Isolation and Identification of Nitrogenous Components in Meat

J. C. ALEXANDER[1] and C. A. ELVEHJEM
Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison, Wis.

Ion exchange and paper chromatography procedures have been used to identify the nitrogenous components in meat. A new system for gradient elution was employed to elute the amino acids from the column. Approximately 100% of the nitrogen contained in meat has been accounted for by 21 amino acids, ammonia, and other nitrogenous constituents such as vitamins, purines, creatine, carnitine, and methylguanidine.

THE NITROGEN accounted for by the quantitative microbiological determination of 18 amino acids in meat in this laboratory [cf. Alexander, Beckner, and Elvehjem, (*2*)] approximated 87% of the total nitrogen, while estimations of the nitrogen contributed by other nitrogenous constituents (*15*) increased the nitrogen value to approximately 95% (Table I). Ammonium chloride also contributed a large part. This brought the apparent amount of total nitrogen accounted for to approximately 100%.

[1] Present address, Miami Valley Laboratories, Procter & Gamble Co., Cincinnati 31, Ohio.

However, because these 18 amino acids were determined microbiologically, and the over-all accuracy of microbiological assays has been estimated as within ±4% it appeared possible that meat contained small amounts of other unidentified nitrogenous constituents, and that these results remained within the experimental error.

Preliminary qualitative analyses of meat hydrolyzates, using two-dimensional paper chromatograms, showed conclusively that these hydrolyzates contain ninhydrin-positive nitrogenous compounds which have not been identified previously. It was assumed that these unidentified constituents contributed significantly to the total nitrogen content of meat.

By the use of ion exchange and paper chromatography the amino acids hydroxyproline, taurine, and β-alanine have been shown to exist in significant quantities in the crude protein of beef muscle (Table I).

Table I. Beef Round Crude Protein
(N × 6.25)

	Grams/100 Grams Protein	N in Amino Acids, %	N, Grams	% of Total N
Alanine	6.2	15.7	0.973	6.08
Arginine	6.7	32.2	2.160	13.50
Aspartic acid	10.0	10.5	1.050	6.56
Cystine	0.9	11.6	0.104	0.65
Glutamic acid	15.8	9.5	1.501	9.38
Glycine	4.6	18.7	0.860	5.38
Histidine	3.5	27.1	0.949	5.93
Isoleucine	5.7	10.7	0.610	3.81
Leucine	7.7	10.7	0.824	5.15
Lysine	8.7	19.2	1.670	10.44
Methionine	2.4	9.4	0.226	1.41
Phenylalanine	4.3	8.5	0.365	2.28
Proline	3.8	12.2	0.464	2.90
Serine	4.4	13.3	0.585	3.66
Threonine	4.4	11.7	0.515	3.22
Tryptophan	1.3	13.7	0.178	1.11
Tyrosine	3.4	7.7	0.262	1.64
Valine	5.2	12.0	0.624	3.90
				87.00
Carnitine				0.09
Creatine (and creatinine)				4.10
Methylguanidine				1.06
Purines				2.00
Vitamins including choline				0.40
				94.65
Ammonium chloride				5.00
				99.65
β-Alanine	0.4	15.7	0.063	0.39
Hydroxyproline	0.7	10.7	0.075	0.47
Taurine	0.4	11.2	0.045	0.28
				100.79

Procedure and Apparatus for Column Chromatography

The method employed in this study was based on that of Stein and Moore (*11*), and on the photometric ninhydrin method which Moore and Stein (*9*) developed for use with starch columns. A procedure employing the hydrogen cycle of the resin was integrated with a device for gradient elution (*5*). In this way simplicity of operation was greatly increased by eliminating stepwise concentration changes. Operating time was reduced and a definite elution curve could be selected over a wide range of concentrations.

Results indicated that a gradual increase in concentration of acid was desired in the initial stages of elution, with a more rapid increase in acid concentration near the end. In order to achieve this with better separation of the amino acids and without delay in elution in the final stages, a system was developed which used a cone placed inside a cylinder (*5*). Concentrated hydrochloric acid (10.5*N*) was put inside the cone, and dilute hydrochloric acid (0.5*N*) was put inside the cylinder. Adequate mixing was accomplished by forcing the contributions from each container simultaneously through a fine capillary tube, with pressure controlled by a Nullmatic regulator (Moore Products Co., Philadelphia 24, Pa.).

The eluate was fractionated with a Technicon drop-counting automatic fraction collector (Technicon Chromatography Corp., New York 51, N.Y.), calibrated to collect 1 ml. per tube at a

flow rate of 4 ml. per hour. The photometric readings were done on the Lumetron photoelectric colorimeter (Photovolt Corp., 95 Madison Ave., New York, N.Y.), which has one exceedingly valuable advantage. When dark solutions were measured in terms of a standard of high transmittance, the readings were often crowded into the lower portion of the slide-wire scale. With the Lumetron the range can be expanded without additional dilution of the samples, and the accuracy increased by using a neutral gray filter as an optical multiplier.

The columns used were made from Pyrex (7740) tubing 9 mm. in inner diameter, with an Alundum disk sealed inside the tube about 2 inches from one end to support 100 cm. of resin. Larger tubing, 2 cm. in diameter, jacketed the column to reduce temperature variation, because operations were at room temperature.

The resin, Dowex 50-X8 of 200 to 400 mesh, was prepared initially and regenerated by a conventional procedure (*1*). The washed resin was then suspended in 1.0*N* hydrochloric acid and poured under regulated pressure.

The meats employed for these determinations were prepared as described by Violante, Sirny, and Elvehjem (*12*). Half-gram samples were hydrolyzed with 20 ml. of 3*N* hydrochloric acid in an autoclave at 121° for 10 hours or such time as is indicated. The hydrolyzate was diluted to the desired volume, and 0.5 ml. was added to the top of the column.

Because the aliquots collected were acidic, the hydrochloric acid was removed by placing the tubes in a vacuum desiccator heated to 100° while it was evacuated by a water aspirator. This greatly improved the consistency of the color developed and permitted better control of pH.

Table II. R_f Values[a] of Amino Acids with Various Solvent Systems and Colors with Ninhydrin and Isatin Sprays

	Solvent Systems				Color	
Amino Acids	Butanol	Lutidine	Methyl ethyl ketone	2-Methoxy-ethanol	Isatin	Ninhydrin
Alanine	0.25	0.32	0.26	0.64	Blue	Purple
α-Aminobutyric acid	0.48	0.43	0.35	0.64	Blue	Purple
γ-Aminobutyric acid	0.46	0.24	0.48	0.71	Gray	Purple
α-Aminoisobutyric acid	0.47	0.41	0.36	0.67	...	Purple
Arginine	0.09	0.06	0.13	0.49	Pink	Purple
Aspartic acid	0.14	0.09	0.18	0.49	Blue	Blue
Canavanine	0.12	0.10	0.17	0.47	Pink	Purple
Carnosine	0.12	0.28	0.13	0.44	...	Purple
Citrulline	0.17	0.15	0.19	0.54	Pink	Purple
Cysteic acid	0.07	0.24	0.06	0.31	Gray	Purple
Cysteine	0.13	...	0.06	0.25	Gray	Brown
Cystine	0.06	0.00	0.07	0.30	Gray	Brown
Djenkolic acid	0.07	0.13	0.06	0.24	Gray	Purple
Dopa[b]	0.20	1.00	0.25	0.57	Gray	Purple
Ethionine	0.60	0.59	0.51	0.76	Pink	Purple
Glutamic acid	0.20	0.11	0.22	0.57	Lavender	Purple
Glycine	0.16	0.23	0.19	0.51	Pink	Purple
Histidine	0.09	0.26	0.12	0.44	Lavender	Purple
Homocystine	0.14	0.17	0.16	0.49	Gray	Purple
Homoserine	0.22	0.34	0.22	0.54	Pink	Maroon
Isoleucine	0.65	0.55	0.52	0.77	Pink	Purple
Leucine	0.68	0.57	0.56	0.80	Pink	Purple
Lysine	0.08	0.14	0.11	0.48	Lavender	Purple
Methionine	0.55	0.52	0.43	0.70	Pink	Purple
Methionine sulfoxide	0.22	0.21	0.19	0.51	Pink	Purple
Norleucine	0.71	0.54	0.55	0.78	Lavender	Purple
Norvaline	0.57	0.48	0.44	0.72	Pink	Purple
Ornithine	0.10	0.11	0.10	0.38	Lavender	Purple
Phenylalanine	0.59	...	0.51	0.73	Blue-gray	Blue
Serine	0.15	0.27	0.17	0.50	Pink	Purple
Threonine	0.22	0.44	0.22	0.59	Pink	Purple
Tryptophan	0.48	...	0.45	0.68	Blue-green	Purple
Tyrosine	0.34	0.00	0.37	0.66	Blue-green	Blue
Valine	0.55	0.49	0.42	0.73	Pink	Purple

[a] Average of at least three values with Whatman No. 1 filter paper at approximately 30° C.
[b] 3-(3,4-dihydroxyphenyl)alanine.

Procedure and Apparatus for Paper Chromatography

One-dimensional paper chromatography was used as an aid in identifying the components of the peaks on the elution curves obtained from the column chromatography. The hydrochloric acid was removed from the tubes as previously described; the amino acid residue was dissolved in 0.2 ml. of 10% isopropyl alcohol and applied to Whatman No. 1 filter paper with small copper wire loops.

The technique of Block (*4*), which combines both ascending and descending chromatography, was employed. At least four different solvent systems were utilized to produce R_f values for identification purposes.

1. 70 volumes of *n*-butyl alcohol and 30 volumes of propionic acid. This mixture was equilibrated against 100 volumes of water immediately before use. Previous reports describing this system have merely mentioned water equilibration, without specifying the ratio. Nevertheless, changes in R_f values were observed when different amounts of water were used.
2. 55 volumes of 2,6-lutidine, 25 volumes of 95% ethyl alcohol, and 20 volumes of water. 2 ml. of diethylamine were added to every 100 ml. of this mixture.
3. 60 volumes of methyl ethyl ketone, 20 volumes of propionic acid, and 20 volumes of water. This is essentially the same solvent system as the one introduced by Clayton and Strong (*6*).
4. 60 volumes of 2-methoxyethanol, 20 volumes of propionic acid, and 20 volumes of water. This is a new water-miscible (noncquilibrium) solvent system which gave good resolution and reproducibility, and was convenient and pleasant to use.

Generally the chromatograms were developed with ninhydrin but both ninhydrin and isatin were employed as sprays on separate chromatograms in cases where additional evidence could be obtained by definite color production (*3, 10*).

The R_f values of the amino acids in the effluent from the column were compared with values of known amino acids with at least two different solvent systems. Once the identity of an amino acid was established securely both from its position on the ion exchange column elution curve and from its R_f value with paper chromatograms, further evidence of its identity could be obtained by chromatographing a mixture of the known amino acid plus the amino acid from the column. This latter procedure was followed whenever there was any doubt as to identity, and for any amino acid which did not have its position on a standard elution curve previously reported in the literature.

Fifty-one amino acids, peptides, and related compounds were chromatographed on paper with the four different solvent systems listed, in an effort to obtain a selection of different R_f values to aid in the identification of unknown compounds present in acid-hydrolyzed meat.

Results and Discussion

The colors produced for both the ninhydrin and the isatin sprays are recorded, along with the R_f values, for all the compounds chromatographed on paper (Tables II and III).

The authors have corroborated published standard hydrochloric acid elution curves for several amino acids (*11, 13,*

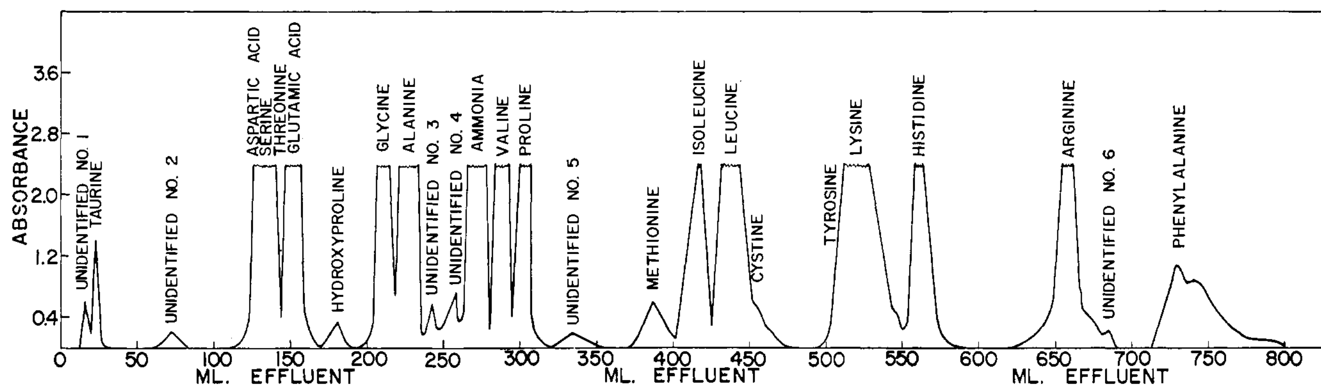

Figure 1. Separation of amino acids

From a 10-hour hydrolyzate of beef round on a column of Dowex 50 operated in the H cycle at room temperature. A system for gradient elution was employed which gave a continuously increasing concentration of hydrochloric acid. Sample contained 6 mg. of nitrogen. Unidentified No. 3 produced β-alanine after 25-hour hydrolysis.

Table III. R_f Values[a] of Peptides and Related Compounds with Various Solvent Systems and Colors with Ninhydrin and Isatin Sprays

	Solvent Systems				Color	
Compounds	*Butanol*	*Lutidine*	*Methyl ethyl ketone*	*2-Methoxy-ethanol*	*Isatin*	*Ninhydrin*
β-Alanine	0.33	0.22	0.32	0.68	Blue-gray	Blue
Asparagine	0.18	0.07	0.20	0.46	Gray	Mauve
Ethanolamine	0.47	0.54	0.45	0.71	Pink	Purple
Glutamine	0.20	0.16	0.18	0.45	Pink	Purple
Glutathione	0.06	0.00	0.06	0.31	Pink	Purple
Glycylglycine	0.17	0.21	0.24	0.55	Pink	Yellow
Glycylglycylglycine	0.14	0.15	0.22	0.57	...	Yellow
Glycyl-L-leucine	0.65	0.51	0.56	0.78	...	Yellow
Glycyl-L-tryptophan	0.52	0.54	0.51	0.74	...	Yellow
Glycyl-L-tyrosine	0.38	0.48	0.38	0.73	...	Yellow
Hydroxyproline	0.20	0.28	0.20	0.53	Blue	Yellow
L-Leucylglycine	0.61	0.53	0.53	0.81	...	Purple
L-Leucylglycylglycine	0.53	0.49	0.45	0.84	...	Purple
L-Leucyl-L-tyrosine	0.72	0.73	0.62	0.87	...	Yellow
Proline	0.37	0.36	0.31	0.64	Blue	Yellow
Sarcosine	0.25	0.36	0.24	0.56	...	Purple
Taurine	0.14	0.42	0.15	0.51	...	Purple

[a] Average of at least three values with Whatman No. 1 filter paper at approximately 30° C.

14); however, no available record showed standard elution curves for several of the more "uncommon" amino acids. The constant position of each amino acid on the elution curves provided a valuable means of preliminary identification.

On analyzing a portion (1 mg. of nitrogen) of a 10-hour hydrolyzate of beef round on a chromatographic column it was found that all the 18 common amino acids (Table I) except cystine and tryptophan could be identified by paper chromatography. Good separation was obtained for all 16 except serine and threonine, which were not separated from aspartic acid. Serine and tyrosine were partly destroyed under the above hydrolysis conditions. Most of the methionine was lost by oxidation to methionine sulfoxide during the eluting process. A peak for ammonia was also present.

The column used in the foregoing analysis was operated below capacity, so maximum resolution for that particular resin was attained. A few unidentified spots showed up on the paper chromatograms, some of which corresponded to minute peaks barely extending beyond the base line of the elution curve. In order to obtain more well defined peaks for these unidentified compounds, and produce more substance for identification purposes, the column resin was overloaded with hydrolyzate (6 mg. of nitrogen), which resulted in some sacrifice of resolution. At least eight ninhydrin-positive unknown spots, which corresponded with peaks on the elution curve (Figure 1), appeared on the paper chromatograms. Other unknown spots appeared which were obscured by known peaks. It was assumed that some of these spots represented less common amino acids, hydrolytic fragments, and peptides.

One peak appearing on the elution

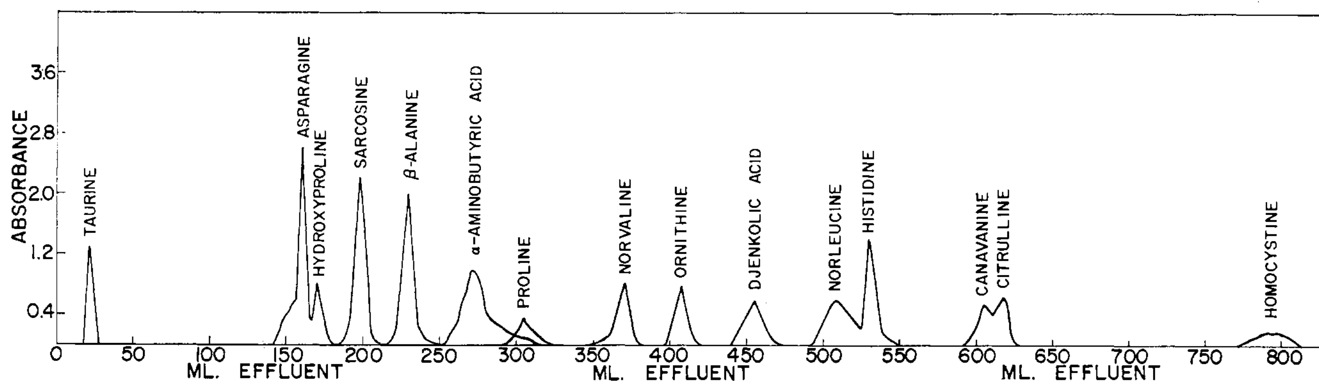

Figure 2. Separation of amino acids and related compounds

From a synthetic mixture on a column of Dowex 50 operated in the H cycle at room temperature. A system for gradient elution was employed which gave a continuously increasing concentration of hydrochloric acid. Sample contained 0.3 mg. of each component.

curve (Figure 1) between glutamic acid and glycine was identified as hydroxyproline, an amino acid previously reported as absent from animal muscle (*8*). The orange-brown color produced by a solution of ninhydrin and hydroxyproline, resulting in high light absorption at 440 mμ, as well as a distinct blue color formed with isatin on paper, greatly facilitated positive identification. As hydroxyproline is known to be a major constituent of the fibrous tissue collagen, it is possible that the animal muscle analyzed by the columns might have contained traces of collagen, as might meat sold for human consumption. The meat protein assayed contained 0.7% hydroxyproline calculated to 16% nitrogen. Hydroxyproline was also discovered in a hydrolyzate of pork ham.

With the overloaded column a peak appeared on the elution curve when approximately 25 ml. of effluent had passed through (Figure 1). A corresponding peak representing taurine was found on a standard elution curve for more uncommon amino acids (Figure 2). Paper chromatography also identified this unknown peak as taurine. The existence of this sulfonic amino acid in animal muscle might be explained by the fact that it is a constituent of mammalian bile, and is readily formed by decarboxylation of cysteic acid, an oxidation product of cysteine. This reaction is known to take place in the liver, catalyzed by a specific decarboxylase.

The beef protein assayed contained 0.4% taurine calculated to 16% nitrogen. Taurine was found also in a pork ham hydrolyzate as 0.5% of the crude protein.

A third unknown peak on the elution curve produced from the overloaded columns for 10-hour hydrolyzates, which came just after the peak for alanine and corresponded to the position for β-alanine, did not give R_f values on paper which corresponded to β-alanine. However, after 25-hour hydrolysis a corresponding peak on the elution curve was conclusively identified as β-alanine. It has been assumed that this peak after 10-hour hydrolysis still represented a peptide of β-alanine, which, although giving different R_f values from β-alanine on paper, produced a corresponding elution peak from the column. The R_f of this "peptide" did not correspond to that of carnosine (β-alanyl-L-histidine) on paper. Anserine (β-alanyl-1-methyl-L-histidine) was not available for comparison. This same peptide of β-alanine was found in a 10-hour hydrolyzate of pork ham.

The 25-hour hydrolyzate of beef protein assayed contained 0.4% of β-alanine calculated to 16% nitrogen. This is many times the quantity of β-alanine which can be accounted for from the pantothenic acid in the meat. On the other hand, both carnosine and anserine are naturally occurring peptides which might have contributed to this significant amount of β-alanine. In microorganisms (*Rhizobium leguminosarum*) there is an amino acid decarboxylase which produces β-alanine from L-aspartic acid (*7*).

Table IV. R_f Values[a] of Unidentified Ninhydrin-Positive Nitrogenous Components[b] of Meat[c]

Unidentified Component No.	R_f Value
1	0.08
2	0.22
3	0.38
4	0.19
5	0.21
6	0.52

[a] *n*-Butyl alcohol–propionic acid solvent system.
[b] cf. Figure 1.
[c] Beef and pork muscle protein after 10-hour hydrolysis with 3*N* HCl at 121° C.

Other peaks on the elution curve (Figure 1) were not identified. These are listed in Table IV along with their respective R_f values on paper with the *n*-butyl alcohol–propionic acid solvent system. Elution curves for different hydrolysis times of 2, 5, 10, and 25 hours were studied. As was expected, more unknown components appeared at the shorter times of hydrolysis. A few transient "unknown spots" appeared at different times, either in insufficient quantities to produce a significant peak on the elution curve or mixed with other amino acids in such small amounts that only a faint spot was perceptible with the ninhydrin spray. Most of these produced a faint yellow color with ninhydrin on paper, which suggested that they were peptides. As these disappeared with longer hydrolysis, no doubt many of them were artifacts from fragments and peptides. The 25-hour hydrolysis was expected virtually to eliminate the possible presence of peptides. Nevertheless, the possibility must not be overlooked that some of these unidentified components may have been actual nitrogenous compounds which were unstable with prolonged acid hydrolysis.

Summary

Approximately 100% of the nitrogen contained in meat has been accounted for by 21 amino acids, ammonia, and other nitrogenous constituents such as vitamins, purines, creatine (and creatinine), carnitine, and methylguanidine.

By the use of ion exchange and paper chromatography, three of the amino acids—hydroxyproline, taurine, and β-alanine—have been shown to exist in the crude protein of beef muscle with values of 0.7, 0.4, and 0.4%, respectively. This is many times the quantity of β-alanine which can be accounted for from the pantothenic acid content of meat. Pork muscle protein has also been found to contain 0.6% hydroxyproline and 0.4% taurine. Hydroxyproline was previously reported as being absent from animal muscle, and no report was found concerning taurine in meat.

Other ninhydrin-positive nitrogenous compounds present in very small quantities in the crude protein of meat have been isolated by chromatography, but remain to be identified.

For eluting the amino acids from the column, a new system of gradient elution gave a continuously increasing concentration of hydrochloric acid, rather than the laborious stepwise increase of other investigators. This resulted in a saving of time and greatly improved operating conditions.

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