remarkably from that obtained by steam distillation. Even higher boiling products with a retention index up to 1700 can be isolated by this method which proves to be very mild and produces practically no artefacts. It has been applied successfully to leek, apples (Dirinck et al., 1975), and tomatoes (Dirinck et al., 1976) and can be used in evaluating natural flavor quality of fruits and vegetables.

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Nitrosopyrrolidine Formation in Fried Bacon

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Experiments involving ^{14}C-labeled amines have shown that proline is more likely to be the precursor of nitrosopyrrolidine formation than either spermidine or putrescine in fried bacon. The data are consistent with a mechanism involving formation of nitrosoproline from sodium nitrite and free proline during processing with subsequent decarboxylation of nitrosoproline during frying.

The existence of the carcinogen, nitrosopyrrolidine (NPYR), in fried bacon is well documented. Crosby et al. (1972) found NPYR in 13 out of 24 samples of fried bacon in concentrations up to 40 ppb. Sen et al. (1973) found NPYR in 8 out of 9 samples of fried bacon in concentrations from 4 to 25 ppb. Higher levels, from 10 to 108 ppb, were reported by Fazio et al. (1973) in eight commercial brands of bacon fried at 350 °F. Nitrosopyrrolidine was not found in raw bacon, indicating that the high temperatures involved in the bacon frying process played a major role in its formation. Another major factor is the amount of sodium nitrite (Sen et al., 1974) added to pork bellies during processing as the reaction of nitrite with some amines is known to produce nitrosamines.

Thus, the amount of NPYR formed can be reduced by the addition of lower quantities of sodium nitrite or by the inclusion of nitrite scavengers such as ascorbic acid (Fiddler et al., 1973) or propyl gallate (Sen et al., 1975). Unfortunately, a combination of improved analytical methodology such as obtained by the use of thermal energy analyzer detectors (Fine et al., 1975) and the apparently unresolvable controversy over whether or not there is a threshold value for carcinogens makes for continuous consumer concern and the possibility of ever more stringent government regulatory action on the use of nitrites. Further reduction in nitrite levels may result in bacon with inferior color and taste as well as bacon with higher amounts of disease-causing microorganisms (Christiansen et al., 1974). As a result, there have been efforts in the recent past to determine what compound(s) react(s) with nitrite to form NPYR so that the mechanism of the reaction could be better understood. With this knowledge, it might be possible to alter processing techniques in order to eliminate NPYR formation without too great a reduction in nitrite.

Bills et al. (1973) found that NPYR was produced from nitrosoproline, pyrrolidine, spermidine, proline, and putrescine in yields of 2.6, 1.0, 1.0, 0.4, and 0.04%, respectively, in a model system containing 1 ml of water in 100 ml of oil heated at 170 °C for 5 min.

Huxel et al. (1974) found that NPYR could arise from heating dry samples of sodium nitrite with the pyrrolidine ring containing compounds: proline, glycylproline, prolylglycine, and pyrrolidine at 170 °C for 2 h. Dry collagen samples formed NPYR when heated with nitrite at 195 °C.

Using a model system similar to that of Bills et al., Gray and Dugan (1975) found that NPYR could be formed as a result of thermal decomposition of collagen to proline with subsequent reaction with nitrite after only 20 min at temperatures as low as 120 °C. Using still another model system, Warthesen et al. (1975) found that putrescine dihydrochloride and ornithine hydrochloride produced NPYR in 22.8 and 1.2% theoretical yields, respectively. Model systems, while useful, do not adequately reflect actual conditions. Our approach, therefore, involved spiking bacon with known amounts of ^{14}C-labeled precursors and determining the yield of NPYR formed after frying.

EXPERIMENTAL SECTION

Apparatus. *Liquid Scintillation Counter.* A Beckman LS-233 scintillation counter equipped with an external standardization system was used. Readings (in counts per minute) were obtained from a full channel (0–1000) window.

GC–MS. A DuPont 21-490 mass spectrometer equipped with mass fragmentography accessory (Pareles and Rosen, 1974) and interfaced to a Varian 2740 gas chromatograph was used. The column was a 3 ft × $^1/_8$ in. i.d. 1% OV-17 on HP Chromosorb W, 80–100 mesh. At column, injector, and detector temperatures of 90, 100, and 225 °C, respectively, and a helium flow rate of 30 cm^3/min, NPYR had a retention time of 4.5 min. Mass fragmentography

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responses were measured at both m/e 100 and 69.

Radioactive Chemicals. Uniformly labeled L-[^{14}C]-proline (sp act. 290 mCi/mmol) and 1,4-[^{14}C]putrescine dihydrochloride (sp act. 63 mCi/mmol) were purchased from Amersham/Searle. 1,4-[^{14}C]Spermidine trihydrochloride (sp act. 110 mCi/mmol) was purchased from New England Nuclear. Uniformly labeled [^{14}C]nitrosoproline (sp act. 0.52 mCi/mol) was synthesized by reacting L-[^{14}C]proline with sodium nitrite in 2 N hydrochloric acid at 10 °C. Uniformly labeled [^{14}C]NPYR (sp act. 0.73 mCi/mol) was synthesized by reaction of sodium nitrite with uniformly labeled [^{14}C]pyrrolidine. The latter was made by decarboxylating [^{14}C]proline in tetralin in the presence of acetophenone as described by Chatelus (1964). Purities of the synthesized ^{14}C-labeled materials (NPYR, 98%; nitrosoproline, 95%) were determined by thin-layer chromatography.

Treatment of Bacon with Sodium Nitrite and Precursors. Smoked bacon, to which no sodium nitrite, sodium nitrate, or ascorbic acid had been added, was obtained from USDA. This bacon was kept frozen at −20 °C until used. After thawing, the bacon was cut into 1-g cubes (ca. 1 cm). Each cube (except those to be subsequently injected with nitrosoproline) was then injected with the appropriate solution of sodium nitrite. This was accomplished by injecting 5 μl of the solution 2 mm apart (100 μl total). The cube was then kept at 4 °C for 48 h and then similarly injected with 100 μl of the respective ^{14}C-labeled precursors. The bacon was then stored at 4 °C for 24 h. It was then cut up into approximately four equal pieces. Two pieces were fried separately and subsequently analyzed for NPYR formation. The other two pieces were each digested in 1 ml of NCS tissue solubilizer (Amersham/Searle) at 50 °C for 24 h followed by neutralization with glacial acetic acid and counting in PPO solution.

The following quantities of precursors per gram of bacon were injected: proline, 4.68 μCi; putrescine dihydrochloride, 3.85 μCi; spermidine trihydrochloride, 4.32 μCi; nitrosoproline, 0.1 μCi. This corresponds to free base precursor concentrations of 2.0, 5.56, 5.85, and 27 648 ppm, respectively.

Frying Conditions. A 250-mg slice (2 mm thick) of bacon was placed in a 100-ml round-bottomed flask fitted with a reflux condenser. The flask was then lowered in an oil bath preheated to 185 °C and frying was allowed to proceed for 5 min.

Analysis for [^{14}C]Nitrosopyrrolidine. The entire contents of the reaction flask were digested with 300 mg of sodium hydroxide in 2 ml of methanol for 3 h. After cooling, the mixture was filtered through glass wool and the filtrate was continuously extracted with methylene chloride for 7 h. The methylene chloride extract was dried over anhydrous sodium sulfate, filtered, and evaporated under reduced pressure to about 500 μl. The solution was then streaked on a 250 μm silica gel precoated (quanta/gram) plate and developed with methylene chloride–ether–hexane (10:7:5). [^{14}C]NPYR was spotted on both sides of the streaked material. Visualization was accomplished by autoradiography using Kodak single-coated Medical x-ray blue sensitive film. Bands corresponding to the R_f value of NPYR were scraped off and eluted with methanol. After evaporation, this fraction was rechromatographed on another TLC plate with methylene chloride–hexane–methanol (10:5:1). Again, [^{14}C]NPYR was spotted on both sides of the spotted material and visualization was accomplished as before. The band corresponding to [^{14}C]NPYR was scraped off and suspended in Cab-O-Sil counting solution.

Table I. Volatilization of NPYR during Frying

Concn in bacon, mg/g	% volatilized
0.107	36.6
0.222	34.5
2.14	22.2
4.88	22.1
8.93	14.2
	Av 25.9

Table II. Percent Yield of NPYR from Various Precursors

	Concn. of $NaNO_2$ in bacon (ppm)				
Precursor	200	100	50	25	0
Proline[a]	0.328	0.277	0.261	0.148	
Nitrosoproline[a]					1.43
Spermidine	ND[b]	ND			
Putrescine	ND	ND			

[a] Average of two determinations. [b] ND = none detected.

RESULTS AND DISCUSSION

Recoveries of [^{14}C]NPYR from bacon using the described method averaged 55% while the GLC method (which uses the same extraction method) averaged 70% (Pensabene et al., 1974; Fazio et al., 1971). The lower recoveries obtained in our procedure are probably due to the fact that the total amount of NPYR present was much smaller or due to losses in evaporation during the two preparative TLC purifications. Since 200 dpm could be determined, the sensitivity of our method (assuming 100% recovery) was 0.0097, 0.0084, and 0.0094% yield for proline, spermidine, trihydrochloride, and putrescine dihydrochloride, respectively. During the course of the recovery studies, it became apparent that a significant quantity (ca. 25%) of the NPYR that had been injected into bacon was volatilized during frying (Table I). Measurement of the trapped volatiles by both liquid scintillation counting and mass fragmentography gave essentially identical results and indicated that NPYR itself, and not its thermal decomposition products, if any, had volatilized. Since NPYR is known to form in a significant number of commercial bacon samples, a potential health hazard exists for employees of dining establishments. It must be remembered, however, that there is no experimental evidence showing that NPYR is carcinogenic upon inhalation.

Of four possible precursors studied, only two, proline and nitrosoproline, gave detectable yields of the carcinogen (Table II).

Confirmation of NPYR formation was made by use of a second thin-layer solvent system in the case of proline and by GC–MS in the case of nitrosoproline. Proline was converted to NPYR in 0.148% yield when only 25 ppm of sodium nitrite was added, while both ^{14}C-labeled spermidine and putrescine failed to produce detectable levels of NPYR even at initial sodium nitrite levels of 200 ppm.

Bacon containing [^{14}C]nitrosoproline gave NPYR in 1.43% yield, a level consistent with that found by Kushnir et al. (1975) in bacon samples after correction for their poor recoveries of nitrosoproline and our poor recoveries of NPYR. Our values for the percent yield of NPYR from proline also fall within the range of values (10–108 ppb) obtained by other investigators for the amount of product found in fried bacon (Fazio et al., 1973; Crosby et al., 1972; Pensabene et al., 1974). If we assume an initial proline concentration of 20 ppm in our bacon (Lakritz et al. (1975) report values of 17.25 to 25.3 ppm), and use a "ballpark" value of 0.3% yield from our data, we calculate the for-

mation of 52 ppb of NPYR. Adjusting for the fact that we calculate our total to also include the amount volatilized (about 25%) and for the difference in average recoveries (55 vs. 70%), we obtain a value of 50 ppb.

The failure of spermidine and putrescine to produce NPYR in our studies does not agree with the findings of Bills et al. (1973) or Warthesen et al. (1975), both of whom found significant quantities of NPYR formation from these two amines in model systems. We feel that our experimental conditions more accurately reflect the true situation in that the reaction was conducted in bacon using realistic frying times and realistic quantities of precursor. For example, Spinelli et al. (1974) determined the concentration of putrescine in pork bellies to be 2.3–36 ppm while the concentration of spermidine was 1.7–14.9 ppm. By the use of ^{14}C-labeled amines, we were able to inject quantities which amounted to concentrations of ca. 6 ppm of spermidine or putrescine in bacon. Injection of [^{14}C]proline added less than 2 ppm to the concentration level, an amount below that found by Lakritz et al. (1975).

The concentration of precursor is of great importance since the rate of formation of nitrosamine depends on it as well as on the square of the nitrite ion concentration (Mirvish, 1975).

Because our [^{14}C]nitrosoproline was of low specific activity, the amount of this precursor present in our bacon was unrealistically high (28 mg/g). However, the thermal decomposition of nitrosoproline to NPYR is a unimolecular process and the percent yield will not be affected by the concentration of starting material.

Other model system studies (Huxel et al., 1974; Gray and Dugan, 1975) have indicated that collagen can be the precursor of NPYR. Both studies, however, were conducted using either too much acid (pH 4) or unreasonably high concentrations (parts per thousand) of sodium nitrite. In the first study, an unreasonably long reaction time (2 h) was used. Nevertheless, our studies in no way disprove the possibility that proline found in collagen is released upon frying to react with nitrite. Our studies, however, tend to support the pathway of free proline converted to nitrosoproline with the latter being decarboxylated during the frying process. Although the overall yield is low, calculations based on the known amount of free proline present and the percent yield of NPYR are consistent with the production of parts per billion concentrations upon frying. Further support for this mechanism comes from the demonstration of the presence of nitroisoproline in processed bacon (Kushnir et al., 1975) as well as the findings of Sen et al. (1974) that the amount of NPYR formed correlated with *initial* nitrite concentration and not with nitrite concentration at the time of frying. Since no NPYR is found in unfried bacon and the frying process is of relatively short duration, it is difficult to imagine a process in which nitrosation, protein hydrolysis, and decarboxylation would take place without the concentration of nitrite present at frying having an effect on NPYR yield.

An alternate mechanism, decarboxylation of proline followed by reaction with nitrite, is also a possibility. Again, this pathway would not be in accord with the results of Sen et al. (1974). Unfortunately, the low specific activity of our [^{14}C]pyrollidine would force us to use unrealistically large amounts of the latter if we were to investigate this question experimentally.

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