THE CHEMISTRY OF THE FLAVOUR PRECURSORS
OF COOKED BEEF

Andrew C. Sharpe

Being a thesis submitted for the degree of Doctor of Philosophy in the University of Surrey

Academic Supervisor: Professor J. Elvidge
Industrial Supervisor: Dr. T. A. Rohan

Research Laboratories
Bush Boake Allen Ltd.

May 1973
SUMMARY

A critical analysis has been made of raw beef to establish the nature of the components which participate in the thermal reactions resulting in cooked beef odour.

Extraction of beef with water or aqueous alcohol gave an extract which, when heated, produced a cooked beef odour whereas the extracted residue did not.

The water soluble material was fractionated into broad groups of constituents by dialysis, gel filtration and ion exchange chromatography, and the various fractions assessed for their cooked beef odour potential.

The more interesting fractions were analysed qualitatively by thin layer chromatography and quantitatively by ion exchange chromatography, colourimetry, U.V. spectrometry, and gas-liquid chromatography.

The water soluble high molecular weight fraction alone did not give cooked beef odour when heated whereas the water soluble low molecular weight material did. Furthermore the components responsible for this aroma were widely distributed in the fractions obtained by gel filtration.

The application of the results to experiments with model systems showed that the precursors of cooked beef odour should include a source of hydrogen sulphide as well as the relevant amino acids and sugars.

The relative weakness of cooked beef odour given by the water soluble material and its fractions, as compared with whole beef, may be related to the virtual absence of the hydrogen sulphide precursor.

As a result of this observation and the evidence obtained from a study of model systems it is felt that sulphur may be provided largely by the meat protein.
INTRODUCTION

The flavour and aroma which characterise Roast Beef are not present in the raw flesh but are produced as a result of cooking action. In consequence it might be assumed that raw meat contains certain components which undergo thermal degradation or reaction, during the cooking process, to give the characteristic flavour. This thesis describes an examination of beef for these components, styled flavour precursors, which should be able, when heated in model systems, to give Roast Beef aromas.

This concept has been applied to several other foodstuffs, notably chocolate, peanuts and bread. The compounds (precursors) which have been identified as contributing to the odour producing reaction of these foodstuffs include sugars and amino acids. Chocolate aroma can be produced by heating valine and glucose (Rohan and Stewart 1968) and peanut aroma by a mixture of aspartic and glutamic acids, glutamine, asparagine, histidine, phenylalanine and a reducing sugar (Newell et al. 1967).

The mechanism by which characteristic aromas are produced from sugar-amino acid mixtures is not fully understood although one aspect, the degradation of the amino acid to an aldehyde, Strecker degradation, has been examined thoroughly. It has been shown that amino acids can be degraded in the presence of any compound of the type:

\[
\begin{align*}
\text{O} & \quad \text{I} \\
\text{-C-C=C-} & \quad \text{n} \\
\text{O} & \quad \text{O}
\end{align*}
\]

\[n = 0, 1, 2, 3...\]

and that the reaction sequence is as follows (Schonberg et al. 1948)
From the knowledge that a reducing sugar can act as a reductone when heated with an amino acid it has been assumed that (during heating) the sugar is degraded to a reductone. This has led to mechanisms being proposed (e.g., Hodge 1967) in which the sugar first condenses with the amino group, then loses water to form a reductone-amino acid compound which then decomposes to yield the aldehyde. Various mechanisms are then proposed to account for the products, such as ketones, other aldehydes and pyrazines. However, only one intermediate has been isolated (Anct and Reynolds 1957), the rearranged sugar-amino acid condensate, an Amadori compound, such as that formed from glucose and glycine, shown below.
The search for the precursors of pyrogenic flavour has been intensified during the past ten years although previously, in studies of the flavour of cooked meat, the possibility that flavours may be produced during cooking was recognised. In a review of work initiated by the United States Department of Agriculture (Howe and Barbella 1937) it was stated that meat flavour consists of the stimuli given to the taste buds by the inherent organic and inorganic substances, such as water soluble extractives, lipids, small amounts of carbohydrates and salts, or compounds produced from these products and proteins by cooking, by enzyme action or by both. This view is now known to be incomplete because the flavour is a combination of the stimuli given to the taste buds, by non volatiles, and the stimuli given to the olfactory epithelium in the nose by volatile compounds.

Beyond these general facts, Howe and Barbella said nothing of the nature of the chemical compounds formed during heating and nothing of the specific compounds or reactions which produce them.

In the first study devoted to cooked meat aromas it was reported that these arise as a result of reactions associated with the fibres rather than the extractable water soluble material (Crocker 1942).

Barylko - Pikielna (1960) was unable to confirm Crocker's observation and found, on the contrary, that the aroma was evolved by reactions associated with water soluble constituents, a conclusion which has subsequently received adequate support.

The identification of meat flavour precursors may be effected either by fractionation of the water soluble fraction and testing the various fractions for flavour potential or by quantitative analysis of the constituents of the water soluble fraction before and after heating.
The latter method has indicated that large amounts of taurine, anserine, carnosine, alanine, glutamic acid, glycine, lysine, serine, cystine, methionine, leucine, isoleucine and methyl histidine were lost and that of the sugars present the most labile was ribose and the least was glucose. (Mabrouk et al. 1964b). However there is no indication given as to which of these compounds give rise to meaty odours.

The first attempt at the elucidation of meat (Beef) aroma precursors by fractionation (Batzor et al., 1960) employed dialysis of the aqueous extract followed by gel filtration and ion exchange separation. The results indicated that the precursors were of low molecular weight and possibly included peptides, carbohydrates and phosphate. In a subsequent paper Batzer and his colleagues (1962) have described the precursor as a glycoprotein. Wasserman and Gray (1965), using much the same methods as those described by Batzer (1960) concluded that the precursors were contained in a fraction which was absorbed by a cation exchange resin and so did not contain free carbohydrate.

Subsequently Wasserman and his colleagues (Zaika et al., 1968) found that the fraction containing the precursors comprised a mixture of sugars and sugar phosphates, amino acids (excluding tyrosine, phenylalanine, taurine and glutamic acid), creatine, creatinine, and the purine derivatives hypoxanthine, inosine and inosinic acid.

The most recent attempt at the identification of beef flavour precursors (Mabrouk et al. 1969) involved similar separation procedures as before, (dialysis and gel filtration), and provided some evidence that sulphur containing amino acids are involved.

A number of attempts has been made to produce flavour reaction mixtures, based on the results of precursor analysis, but with no outstanding success.
However, meaty aromas have been obtained by heating mixtures of compounds. These compounds have usually been of natural origin but have not necessarily occurred in meat. Most of these mixtures are to be found in the patent literature and so there is usually no description of the theoretical considerations which led to the choice of reactants.

**PATENT LITERATURE**

Most of the early patents, describing mixtures of chemicals said to give meaty aromas on heating, were based on cysteine or peptides containing cysteine reacted either with a sugar or a compound which could be produced by the heat degradation of a sugar, e.g. Furans. Typical of these patents was that granted to Unilever (1960) which describes a mixture of cysteine and a monosaccharide, preferably ribose, able to give a meaty aroma when heated in solution under acid conditions.

Other natural sulphur compounds have been claimed to give meaty aromas when heated in the absence of sugars. These mixtures almost invariably contain thiamine, amino acids and cysteine, typified by a patent granted to International Flavours and Fragrances Inc. (1967) in which a sulphur compound (3-acetyl-3-mercaptanol) was heated in the presence of cysteine, thiamine and organic acids.

Finally claims have been made for mixtures which contain no sulphur compounds, and in this context Ajinomoto (1970), have used as reactants a pentose and an amino acid chosen from glutamic acid, aspartic acid, alanine, glycine and proline.

**VOLATILE CONSTITUENTS OF COOKED BEEF AND THEIR PRECURSORS**

The compounds found in cooked beef extracts which are described as having the characteristic aromas are:

4 - hydroxy - 2, 5 - dimethyl - 2, 3 - dihydrofuran - 3 - one. (A) and
4 - hydroxy - 5 - methyl - 2, 3 - dihydrofuran - 3 - one (B) (Tonsbeek et al. 1968) and 2, 4, 5, - trimethyl - 3 - oxazoline (E) and
3, 5 - dimethyl - 1, 2, 4 - trithiolane (D) (Chang et al. 1968)
The Furanone (A) is probably a sugar degradation product, and, as it contains six carbon atoms, is derived from fructose or glucose. Examination of the formulae indicates that the hexose (C\textsubscript{6}H\textsubscript{12}O\textsubscript{6}) would have to lose four hydrogen and three oxygen atoms to form the furan (C\textsubscript{6}H\textsubscript{5}O\textsubscript{3}). Thus, the reaction would have to involve two dehydration and one reduction stages, and such a degradation could be provided by a typical Maillard sugar-amino reaction, as proposed by Hodge (1967), preceded by the oxidative deamination of an amino acid (Strecker degradation) leading to the reduction of the sugar.
The second furanone (B) is most probably a degradation product of ribose. As the difference between the formulae of the furanone and ribose can be accounted for by the loss of two molecules of water from ribose the most probably pathway is that involving the amine catalysed dehydration of ribose.
The trithiolane (D) has been prepared by the reaction of hydrogen sulphide, acetaldehyde and sulphur in the presence of diisobutylamine (Assinger et al. 1959). Acetaldehyde is the Strecker degradation product of alanine, and hydrogen sulphide is evolved by thiols present in meat (Necchi et al. 1964). Thus the trithiolane may be formed by the complex interaction of sulphur compounds, sugars and alanine.

The oxazoline (C) has been prepared by the reaction of acetaldehyde and aceticin with ammonia (Jassmann and Schultz 1963). However, it is possible that the oxazoline could be formed in meat by the interaction of a sugar (fructose) with an amino acid (alanine).
An attempt has been made to characterise the precursors of one of the volatile compounds already described, 4-hydroxy-5-methyl-2,3-dihydrofuran-one (Tonsboek et al. 1969).

Proteins were removed from an aqueous extract of raw beef by precipitation with ethanol and the protein free extract was separated into basic compounds, and acidic and neutral compounds by cation exchange chromatography. Only the acidic/neutral fraction
produced the furanone on boiling and there was no improvement in yield when the combined fractions were heated. The acidic neutral fraction was resolved into acidic and neutral compounds by precipitation of the acids as their barium salts. The acids were recovered from their salts using a cation exchange resin. The neutral fraction was fractionated by precipitation with ethanol and the precipitate and supernatant liquid were treated in the same way as the barium precipitate. Evaluation of samples for the ability to produce the furanone on heating showed that the acids yielded a low amount and that the ethanol precipitate yielded a moderate amount. The ethanol supernatant yielded none.

Analysis of these fractions indicated that the precursor was ribose phosphate and that pyrrolidone carboxylic acid, the lactam of glutamic acid, and taurine catalyse the reaction.
EXPERIMENTAL

Methods of Aroma Evaluation

Heating on aluminium caps: A small sample of the mixed reagents (about 10mg) was heated either wet or dry in aluminium caps (1 inch diameter) on heating block (165°). The aroma was evaluated by smelling the vapours as they were evolved.

Heating in a modified Thunberg tube: The sample to be assessed (about 2ml) was placed in limb of a modified Thunberg tube (fig 1). This was then inserted in the cone of the U tube (B) so that the hole in the cone was aligned with the side arm on the socket. The contents were frozen by immersing the tube in a freezing mixture (solid carbon dioxide/acetone) and the system was evacuated (0.05 torr) by connecting the side arm to a vacuum pump, and sealed by rotating the cone through 90°. The contents of tube A were heated by immersion in a heating block (165°). Simultaneously the end of the U tube was immersed in freezing mixture (solid carbon dioxide/acetone). After the contents were heated to dryness, and browning, but not charring, had occurred the system was opened and the odour of the contents evaluated at room temperature and at 80°.

Boiling under reflux: Solutions (ca 5ml) of the samples to be assessed were boiled, under reflux, for two hours either in round bottomed flasks in isomantles or in tubes in the holes of a heating block (165°). The reaction products were evaporated to dryness and their odours evaluated by flavour profile analysis.

Heating in sealed ampoules: Solutions (ca 2ml) of the samples to be assessed were sealed in ampoules (10ml capacity) and heated in an oven (110° for 2 hr.). At the end of the heating period the ampoules were opened and the odours of the products evaluated. Some of the samples were also evaporated to dryness before evaluation.
Cooking in texturised vegetable protein: Pieces of texturised vegetable protein (T.V.P.) were soaked in an aqueous solution of beef extract and then cooked in a small glass crystallising dish (3 in. diameter) containing a little oil (1 in. deep). The flavour was evaluated by eating.

Fractionation of Water-Soluble Beef Fraction

Aqueous extraction: Topsides of beef (2 kg.) was dissected into fatty and non-fatty tissue. The non-fatty tissue was minced in a domestic mincer and then stirred with an equal weight of water (1700 ml.) The slurry was filtered, the residual solid was re-extracted twice with the same quantity of water, and the combined filtrates were freeze-dried. (Model E.F.I., Edwards, Crawley). The residual solid was redissolved in water (650 ml.) and the solution was centrifuged (10000 rpm, 25000 g., for 1 hr. in an HS 25 centrifuge, MSE Crawley).

An aliquot of the supernatant liquor was freeze-dried and the solid obtained was weighed.

The remaining supernatant liquor was concentrated (125 ml.) by partial freeze-drying and the concentrate was re-centrifuged to give a clear solution. This concentrate was used for gel permeation chromatography.

Dialysis

A freeze-dried aqueous extract of beef (6.9g) was dissolved in distilled water (100 ml). The solution contained in a dialysis bag (1 in. diameter) of cellulose acetate (visking), was dialysed against distilled water (1l) for one day. The water was replaced by the same quantity of fresh water and after a further day's dialysis the two diffusates were combined and freeze-dried to give a pale yellow powder (fraction D) and weighed.

/13....
**Methanolic extraction**: Topside of Beef (1 Kg.) was minced in a domestic mincer and blended with methanol (2.5 l) in a household blender (Sunbeam, model G28 2). The resultant slurry was filtered under reduced pressure and the residue was re-extracted with aqueous methanol (20% water). The filtrate from this second extract was combined with that from the first and concentrated on a rotary evaporator (Buchi) at a low temperature (ca 40°C) and reduced pressure (0.1 torr) until crystals were seen in the solution. This suspension was then extracted six times with its own volume of chloroform to give a clear yellow aqueous solution (Fraction M, Fig. 2). This solution was used in gel permeation chromatography.

**Gel permeation chromatography**: Sephadex G-10 (Pharmacia, Sweden) was stirred occasionally in distilled water for several hours, and then packed into glass columns (45cm. x 5cm. and 100 cm x 2.5cm., Pharmacia) in the method recommended by the manufacturer.

The volumes applied to the columns were 10 ml. (short column) and 2 ml. (long column). Distilled water was used as eluant with the protein-free extracts obtained by dialysis and methanolic extraction, respectively. Samples containing protein were eluted with sodium chloride solution (0.9%) from columns equilibrated with the same solution.

**Cation exchange**: A column (45x2.5cm. Pharmacia) was filled with a sulphonated cross-linked polystyrene cation exchange resin (Amberlite IR 120, 14 mesh), regenerated with 2N sulphuric acid, and washed free of acid with distilled water. The eluate from the column was passed through a pump and UV absorption cell to a fraction collector.

Beef extract (about 5gn. in 10 ml. water) was applied to the column and eluted with distilled water (1ml./min.) until the UV
absorption (234nm) of the eluate fell to near zero. The water was then replaced by ammonia solution (2N) when the absorption increased to a maximum and again dropped to zero, after which the column was regenerated by acid as described above.

**Anion exchange:** A glass column (45 x 2.5 cm. Pharmacia) was filled with a slurry of an anion exchange resin, the quaternary ammonium derivative of cross-linked polystyrene, (Amberlite IRA). The resin was regenerated with 2N sodium hydroxide then washed free of residual alkali with distilled water. The eluate from this column was also passed through a pump and a UV absorption cell to a fraction collector.

The samples for separation, material eluted from the cation exchange column by water (acids/neutral material) and by ammonia (bases and amphoteric compounds), were eluted by water (neutral and basic material respectively) and then by acetic acid (2N) (acids and amphoteric material respectively).

The column was then regenerated as described above.

**Crystallisation:** On concentrating the aqueous solution obtained by methanolic extraction, a white crystalline solid was precipitated which was recrystallised from hot water twice and dried in a vacuum desiccator over solid sodium hydroxide pellets.

**Qualitative Analysis**

**Amino acids:** Samples (0.2ml of purified beef extracts or 0.1ml of a solution containing 1% each of authentic amino acids) were chromatographed on commercially prepared cellulose powder plates supported either on aluminium (D.C. Alufolien Cellulose, Merck) or plastic (M.H.Col 300, Machery and Nagel).

Irrigating solvents used were n-butanol-acetone-ammonia-water (10:10:5:2) and isopropanol-formic acid-water (20:1:5). When two dimensional chromatograms were being prepared the basic solvent was used first.
In addition, a third solvent, n-butanol-pyridine-water (1:1:1) was used in place of n-butanol-acetone-ammonia-water for the separation of glutathione from glutamic acid. Detection of amino acids was by ninhydrin (0.2%) in ethanol containing pyridine (2%).

**Sugars:** Sugars, e.g., in 0.5 µl samples from gel permeation chromatography, were chromatographed on the same cellulose plates as the amino acids. The irrigating solvents used were n-butanol-\(\text{H}_2\text{COO}\)-water (60:15:25), isopropanol-formic acid-water (20:1:5) and n-butanol-acetone-ammonia-water (10:10:5:2). Aldoses were revealed by spraying with aniline-phosphate reagent (aniline: phosphoric acid:acetone:water:acetic acid=2:1:60:20:18 v/v) followed by heating at 100°C. Ketoses were revealed by spraying with naphthoresorcinol-acetone-water-phosphoric acid (2:1000:19:200) followed by heating at 95°C in an oven containing a tray of water.

**Guanidines:** Guanidines (0.1 µl samples of concentrated beef extracts) were chromatographed in the same way as the amino acids but were revealed by spraying with diacetyl reagent (diacetyl: 1-naphthol:water:sodium hydroxide:ethanol=0.1:1:8:100:500), and then heating for 5 min.

**Acids:** Samples, e.g., 0.5 µl of the eluate from gel permeation chromatography of methanolic beef extract, were applied to thin layer plates coated with cellulose powder. They were irrigated with isopropanol-formic acid-water (20:1:5) and were dried in a current of cold air. Acids were detected by spraying with aniline-xylene reagent (xylene:aniline:water:methanol=1:1:3:95), followed by heating at 110°C. Acids were also revealed by bromocresol blue. A solution of bromocresol blue (0.1%) in methanol, acidified until green, was diluted (4x) with acetone and the plates quickly dipped in this and blotted dry.
Nucleotides and derivatives: Nucleotides c.e. in 0.1 ml samples of the material eluted by ammonia from the cation exchange column were analysed by chromatography on prepared silica gel plates with fluorescent backing (D.C. Alufolien Keiselgel F254, Merck) using butanol saturated with water as the irrigating solvent. Cellulose powder plates (MN Polygram Cel 300, UV 254, Machery and Nagel) were also employed and irrigated with isopropanol-formic acid-water (20:1:5 v/v). The nucleotides were seen, under UV light, as dark areas against the fluorescent background.

Imidazole: Before spraying with ninhydrin reagent, amine acid chromatograms were dipped in a solution of iodine (1%) in carbon tetrachloride. Imidazoles were shown as brown coloured regions on a white background, after the iodine had sublimed from the plates.

Phosphates: Analysis for phosphate as in the acidic fraction from ion exchange chromatography (0.2 μl) employed chromatography on cellulose powder plates (MN Cellulose Cel 300, Machery and Nagel) with isopropanol-formic acid-water solvent. Treatment of the plates with polyblic acid reagent (ammonium polyblicate:water:12N-perchloric acid:12N-hydrochloric acid:acetone=1:3:3:3:36) followed by exposure to UV radiation for 30 min., revealed phosphate as a blue coloured zone.

Anthrone test: Anthrone (0.1g), dissolved in concentrated sulphuric acid (10 ml.), is poured carefully down the side of the tube containing the solution to be tested. Sugars are revealed by this test as a blue-green colour at the interface of the two solutions.
Quantitative analysis

Succers: A freeze-dried methanolic extract of fresh beef was dissolved in water (10ml) and deionised by ion exchange on columns of cation and anion exchange resins (amberlite IRA20, hydrogen form, and IRA45, hydroxyl form respectively). The eluate from the columns was analysed for carbohydrates, by anthrone reagent, and that containing carbohydrate was combined and freeze-dried in a tared, plastic-stoppered bottle. This freeze-dried hydroscopic solid (carbohydrate) was treated with hexamethyl disilazane (1.8ml.) and trifluoro-acetic acid (0.2ml.) in the presence of pyridine (2.0ml.) to prepare the trimethyl silyl ethers (Brown and Lott 1966).

An aliquot of the solution (1ml) was injected into a gas chromatogram (type 104/65) and chromatographed on a column (5ft x ½in.) of methyl silicone gum (SE30, 2%) supported on silanised diatomite (100/120 mesh). The flow rate of the eluting gas, nitrogen, was 60ml./min. and detection was by flame ionisation.

Amino Acids: Fractions of the methanolic extract (M3, M4 and M5) (Fig. 2) were diluted (1ml. to 50ml.) and analysed for total amino acids using the method of Moore and Stein (1954) Alanine (1mM) was used as a standard. As the third fraction (M5) had a very low value so its determination was repeated using a lower dilution (1ml. to 5ml.). The fractions were then concentrated (M3 and M4 10 to 1 and M5 by 30 to 1), so that the final concentrations of amino acids were of the order of 5 µ moles/ml. A fourth sample was prepared by diluting an aliquot of one of the concentrated samples (M4) with an equal amount of concentrated hydrochloric acid (12N) and hydrolysed at 105° for 18 hr. in an ampoule. The mineral acid was largely removed by evaporation.

All samples were acidified to pH 3, and analysed for amino acids by ion exchange chromatography using the method of Hamilton (Hamilton 1953) modified by temperature programming, from 40° to 60° at 3/2°/hr.
Creatine: Solutions of creatine in water (0.4g./L.), methenolic extract (7.64g./L.), diacetyl (1ml./L) and naphthalene-1-ol (10g./L.) in sodium hydroxide solution (80g./L.) were prepared in the ratios shown (table 1). The absorbances of the solutions (max 523nm.) were determined after 30 minutes using a spectrophotometer (Unicam SP800). The creatine content of the beef extract was found using a standard graph prepared from the authentic creatine samples.

Flavour Profile Analysis

The evaluation of products in the food industry is becoming increasingly refined as the standardisation of mass produced articles assumes greater importance and as intensified efforts are being made to produce simulated foodstuffs.

In the past, quality control was generally left to the highly subjective assessment of one man whereas today it is made as objective as possible by the use of trained panels.

A method was evolved some twenty years ago (Cairncross and Sjostrom, 1950) which makes use of a panel to evaluate the quality of a foodstuff. Results are presented in the form of a tabulated list of the sensations experienced by the individual members of the panel as the food was being consumed, and are generally quantified. This tabulated list is known as the Flavour Profile.

The Flavour Profile technique has been slightly modified to suit the needs of basic research work on flavour, and eliminates the weakness of the former system whereby the chemist did his own evaluation during the course of his work.

In essence, a panel is trained in the recognition of the principal stimuli, and then used in the definition of the objectives; the panel is subsequently used by the research chemist when he requires organoleptic evaluation at critical stages in his work.
RESULTS AND DISCUSSION

Fractionation and Analysis

Aqueous extraction: The results of previous investigators suggest that the aroma precursors of roast beef are water soluble so the first step in any investigation is the separation of the water soluble components from the insolubles.

The method used is adequate for animal tissues because the cell walls are ruptured by immersion in a solution of low osmotic pressure.

After mixing the minced beef with water and filtering the resultant slurry, the extract was centrifuged to remove cellular debris not retained by the filter. Centrifugation after each concentration stage was necessary because some proteins are precipitated in strong salt solutions which are formed from the salt present in the meat, and it was essential to remove these in order to evaluate the role of the lower molecular weight constituents.

The aqueous extract yielded, on freeze-drying, a pink, slightly hygroscopic solid in 4.1% yield which could be stored, without deterioration, at 4°C for 2 years.

The freeze-dried extract was found to give a beef aroma when boiling and after the removal of the water, a roast beef aroma was obtained.

Exhaustive extraction of a piece of meat with cold water yielded a grey fibrous material which, when assessed in the same way as the extract, was found to give only a very weak aroma, wet or dry, and did not brown at normal cooking temperature. These results, whilst showing that the aroma of roast beef cannot be
obtained from the residue alone, do not prove that the residue, in the presence of the aqueous extract, contributes nothing to the total aroma.

**Gel filtration:** Once it had been shown that a roast beef aroma could be produced by heating the aqueous extract to dryness, a method of fractionating this extract was required. At first, gel filtration of the whole aqueous extract was used, although others who have used this technique have done so after dialysis. It was thought that the dialysis stage could be omitted because gel filtration can achieve the same result, which is the separation of large molecules from small molecules.

However, the first difficulty encountered was the precipitation, on the column, of some component of the extract. This precipitate was assumed to be protein, for not only are proteins precipitated by an increase in salt concentration, but also by a decrease. This identification of the cause of the problem was confirmed by its cure, that is the equilibration and the elution of the column by physiological saline, 0.9% sodium chloride.

The UV absorption trace of the eluate from the gel filtration of the aqueous extract in physiological saline (fig.3) shows several groups of compounds which absorb UV radiation and on this basis, the fractions were bulked as shown (fig.3).

Three of these fractions (W1, W2 and W3) gave a roast beef aroma when heated to dryness. However thin layer chromatography of these fractions (fig.4) showed that the first contained protein, and the second two, even after rechromatography on Sephadex, contained too much salt to make possible the identification of any of the components. Because of this difficulty, recourse was made to dialysis in order to separate the proteins from smaller components before gel filtration. This would make the use of saline solution unnecessary in subsequent gel filtration and would
also prevent possible contamination of the low molecular weight fraction by protein, which is a hazard arising from the high concentration of the protein.

**Dialysis:** A dialysis membrane can separate proteins and other macromolecules from smaller compounds, such as amino acids and sugars, by virtue of its structure which permits the preferential diffusion of the lower molecular weight solutes. Aroma assessment of the diffusate, (the material which passed through the membrane) of the beef extract showed that it retained most, but not all of the aroma producing compounds as evidenced by the weaker aroma produced on heating. This was thought to be due to retention of some of the precursors by protein precipitated during the change in salt concentration within the dialysis tubing.

The diffusate was chromatographed by gel filtration on Sephadex G10. This gel has the smallest pores available in the Sephadex range, and was selected because the range of compounds which the gel is able to fractionate is directly proportional to pore size. The assumption was made that the best separation of low molecular weight compounds would be achieved by using the gel with the smallest pores.

The UV absorption trace of the eluate (Fig. 5) shows that in the region previously occupied by the protein there are constituents of lower molecular weight which have been resolved. The two traces are otherwise very similar. Once again the fractions were combined according to the absorption trace (Fig. 6) and evaluated in the same way as the whole extract.

This evaluation showed that one of the fractions (D4) gave a good but incomplete aroma and that two others (D5 and D6) gave poorer and weaker aromas. The best of these aromas changed, during evaluation, from that of beef broth, through *strong* ammoniacal aroma, just before drying, to an aroma reminiscent not only of roast...
beef but also of fried liver. This fraction was rechromatographed
to remove any components of the adjacent fractions and it was found
that on assessment a much weaker aroma was obtained, probably
because of the partial removal of some precursors, but possibly by
the loss of total material. The former explanation is the more
probable for the combination of the fractions which gave the cooked
aromas gave an aroma better than that of any of the individual
fractions.

Amino acid analysis, by means of thin layer chromatography,
of the fraction which gave the best aroma (D4) was made difficult
by the presence of inorganic salts which caused streaking on the
plates.

Ion Exchange of Fraction D4: Ion exchange resins can be used
for desalting solutions containing amino acids because the amino
acids are more readily displaced from the resin than are stronger
cations. If, for example, the amino acids are in solution with
sodium chloride and the solution is passed down a column containing
particles of sulphonated polystyrene, in the acid form, the amino
acids and sodium ions will be absorbed and will displace protons.
These protons and the chloride ions will then be eluted as hydrochloric
acid. Once the acid has been washed from the column, by water, the
amino acids can be displaced by ammonia solution. The amino acids,
being weaker bases than ammonia, will be eluted largely before the
ammonia, and any protons displaced, being in the presence of
hydroxyl ions, will be eluted as water. The sodium ions, being
stronger bases than the ammonia, will be displaced from the resin
at a very slow rate. Therefore, amino acids will be obtained
uncontaminated by inorganic salts.

This technique was applied to the beef extracts and the
material eluted by ammonia, the basic fraction, was analysed by
T.L.C. for amino acids. This showed (Fig. 7) the presence of, in
approximate order of concentration: - alanine, glutamine, glutamic
aci, leucine and isoleucine, an unknown component possibly taurine, serine, histidine, glycine, threonine, aspartic acid, phenylalanine and methionine.

The original sample was also examined, by thin layer chromatography, for aldehydes, and only glucose was detected (Fig. 9).

Extraction with aqueous methanol: Aqueous extraction of beef was found to be time consuming, and wasteful of the extract because the water soluble proteins made filtration difficult. Relatively large volumes of water were required and had to be removed at a low temperature, and the protein present in the extract had to be removed by dialysis both of which are time consuming processes.

The ideal extraction method would therefore be one which did not extract the protein and which did not use large quantities of water. These requirements are met by aqueous solutions of organic solvents in which most proteins are insoluble.

Solvent extraction has been used before to obtain beef flavour precursors but the solvent used, acetone, gave inconsistent results, possibly because of interaction with amines. However, methanol is a more inert solvent and aqueous methanol (20% water) has been used to extract chocolate aroma precursors from cocoa beans (Rehan and Stewart, 1966).

Because of the successful application of this technique to cocoa precursors, methanol was used for the extraction of beef. Anhydrous methanol was used because beef contains a large proportion of water, sufficient to permit the resulting mixture to act as a solvent for amino acids, sugars and salts. After the removal of the methanol the resultant solution contained a brown-red suspension which was suspected to be a mixture of polar lipids. This was
soluble in aqueous methanol and in chloroform but insoluble in water. Chloroform was successfully employed in removal of the lipids. To prevent the formation of a water-chloroform emulsion, the aqueous solution had first to be concentrated, which resulted in the precipitation of a colourless, crystalline solid, identified as creatine from the I.R. spectrum (Fig.9) of a sample crystallised from warm water and by co-chromatography with an authentic sample on thin layer plates.

By using aqueous methanol the total time for the extraction of 1Kg. of beef was less than 1½ days, whereas to obtain an equivalent solution by aqueous extraction took more than two weeks. The yield of water soluble fractions obtained by freeze-drying the solution was 2.25%.

**Aroma Evaluation of Methanolic Extract:** An improved method was used for the evaluation of this material. Previously, a small aliquot of aqueous solution was heated on an aluminium dish on a hot plate, and the odours were noted as the sample boiled to dryness. Each sample could be evaluated by only one person at a time and the time required for the complete evaluation was inordinately long.

The new method was evolved as a result of other work in this laboratory in which it was found that a concentrated aqueous solution of a boiled beef extract had a roast beef aroma. Flavour profile analysis was used to assess the samples.

A boiled methanolic extract of raw beef (fraction H Fig.3) when concentrated gave a good roast beef aroma, although when submitted to a flavour profile panel, it could be seen that the score for many of the components was lower than that of whole roasted beef extract (table 2). This difference may be due to the presence of protein and fat in the whole beef, each of which might undergo thermal reaction.
Ion Exchange Fractionation of Methanolic Extract: Resolution into its compounds of a fraction of the beef by means of ion exchange chromatography had shown that the amino acids could be obtained free of material which interfered with thin layer chromatography. A similar analysis of the whole methanolic extract was then attempted; to determine the effect of such treatment on the precursors and to determine the role of the sugars.

Using a cation exchange column and a U.V. cell to monitor the eluant, the extract was separated into four fractions as shown on the monitor trace (Fig.10). The first two fractions (A1 and A2) were eluted by water, and were collected separately because, as can be seen from the trace, there was partial resolution. The third fraction (B1) was eluted with ammonia at a pH of 7-9, and did not therefore contain free ammonia. The fourth fraction (B2), eluted at a pH greater than 10, contained ammonia and possibly low concentrations of metallic ions.

It was not expected that any of these fractions alone would give, on heating, a roast beef aroma, for all previous references to precursor mixtures, for any cooked food, have contained both sugars and a basic material. Sugars, having no charge, are separated from amino acids by ion exchange. The first fraction (A1), which should contain the sugars, gave only acidic vapour on heating. The second (A2), of then unknown composition, gave a weak aroma, reminiscent of commercial beef extract (e.g. Bovril), whereas the third and fourth fractions (B1 and B2) gave a reasonable beef aroma. This meaty aroma could be improved by the addition of glucose before heating, but was not as good as the aroma of whole extract, as shown by flavour profile analysis (table 3 and 2).

Because the fraction (B1) gave a reasonable aroma and was eluted free of ammonia it was studied in some detail.
Thin Layer Chromatography of Fraction (B1)

Thin layer chromatography (Fig.11) showed the presence of seventeen amino acids: leucine, iso leucine, phenylalanine, threonine, valine, methionine, tyrosine, proline, alanine, serine, lysine, histidine, glycine, asparagine, glutamine, glutamic acid and aspartic acid. There were also three small ninhydrin positive spots (1, 2 and 3) seen on the chromatogram near the origin, and a large area (4) which gave a brown colour with ninhydrin.

The use of a multiple detection system (Fig.12), U.V. light, followed by iodine dip, then ninhydrin spray and finally diacetyl-naphthol spray, showed that the ninhydrin brown region gave a positive reaction with the iodine reagent and was tentatively identified as an imidazole.

Two imidazolyl peptides, anserine and carnosine, have Rf values similar to the suspected imidazole in the chromatogram and each is known to occur in animal muscle. Confirmation of the presence of these peptides was given by thin layer chromatography of a hydrolysed sample of the fraction (B1) (Fig.12 to 13), which showed that the brown component had been destroyed and that a large histidine spot could be seen. β-Alanine and methylhistidine were also found in the hydrolysate. These amino acids are the hydrolysis products of carnosine (β-alanylhistidine) and anserine (β-alanyl-3-methylhistidine).
The thin layer chromatogram of fraction (B1) (see above) also showed the presence of a streak which was U.V. and diacetyl positive and another streak which was only diacetyl positive. Those were probably creatine and creatinine, which streak because of the large amounts present. Two components which were only U.V. positive, had Rf values similar to those of hypoxanthine and inosine.
The hydrolysed material also showed the presence of a component with similar R_f values to cystine or cysteine which could not be seen in the original and so was presumably the result of the hydrolysis of peptides or protein. The three previously mentioned components found near the origin of the chromatogram were presumed, because of their low R_f values, to be peptides.

The R_f value of a peptide is given by the relationship:

\[ RT \ln \left( \frac{1}{R_f - 1} \right)_p = (n-1)A + B + RT \ln \left( \frac{1}{R_f - 1} \right)_\infty \]

where \( R \) is the molar gas constant, \( T \) is the absolute temperature, \( n \) is the number of amino acids in the peptide and \( A \) and \( B \) are constants (Pardee 1951); the subscripts \( p \) and \( \infty \) refer to the peptide and the amino acids in the peptide respectively. \( A \) is invariably a negative quantity and \( B \) is always numerically smaller than \( A \) so \((n-1)A + B\) is a negative quantity. Examination of the equation will show that the larger the number of amino acids in the peptide, the lower its R_f value and that the R_f value of the peptide is influenced most by those amino acids with R_f values nearest to 1 and 0.

It was thought that as either cysteine or cystine was present in the hydrolysate of the basic fraction (B1) and that peptides were present in the original sample, one of these peptides may have been a cysteinyl or cystinyl peptide. Glutathione (reduced) is a tripeptide which contains cysteine and is known to occur in...
animal tissue, where it has a role in the oxidation/reduction pathways. Glutathione has not been previously reported in precursor studies of beef although it has been found in pork (Macy et al 1964A) and chicken (Bouthillet 1951).

An authentic sample of reduced glutathione (γ-glutamylcysteinyl-glycine) was chromatographed using the same conditions as for the beef fraction and was found to give four ninhydrin positive spots (Fig 14), three near the origin and one in the same position as glutamic acid. The three spots with low Rf values were shown to be artefacts by chromatography of authentic glutathione after treatment with the basic solvent (Fig.15). The high Rf value of glutathione in the acidic solvent, greater than that of cysteine, occurs because glutathione is not a typical peptide. Glutathione retains, from the glutamic acid residue, an amino carboxylic acid group but typical peptides have an amino carboxyamide group and so the theoretically derived equation (Pardee 1951) does not apply.

Thus, it was shown that none of the components near the origin was reduced glutathione, although one of them may have been oxidised glutathione. However, the possibility had been introduced that some or all of the components previously identified as glutamic acid may have been reduced glutathione. In order to establish the presence or absence of glutathione, a sample of the basic fraction of an aqueous extract of beef was chromatographed on Sephadex F 10. As glutathione has a much higher molecular weight than glutamic acid, 307 and 407 respectively, it should be eluted before the glutamic acid. When the eluate from the Sephadex column was analysed by thin layer chromatography it was found that a ninhydrin blue component was eluted by the same volume of water as the ninhydrin brown components, anserine and carnosine (molecular weights 226 and 212 resp.), and before the other ninhydrin blue components (Fig.16). This seemed to be evidence that the material which was originally thought to be glutamic acid had a higher molecular weight and that it might, in fact, have been glutathione.
This possibility was reinforced by the finding that authentic glutathione was eluted from a Sephadox column by the same volume of water as that required for elution of the suspected glutathione. When glutamic acid was chromatographed under the same conditions, it was found to have the same elution volume as glutathione and the apparently high molecular weight amino acid in the beef extract. Therefore, the beef component still remained to be identified. Identification was ultimately made possible by using a new solvent which completely resolved glutathione and glutamic acid (Fig. 17), when it was shown that beef (fraction B1 14) does not contain reduced glutathione, although it does contain a peptide, which, on hydrolysis, gives cystine.

The component, previously thought to be either cystine or cysteine, must have been cystine because thioles are destroyed on ion exchange chromatography or even by contact with strong alkali. This was shown by ion exchange absorption of reduced glutathione, when no glutathione was recovered from the unabsorbed material or the material displaced by ammonia. Also cysteine is never recovered from analytical ion exchange chromatograms.

The material absorbed by the cation exchange column was fractionated on a column of anion exchange resin, and the absorbed material was chromatographed for amino acids and UV absorbing compounds. It was found that the same amino acids were present in this fraction, but no cooked meat aroma could be obtained either from the absorbed or the non-absorbed material, probably because the precursors were completely destroyed. Recombination of the two fractions also failed to produce an aroma.

Both of the UV absorbing compounds were found in the fraction absorbed by the anion exchange resin, so it was at first thought that they must have been nucleotides as these have a phosphoric acid group whereas nucleosides and bases do not. However, thin
layer chromatography showed conclusively that they were inosine, a nucleoside, and hypoxanthine, a purine base.

The material which was eluted by water from the cation exchange resin (A2) but which was partially resolved from the unabsorbed compounds (A1), was found to be only slightly acidic (pH 4-5) even after concentration and, on heating, gave a weak but distinctly meaty aroma. Analysis of this fraction for amino acids by thin layer chromatography (Fig. 18) showed the same amino acid content as the basic material (B1) with the addition of taurine (1-aminocethan-2-sulphonic acid) and O-phosphoserine. Thin layer chromatography for U.V. absorbing compounds (Fig. 19)

\[
\begin{align*}
\text{Taurine: } & \quad \text{H}_2\text{N-CH}_2\text{-CH}_2\text{-SO}_3\text{H} \\
\text{Phosphoserine: } & \quad \text{NH}_2 \quad \text{O} \\
& \quad \text{H-C-CH}_2\text{-O-PO}_4\text{H} \\
& \quad \text{CO}_2\text{H} \quad \text{OH}
\end{align*}
\]

showed the presence of three: inosine, hypoxanthine and inosinic acid. This fraction also contained four sugars three of which were identified as glucose, ribose and fructose by thin layer chromatography (Fig. 20). The fourth was at first thought to be

\[
\begin{align*}
\text{Inosinic Acid: }
\end{align*}
\]
a disaccharide because of its position before glucose on elution from Sephadex G 10, and because of its low Rf. When chromatographed on a thin layer plate (Polygran) and irrigated with a basic solvent, this carbohydrate remained at the origin probably because it is a sugar phosphate, e.g. fructose-6-phosphate.

\[
\begin{align*}
\text{Fructose-6-phosphate} \\
\begin{array}{c}
\text{OH} \\
\text{HO-P-\text{O}} \\
\text{\text{C}} \\
\text{HO} \\
\text{\text{OH}} \\
\text{H} \\
\text{\text{CH}_{2}\text{OH}}
\end{array}
\end{align*}
\]

The partial resolution of this fraction (A2) from the one which preceded it (A1) can be explained by the presence of taurine and inosinic acid in the former fraction (A2). Each of these compounds contains a strongly acidic group and a weakly basic group, and it is only in the presence of strong acids that they can acquire a positive charge and can behave as cations. This condition of low pH exists in the cation exchange resin as protons are displaced from the resin by other cations. However, once the protons and anions are eluted from the resin, dissociation of the acidic group takes place, and the molecule takes on an overall negative charge and so is eluted from the resin.

Inosinic acid is used commercially as a flavour potentiator for meat products, so a sample of the fraction which contained it was neutralised with sodium hydroxide and tasted in a solution with a meat aroma prepared from a model system, which, by itself, had a good aroma but a very weak, sweet taste. No change of aroma was observed but a very strong meaty taste was imparted to the solution. This improvement was better than that obtained by the addition of inosinic acid alone, which may have been due to the presence, in this fraction, of some glutamic acid or to some other
potentiators because the combined effect of the addition of inosinate and glutamate is greater than that of the sum of the parts, (Kuninaka 1960). Taurine, also found in this fraction, had no potentiating property.

Once it had been shown that the ion exchange resins, used to fractionate beef precursors, caused the destruction of glutathione, and possibly other thiols, the use of these resins was discontinued and the aqueous solution from methanolic extraction was separated on a gel filtration column without prior treatment.

The UV absorption trace of the eluate (Fig. 21) was similar to that obtained from the equivalent weight of diffusate from 1 kg. of beef (Fig. 5), although a smaller sample (equivalent to 33 g beef) gave a more detailed trace (Fig. 22). The following analyses were based on this smaller sample. Fractions from the column were not immediately bulked, but were first analysed individually by various detecting reagents after thin layer chromatography. Ninhydrin reagent (Fig. 23) showed that the first ten fractions contained immobile material, undoubtedly peptides; and no ninhydrin positive compounds in the next three fractions. Fractions 14-25 contained ninhydrin brown and ninhydrin blue components and these were identified as carnosine, anserine and glutamic acid respectively. The remainder of the amino acids were found in fractions 24-33 except for leucine and isoleucine, which occurred in fractions 26-36, and methionine in fractions 33-37.

UV light revealed the presence of only one compound (Fig. 24) with the same Rf value as inosinic acid, in the earlier fractions (19-25).

Aniline phosphate reagent (Fig. 25) revealed three components. One of these found in the early fractions (14-16) had a low Rf value in acidic solvents and zero Rf in basic solvents. This had
been tentatively identified in fraction (A2) as a sugar phosphate. The second component which gave a brown colour, was identified as glucose and occurred in fractions 25-31. The third gave a pink colour and was identified as ribose, occurring in fractions 32-38.

Naphthoresorcinol reagent (Fig.26) revealed two compounds, one of which coincided with the tentatively identified sugar phosphate in fractions 14-18, and the other, which was eluted from the Sephadex column together with glucose in fractions 25-31, was identified as fructose.

The pH indicator (Fig.26) revealed the presence of two strong acids. The first acid, found in fractions 14-25, was also phosphate positive and was probably free phosphate as it had the same \( R_f \) as orthophosphoric acid when propanol-formic acid-water was used as an irrigating solvent. The second which had a much higher \( R_f \) value, was identified as lactic acid and was also found in the acid fraction.

Diacetyl reagent showed the presence of creatine (fraction 28-34) and arginine (fraction 28-30), (Fig.25).

These results indicated that the components detected were found in five groups of fractions. The first group (M1; fraction 1 to 10) contained peptides only; the second, (M2; fraction 11-13) appeared to contain little or nothing; the third, (M3; fraction 14 to 23) contained two dipeptides, glutamic acid, one of the sugars, phosphoric acid and most of the monosaccharide; the fourth (M4; fraction 24 to 33) contained most of the amino acids, glucose, fructose, lactic acid and creatine and the fifth (M5) contained the remainder of the amino acids and ribose.

None of the above mentioned compounds was found after fraction 38 but group M5 included fractions 39-43 because these appeared from the UV absorption trace (Fig.22) to be the last fractions of...
one of the peaks. Also phenylalanine in the basic fraction (B') had previously been found in this region (Fig. 16).

Aroma assessment of these grouped fractions showed that the complete aroma was obtained from a combination of groups M3, M4 and M5, and that elimination of any one of these fractions resulted in depreciation in aroma quality and strength.

Quantitative Analysis of Amino Acids

Ion exchange chromatography of fraction groups M3, M4 and M5 respectively revealed the presence of amino acids (Table 4; Figs. 27, 28, 29 and 30).

The second fraction group (M4) was also analysed after hydrolysis because there were two regions on the chromatograms where a simple peptide and an amide co-chromatographed with amino acids.

In the first region (Fig. 28) asparagine (an amido) was eluted with serine and in the second region the peptide anserine was eluted with histidine. Hydrolysis of asparagine and anserine respectively results in products with different chromatographic properties which permit the estimation of the remaining amino acid, and so, by difference, the amount of serine and histidine can be calculated.

A quantitative measurement of asparagine and anserine can be made from the increase in the proportion of one of their hydrolysis products (aspartic acid from asparagine and methyl histidine from anserine). When these values are subtracted from the originals, the quantities of the other components, serine and histidine, respectively can be found.

The only sulphur amino acids seen in the chromatograms were methionine, cystine and taurine. The amount of cystine was very
small and although methionine was present in greater proportion, this amino acid did not give a roast beef aroma when heated with ribose. Taurine, also does not contribute to the aroma and, like methionine, has only once been quoted as a precursor for cooked meat aroma.

It is unlikely that much cysteine was present in any of the fractions because only trace amounts of compounds were observed with a low Rf in the acidic solvent apart from the basic amino acids (Fig. 23).

However, in the first fraction group (33) to be analysed by ion exchange chromatography there were several components present (Fig. 26) which did not have the elution volume of any known amino acid. One of these was found in the position where oxidised glutathione would have been expected. It is possible, therefore, that the sulphur, assumed to be necessary in the production of beef aroma, may be supplied by cystine and oxidised glutathione. It will be shown later that any compound which can give rise to hydrogen sulphide on heating can be a precursor of cooked meat aroma.

Estimation of the Free Sugars, Inosine and Hypeoxanthine in Deionised Aqueous Extract

Quantitative analysis showed that the beef extract after deionisation contained only three sugars; glucose, fructose, and ribose in the proportions shown in Table 5. The gas chromatogram of the trimethyl silyl derivatives showed four peaks (Fig. 31) because glucose is resolved, under these conditions, into its α- and β- anomers.

Examination of the UV absorption traces of the eluate (Fig. 21, 22, 3 and 8) revealed the presence of three groups of components eluted after the amino acids and sugars. Inosine was

/37....
identified in the first group (M6) by thin layer chromatography and UV spectroscopy (Fig. 32) and hypoxanthine was found in the second group (M7) using the same techniques. No identification was possible in the third group but its UV spectrum is shown (Fig. 33).

The inosine content was found by bulking the fractions containing it and determining the absorption at the maximum, 254nm. (Table 5). The hypoxanthine content was found in a similar way (Table 5).

When a sample of the beef extract was hydrolysed, (2M - hydrochloric acid for 16 hr), it was assumed that the inosinic acid content of the starting material could be estimated from the increase in inosine and hypoxanthine (hydrolysis products of inosinic acid). However, the gel filtration was complicated by the change in behaviour of hypoxanthine and inosine on Sephadex in the presence of acid. These two compounds were eluted just after the smallest compounds. Thus only an approximate value could be obtained (Table 5) because complete separation from other UV absorbing compounds could not be assumed.

The analysis procedure for creatine was derived from the detection method for guanidines for it was found that only creatine and arginine reacted with the diacetyl reagent in the cold to give a pink colour. The absorption maximum of this colour was found and used to estimate the quantity of creatine present in a whole extract (Table 5).

Comparison of the results obtained above with those from other reports (Macy et al. 1964a and Bender et al. 1956, Table 6) show that the results are similar to those of Macy with some exceptions, i.e. cystine, phenylalanine and fructose. However, most of the results are not even of the same order as those found by Bender et al, with the exceptions of ascorbic, carnosine, glucose, the purines, creatine and glycine.
Analyses of the fraction groups (M3, M4 and M5), obtained by gel filtration of the methanolic extract, illustrate that Sephadex does not separate small molecules on the basis of size alone. In theory separation by gel filtration occurs because there is, in the gel beads, a distribution of pore sizes. Thus, there are more pores large enough to accommodate the smaller molecules so they are retained for a longer time than the larger molecules (Polidi 1961).

However, it has been found that there are other effects which largely determine the elution order of small molecules (Golette 1959). Results of the investigation of the chromatographic properties of a large number of compounds on Sephadex G25 indicated that under all conditions aromatic and heterocyclic compounds are absorbed by gel more strongly than aliphatic compounds. In the absence of electrolyte in the eluting solution there was a strong absorption of basic substances and an exclusion of acidic compounds from the gel. This was thought to be caused by ionized carboxylic acid groups which would retain basic groups by an ion exchange effect and would reduce the effective size of the gel pores to the acids. Thus, the number of pores available to the acids would decrease and they would be eluted before the non-acidic molecules of the same size.

The effect of the presence of carboxylic acid groups is, most probably, the reason for the early elution of glutamic and aspartic acids. However, the bases are not retained but this may be due to the presence of electrolyte or may only be apparent, the effect on the acids being exaggerated by the smaller gel pores (Sephadex G10 was used whereas the reported effects were observed with G25).

Absorption of heterocyclic and aromatic compounds is to be seen in the retention of inosine, hypoxanthine and, to a lesser extent, phenylalanine and tyrosine. Inosine and hypoxanthine were not retained by the gel at a low pH (about 1); a phenomenon not
hitherto reported.

There were also effects to be seen in the above analyses which were not mentioned by Gelotte (1959). For example cystine and methionine were retained to a greater extent than most amino acids. Also the retention of leucine was slightly greater than that of iso-leucine, which would suggest that the gel is able to differentiate between branched and non-branched structures.

**Model Systems**

At one stage in the examination of beef for aroma precursors there was tentative evidence of the presence of reduced glutathione, which was, in consequence, used in model systems.

The first of these model systems, Sample 1 (Table 6), contained, in addition to reduced glutathione; alanine, the amino acid with the highest apparent concentration; carnosine, the largest ninhydrin positive component; inosine, the only compound in the basic fraction which could have provided carbohydrate; and glucose, whose addition had improved the aroma of the basic fraction.

When this mixture, in the proportions given in Table 6 was boiled in water the aroma was described variously as roast beef, chicken, pork or pork sausage.

By a systematic elimination of certain components of the model system (Table 6) the critical constituents were established to be glutathione and glucose; inosine appeared to be a poor substitute for glucose, and alanine and carnosine had no influence on the aroma. This would suggest that the reaction between a sugar and glutathione was responsible for the meat-like aroma.

When cysteine was used instead of glutathione, the aroma produced was almost identical to that given by glutathione,
suggesting that the role of glutathione is to provide a source of cysteine.

When ribose and hypoxanthine were substituted for inosine, a richer aroma was produced due, possibly, to the greater reactivity of ribose. Elimination of all reactants other than glutathione and ribose did not alter this aroma.

Hitherto, glutathione has not been recognised as a red meat flavour precursor although almost all of the naturally occurring sulphur compounds in meat have been accorded this property e.g. cysteine, cystine, and thiosulphate. Glutathione has been recognised as a source of hydrogen sulphide in chicken meat (Mecchi et al. 1964) and as a possible precursor of chicken aroma (Bouthilet 1950). To increase our knowledge of the part played by glutathione a model system was prepared which contained an inorganic sulphide instead of glutathione, because the most common property of these compounds, which have been claimed to act as precursors of beef aroma, is their ability to release hydrogen sulphide on heating. Sodium sulphide was used for this purpose and was heated with ribose, the most effective sugar in the series of experiments in which glutathione figured.

The reactants (sodium sulphide, ribose and ammonium lactate) were dissolved in a buffer solution, pH 5 to simulate the conditions observed with glutathione. Solutions of pH greater than 4.5 did not produce a cooked meat aroma. Ammonium lactate was chosen as the buffering agent because lactic acid is the most common acid in meat and ammonia is the most simple source of nitrogen for the browning reaction. The solution was heated in a sealed capsule to prevent loss of H₂S and gave an aroma very similar to that observed when glutathione and ribose were heated. It would appear that any source of hydrogen sulphide and a sugar will give a meaty aroma when heated at a low pH in the presence of an amine. Furthermore, xylose, an
aldopentose, could be substituted for ribose.

The aroma obtained from these model systems was not that of roast beef but of boiled beef or yeast. However, a roast beef aroma could be obtained from the previously reacted sulphide/ribose system by boiling down at a pH of 8-9. To maintain this high pH, ammonia had to be added continually to the solution.

The Role of Sulphides and Sugars

The methanolic extract of beef and its aqueous diffusate can produce meaty aromas because they contain cystine, probably oxidised glutathione, and possibly reduced glutathione and cysteine.

The basic fraction, obtained by treatment of the aqueous methanolic extract of beef, with a cation exchange resin, gave, on heating, a recognisable cooked meat aroma. This was despite the absence of free reducing sugars.

The aroma was very weak and was probably produced by the reaction of cystine or oxidised glutathione with the ribosyl moiety of inosine. Additional aromas were probably produced by the amino acids present. The aroma was improved by the addition of glucose.

When the methanolic extract was fractionated by gel filtration seven fractions were collected of which three were required to produce the full aroma. No single fraction contained all of the sugars or thiol and disulphide compounds.

The Possible Role of Protein

Dialysis of the aqueous extract of raw beef showed that good, roast and boiled, beef aromas could be obtained from the diffusate, which appears to confirm the conclusions reached by others who have used this method of separation, that the precursors are low
molecular weight solutes.

The diffusate, however, appeared to have a weaker aroma than the whole extract and this was also reported by Wasserman and Gray (Wasserman and Gray 1964) who found that the diffusate gave only a weak brothy note when boiled, and that on pyrolysis a steak-like aroma could be discerned. The protein, which is unable to diffuse through the dialysis membrane, may therefore contribute to the aroma production. Flavour profile analyses of cooked whole beef, and a methanolic extract of beef, similar in composition to the beef diffusate provided some evidence in support of this hypothesis. Results showed that the whole beef gave more aromas described as meat-like and that they were more intense (Table 2).

The suspected presence of reduced glutathione in the basic fraction of beef extracts resulted in an extension of the work on model systems and the observation that meat-like aromas could be produced by the reaction of hydrogen sulphide, ribose and ammonia at a low pH (4-5).

This evidence was considered sufficient to advance the hypothesis that the protein in the whole beef and in aqueous extracts acts as a source of hydrogen sulphide, with resultant improvement in aroma.

Experiments on chicken flesh have been reported (Mcchi et al. 1964) which show that, although reduced glutathione and cysteine evolve hydrogen sulphide more rapidly than the protein present in the flesh, the sulphur content of the protein is so high as to make it the major source of hydrogen sulphide.

All the experimental work on beef flavour precursors has, to date, been concerned with the low molecular weight, dialysable fraction, but a glycoprotein has been ascribed aroma precursor
properties (Botzer et al. 1962). This glycoprotein was obtained from an aqueous extract of beef by dialysis, followed by gel filtration and anion exchange chromatography. The suspected glycoprotein was not eluted with the void volume from a column of Sephadox G25, and was not absorbed by an anion exchange resin (chloride form). Paper electrophoresis of the material, after separation on Sephadox, showed a component which was displaced towards the negative pole at pH 8.7 and gave a carbohydrate reaction. In the same position was a ninhydrin positive component. Paper electrophoresis at pH 11 again demonstrated the presence of a carbohydrate which migrated to the negative pole, whereas the ninhydrin positive component was now displaced towards the positive.

Analysis of the glycoprotein after ion exchange showed it to be ninhydrin negative but after hydrolysis it was ninhydrin positive. This was thought by Botzer to be additional evidence that the material was a protein. However it is possible that the conclusion was erroneous for the original material was eluted, by water, from an anion exchange column in the chloride form and so would have been acidic. The ninhydrin reaction yields blue colours only at near neutral pH, but after this material had been hydrolysed the hydrolysate was neutralised and so would have been capable of yielding the characteristic blue colour. Paper chromatography of the hydrolysate from the glycoprotein revealed the presence of eight amino acids, two of which were not identified. One of these was estimated to constitute about 80% of the total amino acids present, and was thought to contain sulphur, although its $R_f$ values were not consistent with those of any known sulphur amino acids. Analysis by ion exchange chromatography showed that, of those amino acids eluted with acid buffer from the column, the most abundant was $L$-alanine. The two unidentified amino acids were retained by the resin.
If the two unidentified amino acids are not sulphur-containing then it is possible that they are histidine and methylhistidine which are basic compounds and are retained on the ion exchange resin under the conditions used. Moreover these two have similar Rf values to those of the two unidentified compounds. Furthermore, the large amount of β-alanine indicates that the histidine and methylhistidine were derived from carnosine (β-alanylhistidine) and anserine (β-alanyl-N-methylhistidine).

Thus the most probable interpretation of the analytical data is that the "Glycoprotein" was a mixture of amino acids, peptides and sugars and possibly other meat components.
The major conclusion of this work, based largely on model systems, is that the main precursors of the cooked meat aroma are a source of hydrogen sulphide, an amine and a sugar, preferably a pentose. In the low molecular weight fraction capable of yielding a meaty aroma, the hydrogen sulphide is probably provided by cysteine, either as the free amino acid or as a peptide residue. However in the whole meat most of the hydrogen sulphide is probably provided by proteins.

The model systems produced cooked meat aroma only under rather artificial conditions, a low pH and, where the source of hydrogen sulphide was inorganic, high pressure. In meat, however, the overall pH remains near neutral therefore if similar reactions occur they must either be modified by the presence of other components or there must be present areas of low pH. The latter condition may exist in meat for proteins with carboxylic acid groups, due to aspartic and glutamic acids, would, at relatively low salt concentrations, possess small regions of low pH. These low pH regions are thought to be partially responsible for the activity of some hydrolytic enzymes.
Thus the protein may provide not only hydrogen sulphide but also the correct conditions for the reaction to take place. I therefore feel that a study of the role of the insoluble fraction and the non-diffusible solubles which are largely protein would be rewarding.

Another area worthy of investigation is the mechanism of the reactions between hydrogen sulphide and sugars in the presence of amines for this has not been studied in any detail. Two main types of reaction are possible. The first is the amine initiated degradation of the sugar followed by the reaction of the products with hydrogen sulphide. The second is the reaction of the sugar with hydrogen sulphide, followed by the degradation of this product to odorous substances of lower molecular weight. If the latter is the operative mechanism then the first stage could take place at low pH sites on the protein and the second stage at higher pH in solution.
REFERENCES


Anot, E.F.L.J. and Reynolds, T.N.

Asinger, P., Thiel, H. and Lipport, G.
Liebigs Ann., 627 195 (1959)

Barylko-Pikielna, R.
Przemyśl Sewayscy, 11 (1) 26 (1957)


Batzer, O.F., Santoro, A.T. and Landmann, W.H.

Bender, A.E., Wood, T. and Palgrave, J.A.

Bouthilet, R.J.
Food Research, 16 201 (1951)

Brobst, K. and Lott, G.E.
Cereal Chem. 43 (1) 35 (1966)

Cairncross, S.E. and Sjostrand, L.B.
Food Technol., 4 (8) 308 (1950)

J. Chen. Ind., 1968 (47) 1639

Crocker, E.C.
Food Research 13 179 (1948)

Flodin, P.
J. Chromatog., 5 103 (1961)

Gelotte, B.
J. Chromatog., 2 330 (1960)

Hamilton, P.B.
Anal. Chem. 35 (13) 2055 (1963)

Hodge, J.E.

Howe, P.E. and Barbella, H.G.
Food Research, 2 197 (1937)

International Flavours and Fragrances Inc.
British Patent, 1034619 (1967)

/2....
Jossmann, E. and Schultz, H.
Pharmazie, 18 (8) 527 (1963)

Kaminaka, J.

Nabrouk, I.F., Jarboc, J.K. and O'Connell, L.M.

Nacey, R.L., Neumann, H.D. and Bailey, M.E.
J. Food Sci., 29 136 (1964)

Nacey, R.L., Neumann, H.D. and Bailey, M.E.
J. Food Sci., 29 142 (1964 B)

Nocchi, E.P., Pippon, E.L. and Lineweaver, H.
J. Food Sci., 29 393 (1964)

Moore, S. and Stein, W.H.
J. Biol. Chem. 211 907 (1954)

Newell, J.A., Mason, K.E. and Matlock, R.S.

Pardee, A.B.
J. Biol. Chem. 190 757 (1951)

Rohan, T.A. and Stewart, T.F.
J. Food Sci., 31 202 (1966)

Rohan, T.A. and Stewart, T.F.
Unpublished Results (1968)

Schonberg, A., Houben, R. and Moustafa, A.


Unilever Ltd.
British Patent, 836694. (1960)

Wasserman, A.E. and Gray, R.
J. Food Sci., 30 (5) 801 (1965)

Zeikr, L.L., Wasserman, A.E., Honk, C.H. and Sclay, J.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Creatine Solution</th>
<th>Freeze-dried Extract</th>
<th>Water</th>
<th>Diacetel</th>
<th>Naphthol 1% in 6% NaOH</th>
<th>Absorbance (523 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.04 ml</td>
<td>-</td>
<td>5.16</td>
<td>0.5</td>
<td>0.5</td>
<td>0.275</td>
</tr>
<tr>
<td>2</td>
<td>0.08 ml</td>
<td>-</td>
<td>5.12</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.720</td>
</tr>
<tr>
<td>3</td>
<td>0.12 ml</td>
<td>-</td>
<td>5.08</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.940</td>
</tr>
<tr>
<td>4</td>
<td>0.16 ml</td>
<td>-</td>
<td>5.04</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1.150</td>
</tr>
<tr>
<td>5</td>
<td>0.20 ml</td>
<td>-</td>
<td>5.00</td>
<td>&quot;</td>
<td>&quot;</td>
<td>1.550</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>0.04 ml</td>
<td>5.16</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.190</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>0.08 ml</td>
<td>5.12</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.375</td>
</tr>
<tr>
<td>8</td>
<td>-</td>
<td>0.12 ml</td>
<td>5.08</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.520</td>
</tr>
<tr>
<td>9</td>
<td>-</td>
<td>0.16 ml</td>
<td>5.04</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.735</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>0.20 ml</td>
<td>5.00</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.940</td>
</tr>
<tr>
<td>11</td>
<td>-</td>
<td>-</td>
<td>5.20</td>
<td>&quot;</td>
<td>&quot;</td>
<td>0.070</td>
</tr>
</tbody>
</table>
**TABLE 2**

Flavour Profiles of Boiled Methanolic Extract and Boiled Beef After Concentration

<table>
<thead>
<tr>
<th>Methanolic Extract (M)</th>
<th>Boiled Beef Concentrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Term</td>
<td>Score</td>
</tr>
<tr>
<td>Roast Beef</td>
<td>3</td>
</tr>
<tr>
<td>Boiled Beef</td>
<td>1</td>
</tr>
<tr>
<td>Oily</td>
<td>1</td>
</tr>
<tr>
<td>Potato</td>
<td>½</td>
</tr>
<tr>
<td>Serum</td>
<td>½</td>
</tr>
<tr>
<td>Dripping</td>
<td>¾</td>
</tr>
<tr>
<td>Caramel</td>
<td>¼</td>
</tr>
<tr>
<td>Gravy</td>
<td>½</td>
</tr>
<tr>
<td>Bovril</td>
<td>½</td>
</tr>
<tr>
<td>Mushroom</td>
<td>¾</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### TABLE 2

**FLAVOUR PROFILES OF FRACTION (B1) AND FRACTION (B1) + GLUCOSE**

<table>
<thead>
<tr>
<th>Term</th>
<th>Score</th>
<th>Term</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef/Meat</td>
<td>1½</td>
<td>Boiled Beef</td>
<td>2</td>
</tr>
<tr>
<td>Footid</td>
<td>1</td>
<td>Footid</td>
<td>1</td>
</tr>
<tr>
<td>Amino like</td>
<td>1</td>
<td>Gravy</td>
<td>½</td>
</tr>
<tr>
<td>Sour</td>
<td>½</td>
<td>Dripping</td>
<td>¼</td>
</tr>
<tr>
<td>Caramel</td>
<td>¼</td>
<td>Oily</td>
<td>¼</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nutty</td>
<td>¼</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Acrid</td>
<td>¼</td>
</tr>
<tr>
<td>AMINO COMPOUND</td>
<td>M3</td>
<td>M4</td>
<td>M5</td>
</tr>
<tr>
<td>-------------------------</td>
<td>----</td>
<td>-------</td>
<td>----</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>0.035</td>
<td>0.003</td>
<td>0.049</td>
</tr>
<tr>
<td>Glycero-phosphoethanolamine</td>
<td>0.032</td>
<td>0.004</td>
<td>0.004</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>0.366</td>
<td>0.020</td>
<td>0.386</td>
</tr>
<tr>
<td>Phos-3phoethanolamine</td>
<td>0.274</td>
<td>0.009</td>
<td>0.283</td>
</tr>
<tr>
<td>Scrine</td>
<td>0.006* 0.369*</td>
<td>0.002* 0.377</td>
<td>0.369</td>
</tr>
<tr>
<td>Asparagin</td>
<td>0.003* 0.144*</td>
<td>0.001* 0.148</td>
<td></td>
</tr>
<tr>
<td>Glutamico</td>
<td>0.779</td>
<td>0.007</td>
<td>0.786</td>
</tr>
<tr>
<td>Sarcosine</td>
<td>0.035</td>
<td>0.006</td>
<td>0.634</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>0.535</td>
<td>0.019</td>
<td>0.634</td>
</tr>
<tr>
<td>Proline</td>
<td>0.157</td>
<td></td>
<td>0.157</td>
</tr>
<tr>
<td>Citraline</td>
<td>0.014</td>
<td></td>
<td>0.014</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.014</td>
<td>0.544</td>
<td>0.013</td>
</tr>
<tr>
<td>α Alanine</td>
<td>0.037</td>
<td>2.436</td>
<td>0.018</td>
</tr>
<tr>
<td>Valine</td>
<td>0.023</td>
<td>0.403</td>
<td>0.006</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.0014</td>
<td></td>
<td>0.0014</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.016</td>
<td>0.095</td>
<td>0.111</td>
</tr>
<tr>
<td>iso-Leucine</td>
<td>0.209</td>
<td>0.037</td>
<td>0.246</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.325</td>
<td>0.116</td>
<td>0.441</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.055</td>
<td></td>
<td>0.055</td>
</tr>
<tr>
<td>β Alanine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.004</td>
<td></td>
<td>0.004</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>0.008</td>
<td></td>
<td>0.008</td>
</tr>
<tr>
<td>Ammoniac</td>
<td>0.238</td>
<td>0.819</td>
<td>0.290</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>0.041</td>
<td></td>
<td>0.041</td>
</tr>
<tr>
<td>1-Methylhistidine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lysine</td>
<td>0.248</td>
<td>0.068</td>
<td>0.003</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.113* 0.025*</td>
<td>trace</td>
<td>0.168</td>
</tr>
<tr>
<td>Anasine</td>
<td>0.755* 0.131*</td>
<td>0.003</td>
<td>0.886</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>0.022</td>
<td>0.030</td>
<td>0.002</td>
</tr>
<tr>
<td>Carnosine</td>
<td>5.392</td>
<td>1.215</td>
<td>0.017</td>
</tr>
<tr>
<td>Unknown</td>
<td>0.029</td>
<td></td>
<td>0.029</td>
</tr>
<tr>
<td>Arginino</td>
<td>0.011</td>
<td>0.226</td>
<td>0.237</td>
</tr>
<tr>
<td>Ornithine</td>
<td>0.058</td>
<td>0.029</td>
<td>0.031</td>
</tr>
<tr>
<td>Compound</td>
<td>mg/100g beef</td>
<td>Henry et al.</td>
<td>Bender et al.</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>---------------</td>
</tr>
<tr>
<td>Phosphoserine</td>
<td>0.36</td>
<td>11.08</td>
<td>0.65</td>
</tr>
<tr>
<td>Glycero-phosphoethanolamine</td>
<td>0.02</td>
<td>30.67</td>
<td>0.06</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>0.66</td>
<td>26.70</td>
<td>0.56</td>
</tr>
<tr>
<td>Taurine</td>
<td>9.05</td>
<td>47.43</td>
<td>4.83</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.82</td>
<td>7.10</td>
<td>0.64</td>
</tr>
<tr>
<td>Threonine</td>
<td>1.11</td>
<td>as ser.</td>
<td>3.37</td>
</tr>
<tr>
<td>Serine</td>
<td>7.53</td>
<td>73.56</td>
<td>3.96</td>
</tr>
<tr>
<td>Asparagine</td>
<td>as serine</td>
<td>as serine</td>
<td>1.96</td>
</tr>
<tr>
<td>Glutamine</td>
<td>not est.</td>
<td></td>
<td>11.49</td>
</tr>
<tr>
<td>Sarcosine</td>
<td></td>
<td></td>
<td>0.30</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.63</td>
<td>22.15</td>
<td>9.33</td>
</tr>
<tr>
<td>Prolino</td>
<td>not est.</td>
<td></td>
<td>1.81</td>
</tr>
<tr>
<td>Citrulline</td>
<td></td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.40</td>
<td>7.10</td>
<td>4.29</td>
</tr>
<tr>
<td>Alanine</td>
<td>11.28</td>
<td>36.07</td>
<td>22.19</td>
</tr>
<tr>
<td>Valine</td>
<td>2.99</td>
<td>11.08</td>
<td>5.06</td>
</tr>
<tr>
<td>Cystine</td>
<td>4.37</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.01</td>
<td>5.96</td>
<td>1.66</td>
</tr>
<tr>
<td>iso-Leucine</td>
<td>2.04</td>
<td>6.32</td>
<td>3.23</td>
</tr>
<tr>
<td>Leucine</td>
<td>3.81</td>
<td>17.89</td>
<td>5.79</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>1.85</td>
<td>5.96</td>
<td>1.00</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>1.36</td>
<td>11.36</td>
<td>0.07</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td>Ammonia</td>
<td>as lysine</td>
<td>10.51</td>
<td>2.29</td>
</tr>
<tr>
<td>3-Methylhistidine</td>
<td>4.80</td>
<td></td>
<td>0.69</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.19</td>
<td>2.00</td>
<td>4.68</td>
</tr>
<tr>
<td>Histidine</td>
<td>4.10</td>
<td></td>
<td>2.61</td>
</tr>
<tr>
<td>Inosine</td>
<td>as carnosine</td>
<td>31.81</td>
<td>21.31</td>
</tr>
<tr>
<td>Tryptophan</td>
<td></td>
<td></td>
<td>1.10</td>
</tr>
<tr>
<td>Carnosine</td>
<td>90.14</td>
<td>138.02</td>
<td>127.23</td>
</tr>
<tr>
<td>Unknown</td>
<td>not est.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
<td></td>
<td>3 µ mole/100g</td>
</tr>
<tr>
<td>Ornithine</td>
<td></td>
<td></td>
<td>1.13</td>
</tr>
<tr>
<td>TOTAL amino acids</td>
<td>161.5</td>
<td>503.27</td>
<td>22.77</td>
</tr>
<tr>
<td>Glucose</td>
<td>43.86</td>
<td>59.61</td>
<td>15.68</td>
</tr>
<tr>
<td>Fructose</td>
<td>3.56</td>
<td>as glucose</td>
<td>22.85</td>
</tr>
<tr>
<td>Ribose</td>
<td>1.09</td>
<td>as glucose</td>
<td>1.35</td>
</tr>
<tr>
<td>Creatine</td>
<td>268.7</td>
<td>375.00</td>
<td>22.3</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td></td>
<td></td>
<td>35.0</td>
</tr>
<tr>
<td>Inosine</td>
<td></td>
<td></td>
<td>7.0</td>
</tr>
<tr>
<td>Inosinic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**TABLE 6**

EVALUATION OF CONSTITUENTS OF MODEL SYSTEM.

<table>
<thead>
<tr>
<th>Component</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutathione</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Ribose</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
<td>0.05</td>
</tr>
<tr>
<td>Glucose</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inosine</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carnosine</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fraction A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Neutralized</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Aroma**
- +++ Good
- ++++ Fair-Good
- ++++++ Fair
- ++++ Weak

**Taste**
- + Weak
- ++ Fair
- +++ Fair-Good
- ++++ Good
Appendix

Abbreviations used in figs. 7, 11, 13, 17, 18, 24, and 28 to 31.

<table>
<thead>
<tr>
<th>Abbreviation Trivial Name</th>
<th>Abbreviation Trivial Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>aala alanine</td>
<td>lys lysine</td>
</tr>
<tr>
<td>bala alanine</td>
<td>1-mehis 1-methylhistidine</td>
</tr>
<tr>
<td>ans anserine</td>
<td>3-mehis 3-methylhistidine</td>
</tr>
<tr>
<td>arg arginine</td>
<td>met methionine</td>
</tr>
<tr>
<td>asn asparagine</td>
<td>orn ornithine</td>
</tr>
<tr>
<td>asp aspartic acid</td>
<td>peth phosphoethanolamine</td>
</tr>
<tr>
<td>car carnosine</td>
<td>phe phenylalanine</td>
</tr>
<tr>
<td>cys cystine</td>
<td>pro proline</td>
</tr>
<tr>
<td>eth ethanolamine</td>
<td>pser phosphoserine</td>
</tr>
<tr>
<td>gln glutamine</td>
<td>sar sarcosine</td>
</tr>
<tr>
<td>glu glutamic acid</td>
<td>ser serine</td>
</tr>
<tr>
<td>gly glycine</td>
<td>tau taurine</td>
</tr>
<tr>
<td>gsh reduced glutathione</td>
<td>thr threonine</td>
</tr>
<tr>
<td>gssg glutathione</td>
<td>try tryptophan</td>
</tr>
<tr>
<td>his histidine</td>
<td>tyr tyrosine</td>
</tr>
<tr>
<td>ileu iso-leucine</td>
<td>val valine</td>
</tr>
<tr>
<td>leu leucine</td>
<td></td>
</tr>
</tbody>
</table>
METHANOLIC EXTRACTION

Beef 1Kg

Extraction with methanol 2.5L

Residue

Solution

Concentration and Chloroform extraction

Water Solubles (M) 60 ml

Lipids

Gel filtration of 2 ml

Fractions 1-10 11-13 14-23 24-33 34-43 44-58 59-125 126-207 208-275

(3 ml)

Fig 2
Absorbance (%) at 254 nm

Aqueous extract of beef chromatographed on Sephadex G-10

Fig 3
Thin layer chromatogram of amino acids

Butanol - acetic acid - water

Diffusate  Fraction W2  Fraction W1  Aqueous extract

Shading indicates depth of ninhydrin colour
Fig 5

Diffusate of beef chromatographed on Sephadex G10

Absorbance (%o) at 254 nm

Volume of eluate (ml):
- 0
- 250
- 500
- 750
- 1000

0 50 100
Beef

Extraction with water

Residue

Solution

Centrifugation

Supernatant

Dialysate

Dialysis

Diffusate (D)

Gel filtration

Fractions

D1 D2 D3 D4 D5 D6 D7 D8 D9

1-5 6-9 10-12 13-19 20-26 27-50 51-70 71-82 83-120

Fig 6
Thin layer chromatogram of the basic fraction of beef extract

Fig 7
Thin layer chromatogram of sugars and fructose D4, detected by aniline-phosphomolybdic acid reagent.

Butanol-acetic acid-water
Transmittance(%) 254 nm

Method of extract. M. separated by cation exchange chromatography.

Fig 10
Fig 11

Thin layer chromatogram of fraction B2. Compounds revealed by ninhydrin
Isopropanol - formic acid - water

Thin layer chromatogram of fraction B2. Compounds revealed by reagent sequence: iodine, ninhydrin, diacetyl

Fig 12
Isopropanol – formic acid – water

Thin layer chromatogram of fraction B1 hydrolysed. Compounds revealed by ninhydrin.

Fig 13
TLC of authentic reduced glutathione. Revealed by ninhydrin.

Butanol–acetone–ammonia–water

Fig 14
Illustration of spurious component formation

Fig 15
TLC of fractions from the separation of B1 on Sephadex G10. Revealed by ninhydrin.

Butanol - acetone - ammonia - water
Isopropanol – formic acid – water

TLC of fraction B1/14

Fig 17
TLC of amino acids in fraction A2

Fig 18
TLC of purine derivatives. Detected by UV absorption (254 nm).

Fig 19
TLC of fraction A2. Sugars revealed by aniline-phosphate.

**Fig 20**
TLC of sugars. Revealed by naphthoresorcinol reagent

Fig 21
UV (254 nm) absorption monitored eluate from the chromatography of 10 ml of methanol extract on a column (30 x 5 cm) of Sephadex G10.

Time

Transmittance (%)

Fig. 22
Fig 23

UV (254 nm) absorption monitored eluted from the chromatography of 2 ml of methanol extract on a Sephadex G10 column 100x2.5 cm

Fractions (3 ml)
TLC of fractions of 2 ml of the methanol extract after chromatography on Sephadex G-10. Amino acids revealed by ninhydrin.

Fig 24
As for Fig 24. Cavitated by digest. O Revealed by UV light.

<table>
<thead>
<tr>
<th>40</th>
<th>35</th>
<th>30</th>
<th>25</th>
<th>20</th>
<th>15</th>
<th>10</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Inosinic acid arginine

Cysteine
Fig 26

Reagent: 

TLC as for Fig 24. Sugars revealed by ninhydrin-phosphosphate reagent: ○ Sugars revealed by napthoresorcinol

unknown sugar phosphate

agalactose
fructose
ribose
Fig 27

TLC as for Fig 24. Bases revealed by bromocresol blue. ○ acids revealed by bromocresol blue. □ and by molybdic acid reagent.

Phosphoric acid

Lactic acid
Fig. 28

Ion exchange chromatogram of compounds detected by ninhydrin reagent in beer fractions.
Fig 30

Time hours

0 10 19 18 17 16 15 14 13 12 11
arg orn meth lys
his

NH3

Absorbance

Fraction M4 hydrolyzed

Ion exchange chromatograms detected by ninhydrin reagent of each fractions.
in a detoxified extract of beer

Fig 32 Gas chromatography of the immunofixation derivatives of sugars
Absorbance

Wavelength nm

Fig. 33

UV absorption spectrum of fractions M7 & M8