Effect of Storage and Cooking on Penicillin in Meat

Anneli M. DePaolis, S. E. Katz, and Joseph D. Rosen*

Transformations of benzylpenicillin residues in beef and chicken have been studied under both storage (−2 °C) and cooking (60–85 °C) conditions. The major product formed under storage conditions is α-(1-carboxyethyl) hydrogen benzylpenicilloate (IV), and this is also the major product after the meat is cooked. At higher cooking temperatures, IV isomerizes.

The widespread use of antibiotics for treatment of animal infections and growth stimulation has in turn stimulated much concern over the effects of antibiotic residues in animal foods on public health (Oehme, 1973; Huber, 1971; Swann, 1969). In the case of benzylpenicillin (I), the two major concerns are increased sensitivity to penicillin allergy (Zimmerman, 1959) and the possibility of the development of microorganisms resistant to antibiotics in humans (Huber, 1971). Although there is a lack of firm scientific evidence to support the latter fear in regard to the general population, there has been a report of increased numbers of resistant organisms among members of farm families (Levy et al., 1976).

Further questions have been raised in regard to health effects due to ingestion of penicillin metabolites and/or their thermal (cooking) degradation products. Van Schothorst, as cited by Huber (1971), showed that penicillin activity in meat decreased after heating but the degradation products were not identified. Katz et al. (1974) found that benzylpenicilloic acid (II), a known metabolite of benzylpenicillin as well as a likely thermal degradation product (Clarke et al., 1949), caused increased numbers of antibiotic resistant coliforms in chickens after feeding. We decided, therefore, to determine the chemical structure of the benzylpenicillin thermal degradation products in meat, to approximately determine the extent of their formation, and to synthesize enough of these materials for evaluation of their public health significance.

EXPERIMENTAL SECTION

Materials. All solvents were reagent grade (Fisher Scientific) except acetonitrile which was glass-distilled (Burdick and Jackson, Muskegon, Mich.). Hydromix (Yorktown Research, Hackensack, N.J.) was used as the liquid scintillation counting cocktail. Sodium benzylpenicillinate and benzylpenicillenic acid were purchased from Sigma Chemical (St. Louis, Mo.); procaine salt of benzylpenicillin from National Biochemicals (Cleveland, Ohio); procaine hydrochloride from Pfaltz and Bauer (Flushing, N.Y.); calcium lactate from Mallinkrodt (St. Louis, Mo.). Potassium [^{14}C]benzylpenicillinate (label as shown in Figure 1), sp act. 54 mCi/mmol, 99% purity (TLC), was purchased from Amersham/Searle (Arlington Heights, Ill.). Diazomethane was generated fresh for each methylation (Fales et al., 1973) from *N*-methyl-*N'*-nitro-*N*-nitrosoquanidine (Aldrich, Milwaukee, Wis.). All silica gel TLC plates contained a preadsorbent diatomaceous earth spotting band and were purchased from Quantum Industries (Fairfield, N.J.). Autoradiochromatography was performed on Kodak single-coated medical x-ray film, blue-sensitive SB-54 (Eastman Kodak, Rochester, N.Y.). Freeze-dried hamburger was manufactured by Wilson (Oklahoma City, Okla.) and fresh beef was purchased from a nearby slaughterhouse *immediately* after slaughter; chicken was freeze-dried in our own freeze dryer.

Synthesis of Suspected Degradation Products. Benzylpenicilloic (II), benzylpenilloic (III), and benzylpenillic acids were synthesized according to published procedures (Clarke et al., 1949). α-(1-Carboxyethyl) hydrogen benzylpenicilloate (IV) was synthesized by reacting sodium benzylpenicillinate (17.8 g, 0.05 mol) with calcium lactate (12 g, 0.055 mol) in 100 mL of a 1:1 mixture of water and dimethyl sulfoxide for 0.5 h at room temperature. The reaction mixture was diluted with 110 mL of water, acidified at 0 °C to pH 2 with 10% phosphoric acid and immediately extracted with ether. The ether extract was dried over anhydrous magnesium sulfate, evaporated to dryness, and the resulting oil crystallized upon scratching with a glass rod. Recrystallization attempts failed because of decomposition of the material. However, TLC and HPLC analyses of the unrecrystallized material showed it to be essentially pure. Another synthesis using incorporated [^{14}C]benzylpenicillin showed essentially complete conversion to IV with only trace amounts of unreacted starting material.

Apparatus. A Dupont 21-490 mass spectrometer equipped with both EI and CI sources, a Varian EM-390 nuclear magnetic resonance spectrometer, a Beckman IR8 infrared spectrophotometer, a Beckman LS-233 liquid scintillation counter equipped with external standardization system, and a Tracor 5000 liquid chromatograph equipped with 254-nm detector and a 25 cm × 4.6 mm i.d. stainless steel column packed with 10μ Partisil ODS (Whatman, Clifton, N.J.) were used. The mobile phase for the latter was a freshly prepared mixture of water and acetonitrile (77:23) pumped at a pressure of ca. 500 psi to provide a flow rate of 0.7 mL/min.

Cooking and Spiking Procedures. A solution of procaine hydrochloride was mixed with a solution of potassium [^{14}C]benzylpenicillinate to give a solution of procaine [^{14}C]benzylpenicillinate. Fifty microliters (2.5 μCi) of the latter was diluted with 150 μL of water and added to 100 mg of freeze-dried hamburger or chicken in a small reactor vial. After absorption of the aqueous solution by the meat, the vials were placed in an oil bath (whose temperature was maintained at either 60, 74, or 85 °C) and heated for 30 min. These temperatures correspond to rare beef, well-done beef, and chicken roasting temperatures, respectively. By means of a thermocouple it could be ascertained that the internal temperatures of the meats were about 1–2 °C lower than the oil bath temperatures.

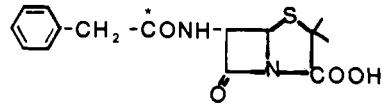
Solutions of procaine [^{14}C]benzylpenicillinate in pH 5.5 phosphate buffer were also heated for various times at temperatures corresponding to those used to cook the meat and chicken. These solutions were prepared by diluting 50 μL of the procaine salt solution (2.5 μCi) with 150 μL of pH 5.5 phosphate buffer.

Department of Food Science (A.M.D., J.D.R.), and Department of Biochemistry and Microbiology (S.E.K.), Cook College, Rutgers University, New Brunswick, New Jersey 08903.

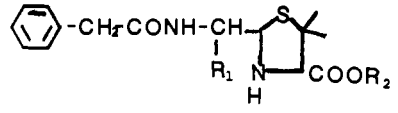
Table I. Comparison of Thermal Degradation Products Obtained (% of Total Radioactivity Recovered) after Heating[a] Penicillin

Material	R_f value	Buffer (25 °C)	Beef (60 °C)	Buffer (60 °C)	Beef (74 °C)	Buffer (74 °C)	Chicken (85 °C)	Buffer (85 °C)
12	1.00	4.06	2.69	4.69	2.36	5.18	2.58	4.44
11	0.92	1.25	0.97	1.96	1.07	2.91	1.36	5.26
10	0.81	84.74	69.14	83.44	63.26	77.36	46.11	66.00
9	0.72	0.86	1.56	2.47	2.61	4.48	3.36	11.81
8	0.62	1.12	1.70	1.41	2.48	3.08	6.73	5.39
7	0.45		14.59		17.58		19.02	
6	0.32		1.84		3.64		10.60	
5	0.30	0.95		1.39		2.80		3.65
4	0.23	5.36	2.81	3.17	4.67	2.17	2.58	1.47
3	0.18	0.66	3.56		1.14		5.69	
2	0.13	0.56	0.43	0.75	0.52	1.09	0.73	0.92
1	0.08	0.43	0.72	0.73	0.67	0.93	1.23	1.05

[a] All samples heated for 30 min.



I



II $R_1 = -CO_2H$, $R_2 = H$

III $R_1 = H$, $R_2 = H$

IV $R_1 = -CO_2-\underset{CH_3}{\underset{|}{CH}}-CO_2H$, $R_2 = H$

V $R_1 = -CO_2-\underset{CH_3}{\underset{|}{CH}}-CO_2CH_3$, $R_2 = CH_3$

Figure 1. Structures of materials discussed in text. Asterisk in structure I denotes position of ^{14}C label.

TLC Analysis and Quantitation. Water was pressed out of the cooked meats and aliquots were spotted directly on a 250 μ TLC plate together with controls of procaine [^{14}C]benzylpenicillinate in water, pH 5.5 phosphate buffer, and uncooked meat. The well-dried plates were developed in a solvent system of ethyl acetate–acetic acid–water (8:1:1, v/v), and the thermal degradation products were located by exposure to x-ray film for a period of overnight to a few days, depending on the amount of radioactivity spotted. In most cases about 6 μL (27 750 dpm/μL) of the meat extracts were sufficient for a 24-h exposure to distinguish all of the major metabolites formed.

The silica gel areas corresponding to the spots on the developed x-ray films were scraped off the plate, suspended in 10 mL of Hydromix and just enough water (2.2 mL) to form a gel and counted in the liquid scintillation counter.

Extraction and Isolation of Degradation Products from Meat. Since preliminary TLC studies indicated the same kind of breakdown pattern for both the procaine and sodium salts of benzylpenicillin, the latter, due to its greater water solubility, was used in attempts to obtain identifiable quantities of thermal degradation products.

Thirty grams of freeze-dried hamburger meat were broken up and soaked in 50 mL of an 8% aqueous solution of sodium penicillinate for 15 min, and the meat was cooked in a 500-mL beaker immersed in an oil bath at 80 °C for half an hour. The cooked meat was cooled and then homogenized with 150 mL of methanol in a Waring blender. The methanol extract was centrifuged and the cloudy supernatant was filtered. The methanol filtrate was concentrated under reduced pressure to about 25 mL and was refrigerated overnight.

The white precipitate that was formed contained neither benzylpenicillin nor its degradation products (TLC). After filtration of these meat constituents, the supernatant liquid was concentrated to near dryness, diluted with 50 mL of water and acidified to pH 2 with 10% phosphoric acid at 0 °C. The resultant acid mixture was extracted into ethyl ether and methylated with diazomethane. Each step was carefully monitored by TLC (using iodine for visualization) to see that the workup procedure had not altered the metabolite pattern.

The methyl esters were first separated by crude fractionation on preparative (1 mm) TLC plates using a benzene–ethyl acetate (1:1) solvent system, and then by high-pressure liquid chromatography (HPLC).

RESULTS AND DISCUSSION

The [^{14}C]benzylpenicillin spiked meat was cooked to temperatures corresponding to well-done chicken and to rare and well-done beef. The aliquots from each sample were resolved by TLC, detected by autoradiochromatography, and quantitated with a liquid scintillation counter. The new type of TLC plate with the preabsorbent layer gave far superior resolution than conventional types of precoated TLC plates. This is due to the fact that the material, although liberally spread on a large spotting area, is concentrated to a narrow band by the developing solvent before entering the absorbing silica gel layer. Phosphate buffer (pH 5.5) solutions of [^{14}C]benzylpenicillin were cooked simultaneously at the same temperatures as the meat samples. This pH was chosen because it represents the approximate pH of meat (Price and Schweigert, 1971). Representative thin-layer chromatograms of the meat extracts and the heated buffer solutions are shown in Figure 2; quantitative data are given in Table I.

Since very small amounts of meat were used in these experiments (resulting in a more thorough distribution of heat than under usual household procedures), the values in Table I should not be misconstrued as being an accurate representation of the distribution of penicillin and its degradation products in cooked meat. The data, however, serve as a good guide as to what products are formed.

The effect of increasing temperature on the buffer solutions was a gradual decrease of benzylpenicillin and benzylpenicilloic acid (the latter being decarboxylated to benzylpenilloic acid) and a gradual increase of several other degradation products. Comparison to the TLC R_f values of components of the heated buffer solutions and meat with those of known metabolites of benzylpenicillin gave presumptive evidence for the presence of benzylpenillic

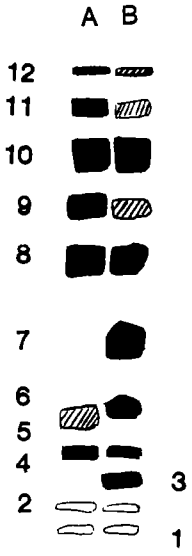


Figure 2. TLC chromatograms of procaine [^{14}C]benzylpenicillin (A) after heating in pH 5.5 buffer and (B) in meat. Eluent = ethyl acetate–acetic acid–water (8:1:1).

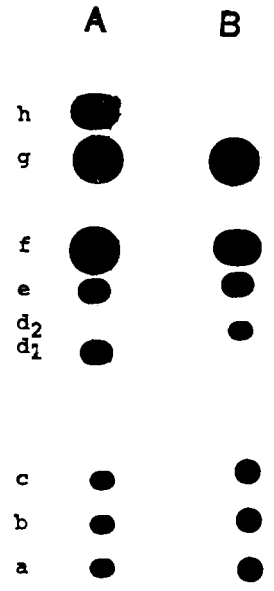


Figure 3. TLC chromatogram of diazomethane-treated (A) meat extract (B) reaction product mixture of heated IV. Eluent: benzene–ethyl acetate (1:1).

acid (spot no. 1), benzylpenicilloic acid isomers (spots no. 3 and 4), benzylpenilloic acid (spot no. 8), and benzylpenicillenic acid (spot no. 11). Material no. 10 was the acid form of benzylpenicillin and material no. 12 was procaine benzylpenicillin salt. Elution with a second solvent system (acetone–acetic acid, 95:5) indicated that spot no. 8 was, in fact, two isomers of benzylpenilloic acid.

The TLC data from the heated, spiked meat indicated that even under the most drastic cooking conditions employed (well-done chicken), about half of the recovered radioactivity was due to penicillin. Although our cooking procedures were not exactly normal, it can be inferred from our data that normal cooking conditions cannot be relied upon to completely destroy penicillin residues. Furthermore, very little benzylpenicilloic, benzylpenillic, and benzylpenicillenic acids were formed in cooked meat but the possibility that these materials could have been bound to meat protein has not been excluded (Levine, 1960). Methanol extraction of the cooked meat (three times) resulted in recovery of only 50.4% of the radioactivity applied.

Of major interest was the formation of two materials (no. 6 and no. 7) in both beef and chicken which did not form in the cooked buffer solutions. One of these materials (no. 7) was formed faster than material no. 6. The concentration of the latter increased with higher temperatures and longer cooking times, while the formation of no. 7 gradually leveled off. Material no. 7 formed in the meat samples even without cooking as shown by storage studies using meat from a steer slaughtered 1 h before spiking. After 6 days at a storage temperature of −2 °C, material no. 7 accounted for 7.95% of the recovered radioactivity. Trace amounts were present even after 1 day at this temperature. Since it takes about 1 week for fresh beef to reach the consumer, it can reasonably be inferred that penicillin contaminated meat will also contain material no. 7.

Diazomethane treatment of the penicillin degradation products extracted from meat gave a mixture of methyl esters. These materials could now be separated on the silica gel plates using a 1:1 mixture of benzene–ethyl acetate to give the chromatogram depicted in Figure 3A. By comparison of this TLC pattern with that of the TLC pattern obtained from diazomethane treatment of an acidified ether extract of a heated buffer solution as well as the R_f values obtained from the methyl esters of I (material h), II (material f), and the isomers of III (materials a and c), it was possible to infer that the material corresponding to spot g was one of the materials of interest. Unfortunately, it was difficult to separate this material from methyl benzylpenicillinate with a variety of solvent systems. Separation of these materials was finally achieved using high-pressure liquid chromatography.

The infrared spectrum of material g exhibited ester and amide stretching at 5.75 and 5.95 μ, respectively, as well as N–H stretching between 2.9 and 3.0 μ. Stretching at 5.6 μ (β-lactam carbonyl) was absent, indicating breakage of the lactam ring. Otherwise, the infrared spectra of material g and authentic methyl benzylpenicillinate were very similar. The chemical ionization (CI) mass spectrum of material g gave an M + 1 ion at m/e 453, indicating that its molecular weight was 452. The electron impact (EI) mass spectrum gave a small molecular ion at m/e 452 (5.3%), and ions at 418 (9), 393 (1), 378 (1.8), 359 (6.2), 333 (6.7), 317 (4.8), 314 (5.7), 291 (11.4), 279 (13.3), 255 (8.6), 215 (11.4), 188 (18), 174 (100), 118 (30), 114 (41), and 91 (63). Also present in the EI mass spectrum was a small 466 fragment, most likely due to methylation of the thiozolidine ring nitrogen. An analogous situation was seen in the mass spectrum of benzylpenicilloic acid which had been treated with diazomethane. In addition to the expected molecular ion at m/e 380, a fragment at m/e 394 was present. The proton NMR spectrum was very similar to that of synthesized dimethyl benzylpenicilloate except for the presence of an additional doublet in the methyl region centered at 1.56 ppm. The spectral data thus indicated that material g was α-[(1-methoxycarbonyl)-ethyl]-4-methyl benzylpenicilloate (V). Confirmation of this assignment was achieved by synthesis of α-(1-carboxyethyl) hydrogen benzylpenicilloate (IV, R_f value identical with that of metabolite no. 7), and treatment of this material with diazomethane to give a material with spectral characteristics (infrared and mass) identical with that of material g.

An aqueous solution of IV gave, on standing, a mixture of II, III, and a material whose TLC R_f value was identical with that of material no. 6. This reaction was accelerated by heating. Better yields of material no. 6 were obtained by heating a chloroform solution of IV in a closed vial for

1 h at 95 °C. Treatment of this reaction mixture with diazomethane gave a number of products with the two most intense spots (after exposure to iodine) corresponding to materials g and f in the benzene–ethyl acetate solvent system (Figure 3B).

Although material f has essentially the same R_f value as dimethyl benzylpenicilloate, TLC analysis of the chloroform reaction mixture before treatment with diazomethane indicated absence of large quantities of benzylpenicilloic acid. Isolation of material f by preparative TLC gave a material whose mass spectrum was essentially identical with V, indicating that material no. 6 is either an isomer or mixture of isomers of IV.

ACKNOWLEDGMENT

The authors are grateful to John W. Turczan, FDA New York District Laboratories for the NMR spectrum of V and to Kurt L. Loening, Chemical Abstracts Service, for naming IV and V.

LITERATURE CITED

Clarke, H. T., Johnson, J. R., Robinson, R., "Chemistry of Penicillin", Princeton University Press, Princeton, N.J., 1949.
Fales, H. M., Jaouni, T. M., Babashak, J. F., *Anal. Chem.* **45**, 2302 (1973).
Huber, W. G., *Adv. Vet. Sci. Comp. Med.* **15**, 109 (1971).
Katz, S. E., Fassbender, C. A., Dinnerstein, P. S., Dowling, J. J., *J. Assoc. Off. Anal. Chem.* **57**, 522 (1974).
Levine, B. B., *J. Exp. Med.* **112**, 1131 (1960).
Levy, S. B., FitzGerald, G. B., Macone, A. B., *New England J. Med.* **295**, 583 (1976).
Oehme, F. W., *Toxicology* 1, 205 (1973).
Price, J. F., Schweigert, B. S., "The Science of Meat and Meat Products", W. H. Freeman, San Francisco, 1971.
Swann, M., "Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine", H. M. Stationary Office, London, 1969.
Zimmerman, M. C., *Arch. Dermatol.* **79**, 1 (1959).

Received for review January 18, 1977. Accepted April 27, 1977. Paper of the Journal Series, New Jersey Agricultural Experiment Station, New Brunswick, N.J. This work was supported by funds from Food and Drug Administration Contract FD-179 and the Charles and Johanna Busch Memorial Fund. Presented in part, Division of Agricultural and Food Chemistry, 172nd National Meeting of the American Chemical Society, San Francisco, August, 1976.

Saccharides of Maturing Triticale, Wheat, and Rye

Robert Becker, Klaus Lorenz,* and Robin M. Saunders

The saccharide content of two tall triticales, two semidwarf "Armadillo" triticales, one spring wheat, one rye, and two durum wheats was determined in kernels of different maturity. Monosaccharides, which were present in grain kernels 4 weeks before maturity, were no longer detectable 2 weeks before the final harvest. Maltose and maltotriose were present in low and unchanging amounts. Sucrose, raffinose, and kestose increased as the grains matured, while the tetrasaccharides decreased. Although the saccharide composition of the kernels from the different cereal grains differed, the trends in triticales were similar to those of the parental species, wheat and rye, during maturation.

Changes in chemical composition of maturing wheat have been studied. The rate of grain filling is similar for wheat, barley, oats, and rye, suggesting a common physiological process (Meredith and Jenkins, 1976b). The decline in percentage moisture observed during the development of cereal grains is due to gain in dry matter (Meredith and Jenkins, 1975), although the yield of dry matter varies considerably (Meredith and Jenkins, 1970).

Changes in carbohydrates, protein and nonprotein nitrogen compounds of maturing wheat have been reported (Jennings and Morton, 1963). Several carbohydrates and their hydrolyzing enzymes were determined (Meredith and Jenkins, 1973a,b, 1975, 1976a,b).

Changes in maturing triticale do not appear to parallel in entirety those changes in other cereal grains (Lorenz and Welsh, 1976). Triticale kernels, which are plump and relatively large in size compared to wheat up to about 3 weeks before maturity, shrivel during the late stages of kernel development, and α-amylase activity tends to be high (Klassen and Hill, 1971; Lorenz and Welsh, 1976).

This study follows the changes in saccharide content during triticale kernel development and compares these changes to those in the maturing parental species, wheat and rye.

Western Regional Research Laboratory, U.S. Department of Agriculture, Agricultural Research Service, Berkeley, California 94720 (R.B., R.M.S.) and the Department of Food Science and Nutrition, Colorado State University, Fort Collins, Colorado 80523 (K.L.).

MATERIALS AND METHODS

a. Sample Identification and Preparation. Two tall spring triticales (6-TA-204 and 6-TA-206), two semidwarf "Armadillo" triticales (RF720009 and RF720011), a hard red spring wheat (Colano), two spring semidwarf durums (RF710066 and 710222), and Prolific spring rye were studied. Plots at the Colorado State University Agronomy Research Center, Fort Collins, were seeded April 10, 1974 and several rows of each cultivar harvested July 8, 22, 29 and August 6 and 12. Moisture contents of the samples at harvest are given in Table I.

Except for the samples harvested July 8, 20 g of each grain sample were frozen approximately 2 h after harvest. All samples were placed in a freeze dryer the following morning. The July 8 samples of necessity were hand harvested which delayed freezing until 8 h after harvest.