odor detected. Reducing sugars, present in muscle, could react with protein and possibly produce the observed aromas. A mixture of gelatin plus glucose was vacuum-pyrolyzed; the results were negative. Gelatin is, however, low in sulfur-containing amino acids, and so the same experiments were repeated with soluble egg albumin powder and with egg albumin plus glucose. The results were again negative. Since lactic acid was isolated in appreciable quantity and pH could have a profound effect on the results of heat treatment (pH of an initial cold water extract on ground beef was approximately 5.5), a model system, consisting of 10 grams of egg albumin, 2 grams of glucose, and 100 mg. of lactic acid, was prepared and treated in the same fashion as the lyophilized lean meat extracts. Some odor was obtained, indicating the lactic acid may be important in developing flavor in cooked meat. However, this odor did not resemble the odor "profile" obtained from the meat powders. Relaxation of rigor with time results in the production of a more desirable flavor than that associated with fresh beef. It may be that as glycolysis continues the increase in lactic acid concentrations may result in this better flavor.

Because the protein systems heated were apparently not the flavor precursors sought, attention was directed to the low molecular weight fraction present in the water extract of lean meat. Dialysis experiments indicated that the material passing through the membrane contained some of the flavor precursors of lean beef. When the white, fluffy powder obtained from this dialyzate by freeze-drying was carried through the vacuum pyrolysis and fractionation procedure applied to the lyophilized water extracts of lean beef and lean pork, a fraction very similar to beef I and pork I was isolated. However, when the free amino acids were separated from a similar dialyzate and heated alone, at both pH 5.5 and pH 8.0, no recognizable meaty odors were obtained. The odors were, in fact, classified as unpleasant, indicating that the free amino acids as such were not flavor precursors.

Conclusions

The identity of the compounds isolated from lean beef and lean pork, as well as the marked resemblance in chromatographic and spectrophotometric behavior of fractions not completely characterized, leads to the conclusion that a similar basic meaty flavor is obtained on heating the lean of beef and pork.

The flavor differences that exist in pork and beef may have their origins in the fat portions of these meats. The fat may not only produce different flavor compounds in different ratios, but perhaps also act as a storage depot for lipide-soluble foreign compounds that, on heating, also contribute to flavor.

Examination of possible precursor systems for the origin of lean meat flavor indicated that the flavor precursors may well be the low molecular weight, water-soluble fractions of lean meats. These produce the characteristic flavor of lean meat, presumably by some kind of interaction between amino acids and the low molecular weight carbohydrates and polypeptides present, and no single compound or class of compounds is responsible for cooked "meat" flavor.

Acknowledgment

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MEAT FLAVOR CHEMISTRY

Precursors of Beef Flavor

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THE INVESTIGATIONS OF MEAT FLAVOR to date consist largely of studies of the volatile constituents comprising an odor or flavor, or studies of the flavorful component of cooked meat. Little is known of the precursors of this flavor in uncooked meat, or of the mechanism of flavor production. One of the first studies of this kind was carried out by Barbella, Hankins, and Alexander (*2*), who observed that meat from lambs, whose growth had been retarded by a nonbalanced diet, did not possess as desirable a flavor as the meat from lambs which were fed a balanced diet. A decrease in an albumin fraction was also observed in the muscle tissue of the retarded lambs. Howe and Barbella (*7*) reported that ripening of meat increases soluble protein products, which, when heated, could play an important part in meat flavor.

Crocker (*5*) suggested that meaty flavor apparently developed in the fiber rather than in the juices of cooked meat, and was released by chewing. Barylko-Pikielna (*3*) separated boiled meat into a water-soluble fraction and a water-insoluble residue. Taste panel evaluation indicated that the insoluble residue contained the flavor more characteristic of roast beef, while the flavor of the water-soluble fraction was not the typical beef flavor but was more intense. Kramlich and Pearson (*8*) worked with press fluids from raw and cooked beef.

Attempts were made to isolate and characterize some of the constituents of beef muscle tissue which evolve the distinctive odor and flavor associated with cooked beef. Beef muscle tissue was fractionated into a number of water-soluble and water-insoluble fractions. Dialysis of the water extract of uncooked ground beef gave a diffusate which, after lyophilization, was capable of producing an odor similar to that of broiled steak when heated with fat, and a beef broth odor and flavor when boiled in water. The components of this fraction were separated by column chromatography and gel filtration. They are of relatively low molecular weight and contain peptides, carbohydrates, and phosphates as yet unidentified.

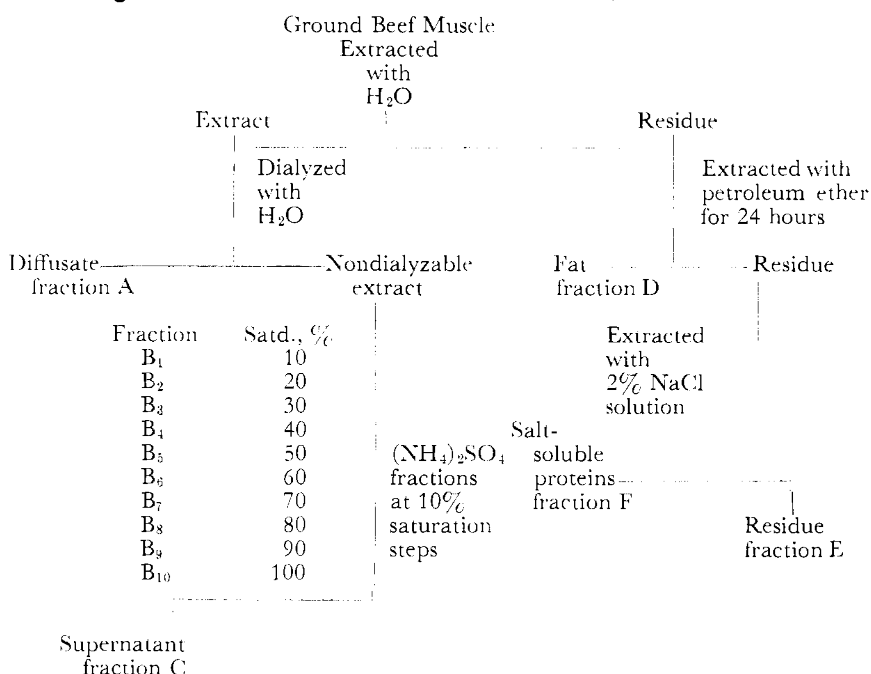
Their results indicated that press fluids of raw meat had a highly concentrated flavor on cooking. Cooking prior to extraction increased the flavor, indicating that full flavor development may be due to heating the juice and the fiber together.

Results of studies on chicken flavor by Bouthilet (*4*) suggested that the flavor was composed of at least two parts—a sulfur-containing material and a fatty acid-type material. The sulfur compound was highly labile and gave off hydrogen sulfide upon standing. Pippin and Eyring (*9*) found that the bulk of the volatile nitrogen components in chicken flavor volatiles was ammonia, which did not constitute part of the characteristic chicken flavor. Volatile flavors were associated with neutral or acidic compounds. The volatile sulfur constituents consisted mainly of hydrogen sulfide.

The complexity of cooked extracts was elegantly demonstrated by Wood and Bender (*11*), who isolated and identified a large number of volatile and nonvolatile compounds in commercial ox muscle extract. Hornstein, Crowe, and Sulzbacher (*6*) heated, in vacuum, a freeze-dried powder obtained from a cold water extract of raw ground beef and trapped the volatiles. Components of the volatile fraction identified were acetone, acetaldehyde, formaldehyde, hydrogen sulfide, ammonia, and methylamine. These authors also reported that the flavor precursors were cold water-extractable.

In the present studies substances have been isolated from raw beef muscle, which, when boiled with water, gave a characteristic beef broth odor and flavor, and when heated with fat, gave an odor similar to broiled steak. The initial observation was made on ground beef which had been serially extracted five times with acetone (1000 grams of beef, 1200 ml. of acetone). The fat obtained from the third acetone extract, when heated, gave the broiled steak type of odor. Analysis of the fat indicated that only normal saturated triglycerides were present. Attempts at duplication of this procedure were not always successful. In some instances, the material from the third acetone extract merely gave the odor of heated fat.

Diagram I. Procedure Used for Fractionation of Beef Muscle



These and other observations led to consideration of the possibility that other substances being carried over in the fat were responsible for the odor produced. On further investigation, the precursor substances were found in the dialyzable portion of a water extract of raw beef.

Procedure

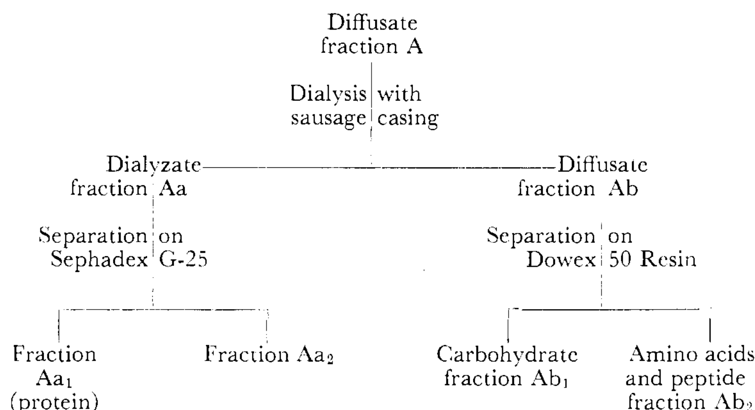
Round of beef (1000 grams), freed from all extraneous fat, was fractionated as outlined in Diagram I. With the exceptions of fractions A and D, all others were dialyzed against running water until a negative sulfate or chloride test was obtained, then lyophilized. Fraction A was lyophilized as the diffusate. Fraction A was obtained by filling cellulose dialysis tubing (Visking) with 200 ml. of distilled water and rotating the tubing in the water extract of beef (2 × 1200 ml. per 1000 grams of meat) for 16 hours in the cold, ~3° C.

Further efforts at separation of fraction A into its constituents were successful when cellulose sausage casing (Visking) was inadvertently substituted for dialysis tubing in one of the experiments. The broiled steak type of odor was not obtained in this case. Thus, by using dialysis tubing which would allow the desired material to enter, and then transferring this diffusate to sausage casing, two more fractions were obtained (Diagram II). The material that remained in the sausage casing (Aa) was primarily protein in nature, while the material that diffused through the sausage casing (Ab) was composed primarily of compounds of small molecular weight such as sugars, amino acids, and small peptides.

Fraction Aa was further separated by using gel filtration on Sephadex G-25 (cross-linked dextran), using the procedure of Porath and Flodin (*10*). Two distinct peaks were obtained by this procedure (Figure 1).

Fraction Ab was further separated by passing the diffusate through Dowex 50 H^+ form 200-400 mesh, 8× linkage. The material that passed through the column (ninhydrin-negative) appeared to be carbohydrate in nature (Ab_1). By passing 2N ammonium hydroxide

Diagram II. Separation Procedure for Fraction A



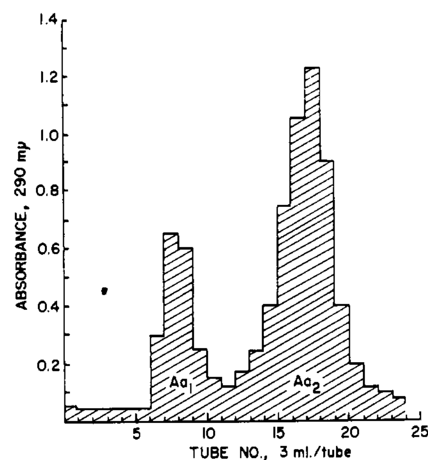


Figure 1. Separation of fraction Aa on Sephadex G-25

through the column, amino acids and small peptides were obtained (Ab_2).

Results and Discussion

When fresh ground beef muscle was fractionated by the procedure outlined in Diagram I and the individual fractions were either boiled in water for 20 minutes or heated in fat, only fraction A produced any characteristic odor or taste. The remaining fractions on boiling produced no odor and were practically tasteless. When heated with fat, they gave a burned protein odor. If ground beef were cooked to an internal temperature of 77° C. and then fractionated, fraction A had a characteristic beef bouillon odor, and fraction E (residue) had a flavor and odor more nearly that of cooked beef. However, the flavor was considerably less intense than that of fraction A. Again, the remaining fractions had no appreciable odor or taste.

Fraction A, as obtained from fresh ground beef muscle, proved very unstable. The material changed from a white granular material to a brown tarry mass, even when kept in an evacuated desiccator. This change was apparent a short while after removal from the lyophilizer and reached the brown tarry stage in about 3 to 7 days. During this period, ammonia and/or amines were given off, and at the end, the material had a stale, old meat odor.

After separation into fractions Aa and Ab by the second dialysis, fraction Aa, which contained the protein material, was fairly stable, but fraction Ab, containing the low molecular weight sugars and amino acids, turned brown rapidly. When fraction Ab was passed through Dowex 50, the eluent (Ab_1) was ninhydrin-negative, but was strongly positive for carbohydrates by either anthrone, triphenyltetrazolium chloride, or aniline acetate tests. On eluting the column with $2N$ ammonium hydroxide, an amino acid fraction was obtained (Ab_2). Both fractions were stable.

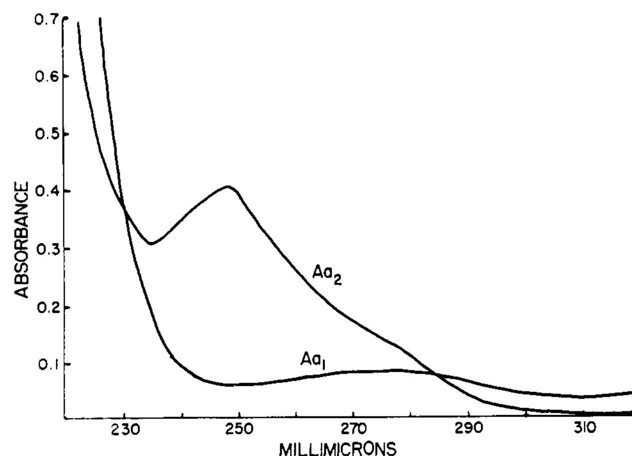


Figure 2. Ultraviolet spectra of fractions Aa_1 and Aa_2

Fraction Aa_1 (~5 to 10 mg. per 1000 grams of beef), as obtained by gel filtration, was a light tan material. Positive ninhydrin and negative carbohydrate tests were obtained. In the ultracentrifuge (60,000 r.p.m.) a 2% solution of this material in water formed a single peak, which, after 3 hours' running time, diffused into a low flat curve in the center of the cell. A spectrum taken in the ultraviolet indicated a normal protein curve (Figure 2). On paper electrophoresis in veronal buffer (pH 8.7, 400 volts, 3 amperes, and 6 hours), the spotted material divided into two spots (one about 1 cm. and the second about 2 cm. toward the positive pole). In acetate buffer at pH 4.0, only one spot appeared toward the negative pole.

Fraction Aa_2 (~10 mg. per 1000 grams of beef) was a white fluffy powder and ninhydrin-positive. A strong positive carbohydrate reaction was obtained. An estimation, using anthrone reagent and glucose as a standard, indicated a carbohydrate content of approximately 16%. However, because of lack of knowledge of the characteristics of the compound, the possible presence of interfering substances, and the exact nature of the carbohydrates present, the analysis is considered, at present, to be an estimate. The material was hydrolyzed on Dowex 50 and analyzed for hexuronic acids, hexosamines, and hydroxyproline, according to the procedure of Anastassiadis and Common (*1*). Negative results were obtained for all three tests. However, when water was added to the test for hexuronic acids (carbazole method), a positive test developed, indicating the presence of hexoses. Readings taken on a Beckman DU spectrophotometer at 520 and 420 mμ gave a 520/420 ratio of 1.06, indicating that the material was not just glucose, which has a ratio of 2.65 with this reagent. Hydrolysis of the material with $6N$ hydrochloric acid for 3 hours, and paper chromatography indicated a minimum of eight ninhydrin spots. The substances represented by these spots have not yet been identified.

The unhydrolyzed fraction Aa_2 gave a faint positive test for phosphate ion, but on hydrolysis with perchloric acid for 1 hour at 175° C., gave a very strong positive test. Estimations indicated a phosphate content of 7 to 7.5%.

The spectrum of the unhydrolyzed material taken in the ultraviolet (Figure 2) indicates a peak at 248 mμ. The unhydrolyzed material was also subjected to ultracentrifugation at 60,000 r.p.m., but no peak was discernible, indicating that the material is of low molecular weight.

On paper electrophoresis in veronal buffer at 8.7 under the same conditions as for fraction Aa_1, the bulk of the ninhydrin-positive material moved toward the negative pole. A heavy spot appeared at 2.5 cm. from the origin and another at 1 cm. A very faint ninhydrin-positive spot moved 9 cm. toward the positive pole. When a duplicate strip was checked for carbohydrate, the bulk of the material also moved toward the negative pole in the same position as the ninhydrin-positive material. One faint sugar spot was located at 12.2 cm. toward the positive pole. When a duplicate strip was checked for phosphorus, the bulk of the material remained at the base line, but some of the material streaked toward the positive pole with a definite spot at around 13 cm.

When this fraction (Aa_2) was run in 0.1*M* sodium carbonate at pH 11, the ninhydrin-positive material moved toward the positive pole. Two spots appeared, one at 4.5 cm. and another at 7 cm. The bulk of the carbohydrate material again moved toward the negative pole in the same position as it did at pH 8.7, but two spots moved toward the positive pole, one at 5.5 cm. and another again at 13 cm. The phosphorus-containing material moved essentially the same as it did at pH 8.7. The fact that a pH of 11 was necessary to cause the ninhydrin-positive material to move to the positive pole, suggested a high content of basic amino acids. However, a Sakaguchi test for arginine was only faintly positive.

Fraction Aa_2, when heated with fat, produced an odor similar to that of broiled steak. However, when small aliquots of fractions Aa_1 (protein) and Ab_1 (carbohydrate) were added to Aa_2 and then heated, the odor, based on subjective evaluation, was equivalent to the odor observed for the original fraction A. At the present stage of this work it appears that fraction Aa_2 contains the material necessary for the "basic" odor of broiled steak, but other constituents—i.e., protein (fraction Aa_1) and carbohydrate (fraction Ab_1)—are necessary for the development of the cooked meat flavor and odor. Natural fat and hydrogenated fat, which were used interchangeably in heating the isolated material, also contribute something to the odor. The same basic odor can be produced if the three fractions are heated in mineral oil, but it is distinguishable from that when natural or hydrogenated fats are used.

The fractionation procedure was tried on chicken and pork loin. The material (fraction Aa_2) isolated in these experiments had the same basic odor as the material isolated from beef. When heated with fat, the odor was similar to that of broiled steak. However there were enough distinguishing characteristics to indicate which material had been isolated from chicken and which came from pork. The 248-mμ peak was present in both materials.

At present, attempts are in progress to identify the various components present in fraction Aa_2, and to attempt to determine the role of these constituents in the production of odor and flavor in meat.

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FEED ADDITIVES MEASUREMENT

Ultraviolet Method for Determination of Streptomycin in Feeds

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THE USE OF ANTIBIOTICS in animal feeds is constantly increasing and state feed control laboratories face problems in determining these drugs. As most feed control laboratories do not have facilities for biological assay procedures, the development of chemical methods for determining antibiotics in feeds is an urgent problem.

All current AOAC methods for antibiotics are microbiological assay procedures. When these AOAC procedures for antibiotics were being studied (*12*), streptomycin was not considered because it was not widely used. Now that its use in poultry and swine feeds is increasing, it is imperative that a suitable chemical method be devised.

The streptomycin molecule can be determined by any one of three basic ways: analysis for the streptose moiety by determining the alkaline degradation product, maltol; for the combined guanidine groups; and for the *N*-methyl-1-glucosamine fraction.

Schenck and Spielman (*14*) showed that heating streptomycin at 100° C. for 3 minutes with 1*N* sodium hydroxide converted to streptose moiety of the streptomycin molecule quantitatively to maltol (3-hydroxy-2-methyl-4-pyrone).

Eisenman and Bricker (*6*) reported the use of this reaction in a spectrophotometric method, in which the color reaction between maltol and ferric iron was employed to estimate the concentration of streptomycin. Boxer, Jelinek, and Leghorn (*4*) used the color reaction between maltol and the Folin-Ciocalteu phenol reagent, as well as the ferric iron-maltol complex, for streptomycin determinations. St. John, Flick, and Tepe (*13*) and Angeles (*1*) adapted the colorimetric maltol procedure for the assay of streptomycin in fermentation broths.

Various investigators determined streptomycin by means of the combined guanidine groups. The two general procedures used were colorimetric estimation with sodium nitroprusside and potassium ferrocyanide (*10*) and colorimetric estimation of the condensation product with biacetyl and 1-naphthol (*2, 3, 7*).