Identification of Components of the Dialyzable Fraction of Beef Adipose Tissue

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The dialyzable fraction from beef adipose tissue, either treated with salt or untreated and stored under either air or nitrogen, was lyophilized, fractionated by column chromatography, and analyzed by chemical procedures or thin layer chromatographic techniques. Column chromatographic profiles of the dialyzable components were unaffected by salt treatments or by storage under air or nitrogen. Amino acid analysis revealed that glycine, glutamic acid, and proline comprised ~46% of the total amino acid residues for the untreated sample as compared to 31% for the sample treated with salt. The diffusate from the salt-treated sample contained more arginine, threonine, serine, isoleucine, leucine, tyrosine, and phenylalanine (22%) than the untreated sample (9%). Only traces of sulfhydryls were evident, but positive tests were obtained for aldoses and free amino groups. The diffusates from untreated adipose tissue contained about five times as many free amino groups and ten times as many aldoses as that from the salt treatment. This suggests that salt stabilized the adipose tissue against breakdown. Thin layer chromatography verified the presence of creatine, creatinine, creatine phosphate, cytosine, uracil, and fluorescent and phosphate-containing compounds in the diffusate, but purine bases, nucleosides, nucleotides, and lactic acid were absent.

Dialyzable components of beef extract were studied by Bender *et al.* (1958) who determined the presence of several amino acids, creatine, creatinine, and reducing sugars. Batzer *et al.* (1960) dialyzed the aqueous extract of fresh beef muscle and fractionated the diffusate into polypeptides, carbohydrates, and amino acids. Further analysis of the polypeptide fraction revealed a glycoprotein which on heating with glucose and inosinic acid produced a meaty aroma (Batzer *et al.*, 1962). Wasserman and Gray (1965) separated the water-soluble fraction of beef muscle by dialysis. The diffusate fraction, collected after elution from the cation exchange resin with ammonium hydroxide, developed a meaty aroma upon pyrolysis. Mabrouk *et al.* (1969) fractionated the water-soluble flavor precursors from lyophilized meat by dialysis and gel permeation chromatography after first extracting the lipids with petroleum ether. Zaika *et al.* (1968) separated and identified many components in the water-soluble fraction of beef muscle using absorption, ion exchange, and gel filtration chromatography. Macy *et al.* (1964) compared the water-soluble diffusates of beef, lamb, and pork and found many compounds common to the three species, but that cysteic acid and ornithine are common only to pork and lamb, and that glutathione was present only in lamb.

In spite of the large amount of work on the water-soluble components of muscle tissue and their relationship to meat flavor and/or aroma, little is known about the contribution of the water-soluble components from adipose tissue. The present study was designed to develop methodology to determine the components in the diffusate from aqueous extracts of beef adipose tissue. Furthermore, the effects of adding 3% NaCl to the ground adipose tissue and storing under either air or nitrogen were also investigated.

EXPERIMENTAL SECTION

Since sausage products often contain high levels of fat and approximately 3% of added NaCl, the ground adipose tissue was treated with 3% added salt or untreated and stored under either air or nitrogen at −23° before extraction with borate buffer. The extracts were subjected to dialysis, and the diffusates were characterized.

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The diffusates were separated from the aqueous extract of beef adipose tissue as previously described by Pepper and Pearson (1971). The diffusates were then concentrated by lyophilization. Immediately prior to analysis, the lyophilized material was taken up in water and filtered and the residue washed with water until approximately 100 ml of filtrate was collected.

For amino acid analyses aliquots (5 ml) of the filtrates were lyophilized. The powder was extracted three times with methyl ethyl ketone (MEK) containing 5% 6 *N* HCl (3 × 5 ml) and filtered through a fine fritted glass filter. The concentrate was obtained by removing the MEK under a stream of nitrogen at 4°. The method described by Smith (1960) was used except that no water was added to the extract prior to MEK removal by nitrogen. Water (2 ml) was added to the concentrate and sufficient concentrated HCl was added to the solution to bring it to 6 *N*. Hydrolysis and amino acid analyses were accomplished as described by Pepper and Pearson (1971). Lyophilized diffusates (1.0–1.5 g) obtained from the salt-containing adipose tissue were extracted twice with MEK containing 5% 6 *N* HCl (2 × 25 ml) and then once with MEK (25 ml). The combined extracts were filtered through a fine fritted glass filter and the filtrate was concentrated under reduced pressure at room temperature in a rotary evaporator until almost dry. Water (3 ml) was added to the concentrate and the mixture was refiltered. Concentrated HCl was added to the filtrate and hydrolysis was carried out as previously described.

The diffusates were separated into various fractions by column chromatography according to Lento *et al.* (1964), except that the resin bed (Dowex I, formate form) was 2.0 × 35 cm and a discontinuous gradient system was employed as the eluent. The pH of the sample was adjusted to 8.0 with 5 *N* sodium hydroxide before application to the column. After applying a 5.0-ml aliquot sample to the column, the diffusate was eluted from the column for 2 hr at a rate of 40 ml/hr with water and then with 0.5, 1.0, and 6.0 *N* formic acid solutions for 3.0, 7.5, 4.5, and 5.0 hr, respectively. The eluent was monitored at 254 nm and collected in 5.0-ml portions. The aliquots of each fraction were combined, lyophilized, and stored in a desiccator at 4°.

Each fraction was subjected to thin layer chromatographic (tlc) analysis. Analysis for creatine and its derivatives was determined according to Zaika *et al.* (1968) using silica gel chromatograms (Eastman chromatogram

Table I. Amino Acid Composition of the Aqueous Diffusate of Various Treatments in Per Cent of Total Amino Acid Residues

	Untreated samples	Salt-treated samples
Lysine	1.77	2.09
Histidine	3.22	3.96
Arginine	0.46	1.22
Aspartic acid	7.35	6.28
Threonine	1.17	3.33
Serine	1.30	4.49
Glutamic acid	21.80	13.46
Proline	6.83	4.93
Glycine	17.50	12.63
Alanine	28.21	27.78
Half-cystine	0.69	0.72
Valine	3.28	4.99
Methionine	0.67	0.93
Isoleucine	1.37	2.49
Leucine	2.66	6.15
Tyrosine	0.48	1.75
Phenylalanine	1.23	2.80

sheet 6061). Lactic acid analyses were carried out using silica gel G chromatograms developed with diethyl ether and 90% formic acid (70:10, v/v), while cellulose MN300 chromatograms were developed with formic acid, 2-butanone, *tert*-butyl alcohol, and water (15:30:40:15, v/v) as described by Zaika *et al.* (1968).

Nucleotides were separated by cellulose MN300 and cellulose powder MN300 Ecteola anion exchange thin layer chromatograms. Cellulose MN300 plates were developed with *tert*-amyl alcohol, formic acid, water (3:2:1), and the Ecteola plates with 0.15 *M* NaCl (Randerath, 1966) and the purine and/or pyrimidine derivatives were detected under ultraviolet light (254 or 366 nm) with and without spraying the chromatograms with 1.0 *N* HCl or 2′,7′-dichlorofluorescein. Tests for carbonyl compounds were run by spraying the developed plates with 3.7% of 2,4-dinitrophenylhydrazine in 2 *N* HCl. Phosphate derivatives were determined by spraying the chromatograms with either 4% ammonium molybdate in 1 *N* HCl (w/v) or 1% aqueous ammonium molybdate followed, after complete drying, by 1% stannous chloride in 10% HCl (Randerath, 1966). Amino acids were detected with either ninhydrin spray (Sigma Chemical Co.) or a cadmium–ninhydrin reagent (Blackburn, 1966).

Sulfhydryl analyses were performed according to procedures described by Pepper and Pearson (1969, 1971), except that 1.0 ml of 8.5 *M* urea was added to 2.0 ml of sample. The aldosaccharide content of the diffusates was determined by the method of Müller (1965, 1967). Free amino groups were detected as described by Florkin and Stolz (1963).

RESULTS AND DISCUSSION

The per cent amino acids in the dialyzable fraction of the salt and salt-free treatments are shown in Table I. Since neither air nor nitrogen significantly influenced the free sulfhydryl groups of adipose tissue (Pepper and Pearson, 1971), all salt-treated and all untreated samples were combined for these analyses. The salt-treated samples contained greater percentages of arginine, threonine, serine, isoleucine, leucine, tyrosine, and phenylalanine than the untreated samples, with the per cent total residues of these seven amino acids comprising about 22 and 9% for the salt-treated and untreated samples, respectively. On the other hand, the untreated samples contained a greater proportion of glycine, glutamic acid, and proline than those treated with salt, comprising approximately 46% of the total amino acid residues as compared to 31% for the samples treated with salt. Although the differences between the treated and untreated samples were appreciable, the limited amount of data did not lend itself to statistical analysis.

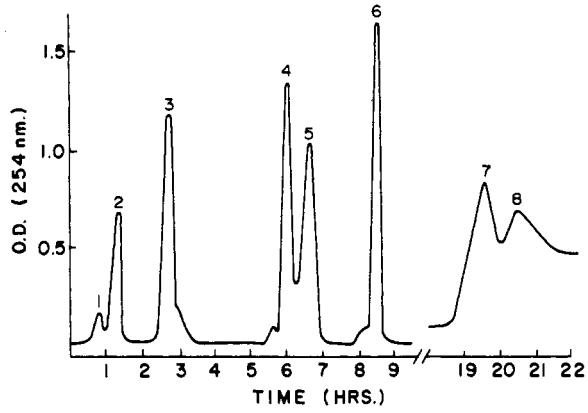

Figure 1. Column chromatographic patterns of untreated (no added salt) diffusates. Elution systems: water (0–2 hr), 0.5 *N* formic acid (2–5 hr), 1 *N* formic acid (5–12.5 hr), 3 *N* formic acid (12.5–17 hr), and 6 *N* formic acid (17–22 hr).

Leach (1966) has shown that acid hydrolysis causes some error in cystine sulfur with some of it being lost in the humin of the hydrolysate. Therefore, the per cent cystine in the samples (Table I) may be in error, but should be relative for both the salt-treated and untreated samples, since both diffusates were hydrolyzed by the same procedure. Data on the hydrolyzed whole extract can be calculated by using these data and the amino acid values presented earlier by Pepper and Pearson (1971).

Figure 1 shows the column chromatographic pattern of the untreated diffusate, which separated into eight separate fractions. The pattern for the salt-treated diffusate is not shown, but separated to give a similar chromatographic profile, with the peaks being eluted at approximately the same times. Since both diffusates had essentially identical chromatographic patterns, only the salt-treated diffusate was utilized in subsequent analyses as it was more concentrated. Chromatographic peaks 1–3 (see Figure 1) contained creatine, creatine phosphate, and creatinine, which were identified by their R_f values on thin layer chromatography or by their color reactions upon spraying with picric acid and NaOH solutions. Chromatographic peak 2 (Figure 1) contained the highest concentration of creatine and its derivatives.

Lactic acid could not be detected in any of the chromatographic peaks (Figure 1) upon employing either silica gel or MN300 plates and their respective solvent systems for thin layer chromatography. On spraying the silica gel plates for phosphates, all of the chromatographic peaks except 4 and 5 (Figure 1) gave positive reactions. Tests for carbonyl compounds were negative for all chromatographic peaks.

Chromatographic peaks 1 and 2 (Figure 1) streaked extensively on thin layer cellulose plates. Chromatographic peak 3 (see Figure 1) possessed a uv absorbing spot, which was also present in peak 4. Peak 3 (Figure 1) showed no fluorescence at a wavelength of 254 nm and fluorescence was barely evident at 366 nm. Chromatographic peaks 4–7 (Figure 1) all continued fluorescing components at both 254 and 366 nm, but the latter two peaks (7 and 8) demonstrated the most intense fluorescence. Upon developing the cellulose plates with water, peaks 4–8 showed an accumulation of yellow fluorescent material near the origin, whereas the yellow fluorescing substances appeared at the solvent front upon development with the methanol–HCl–water solvent system. All other fluorescing components showed light blue fluorescence at both wavelengths. Apparently, most of these fluorescent compounds possessed rather strong electronegative group(s), since they were only eluted with relatively concentrated acid solutions. On

Table II. Chemical Analyses of Water-Soluble Dialyzable Adipose Tissue Components

Sample	Free amino groups, nmol/g of powder[c]	Aldoses, nmol/g of powder[c]
I[a]	273.20	40.23
II[b]	48.24	3.76

[a] Combined diffusates from nitrogen and oxygen treatments without salt. [b] Combined diffusates from nitrogen and oxygen treatments with salt. [c] Lyophilized extract.

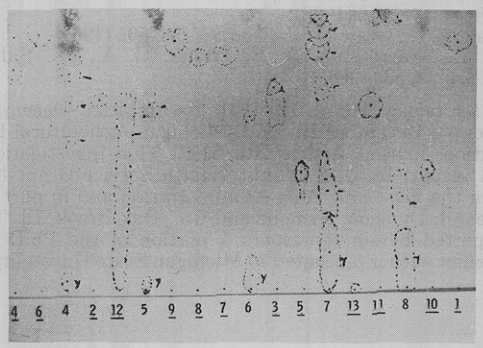

Figure 2. Thin layer chromatograms of standard compounds and the diffusates of the salt-treated components; MN300 cellulose plates; solvent, water: (1) inosine; (2) hypoxanthine; (3) cytosine; (4) cytidine; (5) adenine; (6) uridine; (7) uracil; (8) thymine; (9) thymidine; (10) adenine; (11) adenosine; (12) guanosine; (13) guanidine. All the above underlined numbers in the legend refer to standard compounds. Numbers not underlined in the legend correspond to the peak numbers shown in Figure 1.

the other hand, nucleotides, which possess a phosphate group, are eluted with 0.5 *N* formic acid and 0.2 *N* sodium formate (Lento *et al.*, 1964). Chemical analyses (Table II) revealed the presence of both aldoses and free amino groups, which are necessary for nonenzymatic browning products. Spark (1969) reported that browning products fluoresce under uv light. Therefore, some of these fluorescent components may be browning reaction products.

Figure 2 shows the thin layer chromatogram of various standard compounds. Peaks 4–8 were developed with water and correspond to the fraction numbers in Figure 1. Figure 3 presents a chromatogram, which is identical with that in Figure 2, except it was developed with a methanol–HCl–water solvent system. The dark areas of Figures 2 and 3 were produced by spraying with ninhydrin. Peaks 1–5 demonstrated ninhydrin positive compounds, while peaks 6–8 did not react appreciably with the ninhydrin spray.

Since peaks 6, 7, and 8 (Figure 1) were phosphate positive, they were subjected to thin layer chromatography in an attempt to separate the nucleotides. Figure 4 shows that peak 6 separated into three components. Spot 1 (R_f 0.33) fluoresced but was phosphate negative. Spots 2 and 3 (R_f 0.39 and 0.54, respectively) did not fluoresce but were phosphate positive. Peaks 7 and 8 (Figure 4) resulted in identical patterns with four components being detected in each of the two peaks. Spots 1, 2, and 3 (R_f 0.35, 0.44, and 0.49, respectively) fluoresced but contained no phosphate. Spot 4 (R_f 0.57) appeared to absorb uv light very faintly and gave a fairly intense positive phosphate reaction. Results showed that the components of these peaks that were phosphate positive did not fluoresce, and those that fluoresced were not phosphate positive.

To further verify whether spot 4 (Figure 4) was a nucleotide, peaks 7 and 8 were subjected to anion exchange thin layer chromatography on Ecteola plates prepared according to Randerath (1966) and using 0.15 *M* sodium chloride as the developing solvent. Peak 6 (Figure 4) was also applied to Ecteola plates to confirm the absence of

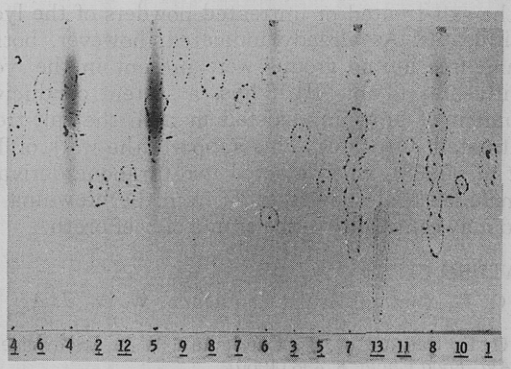

Figure 3. Thin layer chromatograms of standard compounds and salt-treated diffusate components; MN300 cellulose plates; solvent, CH_3OH–HCl–H_2O (65:17:18). See Figure 2 for sample identity.

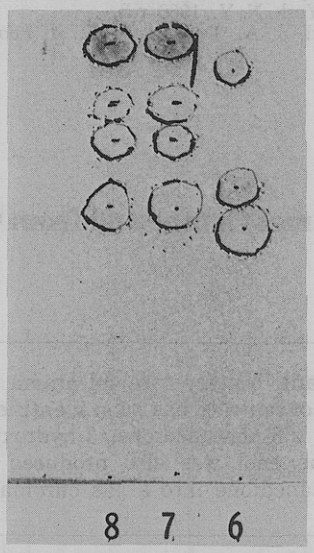

Figure 4. Thin layer chromatogram of salt-treated diffusate components; MN300 cellulose plates; solvent, *tert*-amyl alcohol–formic acid–water (3:2:1). Sample numbers correspond to peak numbers in Figure 1. Please note that the solvent front has been cut off from the chromatographic plate and that R_f values given in the text are actual measurements from the original plate.

nucleotides and separated into two components. The absence of nucleotides was further confirmed since the phosphate positive components did not coincide with the fluorescent compounds.

All thin layer chromatographic systems, except those designed for lactic acid, for creatine and its derivatives, and for nucleotides, indicated the presence of uracil and cytosine in the diffusate. Therefore, thin layer chromatographic analysis indicated the presence of uracil, cytosine, creatine, creatinine, creatine phosphate, and other phosphate-containing compounds, as well as several fluorescent compounds. However, purine bases, nucleosides, nucleotides, and lactic acid were not detected in the diffusate.

Chemical analyses of the lyophilized diffusates are given in Table II. The combined lyophilized diffusates of the salt-treated adipose tissue from both the oxygen and nitrogen atmospheres contained only about one-fifth as many free amino groups and approximately only one-tenth as many aldoses as the diffusates from the untreated tissues. This suggests that the salt treatment stabilized the adipose tissue against breakdown, which is also supported by the protective effect of 3% salt against oxidation of sulfhydryls in the nondialyzable aqueous extract of adipose tissue (Pepper and Pearson, 1971). In the present study, only traces of free sulfhydryls could be detected in

either the salt-treated or untreated powders of the lyophilized diffusates. As already indicated, however, both aldoses and free amino groups were present in the lyophilized diffusates (Table II). Thus, a system conducive to development of browning existed in both the salt-treated and untreated diffusates. This supports the work of Tonsbeek *et al.* (1968), who detected two furanone derivatives and concluded that they resulted from the browning reaction and may contribute to the aroma of beef broth.

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A New Caramel Compound from Coffee

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A pleasant, buttery caramel aroma from a coffee aroma concentrate has been identified as the enol form of 2,3-pentanedione, 3-hydroxy-3-penten-2-one. The enol was also produced by injecting 2,3-pentanedione into a gas chromatograph with a high injection port temperature (300° or higher). The unknown was trapped and identified by ir and nmr. The enol could be stabilized as its trimethylsilyl ether and its structure was further confirmed by mass spectrometry.

During a gas chromatographic fractionation of a coffee aroma concentrate, a particularly pleasant and desirable coffee-caramel aroma was isolated. This was identified by mass spectrometry as 2,3-pentanedione. However, the ir and nmr spectra indicated that this compound was more likely the enol form of the α-diketone.

Numerous reports appear in the literature on the studies of keto–enol tautomerism of carbonyl compounds, triacylmethanes, and cyclodiketones (Koltsov and Kheifets, 1971). Gerbier (1962) compared the enolization of 2,4-pentanedione, 2,5-hexanedione, and 2,3-butanedione as the pure liquid, dilute CCl_4 solutions, and vapor states. He found that enolization occurred in all three states for 2,4-pentanedione, and was complete in the vapor state, although only one C=O group was enolized in each molecule. This evidence was not as clear for 2,5-hexanedione. From this evidence he concluded that the H atom participating in enolization came from a CH_2 group situated between the two C=O groups.

Schwarzenback and Wittwer (1947) reported 2,3-butanedione to be enolized in the pure state, to the extent of 0.0056%. Except for this report, there have been no reports on the enolization of aliphatic α-diketones.

EXPERIMENTAL SECTION

Preparation of 3-Hydroxy-3-penten-2-one. Portions (100 mg) of redistilled 2,3-pentanedione were injected into a Perkin-Elmer Model 800 gas chromatograph, with a flame-ionization detector and effluent splitter, and dual stainless steel columns (⅛ in. × 6 ft) packed with 15% OV-101 on Anakrom ABS, 80–90 mesh size. The operating conditions were as follows: injection port temperature, 300–350°; column temperature, 4 min at 70°, then programmed to 300° at 5°/min; detector temperature, 300°; helium flow rate, 30 ml/min; hydrogen, 30 psi; and air flow rate, 360 ml/min. The injection port temperature had to be maintained at 300–350° in order to facilitate enolization. The component eluted at 3.5 min contained mainly the enol form and had a buttery caramel aroma. It was collected into a melting point tube cooled with Dry Ice. The collected material was dissolved in a small amount of Freon 113 and the enol form was purified by rechromatography of the Freon solution on a Perkin-Elmer Model 900 gas chromatograph, with a flame ionization detector and effluent splitter, and dual stainless steel columns (⅛ in. × 6 ft) packed with 15% stabilized DEGS on Anakrom ABS, 80–90 mesh size. The operating conditions were as follows: injection port temperature, 180°; column temperature, 50–175° at 2.5°/min; detector temperature, 180°; helium flow rate, 17 ml/min; hydrogen, 22 psi; air flow rate, 54 psi. The injection port temperature was kept at 175–180° in order to prevent thermal degradation of the enol form at higher temperatures. The pure enol (R_t of 12.9 min) was trapped into a melting point tube as described above. The unenolized 2,3-pentanedione was eluted at 5.8 min.

2,3-Pentanedione can also be enolized at a lower temperature in the presence of an acid. It was mixed with hydrochloric acid in a ratio of 2:1 in a closed vial and heated at 110° for 1 hr. After being cooled to room temperature, the mixture was extracted with Freon 113. The enol form

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