Identification of Some Beef Flavor Precursors

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Some precursors of beef flavor have been investigated. Those dealt with here appear to be relatively simple water-soluble components of beef muscle tissue. Inosinic acid or inosine and inorganic phosphate have been identified as one of the compounds involved. A glycoprotein of unknown structure is another component. Glucose has been identified as the sugar moiety of the glycoprotein. The amino acid composition is also discussed.

In a previous communication (*1*) the method of obtaining a dialyzable fraction of a water-extract of raw beef muscle was outlined. This fraction, when heated with fat (130° to 160° C.) produced an odor similar to that of broiled steak, and when boiled with water, produced a beef broth odor and flavor. Further separation of the dialyzable material produced a fraction, designated in (*1*) as Aa_2, which contained substances considered to be basic for production of an odor similar to that of broiled steak. Earlier results showed the presence of a material resembling a glycoprotein, a high phosphate content, and some substance absorbing strongly in the ultraviolet with a peak in the 248 mμ region. This paper deals with attempts at identification of the material present in this fraction.

Experimental Procedure

A solution of 60 mg. of Aa_2 in 25 ml. of water was brought to pH 8.5 and passed through a Dowex-1 (200 to 400 mesh, 8×) column, 2.4 × 30 cm., in the chloride form. The glycoprotein material passed through the column, while the ultraviolet-absorbing and phosphorus-containing material was adsorbed by resin. The column was washed with distilled water and then eluted in 10 ml. fractions using a pH gradient from distilled water to 0.006*N* hydrochloric acid (pH ~ 2.2). The absorbance of each fraction was read at 260 mμ on a Beckman DU spectrophotometer and plotted. Several peaks were obtained. Fractions representing individual peaks were pooled and evaporated to dryness under vacuum. Spectra of an aqueous solution of each pooled fraction at pH values of 2.0, 7.0, and 11.0 were taken on a Beckman DK-2 spectrophotometer. Pooled fractions were purified by passing a solution of the dried material in 2*N* hydrochloric acid through a 1 × 20 cm. column of Dowex-50 (200- to 400-mesh, 8×) in the hydrogen form and eluting with the same solvent. This treatment removed most of the contaminating material which interfered with absorbance readings. The solutions representing the major component in each case were read on the spectrophotometer at 250, 260, 280, and 290 mμ at pH values of 2*N* HCl, 2.0, 7.0, and 11.0, respectively. This procedure was repeated with hypoxanthine and inosine (California Biochemicals Corp.) and ratios at 250:260, 280:260, and 290:260 were compared. Paper strip chromatograms were run in the water-saturated 1-butanol system of Hotchkiss (*5*) for further verification by R_f values.

The carbohydrate moiety of the glycoprotein was identified in the following manner. The effluent obtained from the ion exchange column was lyophilized. The dry material was hydrolyzed in 1*N* sulfuric acid for 4 hours at 100° C. The hydrolyzate was neutralized with barium carbonate, centrifuged, and the supernatant solution applied to paper chromatograms which were developed in a number of solvent systems. These included 2-propanol-water, 1-propanol–ethyl acetate–water, phenol-ammonia, ethyl acetate–pyridine-water, 2-propanol-1-butanol-water, 1-butanol–acetic acid-water, ethyl acetate–acetic acid–water (*8*). The chromatograms were sprayed with a 0.3% *p*-amino hippuric acid solution in ethanol, heated for 8 minutes at 140° C., and examined under an ultraviolet light to locate the sugar spots (*3*).

Amino acids were determined by two-dimensional paper chromatography of the hydrolyzate obtained by refluxing the glycoprotein in constant boiling hydrochloric acid for 6 hours. 1-Butanol–acetic acid–water 120:30:50 (v./v.) was used for the first dimension and phenol-ammonia for the second. The spots were located with ninhydrin.

Results and Discussion

The glycoprotein material, as obtained from the Dowex-1 treatment was unusual, since it was negative to ninhydrin, but became ninhydrin-positive after acid hydrolysis. An adaption of the Fiske-Subbarow procedure (*9*) indicated that no phosphate was present. Tests with aniline acetate for carbohydrates gave positive results. No peak was observed at 248 mμ when absorbance spectra were taken in the ultraviolet.

The negative ninhydrin reaction of the intact glycoprotein may be due to a lack of free amino groups. Since glucose is an integral part of the compound, it is possible that the attachment is through the amino groups.

When the sulfuric acid hydrolyzate was chromatogrammed in the various solvent systems used for sugars, two spots appeared. The R_g values in the various solvent systems are defined and listed in Table I. Values for spot No. 2 are sufficiently close to indicate glucose. Mixtures of the hydrolyzate with either fructose, sorbose, mannose, galactose, sucrose or arabinose resulted in the appearance of an extra spot when the various solvent systems known to separate glucose from the added sugar were used. When glucose was added to the hydrolyzate, the usual two spots appeared on the chromatograms run in all of the solvent systems mentioned. Glucostat reagent (Worthington Biochemical Corp.), a coupled enzyme system of glucose oxidase, peroxidase, and a chromogenic hydrogen donor, was also used as a spray to develop the chromatograms. Spot No. 2 gave positive results with this reagent. Spot No. 1 did not represent any known sugar on the basis of its R_g values in the various solvent systems. It did not react with Glucostat reagent, and was ninhydrin-negative. Material from this spot was therefore eluted from the paper, hydrolyzed further, and the second hydrolyzate chromatographed in the same system. This time only one spot appeared which had an R_g value of 100 and also reacted with Glucostat reagent. This suggests that spot No. 1 was unhydrolyzed or partially hydrolyzed material.

When the hydrochloric acid hydrolyzate of the glycoprotein was chromatogrammed in two dimensions for amino acids, eight ninhydrin-reactive

spots were found. By referring to published R_f values for various solvent systems (*7*), it was possible to identify six of the amino acids as proline, isoleucine-leucine, alanine, valine, serine, and β-alanine (Table II). Based on the color intensity with ninhydrin and the relative area, spot X2 appeared to comprise about 80% of the ninhydrin-positive material present. Attempts to identify spot X2, so far, have been unsuccessful. Elementary analysis indicated the presence of sulfur. However, the R_f values differed greatly from those of the common sulfur containing amino acids. Taurine, methionine sulfone, methionine sulfoxide, ergothionine, penicillamine, mercaptoethylamine hydrochloride, mercaptoethylamine disulfide, and thiol-histidine were eliminated when compared to spot X2, on the basis of their R_f values in the various solvent systems. Spot X2 was eluted from chromatograms, hydrolyzed in constant boiling hydrochloric acid for 6 hours, and chromatogrammed. Only one spot appeared with no change in the R_f value. Spot X1 on the chromatograms also has not been identified. Hydrolyzates of the glycoprotein were run on a 150-cm. column of ion exchange resins according to the procedure of Moore, Spackman, and Stein (*6*). Results confirmed the identity of the spots on the paper chromatograms (Table III) with the exception of valine, which was not detected. This was not surprising because the valine spot on the two-dimensional chromatograms was always very faint. The ion exchange procedure also picked up trace amounts of glutamic acid and glycine. These two amino acids were only occasionally observed on the paper chromatograms, and may have been present as contaminants. Spots X1 and X2 were not eluted from this column. Of particular interest is the large amount of β-alanine in comparison to the rest of the amino acids. Attempts are now under way to obtain sufficient material representing spot X2 for quantitative elementary analysis.

The ultraviolet absorbance spectra of the materials isolated by gradient elution from the Dowex column matched published (*4*) spectra of hypoxanthine and inosine. These spectral results and the results obtained by ratio comparison (Table IV) are sufficient to indicate that the compounds are hypoxanthine and inosine. R_f values of the compounds on paper chromatograms in the water-saturated 1-butanol system are in excellent agreement with the published values of Hotchkiss (*5*).

A phosphorus-containing nucleotide, whose ultraviolet absorbance spectrum matched that of known inosinic acid, was obtained from the Dowex-1 column, but when attempts were made to purify it on the Dowex-50 column, only hypoxanthine and inosine were obtained. This was probably due to hydrolysis by the 2*N* acid used in this procedure. The presence of hypoxanthine, inosine, and inosinic acid in commercial ox muscle extracts (*11*), and free and combined hypoxanthine, in fresh ox muscle extract (*2*) has been reported. Wood, in a recent publication (*10*), has stated that all of the compounds isolated and identified from fresh ox muscle extract (*2*) were found to be tasteless with the exception of creatine (bitter) and inosinic acid (meaty). However, when a synthetic mixture of the compounds found in (*2*) were heated in the presence of glucose, the color and flavor that developed were quite similar to those of authentic extracts. Wood concludes that the browning and flavor development of ox muscle extracts are due to a Maillard reaction.

With the identification of inosinic acid and the sugar moiety of the glycoprotein, mixtures of the glycoprotein, inosine, and inorganic phosphate (or inosinic acid) were prepared. When these mixtures were heated in fat, an odor similar to the broiled steak odor was obtained. The decision on whether the odor of cooked meat was present or not was a difficult one and was made by the people working on the project. The judgment—rather subjective—was based on familiarity with the odor of the original fraction *A* which is both characteristic and distinctive. Results were not considered as positive, unless in the estimation of those concerned the odor was identical with that obtained from *A*. When dried hydrolyzates of the glycoprotein (or mixtures of corresponding amino acids including the two unknown components) were used with added glucose, inosine, and phosphate the same odor was evolved. These experiments were not successful every time and it appears that the relative amounts of the various substances are critical. Work on the quantitation of the original fraction (Aa_2) is now in progress.

Some difficulty in obtaining the original Aa_2 fraction has been encountered. One of the necessary steps (*1*) required a second dialysis with ordinary sausage casing. In this step, the low molecular weight material was removed, while the material that gave the odor was retained. The method of obtaining sausage casing (cellulose casing, Visking Corp.) for this operation was to examine a number of rolls for desirable characteristics, by dialyzing some of the water extract. When a roll was found, it could be used for a period of 3 weeks to 6 months. After this time, the char-

Table I. R_g[a] Values of Sugars Obtained as Products of Sulfuric Acid Hydrolysis of the Glycoprotein

Spot	2-Pr Aq[b]	Pr Et Ac[c]	Ph Am[d]	Et Ac Py[e]	2-Pr Bu[f]	Bu A[g]	Et Ac A[h]
1	80–90	110–120	16	92	47	65	...
2	90–95	95–110	98	100	100	100	100

[a] $R_g = \frac{\text{distance substance travels from origin} \times 100}{\text{distance glucose travels from origin}}$

[b] 2-Propanol-water 160:40 v./v.
[c] 1-Propanol–ethyl acetate–water 140:20:40 v./v.
[d] Phenol 160 grams, water 40 ml., ammonium hydroxide, concentrated, 1 ml.
[e] Ethyl acetate–pyridine–water 120:50:40 v./v.
[f] 2-Propanol-1-butanol-water 140:20:40 v./v.
[g] 1-Butanol–acetic acid–water 120:30:50 v./v.
[h] Ethyl acetate–acetic acid–water 140:30:30 v./v.

Table II. R_f Values of Amino Acids from Hydrolyzed Glycoprotein as Obtained by Two Dimensional Chromatograms

	Bu-Ac-H_2O		Phenol-Ammonia		
	Found	Published (5)	Found	Published (5)	Color Reaction with Ninhydrin
Unknown X1	21		88		Light purple
Unknown X2	20		69		Very heavy purple
Proline	40	34	90	90	Yellow
Isoleucine, leucine	68	67	86	85	Light purple
Alanine	31	30	57	58	Purple
Valine	55	51	79	78	Very faint purple
Serine	24	22	35	35	Faint purple
β-Alanine	38	33	65	64	Brownish

Table III. Identity and Approximate Mole Equivalents of Amino Acids of Hydrolyzed Glycoprotein as Obtained by Ion Exchange Chromatography

Identity	Mole Equivalent
Serine	0.130
Glutamic acid	0.010
Glycine	0.025
Alanine	0.100
Isoleucine	0.016
Leucine	0.030
β-Alanine	0.780
Proline	[a]

[a] Not estimated.

Table IV. Comparison of Ultraviolet Absorbance Ratios of Isolated Compounds With Hypoxanthine and Inosine

Compound	Ratios		2N HCl	pH 2.0	pH 7.0	pH 11	R_f 1-Butanol-H_2O Isolated	R_f 1-Butanol-H_2O Known
Inosine	250:260	*i*	1.22	1.58	1.42	1.08		
		k	1.20	1.51	1.45	1.14		
	280:260	*i*	0.10	0.29	0.37	0.28	0.16	0.15
		k	0.21	0.28	0.36	0.25		
	290:260	*i*	0.03	0.11	0.12	0.13		
		k	0.10	0.10	0.19	0.13		
Hypoxanthine	250:260	*i*	1.46	1.36	1.11	0.88		
		k	1.48	1.52	1.33	0.91		
	280:260	*i*	0.01	0.19	0.22	0.21		
		k	0	0.14	0.19	0.16	0.39	0.38
	290:260	*i*	0	0.08	0.13	0.11		
		k	0	0.05	0.11	0.08		

i = Isolated.
k = Known.

acteristics changed so that the desired fraction was no longer retained. This occurred, although the roll of casing was left in a moisture-proof bag and placed in a refrigerator. The molecular weight of the fraction desired is apparently such that a slight increase in the pore size of the casing prevents its retention. Efforts are being made to standardize this phase of the procedure for isolation of fraction Aa_2.

Since all of these compounds can be readily obtained, including the glycoprotein which is easily isolable, it would therefore seem likely that this mixture could be applied to any food material, or meat, prior to cooking to impart a meat flavor, or to enhance the natural flavor of meat. It would also seem likely that a mixture of these precursors could be injected into the blood stream of meat animals just prior to slaughter to enhance the meat flavor as well as to ensure a constant flavor quality.

Conclusions

Some of the precursors of meat (beef) flavor have been found to be a relatively simple mixture of glucose, inosinic acid, and a glycoprotein. While the quantitative relationships among these compounds have not been established, variations in meaty flavor or odor produced on heating in fat or water have been observed when the composition of mixtures of these compounds is varied.

When mixtures of amino acids, found as components of the glycoprotein, are used in conjunction with glucose, inosine, and inorganic phosphate, meaty odors and flavors are also produced upon heating in fat or water. It may therefore be surmised that only certain of the amino acids in the glycoprotein are necessary precursors of meat flavor.

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FAT FLAVOR

Effect of Free Fatty Acid on Flavor of Fat

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SINCE RANCID flavor of highly saturated, low molecular weight fats, such as butter and coconut oil, has been associated with liberation of fatty acids, hydrolysis generally is regarded with disfavor in all edible fats. Yet, in three separate projects of widely different nature, the initial development of free fatty acid has been attended by increased flavor scores for the fat. This relation was observed with fat acidity developed in vivo by nutritional depletion and by feeding wheat to pigs, and in deep-fat frying of potatoes.

Nutritional Depletion in Hogs

When heavy hogs were hauled 50 miles before slaughter and rested for 16 hours without feed, the raw back fat in many cases had higher acid numbers than those found in fed animals, or hogs rested for shorter periods (*1*). The fats with higher acid numbers rated higher flavor scores.

To test the extent of this relation, a scattergram of raw-fat acid numbers was plotted against flavor scores for the cooked fat from all fresh samples from all lots pertaining to feed and rest (Figure 1). Broken lines were drawn parallel to the axes so as to extend the left and upper boundaries of the unoccupied area *D* as far as possible. The absence of points in this area indicated that none of the acid numbers above 0.35 was associated with flavor scores below 6.0. Highest possible score was 7. Acid numbers were milligrams of KOH per gram of fat. With lower acid numbers in areas *A* and *B*, flavor scores ran from lowest to highest, and obviously no relation to acidity