Components Contributing to Beef Flavor

Natural Precursors of 4-Hydroxy-5-methyl-3(2*H*)-furanone in Beef Broth

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The recent identification of 4-hydroxy-5-methyl-3(2*H*)-furanone as a flavor component of beef broth prompted an investigation into its natural precursors. When minced lean shin of beef was extracted with water at room temperature, the extract contained no detectable 4-hydroxy-5-methyl-3(2*H*)-furanone. If it was boiled for 2½ hours, however, the furanone could be isolated, indicating that it is formed from cold water–soluble precursors. These precursors have been identified as ribose-5-phosphate, and pyrrolidone carboxylic acid or taurine or both.

The suggestion that precursors responsible for the flavor of cooked beef are extractable from meat with cold water has been made by Hornstein *et al.* (1960) and Kramlich and Pearson (1958). In addition, heating is claimed to be necessary for production of the flavor. In most cases the development of meaty odors has been associated with a Maillard-type interaction between sugars and amino acids. Macy *et al.* (1964) observed that ribose was the most labile sugar when meat extracts were heated. Wood (1961) studied the browning reaction in model systems and found that ribose and ribose-5-phosphate were particularly reactive. In addition, Batzer *et al.* (1962) described the isolation of a glycoprotein from beef that is supposed to play a role as a flavor precursor.

This article describes the isolation and identification of the precursors of 4-hydroxy-5-methyl-3(2*H*)-furanone, a flavor component recently isolated from beef broth by Tonsbeek *et al.* (1968).

EXPERIMENTAL

Preparation of Aqueous Extract of Beef at Room Temperature. Two kilograms of ground lean shin of beef were suspended in 1 liter of water at room temperature. The mixture was stirred for 15 minutes and then filtered through cheesecloth. The meat residue was extracted twice more with 1 liter of water and the three filtrates were combined. The volume of this extract, corresponding to 2000 grams of beef, was about 3000 ml.

Preparation of Concentrated Aqueous Extract. The aqueous extract prepared from 2000 grams of beef was diluted with 4 volumes of cold ethanol (2° C.) to precipitate the proteins. The suspension was mixed and left to stand at 2° C. for 1 hour. The precipitated proteins were then filtered off. The filtrate was concentrated to a small volume in a Büchi Rotavapor at a temperature of 40° C. The residue was diluted to a volume of 400 ml. with distilled water. The extractable material of 5 grams of beef is then contained in 1 ml. of the concentrated aqueous extract.

Preparation of Fractions I and II. By chromatography over an acidic cation exchange resin (Bio-Rad AG 50W-X8; 50–100 mesh) the concentrated aqueous extract was separated into a fraction I (neutral and acidic components) and a fraction II (basic components). The column (8 × 2.2 cm.) was conditioned by washing with 400 ml. of 6*N* ammonia, and then with distilled water until the effluent was neutral. Next the column was treated with 250 ml. of 6*N* HCl and again washed with water until the effluent was free of chloride. Then 12 ml. of the concentrated aqueous extract was pipeted onto the resin and carefully washed into the column with a small quantity of water. Fraction I was obtained as the effluent after washing the column with 200 ml. of distilled water. The basic compounds such as amino acids, creatine, etc. (but not the inorganic cations), were subsequently eluted from the column with 400 ml. of 6*N* ammonia. The ammonia was removed from the eluate by evaporation at 40° C. under reduced pressure. The residue, dissolved in distilled water, was fraction II.

Preparation of Fractions III, IV, and V. Fraction I was titrated to pH 8 with a saturated solution of barium hydroxide. After concentration under vacuum to a volume of 50 ml., the pH was adjusted to 9.3 with the barium hydroxide solution. The precipitate obtained was removed by centrifugation. The isolated barium salts were shaken with an acidic cation exchange resin (Bio-Rad AG 50W-X8) to replace the barium ions with hydrogen ions. The solution of free acids thus obtained is fraction III. The supernatant was mixed with 2 volumes of cold (2° C.) ethanol and left to stand for half an hour at 2° C. A second precipitate was formed, which was again separated from the supernatant by centrifugation. The supernatant was concentrated to dryness under vacuum at 40° C. and the residue was dissolved in distilled water. The supernatant solution and a suspension of the precipitate were both mixed with an acidic cation exchange resin

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(Bio-Rad AG 50W-X8) to convert the barium salts into the free acids. After filtration the supernatant yielded fraction IV and the second precipitate fraction V.

Preparation of Fractions V-A, V-B, and V-C. The procedure described by Katz and Comb (1963) for the separation of nucleotides was followed. Fraction V was neutralized with 1*N* NaOH solution to a pH of 7.0 and the solution was concentrated to a small volume by evaporation under vacuum. Since a large quantity of material (originating from 12 ml. of concentrated aqueous extract) had to be fractionated, the sample was applied to a larger column (12 × 1.5 cm.; Bio-Rad AG 50W-X8, 400-mesh). Elution was performed with 100 ml. of 0.05*N* HCl. The ultraviolet absorption of the eluate was recorded with an LKB Uvicord. The material of the first peak (fraction V-A) was contained in a volume of 30 ml., whereas the second fraction [fraction V-B, containing inosine-5'-monophosphate (5'-IMP)] had a volume of 70 ml. By washing the column with 50 ml. of distilled water a third fraction [fraction V-C, containing guanosine-5'-monophosphate (5'-GMP)] was obtained. The solutes of each fraction were precipitated by titration with barium hydroxide and addition of 2 volumes of cold ethanol. After being left to stand at 2° C. for half an hour, the precipitates were isolated by centrifugation and reconverted into the free acids with an acidic cation exchange resin (Bio-Rad AG 50W-X8).

Detection of Presence of Precursors. The following procedure was used to check whether the precursors of 4-hydroxy-5-methyl-3(2*H*)-furanone were present in a particular fraction. Each fraction was neutralized with 1*N* aqueous NaOH solution (or with 1*N* HCl for fraction II) to pH 5.5, which is equal to the pH of an aqueous extract of beef. The fractions were then extracted continuously for 20 hours with diethyl ether. The absence of 4-hydroxy-3(2*H*)-furanones was indicated by the failure of the ether extract to show the characteristic ultraviolet absorption at 289 mμ (Tonsbeek *et al.*, 1968). If there was no such absorption maximum, a quantity of each neutralized fraction (pH 5.5) was refluxed for 2½ hours and subsequently extracted with diethyl ether for 3 hours. If the ether extract then showed an ultraviolet absorption maximum at 289 mμ, this indicated that 4-hydroxy-3(2*H*)-furanone(s) had been formed during the refluxing of the fraction. Various combinations of fractions were tested in a similar way.

The presence of 4-hydroxy-5-methyl-3(2*H*)-furanone in ether extracts showing the characteristic absorption peak was ascertained as follows. A 10 × 1 cm. column of polyamide [Machery-Nagel & Co, MN-polyamid (normal)] was prepared in a water-cooled chromatographic tube. The column was washed with ether and light petroleum (b.p. 40° to 60° C.) to remove impurities. The ether extract was concentrated to a small volume. After addition of a small quantity of polyamide, the remaining solvent was removed by evaporation. The remaining powder, containing the solutes, was transferred to the top of the column, which was eluted with 25 ml. of pentane followed by 100 ml. of 10% diethyl ether in pentane. Any 4-hydroxy-5-methyl-3(2*H*)-furanone present was then selectively eluted by washing the column with a 50/50 diethyl ether–pentane mixture. The latter fraction was subjected to GLC analysis under the conditions described by Tonsbeek *et al.* (1968). Only when this analysis revealed the presence of the furanone was it concluded that its precursors were present in the fraction or fraction combination investigated.

Enzymic Hydrolysis of Sugar Phosphates of Fraction V-A. Fraction V-A was neutralized to pH 5.5 and mixed with 5 ml. of a Tris buffer solution, prepared by titrating a 0.1*M* 2-amino-2-(hydroxymethyl)propane-1,3-diol solution to pH 8.8 with a 1*N* NaOH solution. Then 500 μg. of alkaline phosphatase from calf mucosa (Sigma, type II) was added to the buffered solution. A blank, containing only fraction V-A and 5 ml. of the Tris buffer, was also prepared. The sample and the blank were heated for 5 hours at 37° C. Both solutions were then percolated through an acidic cation exchange resin (Bio-Rad AG 50W-X8) column to remove the buffer salt. The effluents were neutralized to pH 5.5 and the presence of the precursors of 4-hydroxy-5-methyl-3(2*H*)-furanone in the two solutions was checked in the manner described above.

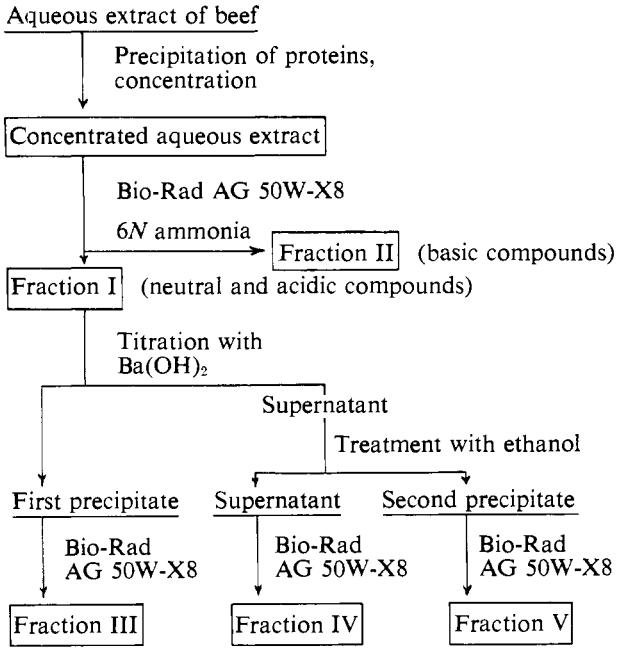
Determination of Sugars in Enzymically Hydrolyzed Fraction V-A. Fraction V-A was hydrolyzed with an alkaline phosphatase as described above. This hydrolyzed fraction was percolated through an acidic cation exchange resin (Bio-Rad AG 50W-X8) and an anion exchange resin (Bio-Rad AG 3-X4; 20–50 mesh) in the free base form to remove basic and acidic components. The neutral fraction, containing the sugars formed from the sugar phosphates, was concentrated to a small volume by evaporation under vacuum at 40° C. The residue was dried over phosphorus pentoxide in a vacuum desiccator. The sugars were then analyzed as their trimethylsilyl derivatives by gas-liquid chromatography by the method of Sweeley *et al.* (1963).

Model Experiments. Varying quantities (2 to 100 mg.) of components identified in fraction IV were dissolved, together with 5 mg. of ribose-5-phosphate in 50 ml. of distilled water. After adjustment of the pH to 5.5, the solutions were boiled for 2½ hours and subsequently analyzed for the presence of 4-hydroxy-5-methyl-3(2*H*)-furanone.

RESULTS AND DISCUSSION

When the aqueous extract of minced lean shin of beef was extracted continuously with diethyl ether for 20 hours, it was impossible to detect even a trace of 4-hydroxy-5-methyl-3(2*H*)-furanone in the ether extract. If, however, the aqueous extract was first boiled for 2½ hours, an ether extract of the resulting solution showed the ultraviolet absorption at 289 mμ characteristic of 4-hydroxy-3(2*H*)-furanone derivatives. The ether extract was then fractionated by adsorption chromatography and the appropriate eluate analyzed by GLC. This was necessary to separate the 4-hydroxy-5-methyl-3(2*H*)-furanone from other substances with the same ultraviolet absorption maximum, such as the 2,5-dimethyl homolog. These experiments confirmed the presence of 4-hydroxy-5-methyl-3(2*H*)-furanone in the refluxed extract and showed that the compound is formed during the refluxing from precursors which are extractable from beef with water at room temperature.

To identify the precursors, the aqueous beef extract was fractionated according to the following scheme:



The concentrated aqueous extract, the fractions derived from it, and various combinations of these fractions were all checked to see whether they contained precursors of 4-hydroxy-5-methyl-3(2*H*)-furanone. Some of the results are tabulated in Table I.

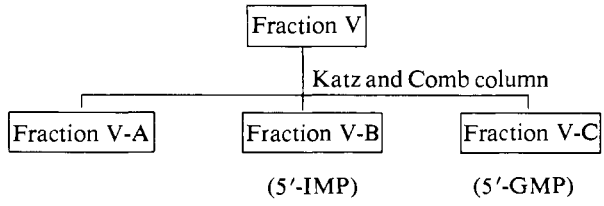
It was concluded from these experiments that 4-hydroxy-5-methyl-3(2*H*)-furanone is not formed by an interaction between sugars (fraction IV) and amino acids (fraction II).

In addition to the results in Table I, it was found that combination of fraction IV with either fraction III or V gave similar appreciable yields of the furanone. Although there appeared to be no clear-cut distinction between the latter two fractions, fraction V was considered the most promising for further investigation. Analysis of fraction V by means of thin-layer, paper, and column chromatography revealed that nucleotides, succinic acid, phosphoric acid, sugar monophosphates, and traces of sugar diphosphates were present. After neutralization to pH 7.0, fraction V was further fractionated on a cation exchange resin column by the method of Katz and Comb (1963).

Table I. Presence of 4-Hydroxy-5-methyl-3(2H)-furanone Precursors in Various Fractions

Solution Investigated	Yield of Furanone after Refluxing[a]
Concentrated aqueous extract	+++
Fraction	
I	+++
II	−
III	+
IV	−
V	++
Fractions	
I + II	+++
II + III	+
II + IV	−
II + V	++

[a] +++ High. ++ Moderate. + Low. − Zero.

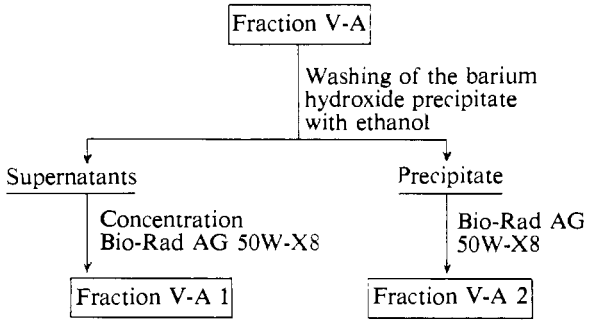


Neither fraction V-B nor fraction V-C contributed to the formation of the dihydrofuranone. It was therefore concluded that 5′-IMP and 5′-GMP do not act as precursors for the flavor component. The precursors were, however, still present in fraction V-A. This fraction contained sugar monophosphates and diphosphates, phosphoric acid, and succinic acid. By separating the monophosphates from the diphosphates on an anion exchange resin column according to Bartlett's method (1959), it was proved that the fraction containing the sugar monophosphates was responsible for the formation of 4-hydroxy-5-methyl-3(2*H*)-furanone. This prompted the idea that a sugar monophosphate is a precursor. This was checked as follows. A Tris buffer was added to a fraction V-A and the resulting solution was divided into two equal parts. To one part 500 μg. of alkaline phosphatase was added, whereas the other served as a blank. After completion of the hydrolysis both solutions were screened for formation of the furanone. The blank still contained the precursors whereas the hydrolyzed solution did not, indicating that an organic phosphate acted as precursor.

In view of the structure of the furanone, it seemed likely that a pentose phosphate was responsible. Since ribose-5-phosphate has often been found to occur in meat and meat extracts, we tried to identify this component in fraction V-A. Attempts to demonstrate its presence by column chromatography according to Jones and Burt (1960) or by paper chromatography according to Bieleski and Young (1963) failed because large quantities of glucose-6-phosphate and fructose-6-phosphate were present. Fraction V-A after enzymic hydrolysis, by which the sugar phosphates are converted to sugars and phosphoric acid, proved to be a better starting material. Phosphoric acid and the buffer salts were removed by treatment on Bio-Rad AG 50W-X8 and Bio-Rad AG 3-X4 ion exchange resin columns and the resulting neutral fraction was analyzed for sugars. Both paper chromatography as described by Scheffer and Kickuth (1962) and gas-liquid chromatography of the trimethylsilyl derivatives by the method of Sweeley *et al.* (1963) indicated the presence of ribose in hydrolyzed fraction V-A. A similar experiment, in which no phosphatase was added, did not reveal any ribose at all. It was concluded that the ribose in the enzymically hydrolyzed fraction originated from a ribose phosphate ester, probably ribose-5-phosphate.

When ribose-5-phosphate was heated in aqueous solution at pH 5.5 for $2^1/_2$ hours, no 4-hydroxy-5-methyl-3(2*H*)-furanone was formed. However, addition of ribose-5-phosphate to fraction V-A prior to refluxing gave rise to a much higher yield of the furanone than with fraction V-A alone. This led to the hypothesis that an interaction between at least two components was necessary for the formation of 4-hydroxy-5-methyl-3(2*H*)-furanone, one of these components being ribose-5-phosphate.

This hypothesis was confirmed by the following experiment. The precipitated barium salts obtained during the preparation of fraction V-A were redissolved in water and again precipitated by the addition of 2 volumes of ethanol and subsequent cooling. Supernatant and precipitate were separated by centrifugation. This procedure was repeated several times. The combined supernatants were evaporated nearly to dryness and percolated through an acidic cation exchange resin (Bio-Rad AG 50W-X8). In this way fraction V-A 1 was obtained. Shaking an aqueous suspension of the precipitate with the same cation exchange resin resulted in fraction V-A 2. This separation is represented by the following scheme:



After adjustment of their pH to 5.5, fractions V-A 1 and V-A 2 were each refluxed for $2^1/_2$ hours. Only the latter fraction yielded 4-hydroxy-5-methyl-3(2*H*)-furanone. Recombination of both fractions, however, resulted in a higher yield of the flavor component. Moreover, if ribose-5-phosphate was added to fraction V-A 1, a high yield of furanone was obtained.

These experiments confirmed that ribose-5-phosphate is only one of the precursors of 4-hydroxy-5-methyl-3(2*H*)-furanone. The other precursor(s) is obviously present in fraction V-A 1, and its barium salt must therefore be soluble in cold 67% ethanol. The ability of fraction V-A 2 to yield the dihydrofuranone is apparently due to adsorption of this salt on the precipitate of insoluble barium salts. In view of the above, the second precursor ought to be present in fraction IV. To check this assumption, ribose-5-phosphate were added to fraction IV; after boiling for $2^1/_2$ hours at pH 5.5, a very high yield of 4-hydroxy-5-methyl-3(2*H*)-furanone was obtained.

Analysis of fraction IV by paper, thin-layer, and gas-liquid chromatography revealed the presence of all those neutral and acidic components which have so far been isolated by other authors from either beef broth or beef extract. In addition to these well-known compounds, fraction IV was also shown to contain pyrrolidone carboxylic acid, a compound already isolated from beef broth at this laboratory (Van der Zijden *et al.*, 1969). Components present in fraction IV are:

Glucose	Lactic acid
Fructose	Pyrrolidone carboxylic acid
Ribose	Phosphoric acid
Inositol	Succinic acid
Glycerol	Hydrochloric acid
Taurine	3-Hydroxybutyric acid
Sugar phosphates	Nucleotides

The presence of small quantities of phosphoric acid, nucleotides, succinic acid, and sugar phosphates in fraction IV is due to the poor separation achieved by precipitation. Each of the components found in fraction IV was heated separately with ribose-5-phosphate at pH 5.5 and the ultraviolet spectra of the resulting solutions were recorded. It was shown that pyrrolidone carboxylic acid and, to a lesser extent taurine, reacted with ribose-5-phosphate to form 4-hydroxy-5-methyl-3(2*H*)-furanone. The other components mentioned in fraction IV failed to give any reaction.

Since neither pyrrolidone carboxylic acid nor taurine precipitate as barium salts in 67% ethanol containing barium ions, it was necessary to explain why fraction V-A 2 alone was capable of yielding the furanone upon boiling. It was possible, however, to ascertain the presence of traces of pyrrolidone carboxylic acid and of taurine in the combined supernatants obtained after repeated washing of the alcoholic precipitate. These traces of pyrrolidone carboxylic acid and/or taurine were sufficient to permit the formation of 4-hydroxy-5-methyl-3(2*H*)-furanone from ribose-5-phosphate.

From the experiments described above it was concluded that the 4-hydroxy-5-methyl-3(2*H*)-furanone present in beef broth originates from a reaction between ribose-5-phosphate and either pyrrolidone carboxylic acid or taurine or both.

Peer and Van den Ouweland (1968) showed that 4-hydroxy-5-methyl-3(2*H*)-furanone can be synthesized in good yield from ribose-5-phosphate in concentrated buffer systems at pH 5.5. The mechanism of this reaction involves a base-catalyzed β-elimination of phosphoric acid from ribose-5-phosphate, followed by an acid-catalyzed dehydration of the resulting 5-deoxyhexose. In a diluted natural medium, such as beef broth, ribose-5-phosphate reacted at pH 5.5 to yield the furanone only in the presence of the nitrogen-containing compounds pyrrolidone carboxylic acid and taurine. This indicated that another reaction mechanism may be responsible.

Investigations into the natural precursors of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone, a compound also found in beef broth by Tonsbeek *et al.* (1968), will be published in due course.

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