Discussion of Method. The accuracy and sensitivity of the method are within the present needs of the industry. The main error seems to result from incomplete extraction of antioxidant from the granule sample. Petroleum ether is a poor solvent for elution from adsorbant material. However, it has other advantages over stronger solvents, which make it desirable for the present use. Chlorinated solvents give rise to corrosive vapors in the flame ionization detector and thus are undesirable for routine use in detectors constructed from metal. Diethyl ether may contain peroxides which could react with small concentrations of antioxidants. Alcohol solvents cause undesirable, excessive tailing with the Apiezon L firebrick column and extract considerable quantities of potato sugars, etc.

The GLC conditions were chosen to give a rapid analysis with a satisfactory separation of BHA and BHT. The Apiezon L column has several advantages over other columns for the present analysis. It is stable and normally has a long life. At the high temperature required, Apiezon L gives a minimum of "bleeding," an essential feature with the sensitivity capabilities of the flame ionization detector. Polar columns give better separation of BHA and BHT, but they cause excessive base line "noise" when used near or above 200° C. at the sensitivity required for the present analyses.

It is important that the Apiezon L column be well "aged"; otherwise it is not satisfactory for BHA. BHA seems to be decomposed to some extent on the column and its peak is smaller than the BHT peak. The extent of decomposition seems also to vary with age of the column. This is no problem if the standard sample is injected immediately prior to the unknown. The decomposition probably occurs on the active centers of the firebrick and it may be advantageous to use one of the several known methods (*1*) of deactivating these centers.

Although the Apiezon L column causes little bleeding, the potato lipide which is injected into the GLC apparatus along with antioxidants is thermally unstable and produces some base line noise, which limits the sensitivity at which the detector can be used.

Because potato lipide is injected into the GLC apparatus along with antioxidants, it would be desirable to use an injection system which can be periodically cleaned with solvent to remove accumulated lipide, which otherwise would eventually result in deterioration of the column. The quantity of lipide injected with each analysis is, however, small (1 mg.). The columns used without periodic cleaning of the injection system had a reasonably long life. It is estimated that columns used under such conditions would last at least 1 month with daily routine use.

Possible Simplification of the Procedure. About the same accuracy can be obtained by a single concentration step to about 5 ml. with the 500-mm. helix-packed column. This concentrate is then transferred directly to a 10-ml. volumetric flask, and 30-μl. samples are injected into the GLC apparatus. Depending upon accuracy required and level of antioxidant used, the volume of concentrate may be changed to simplify the concentration step. Another simplification is to use peak heights instead of peak areas. This seems to be just as accurate, if the standard is injected immediately before the unknown.

A method used by one of the authors (B. N. Stuckey) is to add 2 ml. of iso-octane to the extract and carry out concentration at room temperature using a rotating, high vacuum type evaporator. A convenient volume of iso-octane remains after the relatively rapid removal of the more volatile petroleum ether. The concentrate is transferred to a 10.0-ml. volumetric flask with iso-octane washing and is made up to the mark with iso-octane.

Acknowledgment

The authors are indebted to representatives of the member firms of the Instant Potato Granule Manufacturers Association for continued interest and helpful suggestions, in particular to R. M. Stephenson, American Potato Co.; J. M. Fogelberg, R. T. French Co.; and R. W. Kueneman, J. R. Simplot Co. We also thank R. Teranishi and C. E. Hendel, Western Regional Research Laboratory, for helpful discussion and suggestions.

Literature Cited

(1) Bohemen, J., Langer, S. H., Perrett, R. H., Purnell, J. H., *J. Chem. Soc.* **1960,** 2444.
(2) Filipic, V. J., Ogg, C. L., *J. Assoc. Offic. Agr. Chemists.*, in press.
(3) Jennings, E. C., Curran, T. D., Edwards, D. G., *Anal. Chem.* **30,** 1946 (1958).
(4) Thompson, A. E., *J. Chromatog.* **2,** 148 (1959).

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FEED ADDITIVES

Metal Binding Properties of Bacitracin

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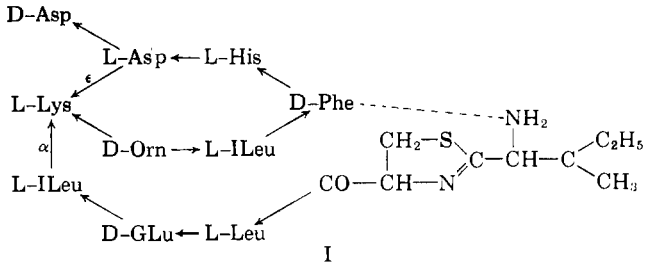
Grain Processing Corp., Muscatine, Iowa

BACITRACIN, a mixture of polypeptide antibiotics, was first isolated by Johnson, Anker, and Meleney (*9*) in 1945 from cultures of *B. licheniformis*. Since its discovery, bacitracin has not enjoyed as widespread pharmaceutical use as other well known antibiotics, but it has been effectively employed in formulated feedstuffs as a growth promoter (*11, 18, 19*).

Two groups of workers, Craig and associates in this country and Newton and Abraham in England, have shown the polypeptide mixture to consist of a main component, bacitracin A, and lesser amounts of bacitracin B, D, E, and F (*2, 13, 16*). Independently, these same two groups (*17*) arrived at the structural formula of bacitracin A (I).

One of the major problems associated with the use of bacitracin in animal feed materials is gradual loss of activity on standing (*3*). More stable forms, such as zinc bacitracin (*6*) and bacitracin methylene disalicylate (*15*), have been prepared, and recently manganese bacitracin has been added to the list of metal bacitracins (*5*). Numerous observations have been published concerning the binding of other antimicrobial com-

The ability of several metal cations toward complex formation with bacitracin was determined in a qualitative way from the pH lowering produced in the titration of metal-bacitracin solutions with alkali. The order found for the tendency toward complex formation was Cu > Ni > (Co, Zn) > Mn. All the metals, except manganese, complexed with the group in bacitracin titrating between pH 5.5 and 7.5. Manganese did not bind bacitracin to any appreciable extent below pH 7. Comparison of the corresponding titration curves of metal-histidine solutions with those of bacitracin and the ultraviolet absorption spectra of zinc and copper bacitracin solutions indicated that the imidazole group of histidine was involved in complex formation.



I

pounds by metallic cations and such binding is the subject of an excellent review by Weinberg (*20*).

The purpose of this investigation was to determine the relative order of the ability of various metals to complex with bacitracin and to attempt a characterization of the chelate linkages involved.

Potentiometric titration is perhaps one of the most useful methods for following the course of chelate formation and it involves measurement of the amount of hydrogen ion released in the reaction

$$M^n + n\ HR \rightarrow MR_n + n\ H$$

where M refers to a metal ion, HR to the chelating agent, MR_n to the metallic chelate, and n is the charge on the metallic cation. The more stable the complex with respect to dissociation, the lower the pH at which it can persist and vice versa.

Table I. pH of Incipient Precipitation of Metal Complex and Metal Hydroxide

Metal	Metal Complex	Metal Hydroxide
Cu	4.4	5.9
Ni	5.2	8.4
Co	5.8	8.3
Zn	5.1	7.1
Mn	7.0	8.0

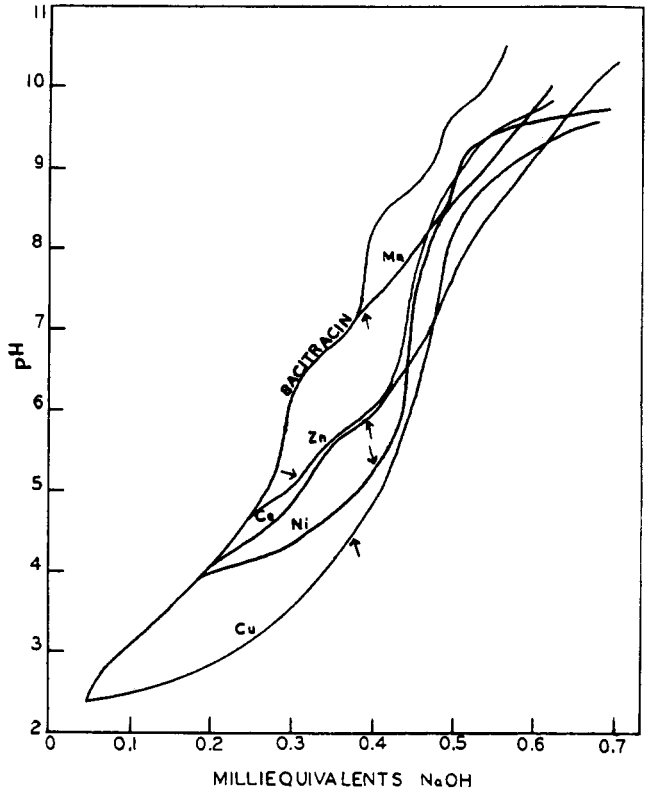


Figure 1. Titration curves of bacitracin and bacitracin in the presence of metal ions, 1 gram atom of metal per 1422 grams of bacitracin

Arrows indicate precipitate formation

Experimental

Materials and Apparatus. U.S.P. bacitracin, labeled Penitracin assaying 66 units per mg., was obtained (S. B. Penick and Co.) as a creamy white powder. A stock solution containing 8.0 mg. per ml. was made up in 0.15M sodium chloride and was kept at 10° C. when not in use.

A stock solution of L-histidine (free base, CFP, California Corp. for Biochemical Research, Los Angeles) was prepared containing 4.42 mg. per ml. in 0.15M sodium chloride.

Stock solutions of metal ions of 0.283M were prepared without further standardization by dissolving the corresponding reagent grade sulfates (Fisher Scientific Co.) in water.

The approximately 0.5N sodium hydroxide, carbonate-free, was standardized against potassium acid phthalate (Mallinckrodt reagent grade). Sulfuric acid, about 0.5N prepared from concentrated reagent acid, was standardized by comparison with sodium hydroxide solution.

All titrations were performed in 100-ml. beakers at 24° ± 0.5° C. using a Beckman Zeromatic pH meter standardized against Beckman's standard buffer solutions at pH 4.0 and 7.0. The solutions were magnetically stirred.

Titration Procedure. Twenty milliliters of bacitracin stock solution (160

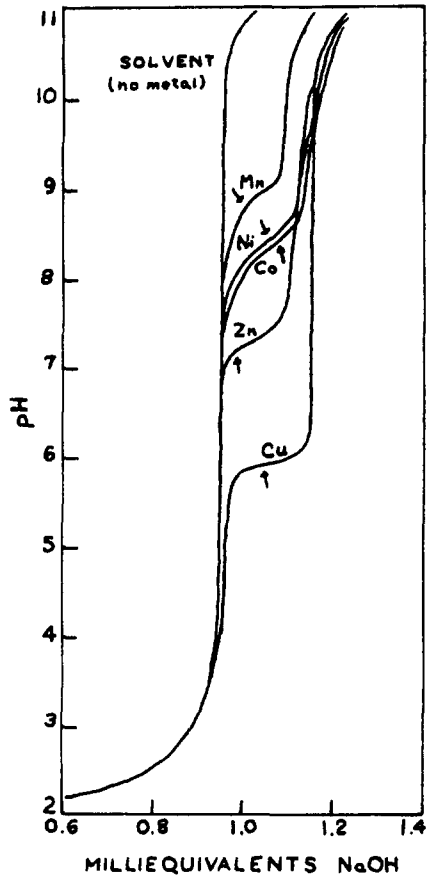


Figure 2. Titration curves of metal ions 0.013M

Arrows indicate precipitate formation

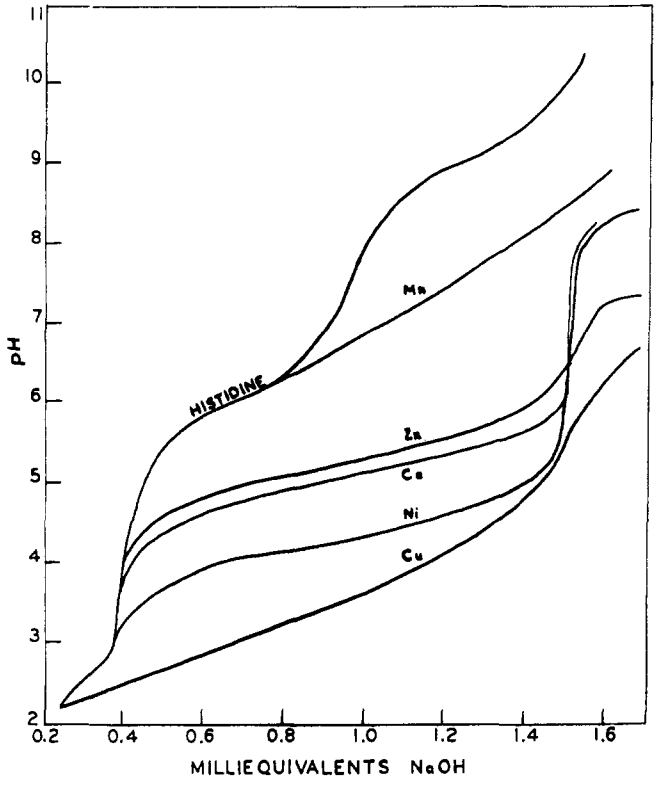


Figure 3. Titration curves of L-histidine and L-histidine in the presence of metal ions, 1:1 molar ratio

mg.), 2 ml. of 0.4780N sulfuric acid, and 0.4 ml. of the metal ion solution were pipetted into a 100-ml. beaker. Assuming the molecular weight of bacitracin to be 1422, the molar ratio of metal ion to bacitracin in all cases was 1 to 1. Sodium hydroxide (0.4932 N) was added in small increments which were further decreased in regions of rapidly changing pH values. The titration curves obtained are shown in Figure 1.

Titrations of metals in the absence of bacitracin were carried out in order to learn the extent to which hydrolysis might interfere in consideration of the regions of complex formation. Results of such titrations are summarized in Figure 2.

Solutions of histidine in the presence of metal ion (1 to 1 molar ratio) containing 20 ml. of histidine stock solution, 1 ml. of standard sulfuric acid, and 2 ml. of metal ion solution were titrated in exactly the same manner as was bacitracin and the titration curves are shown in Figure 3. The titration curves of bacitracin and histidine solutions were corrected graphically from the corresponding solvent blank titration curve.

pH of Precipitation. From titration data (Figure 1), the pH of incipient precipitation of the metal bacitracin complex was determined and tabulated in Table I. Corresponding pH values at which the metal hydroxides precipitated were obtained from Figure 2 and are also given in Table I.

The titration curve for manganese (Figure 2) must be considered as approximate because of oxidation of the metallic ion. Hence, the limiting pH below which no hydrolysis occurred was taken as 8 according to Britton's pH of partial hydroxide precipitation (*1*).

Absorption Spectra. The solutions used for measurement of ultraviolet absorption were prepared from the above stock solutions of bacitracin and metal ions. Each solution, adjusted to pH 5, contained 0.24 mg. of bacitracin per ml. in combination with metal ion in the ratio 1 gram atom of metal ion per 1422 grams of bacitracin. The absorption of metal bacitracin solutions was measured in the Beckman Model DU spectrophotometer against the corresponding metal salt solution containing no bacitracin. The absorption curves are shown in Figure 4.

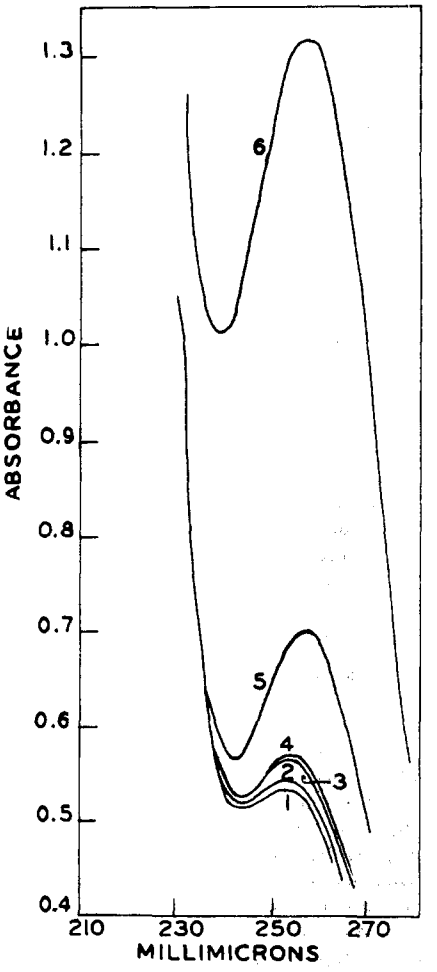


Figure 4. Absorption spectra of metal-bacitracin solutions at pH 5, 1 gram atom of metal ion per 1422 grams of bacitracin

Curve 1. Manganese
Curve 2. Bacitracin—no metal
Curve 3. Zinc
Curve 4. Cobalt
Curve 5. Nickel
Curve 6. Copper

Discussion

The titration curve obtained for bacitracin alone agrees closely with that found by Newton and Abraham (*14*). The buffering between pH 2.5 and 5.5 could reasonably be attributed to the presence of two carboxyl groups, that between 5.5 and 7.5 to the imidazole ring in histidine, that between 7.5 and 9.0 to a free α-amino group, and that between 9.0 and 10.5 to the δ-amino group of ornithine.

Because bacitracin forms insoluble complexes with the cations studied, it is not possible to calculate the corresponding formation constants from titration data. However, the pH lowering produced in regions where no precipitation occurs can be used in a qualitative manner to determine the relative order of their ability to complex bacitracin. In regions where no pH lowering occurs, one may conclude that complex formation does not occur to an appreciable extent.

Metal hydrolysis (Figure 2) offers a serious interference in consideration of complex formation, since the release of hydrogen ions through this process affects the titration curve appreciably. Consequently, the pH values shown in Table I at which the metals begin to hydrolyze set an approximate upper limit to the pH range in which complex formation may be considered to occur. Because copper ion hydrolyzes at about pH 6, determination of the relative order of complex formation for the five metals would have to be considered at a point near or below pH 6.

In view of the above limitations, the relative order of the ability of the metal ions to complex with bacitracin as determined from the titration curves in Figure 1 is:

$$Cu > Ni > (Co, Zn) > Mn$$

A similar order was obtained by the method of Irving and Williams (*8*) using data given in Table I on the pH of incipient precipitation. However, by this criterion zinc is approximately equivalent to nickel in ability to complex with bacitracin.

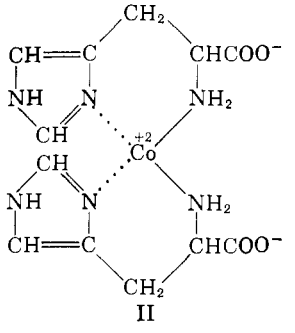
The order found for bacitracin is in good agreement with the order proposed by Mellor and Maley (*12*) for bivalent metal ions irrespective of the nature of the ligands involved. Their order is:

$$Pd > Cu > Ni > Co > Zn > Cd > Fe > Mn > Mg$$

The imidazole group in bacitracin which normally titrates between pH 5.0 and 7.5 forms complexes with Cu, Ni, Co, and Zn. These are decomposed only by hydrogen ions at considerably lower pH values. Manganese appears to complex only with the group in bacitracin whose pK is approximately 8.5 and which is possibly a free α-amino group; whereas copper in addition to complexing with those groups between pH 5.0 and 8.5 reacts with the carboxyl groups of bacitracin in the region of pH 2.5 and 4.5.

These changes are reflected in the color and solubility of the material. In the presence of Cu^{+2}, the solution is pale blue and becomes a deeper blue-green at pH 3 as complex formation begins. At pH 4.4 the complex precipitates. Above pH 9, the solution turns a deep purple and the precipitate redissolves. The cobalt complex forms a white precipitate at pH 5.8 and the solution turns from pink to rose. Above pH 9, the solution turns a violet color which changes to green above pH 10. Nickel, zinc, and manganese showed no unusual color change, but precipitation occurred in all cases. Manganese did undergo rapid oxidation above pH 8 to form brown MnO_2. All the above color changes and precipitate formations were in marked contrast to those observed in the titrations of the metal cations alone.

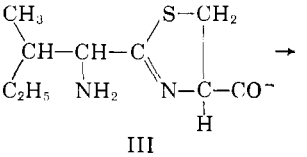
The evidence that an imidazole group in bacitracin is a site of complex formation is further supported by comparisons of titration curves of metal-histidine solutions shown in Figure 3 with those found for bacitracin. The order of complex formation found for the metal histidine complexes is Cu > Ni > Co > Zn > Mn which is in accord with that found for bacitracin. The nature of the structure of the metal complexes of histidine is believed to be similar to that proposed by Hearon (*7*) for the cobalt complex shown in II.



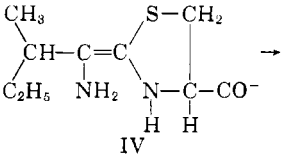
II

The ultraviolet absorption spectrum of bacitracin showed a weak maximum at about 253 mμ (Figure 4). In the presence of copper and nickel, bacitracin exhibited a marked absorption and a shift in position of the maximum to longer wave lengths. Only a slight increase in absorption was obtained with zinc and cobalt and none at all with manganese. These absorption bands are of particular interest because Edsall (*4*) reported that copper complexes of imidazole and histidine showed marked absorption in the region between 240 and 290 mμ and the position of the maximum was displaced in the direction of longer wave lengths. On the other hand, zinc imidazole complexes show no significant absorption in this region. The similarity of behavior in the ultraviolet region of the zinc and copper complexes of bacitracin, histidine, and imidazole strongly suggests that the free imidazole group of bacitracin is one site of complex formation at least in the case of zinc and copper.

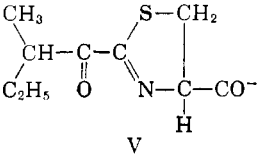
How the free α-amino group in bacitracin is involved in chelation is not entirely clear. Craig (*21*) has shown that bacitracin A is slowly transformed to bacitracin F by a unique type of oxidative loss of ammonia and further proposed (*10*) that this transformation takes place via epimerization at the terminal isoleucine-NH_2 bearing carbon atom. This conversion of bacitracin A to F is shown in the partial formulas III, IV, and V.



III



IV



V

Since bacitracin F is an inactive form of bacitracin, chelation of the α-amino group may be one possible route by which the activity of bacitracin is stabilized by metal cations.

Acknowledgment

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Literature Cited

(1) Britton, H. T. S., *J. Chem. Soc.* **127,** 2100, 2142 (1925).
(2) Craig, L. C., Hausmann, W., Ahrens, E. H., Jr., Harfenist, E., *Anal. Chem.* **23,** 1236 (1951).
(3) Craig, L. C., Weisiger, J. R., Hausmann, W., Harfenist, E. J., Jr., *J. Biol. Chem.* **199,** 259 (1952).
(4) Edsall, J. T., Felsenfeld, G., Goodman, D. S., Gurd, F. R. N., *J. Am. Chem. Soc.* **76,** 3054 (1954).
(5) *Federal Register* **24,** 10062 (1958).
(6) Gross, H. M., *Drug & Cosmetic Ind.* **75,** 612 (1954).
(7) Hearon, J. Z., *J. Natl. Cancer Inst.* **9,** 1–11 (1948).
(8) Irving, H., Williams, R. J. P., *Nature* **162,** 746 (1948).

(9) Johnson, B. A., Anker, H., Meleney, F. L., *Science* **102,** 376 (1945).
(10) Konigsberg, W., Craig, L. C., *J. Am. Chem. Soc.* **81,** 3452 (1959).
(11) Lillie, R. J., Bird, H. R., *Poultry Sci.* **32,** 531 (1953).
(12) Mellor, D. P., Maley, L., *Nature* **159,** 370 (1949).
(13) Newton, G. G. F., Abraham, E. P., *Biochem. J.* **53,**597 (1953).
(14) *Ibid.*, p. 604.
(15) Radomski, J. L., Hagan, E. C., Nelson, A. A., Welch, H., *Antibiotics & Chemotherapy* **4,** 304 (1954).
(16) Sharp, V. E., Arriagada, A., Newton, G. G. F., Abraham, E. P., *Brit. J. Exptl. Pathol.* **30,** 444 (1949).
(17) Swallow, D. L., Abraham, E. P., *Biochem. J.* **72,** 326 (1959).
(18) Terrill, S. W., Becker, D. E., Adams, C. R., Meade, R. J., *J. Animal Sci.* **11,** 84 (1952).
(19) Thomas, O. O., *Ibid.*, **18,** 1175 (1959).
(20) Weinberg, E. D., *Bacteriol. Rev.* **21,** 46 (1957).
(21) Weisiger, J. R., Hausemann, W., Craig, L. C., *J. Am. Chem. Soc.* **77,** 3123 (1955).

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RADIATION EFFECTS ON BEEF

An Investigation of Some Volatile Components of Irradiated Beef

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The volatile components produced by concurrent radiation-distillation at 5 megarads of raw ground beef have been studied in an effort to identify the substances responsible for the characteristic unpleasant odor of beef preserved by radiation. It has been proved that 3-(methylthio)-propionaldehyde (methional) is a major component of the mixture of at least 12 substances detected, and that it makes a major contribution to the unpleasant odor of irradiated beef. The synthesis of methylthioacetaldehyde, ethylthioacetaldehyde, and 2-(methylthio)-propionaldehyde is reported.

A PROBLEM associated with radiation-sterilization of beef is the production of an unpleasant flavor and odor. This investigation has been carried out in an effort to characterize and identify the substances responsible for the characteristic unpleasant odor of irradiated beef.

Prior to this work Burks (*9*) had studied the amines produced by irradiation of beef and found ammonia and at least six other amines, the two major components being methylamine and ethylamine. He concluded that volatile bases are partial contributors to the odor of irradiated beef. Merritt (*21*) studied the volatile components of irradiated beef by means of low-temperature, high-vacuum distillation techniques, gas chromatography, and mass spectrometry. Of the 10 compounds thus identified in a "center cut," methyl disulfide and isobutyl mercaptan alone were present in irradiated beef and absent in nonirradiated beef. The relation of these compounds to the odor of irradiated beef was not directly determined, although it was believed that they probably contribute in part. Odorous compounds were known to be present in the "water fraction."

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Batzer and Doty (*3*) found unusually large amounts of volatile sulfur compounds in irradiated beef. Sribney, Lewis, and Schweigert (*28*) found carbonyl compounds and peroxides.

In the current study, the isolation of volatile compounds has been accomplished by means of concurrent radiation-distillation of raw, ground beef. This procedure was originally developed for milk (*30*). Its advantage is that it makes possible, in theory, the isolation of volatile substances very soon after their production by radiation.

Experimental Procedure and Results

Production of Volatile Components of Irradiated Beef. An aqueous slurry of 10 pounds of ground, raw beef was circulated in the usual manner (*31*) through an irradiation chamber beneath the electron beam of a 1-m.e.v. (General Electric) resonant transformer, to a flash evaporator for the removal of volatile components, and back through the irradiation chamber. The condensate thus obtained was found (*31*) to possess the typical odor of irradiated beef. The conditions of a typical concurrent radiation-distillation of a ground beef slurry are given in Table I.

Investigation of Irradiated Beef Distillates. Carbonyl Compounds. The distillate (approximately 7 liters) was treated with a solution of 2,4-dinitrophenylhydrazine reagent (2 grams per liter of 2*N* hydrochloric acid). A small portion of the neutral, hexane-soluble fraction of the 2,4-dinitrophenylhydrazones obtained was separated by partition chromatography on Celite columns with nitromethane as the stationary phase and hexane the mobile phase. Initial examination of the total mixture indicated, on the basis of its ultraviolet spectrum in chloroform ($\lambda_{max.}$ 354 mμ), that dinitrophenylhydrazones of simple aliphatic aldehydes were present (*17*). Further examination of the fractions showed, by magnesium carbonate fusion, that a number contained sulfur, thus indicating that sulfur-containing aldehydes were present.

Paper chromatographic examination

Table I. Experimental Conditions of a Typical Concurrent Radiation-Distillation of a Slurry of Ground Beef

Conditions	*Quantities*
Quantity of ground beef	10 lb.
Volume of beef slurry	8 l.
Dose	5 megarad
Av. pressure at pump	23 mm. Hg
Av. temperature	32–36° C.
Evaporation rate	6 l./hr.
Total distillate collected	6.5 l.