 ml of methanol. The methanol extract was centrifuged to separate and remove residual lipid material. The clear methanol extract was then dried over anhydrous sodium sulfate and concentrated to a volume of 3 ml in a Kuderna-Danish apparatus equipped with a Snyder column. The extract was further concentrated under a stream of nitrogen to a volume of 1 ml in a Chromaflex sample tube.

Thin-Layer Chromatography. The concentrated extract was subjected to thin-layer chromatography (TLC). Silica gel plates of 250-μm thickness were used with a solvent of ether–methanol (3:1). Following development, the plates were sprayed with modified Griess Reagent (Fan and Tannenbaum, 1971) and then exposed to ultraviolet (UV) light to produce a pink spot for *N*-nitrosamines.

Synthesis of *N*-Nitrosamines. The method of Lijinsky et al. (1970) was used to synthesize 4-hydroxy-*N*-nitrosoproline. Synthesis of 3-hydroxy-*N*-nitrosopyrrolidine was accomplished as follows. Into a 50-ml round-bottomed flask was introduced 2 g of 3-hydroxypyrrolidine (Pfaltz and Bauer), 3 ml of 10 N HCl, 4 ml of water, and a magnetic stirring bar. The flask and its contents were cooled in an ice bath, and then, with constant stirring, 2 g of sodium nitrite dissolved in 4 ml of water was added dropwise. The reaction mixture was allowed to stand for 1 h in the ice bath. The aqueous phase was then saturated with anhydrous sodium sulfate and extracted with four 20-ml portions of anhydrous ethyl ether. The combined extract was dried over anhydrous sodium sulfate and concentrated to a volume of 4 ml in a Kuderna-Danish apparatus equipped with a Snyder tube.

Gas Chromatographic Conditions. A Varian gas chromatograph (GC), Series 1200, equipped with a flame ionization detector and a stainless steel column (0.125 in. o.d. × 6 ft) packed with 1% Carbowax 20M on Chromosorb G was employed. Temperatures of injector, column, and detector were 210, 200, and 265 °C, respectively. The flow rate of the carrier gas was 25 ml/min.

Identification of 3-Hydroxy-*N*-nitrosopyrrolidine. Identification of 3-hydroxy-*N*-nitrosopyrrolidine was based on coincidence of GC retention times, coincidence of TLC R_f values, and comparison of mass spectral fragmentation patterns with those obtained with the authentic compound. The MS was a Finnigan Model 1015C operated under the following conditions: filament current, 400 μA; electron voltage, 70 eV; multiplier voltage, 2.4 kV. Data acquisition and processing were accomplished with a System Industries (System 250) data system. The effluent of the GC was introduced into the MS ion source through a Gholke glass jet helium separator.

RESULTS AND DISCUSSION

When 4-hydroxy-*N*-nitrosoproline was heated in seven trials under the conditions described, a Griess-positive product was observed each time as a pink spot on the TLC plates on which the concentrated extract was spotted and developed. Under the conditions employed, the TLC R_f value for the new compound was 0.45 whereas the original compound (4-hydroxy-*N*-nitrosoproline) was found to

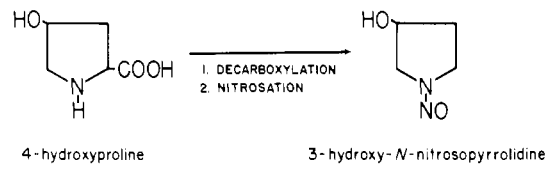

Figure 1. Proposed decarboxylation and nitrosation (not necessarily in that order) of 4-hydroxy-*N*-nitrosoproline to yield 3-hydroxy-*N*-nitrosopyrrolidine.

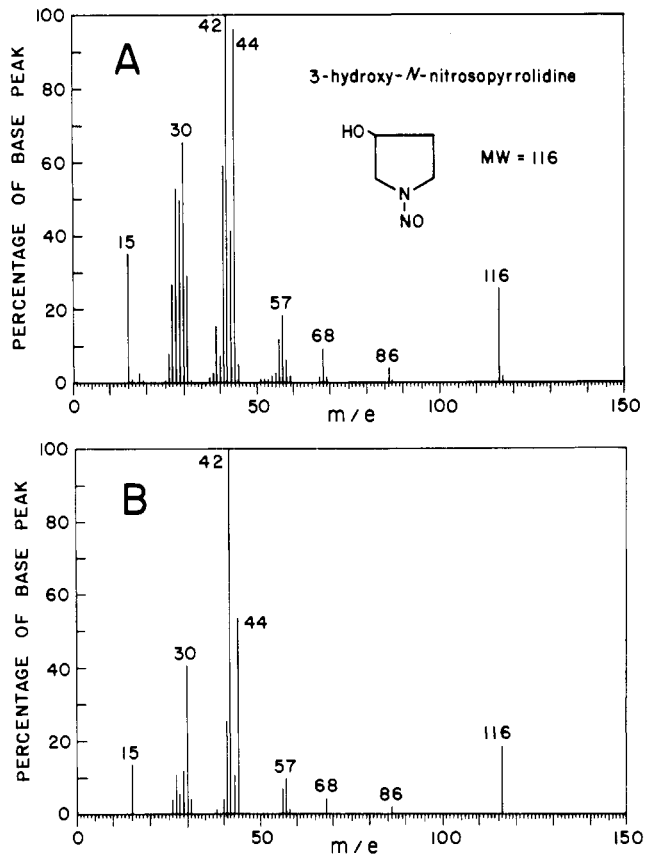

Figure 2. Mass spectra of 3-hydroxy-*N*-nitrosopyrrolidine: (A) authentic compound (synthesized in the laboratory); (B) isolated from heated 4-hydroxy-*N*-nitrosoproline.

elongate only slightly from the point of application. The Griess-positive product at R_f 0.45 was observed when the pH of the aqueous phase of the model system was adjusted to 7.0 or when it was left unadjusted prior to heating. However, 4-hydroxy-*N*-nitrosoproline was completely absent following heating of the systems in which the pH was not adjusted, but was still present to some extent in the heated systems in which the pH was adjusted to 7.0. Also, the yield of the Griess-positive product at R_f 0.45 was lower in the pH adjusted treatment.

When the Griess-positive product was scraped from a TLC plate (which had been developed but not sprayed with Griess reagent), taken up in solvent, and analyzed by GC and MS, it was identified as 3-hydroxy-*N*-nitrosopyrrolidine by comparison of its TLC R_f value, GC retention time, and MS fragmentation pattern with those of the authentic compound which was synthesized as described (Figure 2).

The work described in this communication demonstrated that 3-hydroxy-*N*-nitrosopyrrolidine can be formed from 4-hydroxy-*N*-nitrosoproline under conditions intended to simulate the frying of bacon. The isolation and identification of 3-hydroxy-*N*-nitrosopyrrolidine in fried bacon and in fried-out fat have recently been accomplished in our laboratory and efforts to quantitate the nitrosamine are currently underway. These results will be reported in the near future.

LITERATURE CITED

Bills, D. D., Hildrum, K. I., Scanlan, R. A., Libbey, L. M., *J. Agric. Food Chem.* **21**, 878 (1973).
Crevasse, G. A., Pearson, A. M., Brunner, J. R., McClain, P. E., *J. Agric. Food Chem.* **17**, 356 (1969).
Fan, T. Y., Tannenbaum, S. R., *J. Agric. Food Chem.* **19**, 1267 (1971).
Fazio, T., White, R. H., Dusold, L. R., Howard, J. W., *J. Assoc. Off. Anal. Chem.* **56**, 919 (1973).
Gray, J. I., Dugan, L. R., Jr., *J. Food Sci.* **40**, 484 (1975).
Huxel, E. T., Scanlan, R. A., Libbey, L. M., *J. Agric. Food Chem.* **22**, 698 (1974).
Kushnir, I., Feinberg, J. I., Pensabene, J. W., Piotrowski, E. G., Fiddler, W., Wasserman, A. E., *J. Food Sci.* **40**, 427 (1975).
Lijinsky, W., Epstein, S. S., *Nature (London)* **225**, 21 (1970).
Lijinsky, W., Keefer, L., Loo, J., *Tetrahedron* **26**, 5137 (1970).

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