COMPOSITION OF THE AQUEOUS EXTRACT OF BEEF ADIPOSE TISSUE

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This is to certify that the

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COMPOSITION OF THE AQUEOUS EXTRACT

OF BEEF ADIPOSE TISSUE

presented by

Fred Harold Pepper

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Food Science

A. M. Pearson
Major professor

Date 20 Sept 1969

APPENDIX

COMPOSITION OF THE AQUEOUS SOLUBLE AND INSOLUBLE PROTEIN

by [illegible]

Beef adipose tissue was [illegible] and insoluble (water-salt-ether) [illegible] sulfide evolved upon heating [illegible] yield of hydrogen sulfide [illegible] 8.5 uM for the water soluble, [illegible] spectively. Thus, the value [illegible] hydrogen sulfide, which [illegible] soluble and insoluble [illegible]

The water soluble fraction of [illegible] treatments (air and [illegible] atmosphere). The [illegible] were determined. The [illegible] dialyzable and non-dialyzable [illegible] by pervaporation and dialysis [illegible] protein concentrates were [illegible] until used. The diffusates from the [illegible] heating at reduced pressure or lyophilization.

The dialysates were subjected to [illegible] disc gel and starch gel electrophoresis and [illegible] employing gel filtration chromatography was [illegible] electrophoresis did not give adequate [illegible] separation of the proteins was accomplished [illegible] phoresis. The dialysates were negative [illegible] and lipoproteins, but positive for [illegible]

ABSTRACT

COMPOSITION OF THE AQUEOUS EXTRACT OF BEEF ADIPOSE TISSUE

by Fred Harold Pepper

Beef adipose tissue was separated into the water soluble, salt soluble and insoluble (water-salt-ether) fractions, and the amount of hydrogen sulfide evolved upon heating each of the fractions was determined. The yield of hydrogen sulfide per 100 g of adipose tissue was 17.6, 1.7 and 5.5 µM for the water soluble, salt soluble and insoluble fractions, respectively. Thus, the water soluble fraction contributed 71% of the hydrogen sulfide, while the corresponding contributions from the salt soluble and insoluble fractions amounted to approximately 7 and 22%.

The water soluble fraction of adipose tissue was subjected to four treatments (air and nitrogen atmospheres and 3% sodium chloride under each atmosphere). The sulfhydryl groups and other possible flavor precursors were determined. The aqueous extract from each treatment was divided into dialyzable and non-dialyzable fractions. The dialysates were concentrated by pervaporation and dialysis against a 1 M sucrose solution. The sucrose-protein concentrates were separated into 10 ml aliquots and stored at -23°C until used. The diffusates from the treatments were concentrated by either heating at reduced pressure or lyophilization.

The dialysates were subjected to column chromatographic analysis, disc gel and starch gel electrophoresis and chemical analysis. Separation employing gel filtration chromatography was not satisfactory. Starch gel electrophoresis did not give adequate separation, however, satisfactory separation of the proteins was accomplished utilizing disc gel electrophoresis. The dialysates were negative for glycoproteins, nucleoproteins and lipoproteins, but positive for heme proteins. The sulfhydryl contents

of the nitrogen, air, nitrogen with 3% sodium chloride and air with 3% sodium chloride treatments were 9.05, 8.17, 16.00 and 15.79 μM per g of protein. Thus, there was a slight atmospheric effect on the sulfhydryl contents, since the air treatments for both the salt and salt-free treatments were lower than those under nitrogen. The salt treatments under either air or nitrogen contained almost twice the sulfhydryl content of the salt-free treatments. The increase of sulfhydryls in the salt treatment was also reflected in the disc gel electrophoretic patterns, which revealed a greater number of sulfhydryl containing bands.

Amino acid analysis of the dialysates demonstrated the presence of lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. Glycine, alanine and glutamic acid comprised approximately 25% of the amino acid residues. There was no difference in the amino acid content of the proteins from the various treatments.

The diffusates were satisfactorily separated on Dowex 1 anion exchange resin (formate form), however, Bio-Gel P-2 resin gave inadequate separation. The diffusates from the salt-free treatments under both nitrogen and air atmospheres were similar to those of the corresponding salt treatments. However, the diffusates from the salt treatments were more concentrated because of different procedures of concentrating.

Chemical analyses of all diffusates demonstrated only traces of sulfhydryl groups. Diffusates from the salt and salt-free treatments contained 48.24 and 273.20 μM of free amino groups and 3.76 and 50.23 μM of aldoses per g of lyophilized powder, respectively. All diffusates contained the same amino acids as the dialysates, but glycine, alanine and glutamic acid

comprised over 50% of the amino acid residues. Thin layer chromatography demonstrated that the diffusates contained creatine, creatine phosphate, creatinine, uracil, cytosine, some unidentified phosphate containing components and a number of fluorescent compounds. Purine bases, nucleosides, nucleotides and lactic acid were absent.

A quantitative method for the determination of sulfhydryl and disulfide groups in proteins based upon the ultimate formation of an azo dye was investigated. After reduction of the disulfides, the resulting sulfhydryl groups were nitrosated with nitrous acid. The S-nitroso derivatives were cleaved with mercuric chloride and the resulting nitrosyl chloride was combined with sulfanilamide to form a diazonium salt. The diazonium salt was coupled with N-1-naphthylethylenediamine to form an azo dye. The dye absorbs maximally at 540 nm; and the thiol gives a 1 to 1 stoichiometric relationship with the dye. The only substances interfering with the dye development are those that destroy either nitrous acid or the diazonium salt.

COMPOSITION OF THE AQUEOUS EXTRACT OF BEEF ADIPOSE TISSUE

By

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INTRODUCTION

For years chemical identity of meat flavor has been an intriguing problem. Since the primary criterion to consumer acceptance is meat flavor, studies concerning its identity and origin are most important. The literature contains many controversies pertaining to meat flavor components and their origin.

Some researchers have indicated that whole muscle is responsible for meat flavor (Barylko-Pikielna, 1957), while other workers have concluded that the flavor components are located only in structural proteins (Crocker, 1948). Generally, however, most research has indicated that meat flavor components are located in the water soluble portion of muscle (Kramlich and Pearson, 1958; Hornstein and Crowe, 1964).

While some investigators have suggested that fatty tissues play only a minor role in meat flavor (Pippen et al., 1954; Hofstrand and Jacobson, 1960), other investigators have demonstrated that fatty tissues are responsible for differences in the flavor of meat from various species (Hornstein and Crowe, 1960b; Wasserman and Talley, 1968).

Hydrogen sulfide has been shown to be a major contributor to the characteristic meaty odor, since the aroma of cooked chicken persists as long as hydrogen sulfide is evolved (Bouthilet, 1951a). Subsequent studies on hydrogen sulfide have also implicated sulfur-containing compounds, primarily in the meaty aroma of heated chicken (Minor et al., 1965b). The water insoluble fraction of chicken muscle has been shown to produce twice the quantity of hydrogen sulfide as the water soluble

fraction (Mecchi et al., 1964). Similarly, Hamm and Hofmann (1965) have shown that the myofibrillar fraction is responsible for practically all of the hydrogen sulfide evolved from beef muscle.

The present study was undertaken to determine the amount and source of hydrogen sulfide in beef adipose tissues. In addition, other possible flavor precursors in adipose tissues were identified and studied.

REVIEW OF LITERATURE

Definition of Flavor

Moncrieff (1967) stated that of all the qualities of flavor, taste and odor are of the greatest importance. He indicated that texture (i.e., smoothness, roughness, particle size and solubility) is next in importance, with less important qualities being the hotness of spices and the coolness of menthol. Finally, he indicated that there are the metallic, alkaline and meaty qualities. He also stated that in addition to the four true tastes, i.e., sweet, sour, bitter and salty, there are metallic and alkaline taste sensations.

In a theory that correlated different odor qualities to the physical properties of the compound, Amoore (1967) categorized these qualities as having a minimum of seven characteristic odors, namely, ethereal, camphoraceous, musky, floral, minty, pungent and putrid. He further concluded that molecular shape and/or charge determined the sensory response to different odors.

Flavor Components of Muscle

Muscle can be divided into two principle components. One is water soluble and the other is water insoluble. These components have been studied to learn their role in the production of flavor precursors, and also as to the origin of volatile compounds evolved upon heating. Each will be studied herein.

Water Soluble Components

Meat extracts. Early investigators were concerned with the origin and precursors of meat flavors. In 1956, Wood investigated beef extract by exhaustively extracting it with methanol. The alcohol-soluble compounds were identified as glutamic acid, α-alanine, β-alanine, glycine, proline, methyl histidine, carnosine, anserine, creatine, creatinine, hypoxanthine, inosine, choline, carnitine, betaine, urea and citrulline, as well as other proteinaceous and inorganic materials.

In another study, Wood and Bender (1957) concentrated beef extract to 45% of its initial volume at reduced pressure. Concentration was continued at atmospheric pressure by holding the solution at 75°C. The final concentrate was exhaustively dialyzed against distilled water and the diffusate was electrodialyzed. The compounds in the diffusate were identified by paper chromatography as: methyl histidine, alanine, serine, methionine, leucine, isoleucine, histidine, taurine, carnosine, anserine, creatine, creatinine, hypoxanthine, inosine, carnitine, choline, urea, ammonia, citrulline, lactic acid, glycollic acid, succinic acid and β-hydroxybutyric acid. The main differences between the extracts obtained by two procedures were: 1) different amino acid profiles, and 2) the presence of organic acids in the aqueous extract (Wood and Bender, 1957).

Bender et al. (1958) filtered beef extract, concentrated the filtrate in a rotary evaporator at 40°C and lyophilized the concentrated filtrate. The freeze-dried powder was dialyzed against distilled water at room temperature, and the diffusate was concentrated in a rotary evaporator at 40°C. All of the amino acids detected previously by Wood (1956) and

Wood and Bender (1957) were identified, plus aspartic acid, lysine, phenylalanine, threonine, tyrosine and valine. Creatine and creatinine were determined. It was also noted that creatine was found in much larger quantities than creatinine. The extract was also shown to contain 2.1% of reducing sugars.

Wood (1961) prepared a diffusate by dialyzing beef extract, but upon lyophilization, the anhydrous powder rapidly absorbed moisture from the air and turned brown. Subsequently, the beef extract was treated with perchloric acid and filtered. The filtrate was neutralized, lyophilized and solubilized with a small quantity of water. The slightly soluble potassium perchlorate was removed by filtration, and the filtrate was lyophilized and stored over phosphorous pentoxide at 0°C until analyzed. Purine and pyrimidine derivatives were identified as adenosine mono-, di- and triphosphate, adenine, hypoxanthine and inosine. Glucose-6-phosphate, fructose-6-phosphate, ribose, glucose and fructose were identified as the carbohydrates present along with ribose-5-phosphate, which was only tentatively identified.

Bender and Ballance (1961) concentrated and heated commercial beef extract. The volatiles evolved were gas chromatographically analyzed. Many carbonyl compounds were identified, as well as a number of sulfur-containing compounds, i.e., methyl mercaptan, ethyl mercaptan, methyl sulfide and hydrogen sulfide. They concluded that raw meat has little flavor and showed that the potential for flavor development resides in the water soluble extract.

Meat flavor precursors. Bouthilet (1951b) declared that the "meaty" flavor of chicken is found in the water extractable portions of the meat fibers and not in the organic soluble fraction of the fatty tissues. Pippen et al. (1954) further demonstrated that chicken flavor precursors could be extracted from raw chicken with cold water.

Batzer et al. (1960) extracted fresh beef muscle with water, dialyzed the extract and concentrated the diffusate. The concentrate was redialyzed with dialysis tubing of a smaller pore size. This separated the original diffusate into a high molecular weight fraction and a low molecular weight fraction. They further separated the high molecular weight fraction into two parts using a Sephadex column. One was composed of proteins, while the second contained smaller polypeptides. The low molecular weight fraction was divided into two portions by a Dowex ion exchange resin. One fraction contained carbohydrates, while the second was composed of amino acids. When they heated the polypeptide fraction with fat, a broiled steak aroma was evolved. When the protein, polypeptide and carbohydrate fractions were combined and heated in fat, a meaty aroma of the same intensity as the original diffusate was evolved.

Batzer et al. (1962) further analyzed the polypeptide fraction and isolated a glycoprotein. On heating the glycoprotein with glucose and inosinic acid, a meaty aroma resulted. Hydrolysis of the protein yielded the following amino acids: α-alanine, β-alanine, isoleucine, leucine, proline, serine, valine and two unknown ninhydrin-positive compounds. One compound, comprising 80% of the unknown fraction gave a positive

test for sulfur. Doty (1961) working in the same laboratory reported that heating of the isolated polypeptide fraction from chicken and pork loin resulted in almost the same odor as that from beef muscle.

Macy et al. (1964a) compared the water soluble diffusates of beef, lamb and pork. The diffusates of all three species contained alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, glycine, histidine, hydroxyproline, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine, valine, anserine, carnosine, glutamine, glycerophosphoethanolamine, 1-methyl histidine, phosphoethanolamine, phosphoserine, taurine, urea, ribose, fructose, glucose, inosine, creatinine, hypoxanthine and possibly maltose. The diffusate from the three species differed in the fact that cysteic acid and ornithine were only obtained from pork and lamb, while glutathione was found only in lamb.

Wasserman and Gray (1965) fractionated the water soluble diffusate of defatted beef muscle. The double dialysis method of Batzer et al. (1962) was utilized, but analysis showed that the dialyzate and diffusate of the second dialysis contained the same components. Thus, separation by this procedure was unsuccessful. The dialysate was separated into three fractions employing a Sephadex column. Only one fraction possessed a meaty aroma on heating. This fraction was lyophilized, but the dried powder absorbed water and turned brown in a very short time. The dark brown solution developed the aroma of commercial beef extract. The diffusate was separated into three components by a cation exchange column. Two components were eluted with water, while the third was released with

ammonium hydroxide. After the components were lyophilized, the fraction eluted with ammonium hydroxide was the only one that developed a meaty aroma upon pyrolysis.

The ammonium hydroxide-eluted fraction was separated further by these investigators (Wasserman and Gray, 1965) with an anion exchange resin. This resulted in three additional components, which were eluted with water, 6 mM and 2 M HCl, respectively. The first two components gave off a meat-like aroma, but combining these two fractions did not enhance the aroma. The third fraction contained only hypoxanthine. The components eluted with ammonium hydroxide contained both free amino acids and low-molecular-weight water-soluble proteins. Wasserman and Gray (1965) readily determined the presence of free amino acids, but the water-soluble peptides were not detected until acid hydrolysis revealed an increase of free amino acids. Of all the amino acids, only tryptophan and serine showed decreases. The authors expected a decrease in tryptophan, since it is destroyed by acid hydrolysis. A decrease in serine was also expected, since glutamine, asparagine and serine were eluted simultaneously, thus, masking serine. They explained that acid hydrolysis converted glutamine and asparagine to their respective amino acids, so that only serine itself was determined.

Seven amino acids were found in the diffusate by paper chromatography (Wasserman and Gray, 1965). However, eighteen amino acids were found using the amino acid analyzer. The latter discrepancy could not be explained. The authors concluded that meaty aroma is primarily due to amino acids, polypeptides and possibly hypoxanthine.

In a subsequent investigation, Zaika et al. (1968) fractionated the low-molecular-weight water-soluble diffusate of beef muscle utilizing

column chromatographic resins. Separation was achieved by gel filtration (Bio-Gel P-2), adsorption (Amberlite XAD-2) and ion exchange (DEAE-Sephadex) chromatography. Many compounds were identified which evolved a meaty aroma upon heating. At least a score of compounds were isolated in the odoriferous fractions. Many of these were present only in trace amounts. The authors did not elucidate the responsible compounds, but they demonstrated that many of them could be removed without seriously affecting the aroma. Such compounds were identified as: tyrosine, phenylalanine, taurine, glutamic acid, inosinic acid, inosine, hypoxanthine, and possibly creatine and creatinine.

Zaika et al. (1968) obtained seven different fractions by ion exchange chromatography. Only three of them produced aromas, although none were characteristic meaty aromas. Fraction 1 possessed an amine like odor; fraction 3 had a potato-like aroma and was pungent, while fraction 4 produced a faint but sweet and acrid odor. When fraction 4 was combined with either fractions 1 or 3, the aroma became more meaty. When all three fractions were combined, the aroma was very similar to that of the original diffusate.

Zaika et al. (1968) reported that fraction 4 contained tyrosine, glucose, fructose, ribose, inosine and hypoxanthine, but stated that tyrosine, inosine and hypoxanthine could be eliminated without seriously affecting meaty aroma. In a previous investigation, Wasserman and Gray (1965) had reported that no carbohydrates were found in the fraction responsible for meaty aroma. These results are completely contradictory, since Zaika et al. (1968) had reported that fraction 4 is necessary for

meaty aroma, yet all of the compounds in this fraction were eliminated as contributors to meaty aroma (Wasserman and Gray, 1965; Zaika et al., 1968).

Koehler and Jacobson (1967) separated chicken into light and dark meat fractions, slurried each with water and dialyzed the slurries. The non-dialyzable portions were filtered. Unheated diffusates and filtrates had similar "raw meat" and "salty" tastes, but heated diffusates of light and dark muscles possessed a much stronger chicken flavor than their respective filtrates.

The dialysates were lyophilized and separated into four fractions by gel filtration. The second fraction from the light meat dialysate possessed a characteristic chicken flavor, both before and after heating, whereas, similar treatments of the corresponding dark meat fraction resulted in a less pronounced chicken flavor. No chicken flavor was detected in any of the remaining fractions.

Recombined unheated light meat fractions possessed a weak chicken flavor, which was intensified upon heating. However, recombination of the dark meat fractions produced "brothy", "meaty", "rich" and "sulfury" flavors, which were more fully developed with heat, but in neither case were flavors produced that resembled chicken. These findings substantiated the earlier results of Minor et al. (1965b; 1966), who demonstrated that dark meat was responsible for "meaty" aroma, while light meat possessed a characteristic "chickeny" aroma.

The second fraction of white meat contained sixteen amino acids, ribose, fructose, glucose, lactic acid, inosine, IMP (inosine monophosphate),

GMP (guanosine monophosphate), carbonyls, sulfhydryls and small polypeptides. The corresponding dark meat fraction contained all the components of the light meat fraction except arginine, leucine and/or isoleucine, threonine, tyrosine, valine and polypeptides. The pH of the light meat fraction was 5.8 compared to 7.2 for the dark meat.

Meat flavor volatiles. Bouthilet (1950) steam distilled a chicken-water slurry and concentrated the distillate. The concentrate had an amine type odor until the pH was lowered by adding monobasic potassium phosphate. The lowering of the pH eliminated the amine odor and produced a strong distinct chicken aroma. The author then concluded that broth concentrate could be used to determine the chemical properties of chicken flavor.

Kramlich and Pearson (1958) observed that the water soluble components from fresh beef muscle produced a meaty aroma upon heating. They were the first to state that the flavor constituents of both cooked and raw meat appeared to be in the water soluble fraction. The difference between the aroma of the water soluble cooked fraction and the heated water extract of raw meat was only one of intensity.

Hornstein et al. (1960a) water extracted beef muscle and filtered the slurry. The filtrate was lyophilized and placed in an evacuated system, which was heated to 100°C. The volatile components were collected in a trap cooled with liquid nitrogen and identified as: acetone, formaldehyde, acetaldehyde, ammonia, hydrogen sulfide and possibly methyl amine. These investigators observed that dried beef powder gave off an aroma similar to roast beef upon heating, but upon boiling, a characteristic boiled beef odor was evolved.

In a continuation of the previous study, Hornstein and Crowe (1960b) fractionated the volatiles of beef and pork muscles. The volatiles collected in a cold trap were separated into a volatile and non-volatile fraction by allowing the cold trap to come to room temperature. The volatile fraction was isolated by collection in an adjacent cold trap, while the non-volatile fraction remained in the original trap. Hydrogen sulfide, ammonia, carbon dioxide, acetone, formaldehyde and acetaldehyde were found in the volatile fraction of water extracts from both species. Upon analyzing the non-volatile fraction from both species, a maximum absorbance at 290-295 nm was determined. Infra-red spectral analysis of the non-volatile material showed beef and pork samples to be identical, while tests for phosphorous and sulfur were negative. It was also noted that the odor characteristics from the non-volatile fractions of both species were the same. The authors stated that the ultra-violet absorbing compound(s) from both samples may be responsible for the "meaty" aroma.

In elucidating their theory that the non-volatile fraction was responsible for "meaty" aroma (Hornstein and Crowe, 1960b), Hornstein et al. (1963a) studied the water-soluble fraction of whale muscle. The whale muscle extract was heated and fractionated as previously described (Hornstein and Crowe, 1960b). The non-volatile fraction had an amine type odor, rather than the "meaty" aroma that was anticipated. However, upon heating the fraction to 70°C to remove the trimethylamine, a similar "meaty" aroma was evolved. Gas chromatographic profiles of beef, pork and whale were the same, except for the trimethylamine peak found in the whale volatiles.

Hornstein and Crowe (1963b) subjected lamb to the same analysis as beef and pork (Hornstein and Crowe, 1960b). In addition to analysis of the collected volatile fractions, the original heated solution was also investigated for any residual aromas, but no appreciable aroma was detected. The collected volatile fraction from lamb had only a very weak sulfide odor with hydrogen sulfide being barely detectable, whereas, beef and pork had a high sulfide content. The non-volatile (room temperature) fraction from lamb demonstrated absorption at 290-295 nm and had a "meaty" aroma very similar to beef and pork.

Yueh and Strong (1960) slurried ground lean beef with water and heated the slurry. The volatiles were collected and identified as hydrogen sulfide, ammonia, acetaldehyde, acetone and diacetyl. In addition, formic, acetic, propionic, butyric and isobutyric acids were tentatively identified along with methyl sulfide. No alcohols or esters could be detected, while hydrogen sulfide, ammonia, diacetyl and acetone appeared to be the major components responsible for cooked beef aroma.

Jacobson and Koehler (1963) water extracted and dialyzed cooked and raw lamb. The same amino acids and sugars were found in both diffusates. The volatile compounds identified in cooked lamb were carbonyls, ammonia and hydrogen sulfide. Results led them to conclude that variations in carbonyl compounds may be responsible for the aroma of lamb.

Kramlich and Pearson (1960) refluxed a beef-water slurry and swept the volatiles into a cold trap with nitrogen gas. The volatiles were gas chromatographed and identified as acetone, acetaldehyde, methyl mercaptan, hydrogen sulfide and possibly methyl sulfide.

Macy et al. (1964b) quantitatively determined the components of beef, lamb and pork diffusates both before and after heating. The lyophilized diffusates were dissolved in water and divided into two aliquots. One aliquot was analyzed for various components upon solubilization, while the second was heated in a boiling water bath for 1 hr. The heated aliquot underwent extensive browning and evolved a "brothy" odor in all cases. Quantitatively, the amino nitrogen compounds of all species seemed to be quite similar. Taurine, anserine-carnosine and alanine were the major constituents in both the heated and unheated samples. Other constituents degraded during heating were glutamic acid, glycine, lysine, serine, cystine, methionine, leucine, isoleucine and methyl histidine. Phosphoethanolamine increased in beef, pork and lamb, whereas, histidine increased only in beef and pork and methionine only in pork.

Macy et al. (1964b) also found that the carbohydrate content of all three species was about the same. Glucose was present in the highest quantity, followed by fructose, ribose and an unknown fraction. Ribose appeared to be the most heat labile, while fructose showed the greatest heat stability.

Water Insoluble Protein Fractions

Crocker (1948) stated that meat flavor resides in the meat fibers of cooked meat and not in the expressible fluid. He also indicated that flavor is not derived from the fat, but is probably obtained upon hydrolysis of protein. However, Barylko-Pikielna (1957) indicated that the three fractions of cooked beef, i.e., meat fibers, denatured water-soluble proteins

and extracted substances, all participate in the development of full meaty flavor.

Greenwood et al. (1951) reported that the amino acid content of crude protein was similar in different grades of beef and also in different cuts. The amino acids in these cuts were stable to cooking. The cooked samples contained the same percentage of amino acids as the raw samples, except that isoleucine, histidine and serine all decreased by approximately 10% in the cooked samples.

Hornstein (1967) water extracted beef muscle, centrifuged the slurry and retrieved the protein residue. The residue was heated to 100°C under vacuum and the volatiles were collected in a cold trap. Only traces of ammonia could be detected and no meat-like aromas were discernible. The author also stated that heating of the original aqueous extract resulted in meaty aromas. This provided proof that regardless of their origin, the flavor precursors are water soluble, while the insoluble substances make no contribution to flavor.

Fatty Tissue Flavor Components

Barbella et al. (1936) found that lambs on a restricted ration yielded less desirable flavor than similar lambs full fed on the same ration. The amount of fat and protein was less for the lambs on the restricted diet, so the authors concluded that the desirability of flavor varied directly with fatness.

In further investigations, Howe and Barbella (1937) observed that a characteristic flavor was produced by the fatty tissues. The flavor became

more pronounced at higher temperatures, especially when browning occurred. The authors postulated that the flavor characteristics reside chiefly in the fatty tissues. In agreement, Jones (1952) reported that lean contributes very little to meat flavor, while fat is mainly responsible for flavor development. On the other hand, Pippen et al. (1954) stated that the flavor precursors of chicken are water extractable and that fat only contributes to the aroma of the broth.

Hornstein and Crowe (1960b) stated that there are no characteristic flavor differences in the water-soluble fractions of beef and pork. Thus, they suggested that the species characteristic flavor differences reside in the fatty tissues. They melted and filtered fatty tissues from both species. The filtrate was taken up in petroleum ether and passed over an ion exchange resin to isolate the fatty acids. The fatty acids were then measured quantitatively.

Hornstein and Crowe (1960b) also heated fatty tissues under reduced pressure and in the presence of air. The fatty acids found upon heating adipose tissue under vacuum were the same as those retained by the ion exchange column. Upon heating in air, however, large concentrational changes were noted in fatty acids due to the hydrolytic effect of water on glycerides. They found that the fatty acid gas chromatographic profiles varied both qualitatively and quantitatively between beef and pork fatty tissues.

Similar results were obtained for carbonyl compounds by Hornstein and Crowe (1960b). Heated adipose tissue showed a low carbonyl content. However, the fatty tissues heated in air evolved a much greater quantity

of carbonyl compounds due to oxidation. They concluded that similar meaty flavors are obtained upon heating lean from both beef and pork, but that flavor differences have their origin in the fatty portions. They further concluded that fatty tissues may have different ratios of compounds and different lipid soluble components which may contribute to flavor.

Hornstein et al. (1963a) analyzed the fatty acid fraction from fatty tissues of the whale. The gas chromatographic profile was dissimilar to the same fraction from beef and pork. Whale fat had a high percentage of long chained polyunsaturated fatty acids. Since the water soluble fractions of all three species were similar but the fatty fractions differed, the authors concluded that the species characteristic flavor originated from differences in fat composition.

Hornstein and Crowe (1965b) cooked lamb adipose tissue in water at 100°C and noted a very strong mutton odor. They theorized that carbonyl compounds in the fat were responsible for mutton odor, even though only a small quantity of these compounds was found in comparison to beef and pork. On the basis of several studies, Hornstein and Crowe (1964) concluded that species flavor differences are determined by components in the fatty tissues.

Flavor Components Due to Browning

Howe and Barbella (1937) observed that the characteristic lamb flavor is produced in the fatty tissues, but becomes more pronounced at higher temperatures, especially upon browning. Herz and Shallenberger (1960) reported that heating of glucose with free amino acids resulted in a

variety of aromas. They indicated that the aromas vary not only with different amino acids, but also at different reaction temperatures. When cystine and cysteine were heated with glucose, only sulfide aromas were detected. Upon using methionine, it resulted in evolution of a potato-like aroma. However, no meaty aroma was developed with any of the amino acids. Pearson et al. (1962) spectrophotometrically measured the colored components produced by browning of lean pork. They found that the color developed was closely correlated to the level of reducing sugars in the tissues. However, there was little correlation between color and free amino nitrogen. In a subsequent paper, Pearson et al. (1966) concluded that browning of pork muscle was primarily due to the Maillard reaction with some of the brown color originating from caramelization.

Lobanov and Wolfson (1958) concluded that degradation products of free amino acids and reducing sugars in meat products brought about the formation of unsaturated melanoidins, which they stated are responsible for the yellow tint of meat broths. Upon refluxing free amino acids and reducing sugars, melanoidins were formed that smelled, tasted and had the color of meat broths.

Wood (1961) reported that ribose-5-phosphate reacted more readily with amino acids than other reducing sugars, producing the browning reaction. Browning products of glucose and a number of amino acids possessed a bitter flavor, but upon heating glucose with a combination of components found in meat extracts, a meaty flavor was obtained. The author then stated that heating developed the brown color and flavor, which are characteristics of the Maillard reaction.

Macy et al. (1964b) stated that the interaction of carbohydrate and amino nitrogen during heating is one of the most important aspects in the production of desirable flavors and odors in meat. Hornstein and Crowe (1964) separated a reducing sugar fraction and an amino acid fraction from the water-soluble portion of beef muscle. They noted that no meaty aroma was evident upon heating each individually. However, when both fractions were recombined and heated, a meaty aroma occurred.

Wasserman and Gray (1965) separated the components of an aqueous beef extract and obtained a specific fraction which was responsible for the meaty aroma upon heating. The fraction was analyzed and contained aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine, tryptophan and ammonia. However, glucose and ribose, which are associated with the Maillard reaction, were not found.

Tonsbeek et al. (1968) reported the presence of 4-hydroxy-5-methyl-3(2H)-furanone (I) and its 2,5-dimethyl homolog (II) in an ether extract of beef broth. Compound I had a caramel type color, and component II had the aroma of roast chicory root. These compounds could be synthesized by combining reducing sugars and amines. Both compounds showed strong absorption at approximately 287 nm. The authors concluded that these odorous dihydrofuranones contribute to the composite flavor of beef. Since Hornstein and Crowe (1960b) isolated unidentified compound(s) from water extracts of beef having similar absorption patterns (270-295 nm), it is probable that they isolated the same or similar compounds.

Flavor Components Involving Hydrogen Sulfide

Many papers have been published on the detection of hydrogen sulfide in the volatile fraction of chicken (Sadikov et al., 1934; Bouthilet, 1949, 1950, 1951a, 1951b; Pippen and Eyring, 1957; Lineweaver and Pippen, 1961; Kazeniac, 1961; Mecchi et al., 1964; and Minor et al., 1965a, 1965b, 1965c, 1966), beef (Stahl, 1957; Yueh and Strong, 1960; Kramlich and Pearson, 1960; Hornstein et al., 1960a; Hornstein and Crowe, 1960b; Hamm and Hofmann, 1965), lamb (Hornstein and Crowe, 1963b; Jacobson and Koehler, 1963), and pork (Hornstein and Crowe, 1960a).

Sadikov et al. (1934) reported that hydrogen sulfide was produced upon heating chicken muscle and appeared to be due to complete decomposition of glutathione, cystine and methionine. The authors postulated that some of the hydrogen sulfide was reabsorbed by the protein and oxidized to free sulfur. They also stated that glutathione was an important chicken flavor precursor. Similarly, Bouthilet (1951b) reported that glutathione was a possible hydrogen sulfide precursor in chicken volatiles. Mecchi et al. (1964) observed that although glutathione released hydrogen sulfide about 160 times as fast as chicken muscle, there was not enough glutathione to play a significant role in hydrogen sulfide production. Therefore, they stated that sulfur-containing proteins were the precursors of hydrogen sulfide.

Minor et al. (1965a, 1965b, 1965c, 1966) refluxed chicken-water slurries for various times under different atmospheres and compared the volatile fractions of light and dark muscles. Hydrogen sulfide was detected under all conditions at temperatures greater than 80°C. They

reported that upon heating older chickens and dark muscles more hydrogen sulfide was evolved than from younger chickens or light muscles. Minor et al. (1965b) also reported that breast muscle volatiles had a typical "chickeny" aroma, whereas, leg muscle possessed a "chicken-like" aroma, but with a definite "meaty" aroma reminiscent of roast beef. Upon passing the volatiles through various solutions, they found that elimination of sulfur components resulted in an almost complete loss of "meaty" odor, while removal of the carbonyl compounds eliminated the "chickeny" odor, but intensified the "meaty" aroma. They thus stated that sulfur compounds were important to meaty odors.

Mecchi et al. (1964) reported that during 2 hr of boiling, leg and breast muscle produced hydrogen sulfide at about equal rates. Isolated leg and breast muscle proteins produced 75 and 84 % as much hydrogen sulfide as their respective intact muscles. Nonprotein muscle fractions evolved no detectable amounts of hydrogen sulfide during the 2 hr cooking period. They also stated that 9-12 μg of hydrogen sulfide are evolved per min for every 100 g of chicken muscle. This is a significant amount since hydrogen sulfide can be detected in concentrations of as little as 1.1 μg per liter of air (Fieldner et al. 1931).

Hornstein et al. (1960a) determined that 0.1 mg of hydrogen sulfide was evolved per gram of lyophilized aqueous beef muscle extract. They also noted that dried beef powder evolved a roast beef aroma upon heating. On the other hand, boiling of the powder resulted in boiled beef aroma.

Hamm and Hofmann (1965) reported no loss in free sulfhydryl groups upon heating beef myofibrills to 70°C, but upon attaining a temperature

of 80°C hydrogen sulfide was detected in the volatile fraction. When myofibrils were heated to 120°C for 30 min, there was a decrease in sulfhydryl groups. All of the decrease in sulfhydryl groups could be accounted for by an increase in disulfide groups except for 7%. They also found that heating the myofibrils under a nitrogen atmosphere did not significantly decrease the free sulfhydryl groups. They reported that the formation of hydrogen sulfide increased with temperature and cooking time. Upon heating myofibrils at 120°C for 5 hr, the quantity of both free sulfhydryls and disulfides declined. Whole beef muscle and beef myofibrils contained essentially the same quantity of hydrogen sulfide per unit myofibrillar protein, which suggests that all hydrogen sulfide originates in the myofibrillar fraction. This led the authors to conclude that hydrogen sulfide originates from the readily available free sulfhydryls of the structural proteins and not from the water soluble fraction of beef muscle.

Organoleptic Evaluations of Meats and Model Systems

In the investigation of chicken flavor, Minor et al. (1966) devised three model systems, which were primarily composed of compounds found in chicken broths and distillates. The first system was composed of sodium sulfide, lactic acid, water and in some cases carbamyl phosphate. The second system contained all the compounds present in the first system plus glutathione and diacetyl. The third system consisted of methionine in addition to all compounds in the second system. The systems were heated to 180°F. They organoleptically evaluated the systems after heating.

The first system possessed an egg-like odor without carbamyl phosphate and an odor similar to chicken volatiles upon addition of carbamyl phosphate.

The second system used by Minor et al. (1966) had a chicken odor and an egg-like taste. The third system yielded a strong sulfide, oily butter-like aroma with the connotation of breast meat broth odor. The investigators reported that the addition of monosodium glutamate (MSG) improved the taste of the first and third systems. An additional improvement was noted when disodium inosinate (DSI) and disodium guanylate (DSG) were added to the above systems in addition to MSG. However, the systems with the added flavor enhancers did not produce a true chicken broth flavor. The addition of taurine to the third system plus MSG, DSI and DSG imparted a serum-like taste more similar to dark muscle broth. No change was noted when creatine was added.

Klose et al. (1966) were somewhat skeptical of the conclusion drawn by Minor et al. (1965b, c) that removal of the volatile carbonyls eliminated the "chickeny aroma", because the acidic hydrazine solution would also remove basic compounds. In subsequent studies, these authors conducted odor evaluation tests on heated chicken volatiles by utilizing solid absorbents, which would neither chemically modify nor concentrate the effluent gases. They found that more hydrogen sulfide and ammoniacal components were produced by dark meat than light meat. Results indicated that chicken aroma was due to the blending of hydrogen sulfide with the ammoniacal compounds and another fraction with an unpleasant aroma was not characterized. Upon recombination of various effluents, they could

not duplicate the original chicken aroma, although chicken aroma was developed upon heating the solid absorbent and purging it with nitrogen gas.

Wasserman and Talley (1968) conducted organoleptic identification experiments on beef, lamb, pork and veal. When the panel was given samples without standards for comparison, only 33% of the panelists identified all four meats correctly. Upon repeating the same experiment, but with known samples present for comparison, 66% identified all four unknowns.

Upon conducting identification investigations on beef, Wasserman and Talley (1968) reported that panelists identified roasted beef roasts, with all visible covering fat removed, as beef 82.0% of the time. Lean ground and roasted beef (devoid of fat) was identified as beef by 42.5% of the panel, while naturally trimmed roasts were ground, roasted and correctly identified 71.5% of the time, whereas, a mixture of lean roast beef combined with 10% of beef adipose tissue was ground, roasted and then identified correctly by 90.2% of the panel. These results led the authors to investigate the role played by fatty tissues in species characteristic flavors. They combined ground lean veal, a bland meat, with 10% beef adipose tissue. The panel identified it as beef in 40.8% of the cases. Beef adipose tissue was extracted according to Folch et al. (1957) and the solvent-free fat extract was added to lean veal to a level of 10% fat. The panel identified the mixture as beef 38.5% of the time.

On investigating pork and lamb flavor, Wasserman and Talley (1968) found that compound(s) in the fatty tissues imparted a species specific flavor. In pork, the fatty tissue flavoring factor(s) were water soluble, whereas, in lamb the factor(s) were due to component(s) of the fat or fat soluble fractions.

Investigation of Analytical Techniques in Sulfhydryl and Disulfide Determination

Numerous methods have been developed for the determination of free sulfhydryl and disulfide groups in proteins and other biochemical compounds. The advantages and disadvantages of the available methods will be discussed herein.

General sulfhydryl analytical techniques. In a review concerning the estimation of thiol and disulfide groups, Leach (1966) discussed the advantages and disadvantages of several methods. He indicated that iodine, ferricyanide, hydrogen peroxide and other oxidizing reagents should be avoided since they lack sulfhydryl specificity. He further noted that metal salts of copper, mercury, silver and zinc combine specifically with thiols when used in very low concentrations. It was also noted that the concentration and pH of alkylating agents such as iodoacetate, iodoacetamide, methyl bromide and N-ethylmaleimide (NEM) must be carefully controlled to prevent reactions with $-SCH_3$, $-NH_2$ and -OH sites, however, these alkylating agents are more -SH specific than either oxidizing agents or metal salts. He finally stated that organic mercurials (RHgX) are the most -SH specific compound available, but he also acknowledged that simple alkylmercuric halides and alkylating agents reacted with labile disulfides. Thus, these methods gave high sulfhydryl values due to slow hydrolysis of the disulfide bonds.

General colorimetric, amperometric and spectrophotometric techniques. Leach (1966) designated three general techniques for determining sulfhydryls: colorimetric, amperometric and spectrophotometric. He classified the colorimetric methods as qualitative. Three common colorimetric methods exemplified by Leach (1966) were sodium nitroprusside, dithizone and thiofluorescein. Upon using sodium nitroprusside, he stated that development of a pink-violet color indicated the presence of thiols. Dithizone solutions changed from purple to yellow to confirm a thiol content, while thiofluorescein changed from colorless to blue in thiol-containing solutions.

The second general technique for sulfhydryl determinations outlined by Leach (1966) was amperometric titration. This method is particularly useful for determining sulfhydryl groups in colored compounds and substances that are partially or completely insoluble.

Leach (1966) stated that the disadvantages of titrating with silver nitrate or mercuric chloride were their tendencies to complex with the titrated mercaptides and the limited specificity of silver nitrate. He listed two types of electrodes for use in amperometric titrations - rotating platinum and dropping mercury electrodes. The advantages of the rotating platinum electrode are its simple, inexpensive construction, its sensitivity (10^{-5} M -SH) and its use with different metal ions (Cu, Ag, Hg). The disadvantages are its tendency to become "poisoned", micro-fractures at the glass-platinum joint, low over-voltage for hydrogen discharge (thus limiting the working plateau) and the inability to titrate disulfide groups with mercuric chloride. The advantages of the dropping mercury electrode are its trouble-free service and its high over-voltage. The main disadvantage is its low sensitivity (10^{-4} M -SH).

The third general sulfhydryl technique cited by Leach (1966) was spectrophotometric analysis. These methods, as with amperometric titrations, are used for quantitative analysis. He reported that using p-mercuribenzoate as the sulfhydryl reagent gives relatively sensitive (10^{-4} -10^{-5} M -SH) and specific results. The disadvantages of this method were the common absorption of the reagent, products and protein, which all absorb in the same ultra-violet region (250-255 nm). The molar absorptivity was affected by pH, urea and ethylenediamine tetraacetate. Also, the wavelength was read on the slope of the absorption peak, thus, small spectral shifts resulted in large absorbance errors. Finally, Leach (1966) stated that other disadvantages were the limited reagent specificity (steric hinderance) and its instability in solution.

In discussing the use of NEM to determine sulfhydryls, Leach (1966) stated that the only advantage was that protein precipitation did not interfere with the analysis. He pointed out several disadvantages. The product had a low molar absorptivity, reactivity was low and the reagent was unstable in alkaline solutions. Absorbance was read at 300 nm. The low sensitivity of the reagent and the necessary high protein content resulted in high blank absorbance.

Fluorescence. Fluorescence, either development or quenching, for determination of sulfhydryl or disulfide groups has the advantage of great sensitivity. However, the methods suffer from limited application and narrow experimental conditions.

McNeil and Beck (1968) developed a fluorometric determination of glutathione employing o-phthalaldehyde (OPT). However, they reported the method to be specific only for glutathione.

Karush et al. (1964) found that fluorescein mercuric acetate (FMA) had a molar absorptivity of 7.8×10^4 at 497 nm in 1.0N NaOH. They also stated that at a neutral pH sulfhydryls quench the fluorescence while disulfides do not. However, at a higher pH, both groups quench the fluorescence. The pH of the solution, the concentration of the sulfur-containing compounds and the FMA concentration must be carefully controlled, since a stoichiometric relationship between quenching and disulfide concentration exists only over very low disulfide concentrations.

Ionic chromophores. The use of chromophore producing disulfides is advantageous because of the specificity in sulfhydryl determinations and the existence of a molar absorptivity, thus, eliminating the construction of a standard curve. Ellman (1959) employed DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] to determine free sulfhydryl groups. Grassetti and Murray (1967) employed the same principle for thiol determinations, but the compounds used were either 2,2'- or 4,4'-dithiodipyridine. The principle disadvantage of these methods is the inability to determine disulfide groups. However, Cavallini et al. (1966) used DTNB to determine disulfides in proteins after reduction with sodium borohydride, but the method is quite laborious.

Zahler and Cleland (1968) described dithioerythritol (DTE) as a sensitive and specific reducing agent for disulfides. After disulfide reduction, the sulfhydryl groups were determined with DTNB. When DTE was used to

reduce the disulfide bonds of bovine serum albumin, however, the results were not satisfactory. DTE was useful as a disulfide reducing agent for low molecular weight disulfides.

Ninhydrin as a cysteine specific reagent. Gaitonde (1967) determined cysteine with an acid ninhydrin reagent. He reported that the chromophore produced had a molar absorptivity of 2.8×10^4. Cystine was determined after reduction with dithiothreitol, which did not interfere, since the determination is specific for cysteine. The disadvantage of the method is its inability to determine cysteine residues in proteins.

Indirect sulfhydryl analysis by spectrophotometric methods. Saville (1958) developed a method for the indirect determination of thiols, and Liddell and Saville (1959) slightly modified the method for the determination of cysteine and reduced glutathione. The method was based on the determination of nitrous acid, which was quantitatively cleaved from the S-nitroso derivative of the thiol. The thiol was reacted with an excess of nitrous acid, which formed a stable S-nitroso derivative. The excess nitrous acid was eliminated by treating the solution with ammonium sulfamate. The S-nitroso compound was then combined with a solution containing mercuric chloride and sulfanilamide. The mercuric chloride complex ($HgCl_4^{-2}$) cleaved the S-N bond to produce a mercuric mercaptide and nitrous acid. The sulfanilamide was added simultaneously with mercuric chloride because it favorably competed with ammonium sulfamate for the liberated nitrous acid. In an acid medium, the nitrous acid combined with sulfanilamide to form p-sulfamoylbenzenediazonium chloride. N-1-naphthylethylenediamine dihydrochloride was added to the diazonium salt solution and the electrophilic

diazonium salt was rapidly coupled to the nucleophilic aromatic amine to form the intense reddish-violet azo dye p-{4-[(2-aminoethyl)-amino]azo} benzenesulfonamide.

Liddell and Saville (1959) stated that the only critical points to be observed were the initial amount of thiol to be determined and the final dilution volume. They indicated that the timing, accuracy of other reagent additions and concentration of acids are only of secondary importance. If the concentration of nitrous acid was doubled, the calibration results never deviated more than 2%. By allowing the excess nitrous acid to react with the thiols up to 6 min, and the ammonium sulfamate to react up to 5 min, little change was noted in the results. Upon increasing the acid content tenfold in the sulfanilamide and naphthylamine solutions, the time for color development was increased to 20 min, while the final results were practically unchanged.

The possibility of interference in the thiol determination by various substances was investigated by Liddell and Saville (1959). Secondary amines, which form N-nitroso compounds, were added to the reaction mixture. Up to 5 mM dimethylamine was added with no notable effect. A 1000-fold excess of glycine, alanine, tyrosine, arginine, valine, lysine, leucine, isoleucine, histidine, threonine, serine and proline produced no interference. They indicated that nitrosation of cysteine was completed before any deamination of the excess amino acids occurred. Cystine, aldehydes, ketones and alcohols did not interfere with the reaction. Chloride and bromide ions did not interfere, but large amounts of mercuric, silver and cupric ions caused serious interference. Zinc ions did not interfere, thus, cystine can be determined as cysteine after reduction with zinc and HCl.

Leach (1966) stated that alkaline hydrolysis degraded both -SH and -SS-groups and that acid hydrolysis destroyed -SH groups. Thus, the main disadvantage of this method is its inability to determine cystine residues in proteins without hydrolysis.

METHODS AND MATERIALS

Beef adipose tissue was analyzed and fractionated to determine the amount of hydrogen sulfide evolved upon heating, to ascertain the principle fraction responsible for hydrogen sulfide production, and to determine hydrogen sulfide precursors and other flavor precursors in this fraction.

Adipose Tissue Sample Collection and Preparation

The trimmable subcutaneous and intermuscular fatty tissues from three U.S. Choice grade steer carcasses were combined and ground twice at 4°C through a 12.7 mm plate, and twice through a 3.2 mm plate with thorough mixing between each grinding. The samples were placed in Cry-o-vac bags, evacuated, purged with nitrogen, reevacuated and stored at -29°C until used. Samples were used to determine the amount of hydrogen sulfide evolved and its primary source (Study I).

A second study (Study II) was designed to analyze for hydrogen sulfide and other flavor precursors. The adipose tissue was collected from three different U.S. Choice grade steer carcasses and coarsely ground twice at 4°C with thorough mixing. The ground tissue was mixed for 1 min in an evacuated Hobart vertical cutter-mixer, and the mixer was brought to atmospheric pressure with nitrogen.

The adipose tissue was separated into four portions and divided into 500 g aliquots. The first portion was placed in Cry-o-vac bags. Each bag purged with nitrogen several times, evacuated and sealed (N_2). The second portion was similarly treated except that the aliquots were purged with air

(0_2). The third portion was prepared the same as portion one, except that 3% of solid sodium chloride was added (N_2S). Portion four was prepared the same as portion two, except that 3% of solid sodium chloride was added (0_2S). All samples were stored at -23°C for a two month period.

Fractionation of Adipose Tissue Samples

The fractionation procedures utilized in Study I are shown in Figure 1. All water used in this study was deionized and distilled. Aliquots of adipose tissue (2 x 75 g) were mixed with cold (4°C) water in a 250 ml stainless steel homogenizer flask and homogenized for 1 min with a Virtis "45" homogenizer, while being cooled in an ice-water bath. The homogenate was placed in centrifuge bottles from which the necks and shoulders had been removed, and was centrifuged in an International Refrigerated Centrifuge (Model PR-2) at 1600 x g at 0°C for 30 min. Centrifugation separated the homogenate into three phases: (1) the bottom phase - composed of a small amount of mucous-like material, which was discarded; (2) the middle phase or the aqueous layer; and (3) the top phase or the fatty fraction containing the fat and remaining insoluble protein. The aqueous phase was decanted and the bottom phase was discarded. The top phase was extracted with water and centrifuged as previously described until almost no color remained in the aqueous layer. The aqueous extracts were combined, filtered through a milk filter to remove all fatty particles, lyophilized and stored in a dessicator at 4°C until used.

The top phase was extracted and centrifuged as previously described using 0.45 M sodium chloride instead of water. The combined salt soluble extracts were pervaporated and dialyzed against water at 4°C until no salt

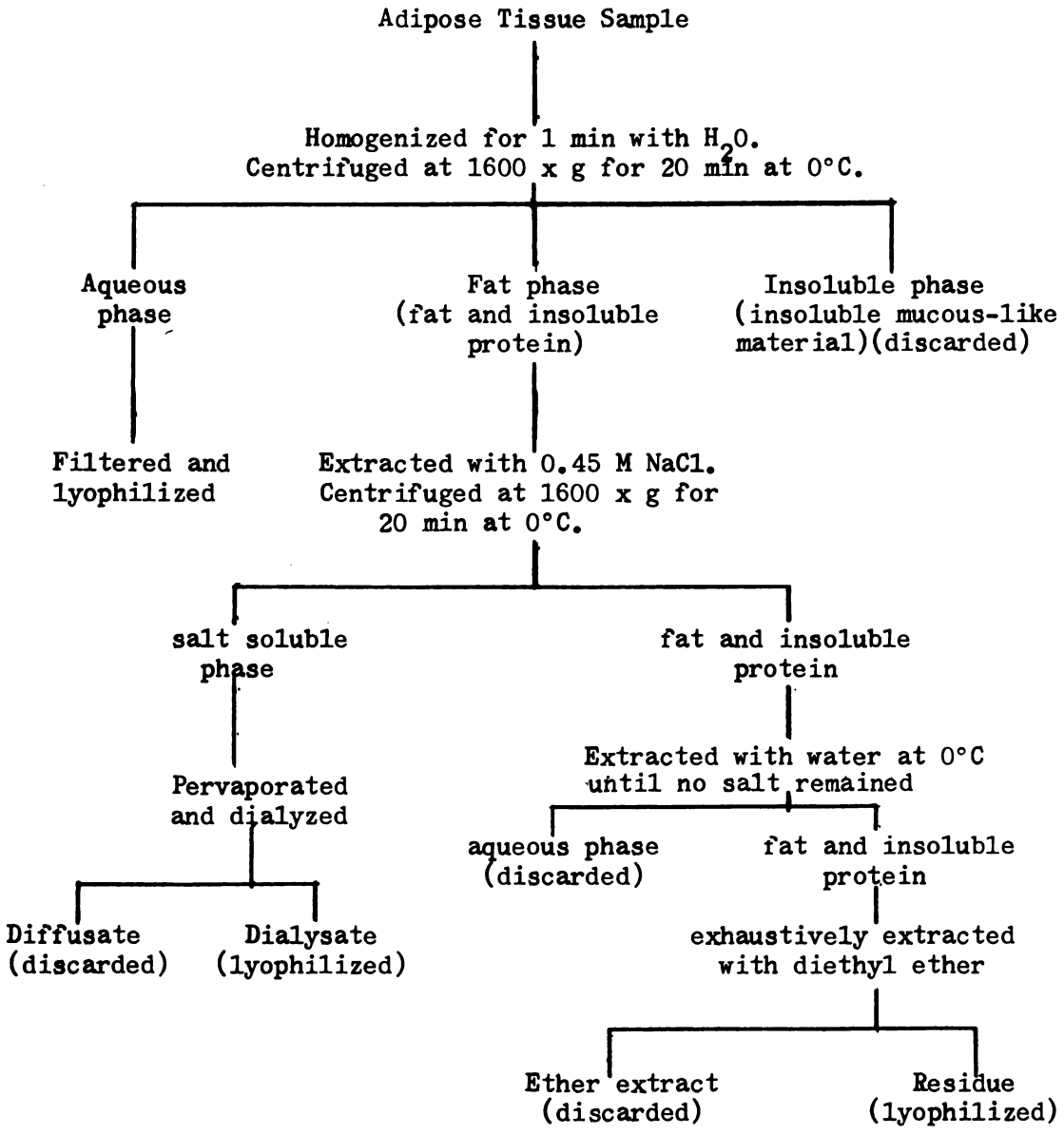


Figure 1. Fractionation of beef adipose tissue.- Study I

remained. The dialysis tubing had previously been boiled in water for 2 hr. The salt soluble fraction was then slurried with water, lyophilized and stored in a dessicator at 4°C.

The top phase was extracted with water and centrifuged until all salt was removed. The salt-free fatty residue was extracted with cold (4°C) diethyl ether until no further fatty material was apparent. The excess ether was removed under reduced pressure and the insoluble (water-salt-ether extracted) fraction was slurried with water, lyophilized and stored at 4°C in a dessicator.

Fractionation procedures for Study II are outlined in Figure 2. All water used in this study was deionized, distilled and saturated with toluene to prevent microbial growth. Aliquots of adipose tissue samples (100 g) were successively homogenized with 125, 75 and 50 ml portions of borate buffer (pH 7.8; μ = 0.1) containing 2 mM EDTA (disodium ethylenediaminetetraacetate) for 1 min under the conditions previously described.

Borate buffer was used because it does not interfere with nitrogen or phosphorous determinations. An ionic strength of 0.1 and a pH of 7.8 was selected since Ellman (1959) had previously suggested this concentration and approximate pH for free sulfhydryl determinations. Free sulfhydryl groups are stable at pH 7.8, and the low salt concentration would have little affect on the water soluble proteins, while the salt soluble fraction would not be dissolved. A boric acid sodium borate buffer was prepared by calculating the necessary concentrations to give an ionic strength of 0.1 and a pH of 8.0. Actually a pH of 7.8 resulted due to the low pH of the water.

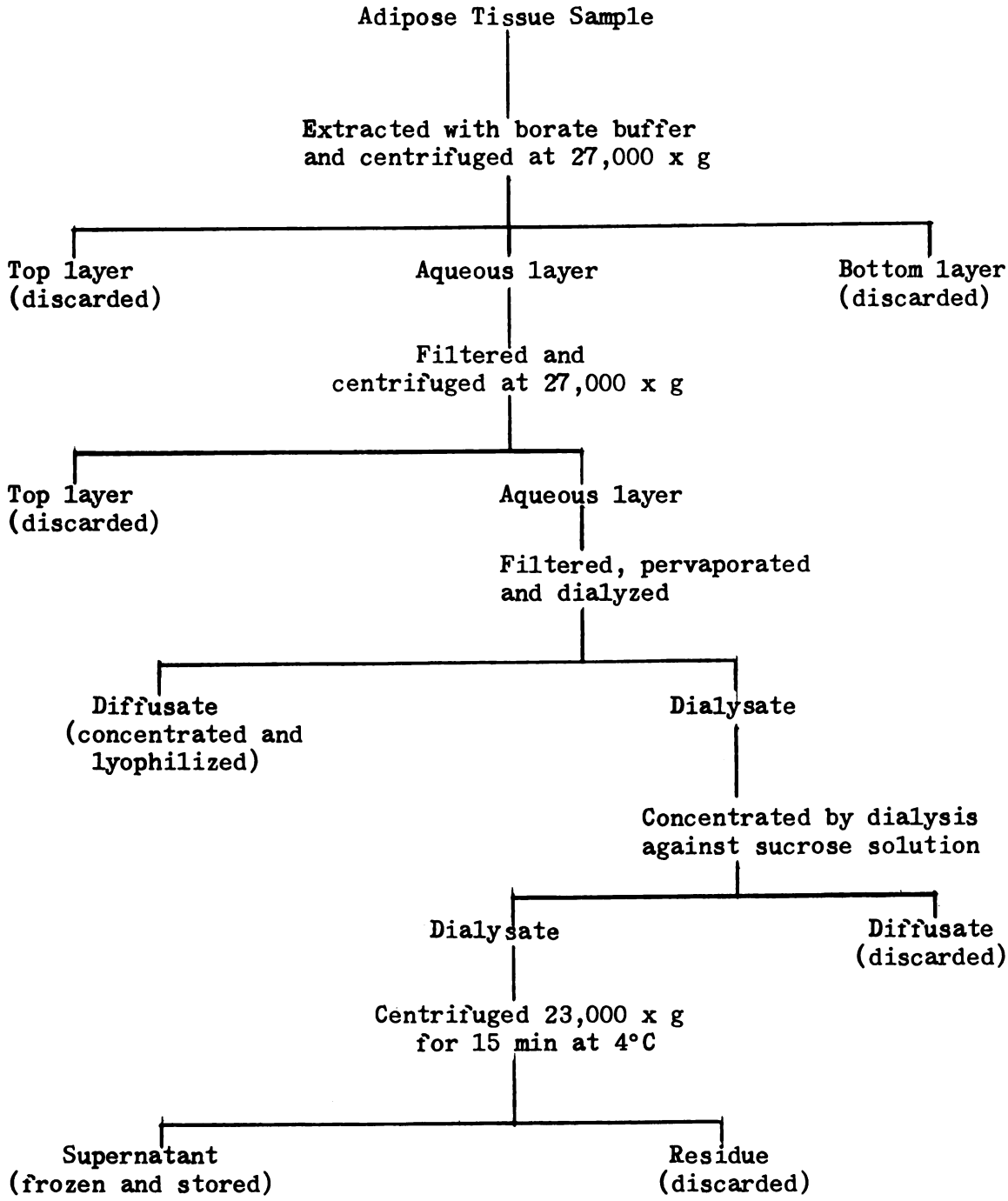


Figure 2. Fractionation of adipose tissue for flavor precursor analysis - Study II.

The extraction solvent contained 2 mM EDTA, since Scopes (1968) had previously utilized this concentration for extracting muscle. EDTA was used since it complexes with and removes any divalent cations, which may act as catalysts for oxidation of free sulfhydryl groups in the sarcoplasmic proteins from muscle.

The aqueous solution was decanted after each homogenization. After homogenizing, the solutions and fatty tissues were combined, placed in stainless steel centrifuge cups and centrifuged at 0°C for 20 min at 27,000 x g in a Sorvall (Model RC2-B) centrifuge equipped with a GSA rotor (5.25 cm radius). The aqueous layers were collected, combined and filtered through a milk filter to remove any large particles. The other layers were discarded. The filtrate was then centrifuged. The fatty material was concentrated in the top layer and solidified in the cold (0°C) centrifuge. The layer of fatty material was removed with a spatula, and the aqueous layer was filtered through Whatman No. 2 filter paper. The filtrate appeared slightly turbid indicating the presence of suspended fatty particles.

Further filtration through various types of filter paper (Whatman Nos. 5, 40, 50) did not eliminate the turbidity. Ultracentrifugation at 95,000 x g (average) in an International Preparative Ultracentrifuge (Model B-60) with an A-170 type rotor also failed to remove the turbidity. However, the turbidity was removed by suction filtration using a filter pad (1.5 cm in thickness) made by pulverizing Whatman No. 40 filter paper trimmings with deionized distilled water in a Waring blender. A disc of Whatman No. 2 filter paper was placed in a filter funnel attached to a Büchner filter flask and moistened with deionized distilled water. The paper-water slurry was

poured into the funnel and compressed into a pad by suction filtration. The pad was covered with a disc of Whatman No. 2 filter paper and the aqueous extract was filtered into a Büchner filter flask cooled in an ice-water slurry. When filtration was completed, the pad was rinsed with 25 ml water. One drop of toluene was added to every 150 ml of filtrate to prevent subsequent microbial growth.

Preparation of Muscle Sample

A sample of beef muscle was prepared in order to compare the water soluble extracts from adipose tissue with those from muscle. Beef muscle (10.0 g) was trimmed of all fat and combined with a solution of 0.3 M sucrose in the borate buffer (pH 7.8)(20 ml) previously described herein. The mixture was blended at 4°C for 1 min in a Waring blender. The slurry was centrifuged in a Sorvall centrifuge (Type SS-1) at 4°C for 15 min at 19,400 x g. The extract was stored at 4°C and used within two days to develop disc gel and starch gel electrophoretic patterns.

Preparation of Dialyzable and Non-Dialyzable Components of Adipose Tissue

The filtrate, which contained the water soluble components, was poured into 2.1 cm (diameter) dialysis tubing. The dialysis tubing was prepared by boiling in a 2 mM EDTA solution for 20 min, and thoroughly rinsing with water. It was then boiled twice for 20 min with deionized distilled water, and thoroughly rinsed. The filtrate was pervaporated and concentrated at 4°C to a final volume of 50-75 ml. During pervaporation, the tubing was kept wet with the internal solution in order to prevent drying and cracking.

Upon completion of the pervaporation, the dialysis tubing and its contents was placed in a one liter Erlenmeyer flask and dialyzed against water. The water was changed at 12 hr intervals, until approximately four liters of diffusate had been collected. The non-dialyzable material was transferred to 1.53 cm dialysis tubing and concentrated by dialysis for 24 hr at 4°C against one liter of 1 M sucrose solution, which contained borate buffer and EDTA as previously described herein. The sucrose solution had previously been decolorized with activated charcoal.

Centrifugation of the concentrate at 23,000 x g for 15 min at 4°C in a Sorvall centrifuge (Type SS-1) produced a supernatant solution and a small amount of precipitate. The precipitate was discarded, and the supernatant solution was placed in 6 oz Whirl-Pak bags, frozen and stored at -23°C.

The diffusates were concentrated by either lyophilization or heating in a flash evaporator at 40°C under reduced pressure. The flask evaporator was not allowed to rotate, since rotation resulted in the formation of fine protein strands. The precipitate resulting from concentration in the flash evaporator was removed by filtration through Whatman No. 2 filter paper. The residue was washed with 10 ml of 4°C water, giving a final diffusate volume of 90-95 ml. A 10 ml aliquot was lyophilized, and ultimately utilized for detailed chemical analysis.

Concentration by lyophilization was accomplished by freezing a four liter sample on a 45.5 cm x 60.5 cm x 4.5 cm stainless steel tray. The tray was covered with cheese cloth to prevent loss of the dried powder, and placed in a RePP Sublimator (Model FFD 42 WS). After concentration, a 150 ml portion of water was swirled in the tray to rinse it and to dissolve the

powder. The tray was rinsed again with 50 ml water. Some powder adhered to the cheese cloth and could not be recovered. The rinses were combined and lyophilized using a laboratory constructed lyophilization apparatus. The dried powder was slurried with 90 ml water, and the precipitate was removed and washed as previously described. The final volume was approximately 95 ml.

Adipose Tissue Analysis

The moisture, fat and total nitrogen analysis were carried out the same for all samples, except that the digestion procedure for nitrogen was carried out at a lower temperature and for a longer time for the sucrose-protein concentrates. The modification prevented the charred sample from bubbling into the neck of the micro-Kjeldahl flask.

The fat and moisture content was determined according to Benne et al. (1956). Aliquot samples (10 g) of beef adipose tissue were weighed in disposable aluminum dishes and placed in a drying oven (approximately 100°C) for a period of 24 hr. The dishes were removed from the oven and allowed to cool to room temperature in a dessicator. The dried samples were weighed and the percent moisture was calculated.

Fat content was determined by ether extracting the dried samples using a Goldfisch fat extractor. After the samples had been extracted for 3.5 hr, the ether was removed and the extractor flasks containing the ether soluble material were placed in the oven to remove any residual moisture. The previously weighed flasks were reweighed after drying, and the fat content was calculated.

Total nitrogen analyses were performed according to the micro-Kjeldahl method (American Instrument Company, 1961). The equipment included a rotary digestion unit, 100 ml micro-Kjeldahl flasks and a steam distillation-condensation apparatus. No attempt was made to differentiate between protein and non-protein nitrogen.

Hydrogen Sulfide Determinations

All glassware used in this determination was rinsed successively in chloroform, acetone, tap water and 6 N HCl. It was then washed with Alconox detergent and rinsed several times with water. After cleansing in this manner, no residual zinc sulfide could be detected.

Hydrogen sulfide was determined according to the method of Sands et al. (1949). A standard curve was prepared using sodium sulfide as the source of the sulfide ion. One or two sodium sulfide crystals were rinsed with water to remove any residual sulfides, dried between acid washed filter paper, weighed and made to volume with water. The sulfide concentration was determined by adding an excess of standardized iodine solution to an aliquot of the sodium sulfide solution, and back titrating with a standardized sodium thiosulfate solution. The calibration curve was determined from serial dilutions of the standardized sodium sulfide solution, but otherwise employing the method of Sands et al. (1949).

The cooking apparatus was essentially the same as that described by Mecchi et al. (1964). The collection apparatus was equipped with the radial manifold-trap system of Minor et al. (1965b). A series of traps and a Pyrex brand flow meter, using the 1/4 mm orifice, were attached to the radial manifold. The flow rate of nitrogen gas was maintained to give a pressure differential of 120-125 mm of ethylene glycol.

A slurry of 150 g adipose tissue and 300 ml water was gently refluxed for a period of 7 hr, starting from the time of turning on the glass-col heating mantle. The volatiles were swept through each trap with nitrogen gas previously purified by scrubbing with an aqueous solution containing 2% $KMNO_4$ and 3% $HgCl_2$. Each trap contained 50 ml of 2% zinc acetate. The volatiles were bubbled sequentially through each trap for a period of 1 hr. The nitrogen gas flow rate and the heating rate were selected to give a faint turbidity due to zinc sulfide precipitating in each trap after 1 hr or heating.

After 7 hr of refluxing, each trap was emptied and rinsed with 2% zinc acetate. The trap contents and its rinsings were combined and made to a volume of one liter by adding the necessary amount of 2% zinc acetate solution. To quantitate the amount of zinc sulfide in each trap, 50 ml aliquots of the collected zinc sulfide solutions were added to 125 ml Erlenmeyer flasks and cooled to approximately 0°C in an ice-water bath. To this solution, 5.0 ml of a 0.1% solution of N,N-dimethyl-p-phenylenediamine sulfate in 24 N sulfuric acid were added and gently stirred. Then 1.0 ml of ferric chloride solution (2.7 g of $FeCl_3.6\ H_2O$ dissolved in 50 ml concentrated HCl and diluted to 100 ml) was added. The resulting solution was gently swirled and allowed to stand for approximately 30 min until maximum color was developed. Absorbance was determined at 745 nm.

The residual zinc sulfide adhering to each trap was determined by adding 2% zinc acetate (50 ml) and developing the color in each trap. The analysis was carried out as outlined above. Total hydrogen sulfide was obtained by adding the hydrogen sulfide in the trap contents and rinsings to that adhering to the trap. Hydrogen sulfide was determined for the water soluble,

salt soluble and insoluble lyophilized fractions in the same manner. The slurry in all three cases was composed of 2 g lyophilized powder in 300 ml water.

Amino Acid Analysis of Dialyzable and Non-Dialyzable Components

Amino acid analyses were conducted on the non-dialyzable and diffusate fractions. Aliquots (10 ml) of the sucrose-protein concentrate were placed in 2.1 cm dialysis tubing and tied to allow a minimum of sample space. Dialysis was performed against one liter of water for 12 hr, and the water was changed once. Amino acid analysis was conducted according to the method of Moore et al. (1958), except that the proteins were hydrolyzed for 22 hr. Qualitative and quantitative amino acid determinations were accomplished by using a Beckman (Model 120C) amino acid analyzer.

For determination of amino acids in the diffusate, an aliquot (5.0 ml) was lyophilized. The dried powder was extracted three times with methyl ethyl ketone containing 5% 6 N HCl (3 x 5 ml), and the extract was filtered through a fine frited glass filter. The method described by Smith (1960) was used except that no water was added to the extract before it was removed at 4°C under a stream of nitrogen gas. Two ml of deionized distilled water was added to the concentrate. The lyophilized diffusate (1.0-1.5 g) obtained from the salt-containing adipose tissue was extracted twice with methyl ethyl ketone containing 5% 6 N HCl (2 x 25 ml), and then once with methyl ethyl ketone (25 ml). The extract was filtered through a fine fritted glass filter and concentrated at room temperature in a rotary evaporator until almost dry. Three ml of deionized distilled water was added and the concentrate was refiltered.

Sufficient HCl was added to the concentrated diffusate to bring it to 6 N. The concentrates were hydrolyzed and amino acid determinations were carried out as previously described herein.

Detection of Various Non-Dialyzable Components by Disc Gel and Starch Gel Electrophoresis

The method and system of Davis (1964) was used with minor modifications. Cyanogum (E. C. Apparatus Co.) was substituted for acrylamide and N,N'-methylenebisacrylamide. Riboflavin was utilized for photopolymerization of the gels, but no sample gel was used. A modification of the method of Jolley et al. (1967) was used to prepare the running gel solutions. A 7.5% running gel solution was made by combining three solutions (A, B, C). Solution A consisted of 6 ml 2 N HCl, 9.1 g Tris [Tris (hydroxymethyl) amino methane], 0.06 ml TEMED (N, N, N,' N'-tetramethylethylenediamine) and deionized distilled water to bring the final volume to 25 ml. Solution B was a 30% (W/V) aqueous solution of Cyanogum, which was filtered through Whatman No. 1 filter paper before using. Solution C was made by dissolving 1 ml riboflavin in 25 ml deionized distilled water. The final running gel solution was composed of A, B, C and deionized distilled water in a ratio of 1.25:2.50:1.25:5.0, respectively. In making a 5% running gel solution, solution B contained 20% Cyanogum. In making a 5-10% linear gradient gel, a 5% gel solution was placed in one chamber and a 10% gel solution was placed in the second chamber of a two chamber gradient device. The 10% gel solution consisted of a 40% Cyanogum solution (Solution B). The running gel was produced by degassing the gel solution, and filling the glass tubes

(6.5 cm x 0.5 cm I.D.) to a depth of 5 cm. The gel solution meniscus was leveled with deionized distilled water and photopolymerized with fluorescent light on both sides for 15 min.

The spacer gel consisted of three solutions (I, II, III). Solution I was prepared by mixing 4 ml 2 N HCl, 1 g Tris and 0.06 ml TEMED, and was diluted to 80 ml with deionized distilled water. Solution II was a 33.3% aqueous solution of Cyanogum. Solution III was the same as Solution C, which was described earlier herein. The three solutions were combined to give ratios of 4.0:1.0:1.67 for solutions I, II and III, respectively.

After the running gel had been polymerized, the water was completely removed. The spacer gel solution was degassed and immediately placed on top of the running gel to a depth of 0.75 cm. Upon leveling the surface with water; the gel solution was photopolymerized for 20 min as described for the running gel.

After removing the leveling water and placing the tubes in the electrophoresis apparatus, 550 ml of tank buffer was poured into the lower reservoir and 450 ml tank buffer with two drops 0.01% aqueous Bromphenol Blue was added to the upper reservoir. A stock solution of tank buffer was made by combining 6.0 g Tris, 28.8 g glycine and deionized distilled water to a final volume of one liter. The stock solution was diluted tenfold for electrophoresis.

A 0.025 ml aliquot of sucrose-protein concentrate was carefully layered on the surface of the spacer gel. A current of 2 ma was applied to each gel, with the positive carbon electrode in the lower reservoir and the negative carbon electrode in the upper reservoir. The power supply was a Heathkit

Model PS-4. When the blue dye front reached the bottom of each tube, the gels were removed by rimming with a 26 gauge hypodermic needle while injecting water. After the gels were removed, they were placed in their respective staining solutions.

Attempts to separate the water soluble proteins in adipose tissue were made employing a system described by Rampton (1969). The spacer gel was the same as described previously herein. The 7.5% running gel was prepared by combining solutions A, B and C (6.4:1.6:2.67). Solution A was made by combining 4 ml 2 N HCl, 6.1 g Tris, 0.08 ml TEMED and diluting the solution to 80 ml with distilled water. Solution B was composed of 50% (W/V) aqueous Cyanogum. Solution C consisted of 1 mg riboflavin in 50 ml distilled water.

Urea gels were also used to separate the water soluble proteins in adipose tissue (Rampton, 1969). A 5% spacer gel was produced by combining solutions 1, 2 and 3 (1.6:0.4:0.67). Solution 1 was composed of 4 ml 2 N HCl, 1 g Tris, 0.06 ml TEMED, 65 ml 10 M urea and distilled water to give a total volume of 80 ml. Solution 2 contained 33.3 g Cyanogum, 25 ml 10 M urea and was made to 100 ml with distilled water. Solution 3 consisted of 1 mg riboflavin, 35 ml 10 M urea and distilled water to bring the volume to 50 ml.

A 6.5% running gel (Rampton, 1969) was made by mixing solutions I, II and III (6.4:1.6:2.67). Solution I contained 4 ml 2 N HCl, 6.1 g Tris, 0.08 ml TEMED, 65 ml 10 M urea and distilled water to a final volume of 80 ml. Solution II consisted of 43.3 g Cyanogum, 25 ml 10 M urea and was made to 100 ml with distilled water. Solution III was identical to solution 3 utilized in preparation of the spacer gel. Polymerization, sample application and electrophoresis were carried out as previously described herein.

Starch gel electrophoresis was carried out using an apparatus similar to that described by Smithies (1959). The gel former was circumscribed with removable Plexiglas blocks which defined the starch gel bed. Platinum electrodes were used to conduct current from the power supply (Heathkit Model PS-4).

Horizontal starch gel electrophoresis was carried out in a discontinuous buffer system at 4°C. The starch gel was made according to a modified method of Scopes (1964). The starch gel solution was produced by combining 30 g hydrolyzed starch (Connaught Medical Research Laboratories, Toronto) with 55 ml buffer (0.012 M Tris -0.002 M diethylenetriaminepenta-acetic acid, pH 8.25 at 4°C), and then adding this mixture rapidly to 195 ml of heated (95°C) buffer. The resulting viscous solution was shaken vigorously for 15 sec, degassed for 2 min and poured into the gel former. A slot former was placed 11.5 cm from the end of the gel and the gel was allowed to cool at least 90 min before use. After removing the slot former, samples were placed in the slots and covered with Saran Wrap to exclude air bubbles in the sample. The gel was connected to the buffer (0.06 M Tris -0.05 M Boric acid, pH 8.6 at 4°C) in the electrode chambers with filter paper wicks. A current of 10 ma at 400V was applied to the system. Electrophoresis was carried out at 400V for 6 hr at 4°C.

The gel was stained for 5 min in a dye solution (250 ml distilled water, 250 ml methanol, 50 ml glacial acetic acid and 2 g Amido Black 10B) and destained overnight in a solution containing 1.0 liter distilled water, 1.0 liter methanol, 200 ml glycerin and 200 ml glacial acetic acid. Destaining was continued in 7% aqueous acetic acid.

Staining the Disc Gel Electrophoretic Patterns of the Non-Dialyzable Components

Disc gel electrophoretic patterns were stained for proteins, hemoproteins, sulfhydryl groups, glycoproteins, nucleoproteins, ribonucleic acids, lipoproteins and esterase activity. Various dye producing reagents were studied to determine their intensity and contribution to background staining.

Proteins were stained by immersing the gels in an Amido Black 10B solution for 20 min. The staining solution was prepared by combining 250 ml deionized distilled water, 250 ml methanol, 50 ml glacial acetic acid and 2 g Amido Black 10B. The gels were destained and stored in 7% aqueous acetic acid.

The Coomassie Blue method of Crambach et al. (1967) was also used to stain proteins. The gels were immersed in 12.5% TCA for 30 min, then in a solution composed of one part 1% aqueous Coomassie Blue and 20 parts 12.5% TCA for 30 min. The gels were destained in 10% TCA.

Hemoproteins were detected in the gels using a benzidine test. The gels were immersed in a solution composed of benzidine (0.2 g), 30% hydrogen peroxide (0.2 ml) and glacial acetic acid (0.5 ml) and made to a final volume of 100 ml with deionized distilled water (Smithies, 1959b). Staining required a total of 10 min. Destaining was carried out in distilled water.

Sulfhydryl groups were determined according to the DDD (2,2'-dihydroxy -6,6'-dinaphthyl disulfide) method of Zwaan (1965) as modified by Pitt-Rivers and Schwartz (1967). The following solutions were prepared: A) 25 mg DDD were dissolved in 15 ml absolute ethanol at 50°C. Then 35 ml of 0.04 M sodium barbital-acetic acid buffer (pH 8.5) was added and the mixture was

heated to 50°C; B) deionized distilled water was acidified with one drop glacial acetic acid per liter (pH 4.0-4.5); C) 60% ethanol; D) 95% ethanol; E) 50 mg of either Fast Black K, Fast Blue RR or Fast Violet B were dissolved in 50 ml 0.04 M barbital-acetic acid buffer (pH 7.0). The gels were incubated in solution A for 4 hr at 50°C and washed two times for 15 min each in solution B. Then the gels were washed and dehydrated three times in solution C and once in solution D for 15 min periods. After the gels were rehydrated in deionized distilled water, they were stained for 50 min in solution E. The gels were destained and stored in deionized distilled water and kept in the dark. Staining with Fast Black K resulted in black bands with a very dark background. Fast Blue RR gave blue-purple bands with a fairly dark background, however, Fast Violet B yielded red-violet bands with practically no background color. Therefore, Fast Violet B was used in all subsequent staining procedures.

Glycoproteins were stained by Alcian Blue (Gifford and Yuknis, 1965). The gels were stained overnight in a 0.2% solution of Alcian Blue in 15% acetic acid. Destaining was accomplished in 15% acetic acid.

The Acridine Orange method of Richards et al. (1965) was used to detect nucleoproteins. Acridine Orange staining solution was made as follows: 2.5 g of lanthanum oxide (La_2O_3) was combined with 75 ml glacial acetic acid and dissolved at 90°C. Deionized distilled water (75 ml at 90°C) was added. Clearing of the solution indicated the formation of the required lanthanum acetate [$La(C_2H_3O_2)_3$]. The solution was cooled and diluted to 490 ml with deionized distilled water, and 10 g of Acridine Orange was added. The gels were stained overnight and destained in 7% acetic acid.

Ribonucleic acid was determined by the Methylene Blue staining method of Peacock and Dingman (1967). The staining solution was a 0.2% solution of Methylene Blue in a 0.2 M sodium acetate-0.2 M acetic acid buffer. Gels were immersed in 1 M acetic acid for 15-20 min, stained in the staining solution for 2 hr and destained in water.

Lipoproteins were stained by Sudan Black B according to Gifford and Yuknis (1965). The gels were placed in 15% acetic acid for 30 min, stained overnight in a solution of 40% ethanol saturated with Sudan Black B and destained in 40% ethanol.

Lipoproteins were also stained by the Oil Red O method of Beaton et al. (1961). The staining solution was made by saturating warm methanol with Oil Red O, cooling and filtering. This solution was added to 20% TCA (1:1) and thoroughly mixed. The gels were stained overnight in the staining solution and destained in a methanol-distilled water-acetic acid (50:50:10) solution.

Esterase activity was detected in the gels according to the method of Allen et al. (1965) with slight modifications. The substrate, coupling, developing and rehydrating solutions are described as follows: A (substrate solution)-40 mg α-naphthyl butyrate (or acetate) in 100 ml 0.04 M Tris-HCl buffer (pH 7.1); B (coupling solution)-70 mg Fast Blue RR in 100 ml 0.04 M Tris-HCl buffer (pH 7.1); C (developing solution)-absolute ethanol: 10% glacial acetic acid (3:2); D (rehydrating solution)-10% glacial acetic acid. The gels were immersed in a mixture of solutions A and B (1:1) for 1 hr at 37°C, placed in solution C overnight and rehydrated and stored in solution D. The α-naphthyl butyrate substrate solution yielded darker, more intense bands than α-naphthyl acetate. Therefore, α-naphthyl butyrate was used in all determinations.

Column Chromatographic Separation of Non-Dialyzable and Dialyzable Components

Attempts were made to separate the sucrose-protein concentrates into various molecular weight fractions. All gel filtration resins were equilibrated with borate buffer (pH 7.8, μ = 0.1). Aliquots (5 ml) of the sucrose-protein concentrates were applied to 2.5 x 37 cm columns packed with either Bio-Gel P-30, Bio-Gel P-100, Bio-Gel A-1.5 m, Bio-Gel A-15 m, or Bio-Gel A-50 m. Gel filtration eluants were monitored by an Isco column monitor recorder at 254 nm during the collection of 10 ml fractions.

The dialyzable components were separated into various fractions by column chromatography. A 5 ml aliquot was applied to the columns composed of Bio-Gel P-2 (2.5 x 60 cm) and Dowex 1 anion-exchange resin (2.0 x 35 cm).

The Bio-Gel P-2 resin was equilibrated with deionized distilled water and the fines were removed. The flow rate was approximately 40 ml per hr, and 5 ml aliquots of the eluant were collected. The eluant was monitored at 254 nm as described previously.

Dowex 1 anion-exchange resin was converted to the formate form by washing the resin with 1 N NaOH and removing the excess alkali with deionized distilled water. The resin was then washed with three bed volumes of 6 N formic acid. The resin was poured into the column, allowed to settle and then the column was washed with deionized distilled water until no ultraviolet absorbing materials appeared in the effluent (Lento et al., 1964). Two elution systems were used. The first system described by Lento et al. (1964) was a gradient system composed of water, formic acid (0.5 N) and

sodium formate (0.2 N). The second system was composed of water, 0.5 N formic acid, 1 N formic acid, 3 N formic acid and 6 N formic acid. The pH of the sample was adjusted to 8.0 with 5 N NaOH before application to the column. The effluent was monitored at 254 nm and collected in 5 ml aliquots. The column flow rate was approximately 40 ml per hr.

The fractions were collected, concentrated by lyophilization and stored in a desiccator at 4°C until used. The lyophilized fraction was solubilized in either 1 or 2 ml of deionized distilled water for thin layer chromatography.

Thin Layer Chromatographic Analysis of Dialyzable Components

The aqueous solutions obtained by column chromatography were applied to thin layer chromatographic plates in 1 µl aliquots until sufficient material had been applied to detect the various components.

Purine and pyrimidine derivatives were detected by several thin-layer chromatographic methods (Randerath, 1966; Bolliger et al., 1965). Purine and pyrimidine bases and nucleosides were chromatographed on thin-layer plates coated with cellulose (Eastman Chromatogram Sheet 6064), Cellulose Powder MN300 and MN300G (Laboratory prepared) and Silica Gel G (Eastman Chromatograph Sheet 6061 and laboratory prepared) using water as the developing solvent for both the Silica Gel G and cellulose plates. A solvent consisting of methanol, concentrated HCl and water (65:17:18) was also used to develop the cellulose plates.

The Cellulose Powder (MN300 and MN300G) chromatograms were prepared by combining 15 g of powder with 90 ml distilled water in a Waring blender and blending for 1 min. The cellulose layers were approximately 0.35 mm in thickness.

Silica Gel G thin-layer chromatograms (0.25 mm thick) were produced by spreading a Silica Gel G and distilled water (1.0:2.0) slurry over the glass plates. Methanol, concentrated HCl and distilled water (65:17:18) and distilled water were used as the solvent systems to chromatographically separate purine and pyrimidine bases and nucleosides on the cellulose chromatograms. Distilled water was employed as the mobile phase to develop the Silica Gel G chromatograms.

Nucleotides were detected by using MN300 and MN300G cellulose powder MN300 ECTEOLA anion exchange chromatograms. MN300 cellulose powder chromatograms were developed with a solvent consisting of n-butanol, acetone, acetic acid, 5% ammonium hydroxide and distilled water (4.5:1.5:1.0:1.0:2.0). The solvent system used to develop the MN300G cellulose powder chromatograms consisted of tert. amyl alcohol, formic acid and water (3.0:2.0:1.0). MN300 cellulose ECTEOLA chromatograms (0.35 mm thick) were prepared by combining 8 g of exchange material, 2 g of unmodified cellulose powder MN300 and 90 ml distilled water. The mixture was blended for 1 min in an electric mixer and spread on the glass plates. The chromatograms were developed with a 0.15 M aqueous NaCl solution.

Purines, pyrimidines and their derivatives were detected with and without spraying with 1.0 N HCl under ultra-violet light (254 nm) and by spraying with 2',7'-dichlorofluorescein prior to exposure with ultra-violet light. Tests for carbonyl compounds were run by spraying the chromatogram with 3.7% 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 drying completely

by 1% stannous chloride in 10% HCl (Randerath, 1966). Amino acids were detected by spraying with either ninhydrin (Sigma Chemical Co.) or a cadmium-ninhydrin reagent (Blackburn, 1966). The cadmium-ninhydrin reagent was prepared by combining 1 g cadmium acetate, 100 ml water, 20 ml acetic acid and 1 liter acetone. The cadmium-ninhydrin treated chromatograms were allowed to develop at 4°C over sulfuric acid, whereas, the ninhydrin treated chromatograms were developed from 3-5 min at 100°C.

Creatine, creatine phosphate and creatinine were detected using Silica Gel chromatograms (Eastman Chromatogram Sheet 6061) as the stationary phase, and using isopropanol and ammonia (70:30 V/V) as the mobile phase. The developed chromatograms were sprayed with 1% aqueous picric acid, dried for 1 hr at 110°C and then sprayed with 4% aqueous sodium hydroxide (Zaika et al., 1968).

Analyses for lactic acid were carried out by thin layer chromatography on Silica Gel G and Cellulose MN300 plates. The Silica Gel G chromatogram was developed with diethyl ether and 90% formic acid (70:10, V/V), while the Cellulose MN300 chromatogram was developed with formic acid, 2-butanone, tert. butanol and water (15:30:40:15, V/V). The chromatograms were sprayed with 0.3% bromphenol blue and 0.1% methyl red in 95% ethanol (Zaika et al., 1968).

Chemical Analysis of Dialyzable and Non-Dialyzable Components

Free sulfhydryl group determinations were conducted according to the method of Ellman (1959) with modifications. DTNB [5,5'-dithiobis (2-nitro-benzoic acid)] was reoxidized and crystallized, m.p. 239°C (237-238°C reported). DTNB (39.6 mg) was made to a volume of 10 ml with 90% ethanol.

Sulfhydryl determinations in study I were performed only on the water soluble extract before and after heating. The lyophilized water soluble fraction of adipose tissue (0.1 g) was diluted to 10 ml with phosphate buffer (pH 8.0; μ = 0.1). A 1 ml aliquot was placed in a 1 cm square Pyrex cuvette. The aliquot was combined with 2 ml 8.5 M urea (recrystallized from water and ethanol) and 0.02 ml DTNB solution. The cuvette was sealed with Parafilm, and the contents were mixed by gently inverting the cuvette several times until all schlieren lines were eliminated. The mixture was then allowed to stand at room temperature for 45 min before absorbance was measured. The sulfhydryl concentration was determined using a molar absorptivity value of 13,600 at 412 nm. Absorbance was measured using a Beckman, Model DU, spectrophotometer equipped with a Gilford (Model 220) absorbance indicator. After heating, the protein-water slurry was lyophilized and the free sulfhydryl groups were determined in the same manner. The salt-soluble and insoluble fractions were not soluble in the buffer-urea mixture, so that their sulfhydryl content could not be determined.

In study II, free sulfhydryl groups were determined as described above, except that the non-dialyzable fraction (1 ml) was an aliquot of the sucrose-protein concentrate, and the urea was crystallized from water and ethanol and then passed over an Amberlite MB-3 mixed bed resin.

To determine the sulfhydryl content of the diffusate, isolated in study II, 1 ml of urea was added to 2 ml of sample. The determination was then carried out as previously described herein.

The aldosaccharide content of the diffusate was determined according to the method of Müller (1965). The reagent is composed of o-toluidine

(6%, V/V) and thiourea (0.15%, W/V) in glacial acetic acid. The sample (0.5 ml) was mixed with the reagent (3.5 ml), heated in a boiling water bath for exactly 8.0 min and cooled in running tap water. Absorbance was measured at 630 nm. The color developed was stable for 1 hr. The addition of trichloroacetic acid (TCA) and centrifugation were omitted from the procedure, since the protein had already been removed by dialysis.

Free amino groups in the diffusate were determined by the method of Florkin and Stolz (1963). Samples (0.1 ml) containing 0.2-1.0 mg protein per 0.1 ml solution were combined with ninhydrin reagent (1 ml), covered with aluminum foil caps and mixed. The mixture was heated for 20 min in a boiling water bath, cooled and mixed rapidly with 5 ml of 50% aqueous isopropanol. Absorbance was measured at 570 nm within 15 min. Serial dilutions of leucine were used to construct the standard curve. The ninhydrin reagent was composed of citrate buffer and a ninhydrin solution. The citrate buffer (pH 5.0) was made by dissolving 21 g citric acid in 200 ml 1 N NaOH and diluting the mixture to 500 ml with deionized distilled water. The ninhydrin solution was prepared by dissolving 20 g ninhydrin in 500 ml redistilled methyl cellosolve. The ninhydrin reagent was produced by combining the citrate buffer and ninhydrin solution (1:1 V/V).

Sulfate was detected in the diffusates by combining approximately 0.30 ml of the concentrated diffusates with approximately 10 ml of 10% barium chloride in 1 N HCl. The mixture was boiled for approximately 15 sec and allowed to cool. The formation of a white precipitate indicates the presence of the sulfate ion in the diffusate.

Development of a Method to Determine Sulfhydryl and Disulfide Groups

After reviewing several sulfhydryl analytical techniques, a method described by Saville (1958) and Liddell and Saville (1959) appeared to be particularly adaptable for the determination of sulfhydryl and disulfide groups. They determined low molecular weight thiols (cysteine and glutathione) indirectly, employing the formation of an azo dye.

Several solutions were employed by Liddell and Saville (1959) for the thiol determinations. The thiol concentrations ranged from 1 to 6 x 10^{-4} M. Solution A was composed of a 0.01 M sodium nitrite solution (5.0 ml) in 1 N H_2SO_4 (35 ml) and water to a final volume of 100 ml. Solution B was 0.5% aqueous ammonium sulfamate solution. Solution C was composed of a 4:1 (V/V) mixture of 3.44% sulfanilamide in 0.4 N HCl and a 1.0% mercuric chloride solution in 0.4 N HCl, respectively. Solution D was produced by adding 1.0 g N-1-naphthylethylenediamine dihydrochloride to 1.0 liter of 0.4 N HCl.

Liddell and Saville (1959) allowed the thiol (1.0 ml) to react with 5.0 ml of solution A for 4-5 min. This solution was in turn allowed to react with 1.0 ml of solution B for 1-2 min. Solution C (10 ml) was rapidly added to the reactants, and after a 2 min period the solution was diluted to 25 ml with solution D. The colored solution was read at 540 nm after 2 min.

The first modification of the Liddell and Saville (1959) method involved reduction of the total volume of the various reagents and investigation of the applicability of the method for disulfide analysis. Solutions A, B, C and D were the same concentrations as described previously. The reducing

agent was 5% potassium cyanide in 1 N ammonium hydroxide. The source of the thiol groups was DTNB. This disulfide was satisfactorily reduced by the cyanide reducing agent to produce two thiols per disulfide as found by determining the thiol concentration at 412 nm.

DTNB (0.5 ml) was combined with the reducing agent (0.1 ml) and allowed to react for 45 min. Solution A (0.5 ml) was added to the reduced thiol solution. After 2 min, 0.2 ml solution B was added to the mixture, and 5 min was allowed for the destruction of excess nitrous acid. Solution C (1 ml) was combined and mixed with the S-nitroso solution. Solution D (1 ml) was added 2 min later, and 2 min was allowed for color development. The resulting dye was read at 540 nm.

Catsimpoolas and Wood (1966) stated that both cyanide and sulfite reduction incompletely reduced disulfides in some proteins. Hamm and Hofmann (1965) used 0.6 M sodium borohydride in 8.0 M urea to reduce protein disulfides. However, upon substituting the sodium borohydride reducing agent for the cyanide solution in the present study, unsatisfactory results were obtained. The borohydride solution generated hydrogen gas, which made accurate pipetting impossible. After decreasing the borohydride concentration to 0.1 M in 8 M urea and adding 0.5 ml of the reducing agent to the disulfide solution, results were still unsatisfactory. Since urea destroys nitrous acid, a 0.1 M aqueous borohydride solution was used. However, the sodium borohydride was less stable in water than in urea, still giving unsatisfactory results.

Several compounds were used to destroy the excess borohydride (ascorbic acid, acetone, dihydroxyacetone, uric acid, HCl). In order to destroy the

borohydride, either HCl had to be added to bring the pH to 2.0 or else high concentrations of the other reagents were required. The concentrations were determined by adding the reagents to the borohydride and then adding a solution of DTNB. The development of a yellow color indicated incomplete borohydride destruction.

To study the effect of various reagent concentrations, three of the four reagents (A, B, C and D) were held at 2×10^{-3} N except the reagent under investigation. However, ammonium sulfamate was held at 4×10^{-3} N in all cases except when its concentration was under investigation. This insured complete destruction of excess nitrous acid, regardless of the concentrations of nitrous acid or the other reagents. The investigation confirmed the findings of Liddell and Saville (1959) that the only reagent detrimental to the reaction was a large excess of the mercuric salt. However, much lower concentrations of mercuric chloride than recommended were found just as effective.

In order to simplify the method and thus insure greater repeatability, the reagent concentrations were modified so that 0.5 ml of each reagent could be added to the cuvette with 0.5 ml pipets (TD). This meant that 0.5 ml aliquots of the thiol, reducing agent and reagents A, B, C and D could be added to the approximately 3.5 ml cuvettes, leaving sufficient space for adequate mixing.

Kobayashi (1966) reported a molar absorptivity of 3.93×10^4 for the azo dye, p-{4-[(2-aminoethyl)-amino] azo} benzenesulfonamide. Sawicki et al. (1963) found that the molar absorptivity of the azo dye was approximately 4.0×10^4. In the previous two reports, the method of determining the molar

absorptivity was not elucidated. Liddell and Saville (1959) did not report the molar absorptivity of the dye, but they reported the absorbance of various thiol concentrations and the light path length through the solution. These values plus the volumes of the thiol and reagents, which gave the dilution factor, made determination of the molar absorptivity possible. The resulting molar absorptivity was approximately 3.84×10^4. However, this is not the true molar absorptivity of the dye, since this value is dependent upon the yield of all the various reactions.

By using the modified Liddell and Saville (1959) method, the thiol content, absorbance, light path length and dilution factor were determined. The calculated molar absorptivity was approximately 4.20×10^4. Therefore, it was necessary to determine the actual molar absorptivity of the dye.

Approximately 1.0 g of the dye was synthesized by reacting sufficient sodium nitrite in 1.0 N HCl with excess sulfanilamide in 0.4 N HCl, which gave the diazonium salt. The resulting diazonium salt solution was then reacted with excess N-1-naphthylethylenediamine dihydrochloride in 0.4 N HCl to give the azo dye. Upon neutralization of the reddish-violet dye solution with sodium bicarbonate, the solution turned orange and the neutralized dye formed a precipitate. The precipitated dye was quite soluble in ethyl acetate but much less soluble in ethyl ether. It was decided that a much higher recovery of the dye could be achieved by ether extraction. Therefore, the aqueous solution containing the precipitated dye was subjected to liquid-liquid extraction with ethyl ether over a four day period. Golden cyrstals of the dye were isolated in the ether solution and were recovered upon removal of the ether under reduced pressure in a Rinco rotating evaporator.

After storing the dye in an evacuated dessicator overnight, the molar absorptivity was determined by solubilizing a known amount of dye to a final known volume of 0.4 N HCl. The volume of HCl added was adjusted to give an absorbance of approximately 0.300 at 540 nm. The 540 nm wavelength was determined by noting the wavelength at which the highest absorabance of the dye (acid form) occurred using a Spectronic 505 recording spectrophotometer. The molar absorptivity of the dye was calculated to be approximately 4.93×10^4.

Theoretically, the coupling reaction of the diazonium salt and N-1-naphthylethylenediamine could result in three products, since coupling could take place in the ortho-, para- or both positions. Since the secondary free amino group on the coupling agent is in the α-position and is a stronger para- than ortho-directing group, the majority of the coupling reactions should occur in the para-position. However, all these theoretical assumptions appear to be pre-empted by the fact that coupling occurs in an acid medium. Since coupling occurs in an acid medium, equilibrium must exist between the free amine and amine hydrochloride.

The question as to why coupling occurs was resolved by titrating a solution of N-1-naphthylethylenediamine (pH 1.15) with sodium hydroxide and plotting the amount of sodium hydroxide used against the pH of the solution. The pKa of the aromatic secondary amine group was found to be approximately 3.1. Thus, coupling could readily take place in the acidic medium since approximately 50% of the free amine form is available for coupling at this pH. Coupling could occur fairly rapidly since the reaction would be driven to the right.

In order to determine if coupling had occurred in any position other than the para postion, the neutralized dye was subjected to thin layer

chromatography employing a modification of the method of Ganshirt et al. (1965). Alusil (Silica Gel G:Alumina G, 1:1) was the stationary phase. Three mobile phases were used in the separation of the dye components. The first mobile phase consisted of ethyl acetate, methanol and 5 N ammonium hydroxide (60:30:10). The second system consisted of ethyl acetate, tetrahydrofuran, methanol and 5 N ammonium hydroxide (60:20:10:10), while the third phase was a combination of ethyl acetate, tetrahydrofuran and 5 N ammonium hydroxide (60:30:10). All three solvent systems separated the dye into three components, one major and two minor ones. All three components had different R_f values in the three different solvent systems. The most polar solvent system gave the highest R_f values for all three components.

After the chromatography was completed, the plates were dried at approximately 55°C and the orange spots were sprayed with 0.4 N HCl. All three components were converted to reddish-violet azo dyes. The minor components were not subjected to spectrophotometric analysis to determine their molar absorptivities.

To prevent the formation of variously coupled dyes, a suitable coupling agent was sought. Since none was available or reported in the literature, the coupling agents had to be synthesized. N-1-acenaphthylethylenediamine was chosen as a suitable coupling agent. Although the para position is occupied, coupling should take place readily at the 4-position, i.e. ortho to the amine group (Zollinger, 1961).

Cava et al. (1965) reported the synthesis of 5-aminoacenaphthene via the reduction of 5-nitroacenaphthene. 5-nitroacenaphthene was formed by adding 125 ml of 70% aqueous nitric acid over a 15 min period to a mixture of 100 g acenaphthene in 800 ml of glacial acetic acid. After the addition

of nitric acid was completed, the mixture was cooled to approximately 20°C and added to 2 liter of distilled water. The precipitate was filtered, employing a Büchner filter fitted with Whatman No. 1 filter paper, and was dried at 50°C. The yellow precipitate was cyrstallized from either methanol or petroleum ether, m.p. 103°C. Cava et al. (1965) reported a m.p. of 104°C for 5-nitroacenaphthene.

5-aminoacenaphthene was synthesized according to the method of Cava et al. (1965) by combining 50 g of 5-nitroacenaphthene, 10% Pd-C (2.5 g), 60 ml of 85% hydrazine hydrate and 1.0 liter of 95% ethanol. The mixture was refluxed for 45 min and the hot solution was filtered through Celite. Distilled water was added to the filtrate until the precipitation of 5-amino-acenaphthene was complete. The precipitate was recovered by filtration and crystallized from petroleum ether. In the present study, a m.p. of 105°C was found for the white cyrstals of 5-aminoacenaphthene, which compares to the m.p. of 104°C reported by Cava et al. (1965).

N-1-acenaphthylethylenediamine was prepared by a modification of the methods of Newman (1891), Ing and Manske (1926) and Bratton and Marshall (1939). 5-aminoacenaphthene was fused with a slight excess of β-bromoethyl-phthalimide at approximately 100°C (heating to higher temperatures caused charring of the reactants). The liquid was washed with several volumes of hot (~90°C) distilled water. The β-(5-aminoacenaphthyl) ethylphthalimide was then refluxed for 2 hr with 50 ml of 85% hydrazine hydrate in 200 ml of 95% ethanol. Excess HCl was added to the mixture and refluxing was continued for an additional hour. Phthalylhydrazide was removed by filtration and the filtrate was concentrated to 50% its original volume at reduced pressure

employing a Rinco rotating evaporator. The residual phthalylhydrazide was removed by filtration and 100 ml of distilled water was added to the filtrate. The ethanol was removed at reduced pressure and the solution was neutralized with aqueous potassium hydroxide. The free amine compound was extracted with ethyl ether, dried over anhydrous sodium sulfate and, after removal of the sodium sulfate, was precipitated from the ether solution by the addition of concentrated HCl. The hydrochloride was dissolved in a minimum of methanol and precipitated with ethyl ether. A white precipitate was formed that rapidly changed to red.

Methanol, containing a trace of HCl, was added to the ether solution and the precipitate was redissolved upon removal of the ether. Activated charcoal was added to the methanolic solution but failed to decolorize the precipitate. The precipitate was added to an acidic solution of p-sulfamoylbenzenediazonium salt, and an intensely purple solution resulted. This indicated that the precipitate possessed the properties of a coupling agent. Further studies to determine the usefulness of this compound as a coupling agent will be carried out in the future.

Presently, the method has been utilized only for low molecular weight disulfides. However, studies have shown that use of a 1% aqueous solution of sodium lauryl sulfate as a protein denaturant in place of urea produced no undesirable side reactions. But the N-1-naphthylethylenediamine solution (D) must be composed of 0.1950 g of N-1-naphthylethylenediamine dihydrochloride in 10 ml of 0.4 N HCl and made to a final volume of 25 ml with acetone. The acetone not only destroys the foam resulting from the agitation of the sodium lauryl sulfate solution but also introduces a bathochromic effect, which changes the maximum absorption from 540 to 550 nm.

Since alkaline cyanide, sulfite and sodium borohydride solutions are not suitable reducing agents, aqueous sodium trimethoxy borohydride and tetramethylammonium borohydride are presently being investigated. Neither reducing agent produces profuse hydrogen gas generation when combined with water, but sodium trimethoxy borohydride appears to react very slowly. Therefore, tetramethylammonium borohydride appears to be the most desirable reducing agent. However, tetramethylammonium borohydride should be almost as alkaline as sodium borohydride, so it may be necessary to decrease the pH of the reducing solution to 9.0 to prevent any possibility of alkaline scission of the disulfide bond.

Nitrous acid is used for two purposes in the method. First, nitrous acid is used to destroy the excess reducing agent. Secondly, the residual nitrous acid is employed in the formation of the S-nitroso derivatives of the sulfhydryl groups. The amount of nitrous acid added must be regulated by the amount of reducing agent to be destroyed, by the amount of thiols to be nitrosated and by the amount of ammonium sulfamate added. Since the ammonium sulfamate destroys the excess nitrous acid after nitrosation has occurred, the nitrous acid content must be held at a lower concentration than that of the ammonium sulfamate. It is not advisable to increase the concentration of ammonium sulfamate, since the excess sulfamate would more favorably compete with the sulfanilamide for the cleaved nitrous acid. Thus, the sensitivity of the determination would be decreased.

RESULTS AND DISCUSSION

Adipose Tissue Analysis

In study I, the moisture, fat and protein content of raw beef adipose tissue averaged 15.36, 79.20 and 4.84%, respectively. The corresponding values for the raw adipose tissue utilized in study II were 10.35, 86.07 and 3.27%.

Hydrogen Sulfide Determinations

The appearance of a white zinc sulfide precipitate in the collection traps indicated that hydrogen sulfide was evolved upon heating the whole adipose tissue, and by the water soluble, salt soluble and insoluble fractions of adipose tissue. The precipitate was first noted when the heated slurries reached a temperature of approximately 80°C. The temperature at which hydrogen sulfide commenced to evolve is in agreement with that reported by Hamm and Hofmann (1965), who stated that hydrogen sulfide was initially evolved from heated beef myofibrils at 80°C.

The amount of hydrogen sulfide evolved upon cooking the whole adipose tissue and its various fractions for a 7 hr period is shown in Table 1. The hydrogen sulfide evolution rate upon cooking whole adipose tissue as compared to the water soluble fraction is shown in Figure 3. The amount of hydrogen sulfide obtained from the salt soluble and insoluble fractions could not be plotted against time, because the total amounts obtained from each fraction over a 1 hr period were too small to be accurately measured. Therefore, the

hydrogen sulfide generated from a single fraction was collected in only one trap over the 7 hr period in order to sufficiently increase the total amount and allow accurate measurement.

Table 1. Hydrogen sulfide evolved upon cooking adipose tissue and its various fractions for 7 hr.

Source	Protein in adipose tissue g/100 g	H_2S/100 g adipose tissue μM	H_2S/g protein μM
Adipose tissue (whole)	4.84	42.74	8.83
Water soluble fraction	0.43	17.63	41.01
Salt soluble fraction	1.18	1.71	1.45
Insoluble fraction[1]	3.23	5.46	1.69

[1]Insoluble in water, 0.45 M NaCl and ethyl ether.

The sulfhydryl content of the water soluble fraction before cooking was 9.66 μM (micro moles) of sulfhydryl per g of protein, but declined to 0.85 μM of sulfhydryl per g protein after cooking. The salt soluble and insoluble fractions were not soluble in the buffer-urea mixture, so sulfhydryl analyses could not be made.

The rate at which hydrogen sulfide was evolved from the heated water soluble fraction rapidly reached a maximum and then decreased. The amount of hydrogen sulfide evolved was approximately 5.43 μM per g of protein during the first hr of cooking, and was approximately 6.12 μM per g of protein during the second hr. More hydrogen sulfide was collected during the second hr of cooking than during the first. However, it should be borne in mind that the first hr of cooking started upon connecting the heating

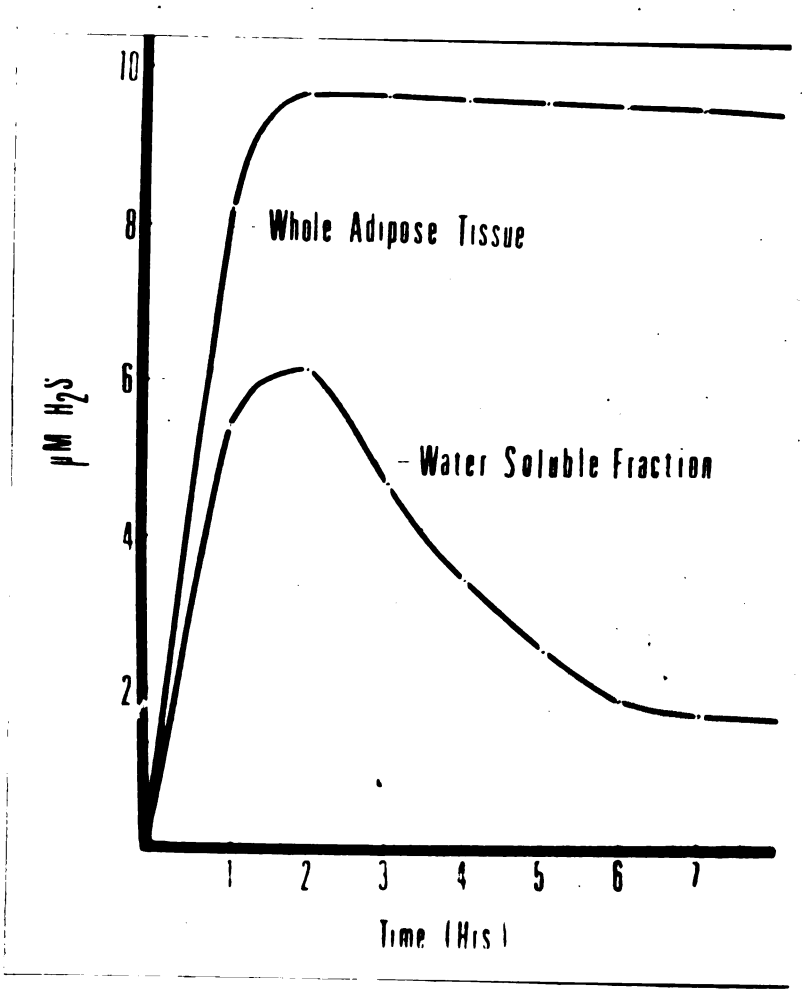


Figure 3. Amount of hydrogen sulfide evolved with increased cooking time. Values are for 150 g of whole beef adipose tissue and 0.64 g of lyophilized water soluble fraction. The latter figure corresponds to the amount of water soluble substance in 150 g of whole adipose tissue.

mantle. The time required to heat the solution to 80°C was approximately 20 min. Therefore, the actual hydrogen sulfide evolution time during the first hr of cooking was approximately 40 min. If the evolution time (40 min) is extrapolated to 60 min, the hydrogen sulfide evolved would be approximately 8.15 μM of hydrogen sulfide per g of protein. Since a total of approximately 6.12 μM of hydrogen sulfide was collected during the second hr of cooking, the rate of hydrogen sulfide evolution would be greater during the first hr than during the second.

Figure 3 shows a decrease in hydrogen sulfide evolution after the second hr. During the first 2 hr of collection, the amount of hydrogen sulfide obtained was 11.55 μM. Using the extrapolated value of 8.15 μM of hydrogen sulfide during the first hr, it seems likely that hydrogen sulfide began to decline during the second hr. The reason for assuming that free sulfhydryls are responsible for the initial evolution of hydrogen sulfide is the decline in free sulfhydryls from 9.66 μM per g protein before cooking to 0.85 μM per g protein after cooking.

Hydrogen sulfide was evolved at approximately the same rate during the first hr of cooking from both the heated whole adipose tissue and an equivalent amount of water soluble protein (the quantity of water soluble protein in the same amount of whole adipose tissue). After the first 2 hr of heating, the amount of hydrogen sulfide from the whole adipose tissue continued to be evolved at almost a constant rate, whereas, the hydrogen sulfide from the water soluble fraction steadily declined. This is probably due to the limited exposure of the free sulfhydryl groups entrapped in the fat and tissue matrices. The free sulfhydryl groups of

the freeze-dried, water soluble fraction were apparently much more accessible, and therefore, more susceptible to degradation during heating.

Using chicken as substrate, Mecchi et al. (1964) reported that the water insoluble fraction was responsible for twice as much hydrogen sulfide as the water soluble fraction. Hamm and Hofmann (1965) found that almost all of the hydrogen sulfide released upon cooking whole beef muscle was derived from the structural proteins and not from the water soluble substances. With adipose tissue, the reverse was found to be true. Of the protein fractions isolated from adipose tissue, the water soluble fraction evolved 71% of the hydrogen sulfide, while the salt soluble and insoluble fractions yielded only 7 and 22%, respectively. Therefore, the water soluble fraction contributed nearly 2.5 times as much hydrogen sulfide as the combined water insoluble fractions.

Analysis of Dialyzable and Non-Dialyzable Components of the Water Soluble Fraction of Adipose Tissue

There are four reasons for studying the water soluble fraction of adipose tissue. First, the components responsible for meat flavor are located in the water soluble portion of muscle (Kramlich and Pearson, 1958; Hornstein and Crowe, 1964). Secondly, fatty tissues have been reported to be responsible for differences in the flavor of meat from various species (Hornstein and Crowe, 1960b; Wasserman and Talley, 1968). Thirdly, it has been reported that hydrogen sulfide plays a major role in the meaty aroma of chicken (Minor et al., 1965b). Fourth, the present study indicates that the water soluble fraction of adipose tissue is primarily responsible for hydrogen sulfide production upon heating.

The adipose tissue was stored under air and nitrogen to determine if these atmospheres would affect the sulfhydryl content of the fatty tissue. The adipose tissue was mixed with solid sodium chloride to give a 3% salt concentration and stored under air and nitrogen. A 3% salt concentration was selected, since most sausages contain approximately this level of salt.

Amino acid analysis. The percent of the amino acids present in the dialyzable and non-dialyzable water soluble fractions of the various treatments (O_2 - air atmosphere; N_2 - nitrogen atmosphere; O_2S - air atmosphere and 3% salt; N_2S - nitrogen atmosphere and 3% salt) are given in Table 2. Since only trace amounts of free sulfhydryl groups were determined in the diffusates of all treatments, the diffusates from treatments N_2 and O_2 were combined, as well as those from N_2S and O_2S and designated as I and II, respectively.

There was no major difference in the amino acid composition of the proteins isolated from the various treatments, except for the high glutamic acid and low threonine and serine contents in the N_2S treatment. These values cannot be explained.

Alanine, glycine and glutamic acid composed over 50% of the amino acids found in the diffusates, whereas, the same amino acids accounted for only approximately 25% of the amino acid residues in the protein fractions (Table 2). The half cystine content of the dialysates was approximately three times that found in the diffusates. Under acid hydrolysis, some cystine sulfur appears in the humin of the hydrolyzate (Leach,

Table 2. The amino acid composition of dialyzable and non-dialyzable aqueous fractions of the various treatments.

	Percent amino acids					
	N_2[1]	O_2[2]	O_2S[3]	N_2S[4]	I[5]	II[6]
Lysine	9.10	9.00	9.24	9.52	1.77	2.09
Histidine	2.80	2.67	2.84	3.41	3.22	3.96
Arginine	3.85	3.77	4.06	4.10	0.46	1.22
Aspartic acid	10.43	10.48	10.56	10.96	7.35	6.28
Threonine	5.99	5.92	6.11	1.80	1.17	3.33
Serine	6.24	5.75	5.86	0.43	1.30	4.49
Glutamic acid	12.54	12.79	12.72	16.55	21.80	13.46
Proline	5.12	5.07	5.11	6.27	6.83	4.93
Glycine	5.64	5.43	5.80	6.32	17.50	12.63
Alanine	8.44	8.06	8.04	8.80	28.21	27.78
Half cystine	2.90	3.23	2.80	2.28	0.69	0.72
Valine	6.83	6.93	6.88	7.83	3.28	4.99
Methionine	0.25	1.07	0.66	0.90	0.67	0.93
Isoleucine	3.27	3.29	3.34	3.36	1.37	2.49
Leucine	9.82	9.87	9.58	10.70	2.66	6.15
Tyrosine	2.55	2.53	2.42	2.21	0.48	1.75
Phenylalanine	4.24	4.12	3.99	4.54	1.23	2.80

[1]Adipose tissue stored under nitrogen.
[2]Adipose tissue stored under air.
[3]Adipose tissue with 3% NaCl stored under air.
[4]Adipose tissue with 3% NaCl stored under nitrogen.
[5]Combined diffusates from N_2 and O_2 treatments.
[6]Combined diffusates from N_2S and O_2S treatments.

1966). Therefore, the percent of cystine found in the hydrolyzed solution is not an absolute but a relative percent, since all hydrolyzed substrates were subjected to identical treatments.

Starch Gel Electrophoresis of Non-Dialyzable Components

The starch gel electrophoretic patterns obtained from the water soluble components of beef muscle and adipose tissue are shown in Figure

4. Patterns 1, 3, 4 and 6 were obtained from adipose tissue extracts (0_2), while 2 and 5 are patterns of the beef muscle extract. The patterns for the two substrates are not comparable, since the adipose tissue extracts were not satisfactorily separated even though good separation was achieved on the muscle extracts. These results were not surprising, however, since proteins from different substrates may not be completely resolved by one electrophoretic system. Since starch gel electrophoresis resulted in poor resolution of the water soluble components from adipose tissue, the method was abandoned.

Disc Gel Electrophoresis of Non-Dialyzable Components

Both disc gel electrophoretic methods (urea gels and aqueous gels) described by Rampton (1969) for the separation of rabbit muscle contractile proteins did not adequately separate the water soluble proteins of adipose tissue. However, a gel system described by Jolley et al. (1967) gave good protein resolution for both the muscle and adipose tissue extracts.

Figure 5 compares the proteins in both muscle and adipose tissue (0_2) extracts, and also shows the sulfhydryl distribution for the two substrates. The sulfhydryl distribution is more closely related to the protein distribution in the muscle extract than in the adipose tissue extract. Both the protein and sulfhydryl patterns, however, show the dissimilarity between the two substrates. At 3.7 cm a very broad protein band (band A) was present in the muscle extract, while it was absent in the adipose tissue substrate.

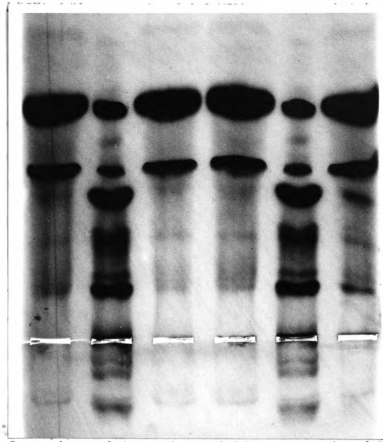


Figure 4. Comparison of the water soluble components of beef muscle and adipose tissue. (1-r, 1-6) 1, 3, 4 and 6 beef adipose tissue extract; 2 and 5 beef muscle extract.

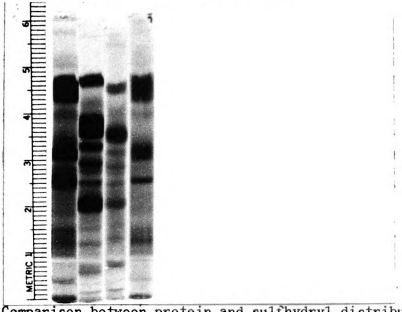


Figure 5. Comparison between protein and sulfhydryl distribution in muscle and adipose tissue extracts. (1-r. pattern 1-adipose tissue protein; 2-muscle protein; 3-muscle SH; 4-adipose tissue SH.)

When the gel from the muscle extract was stained with Benzidine reagent (Figure 6), band A (3.0 cm) stained very strongly. One other band (band B) at 2.5 cm was also stained but less intensely. This stain is specific for heme proteins. Scopes (1968) showed that rabbit myoglobin migrates faster than hemoglobin. Therefore, band A appears to be myoglobin. The slower migrating smaller band B is probably hemoglobin. When the adipose tissue gel was stained with benzidine reagent, a very lightly stained band was observed, which had the same migration rate as band B from the muscle extract. The staining and migration characteristics of this band indicate that it was probably hemoglobin. This is not unreasonable, since at least a small quantity of residual blood was probably present in the adipose tissue.

Gels 1, 2 and 4 in Figure 6 show positive tests for ribonucleic acids, nucleoproteins and glycoproteins, respectively. Gel 3 (Figure 6) indicates that esterase activity is also present in the same general area of the gel. To determine whether glycoproteins and nucleoproteins are present in this region, the running gel was made of 5% Cyanogum. The resulting gel patterns are shown in Figure 7. All proteins migrated at a faster rate in the 5% running gel than in the 7.5% running gel. The esterase migrated from 1 mm in the 7.5% gel to 4 mm in the 5% gel, however, the substances that stained positive for glycoproteins and ribonucleic acids did not migrate further with the 5% gel. Thus, these substances appear to be ribonucleic acids. They stained extremely lightly with Amido Black, so they contain practically no protein characteristics. However, they stained positively with methylene blue, which is specific for ribonucleic acids.

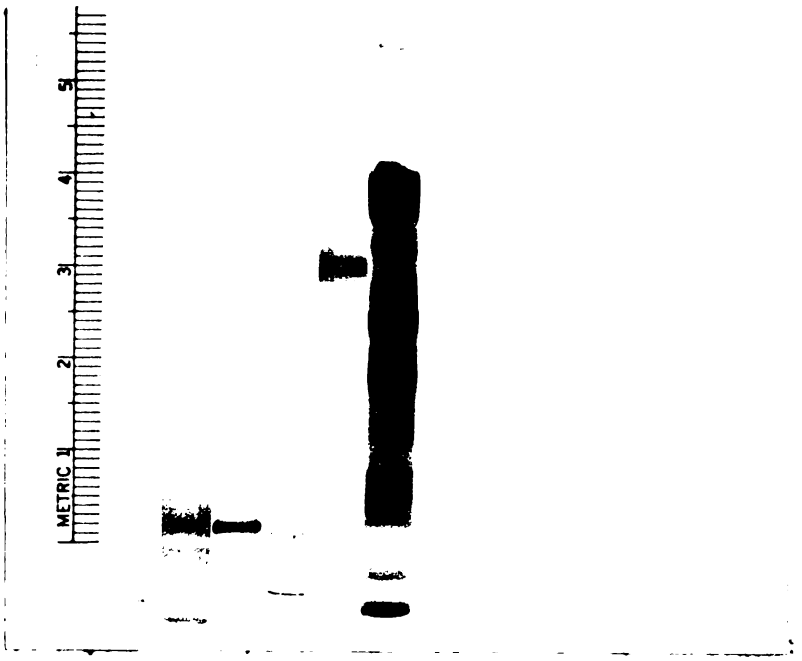


Figure 6. Staining of various groups from adipose and muscle tissue extracts. [1-r. adipose tissue (0_2) extract patterns, 1-ribonucleic acids; 2-nucleoproteins; 3-esterase; 4-glycoprotein; 6-protein; 5-heme proteins in muscle extract.]

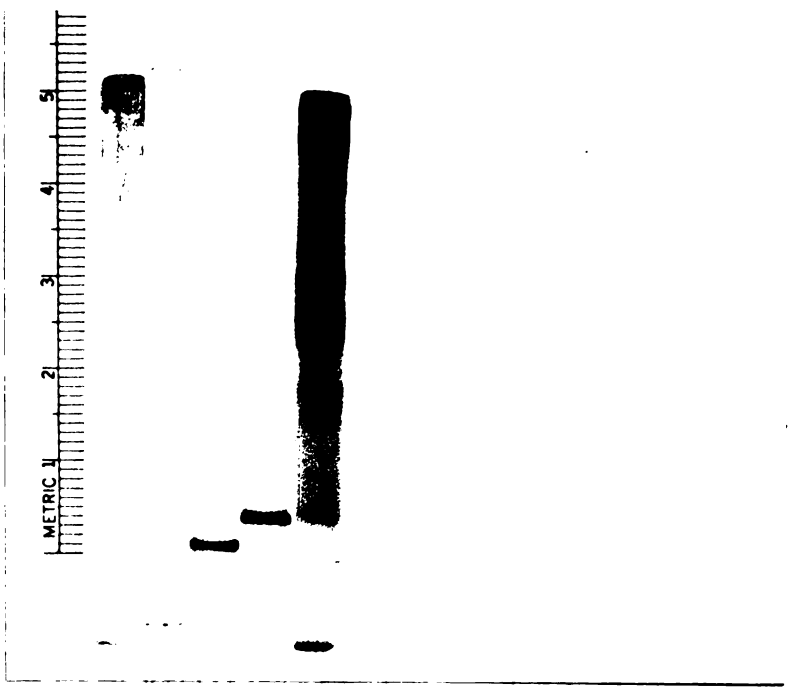


Figure 7. Gel patterns of the water soluble fraction of adipose tissue employing 5 and 7.5% running gels. [1-r. Adipose tissue (0_2) extract patterns, 1-ribonucleic acids; 2-glycoproteins; 4-esterase activity; 5-proteins; 3-esterase activity in a 7.5% running gel.]

Although the specific esterase was not identified, its activity demonstrated that the extraction and concentration procedures used on the tissue were relatively mild. Therefore, the results are believed to represent the water soluble proteins indigenous to adipose tissue, and are probably not breakdown products.

Figure 8 illustrates the protein and sulfhydryl distribution in all four treatments (N_2, O_2, N_2S and O_2S) using 7.5% running gels. Differences could not be ascertained between the protein and sulfhydryl distribution in either the N_2S and O_2S treatments or between the N_2 and O_2 treatments. Therefore, neither nitrogen nor air appeared to have any affect on the proteins or sulfhydryl groups in the water soluble fractions of adipose tissue. However, upon comparing the protein and sulfhydryl patterns between the salt and salt-free treatments, definite differences are apparent. The protein and sulfhydryl bands at 2.1 cm in Figure 8 are much more intense in the salt treatments than in the salt-free treatments. Conversely, the protein and sulfhydryl bands at 2.6 cm are more distinct in the salt-free treatments than in the salt treatments (Figure 8). There are also more distinct bands at 1.2 cm in the gels of the salt-free treatment than in the salt treatments (Figure 8). The band at 5.8 cm appears to be more intensely stained in the protein gels of the salt treatment than in the corresponding gels of the salt-free treatment. However, when the band at 5.8 cm was stained for sulfhydryl groups, the results were negative for all treatments (Figure 8).

Both Amido Black 10B and Coomassie Blue were used to stain the gels for proteins. The protein bands stained with Coomassie Blue became

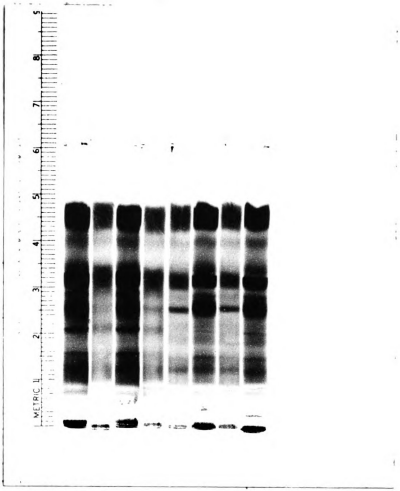


Figure 8. Gel patterns of the water soluble fractions of adipose tissue subjected to N_2, O_2, N_2S and O_2S treatments. (1-r. Adipose tissue extract patterns, 1-N_2S protein; 2-N_2S SH; 3-O_2S protein; 4-O_2S SH; 5-O_2 SH; 6-O_2 protein; 7-N_2 SH; 8-N_2 protein.)

discernible in a much shorter time than those stained with Amido Black, but they were not as intensely stained. However, approximately the same destaining time was required to obtain distinct bands for both stains. Since both stains gave identical protein patterns and Amido Black gave a more intense color, the latter stain was used in all subsequent studies.

Lipoproteins appeared to be absent in the water soluble fraction of adipose tissue in all treatments. No bands could be detected when the gels were stained with either Sudan Black B or Oil Red O.

The disc gel electrophoretic patterns from the water soluble protein fraction of adipose tissue employing a 5-10% gel gradient were not as satisfactory as those obtained from the 7.5% gels. Although many bands were detected, the proteins failed to migrate into the more concentrated portions of the gel, whereas, the protein bands were dispersed throughout the 7.5% gel. Therefore, the 7.5% gel gave more numerous and better separated bands.

Column Chromatographic Separation of Non-Dialyzable and Dialyzable Components

Separation of the water soluble proteins of adipose tissue could not be accomplished employing any of the gel filtration resins. The proteins were eluted in a single peak using columns packed with Bio-Gel P-30, Bio-Gel P-100, Bio-Gel A-1.5 m, Bio-Gel A-15 m and Bio-Gel A-50 m.

Several peaks were observed when Bio-Gel P-2 resin was used to separate the diffusate of the water soluble components from adipose tissue. However, the peaks were not separated sufficiently to give adequate resolution of the components.

The formate form of Dowex 1 anion exchange resin yielded satisfactory separation of the diffusate. Diffusate I (N_2 plus O_2) was separated into five peaks when the gradient system described by Lento et al. (1964) was used. However, a discontinuous gradient system composed of water, 0.5 N formic acid, 1 N formic acid, 3 N formic acid and 6 N formic acid divided diffusate I into eight peaks (Figure 9). Therefore, this elution system was employed to separate diffusate II (N_2S plus O_2S). Diffusate II was also separated into eight peaks, which were eluted at approximately the same elution time using the same system. The principle difference was the increased absorbance of the peaks, which indicated that diffusate II was more concentrated. This was expected because of the different method of concentration.

Diffusate I was concentrated in a flash evaporator at 40°C. However, fine white strands of material appeared in the solution, so rotation was stopped and concentration was completed under reduced pressure at 40°C. When a volume of approximately 100 ml was reached, appreciable amounts of precipitate were present, which was removed by filtration. It is believed that agitation and heat may have exerted adverse affects on the diffusate components. Since a RePP Sublimator became available for remaining studies, diffusate II was concentrated by lyophilization, which is a much milder method of concentration. Upon solubilizing diffusate II in a minimum amount of water, a very light yellow solution was obtained. Whereas, the concentrate from diffusate I was a much darker yellow.

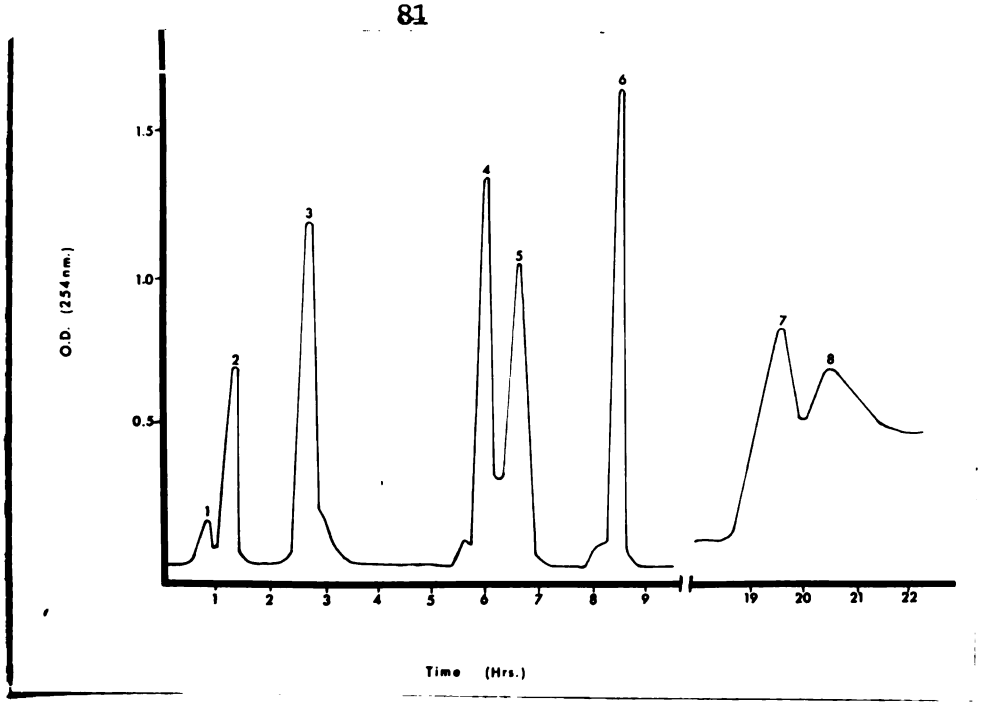


Figure 9. Column chromatographic pattern of diffusate I components. [Elution systems, water (0-2 hr), 0.5 N formic acid (2-5 hr), 1 N formic acid (5-12.5 hr), 3 N formic acid (12.5-17 hr), 6 N formic acid (17-22 hr)].

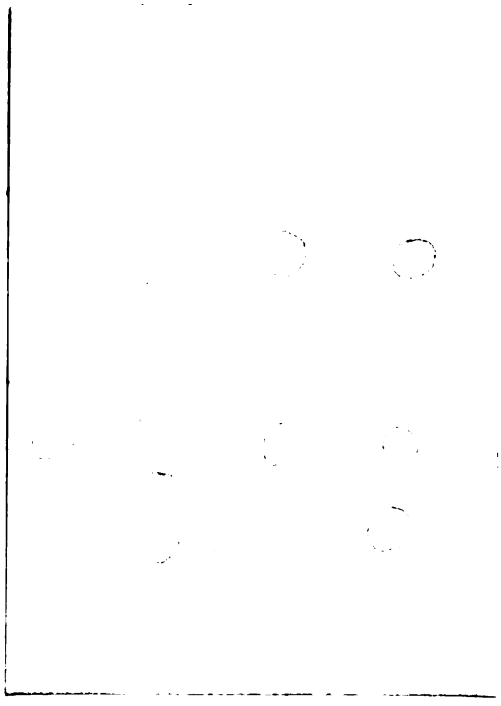


Figure 10. Thin layer chromatographic patterns of diffusate II and standard samples of creatine phosphate, creatine and creatinine. (1-r, 1-4) 1-creatine, 2-diffusate II, 3-creatine and creatinine, 4-creatine phosphate, creatine and creatinine.

Thin Layer Chromatographic Analysis of Dialyzable Components

Preliminary investigations showed that peaks 1-3 contained creatine and creatine derivatives in both diffusates I and II. Peaks 1-6 of both diffusates contained ninhydrin positive components. Peak 2 gave the most intense ninhydrin reaction, while peak 6 gave the least intense. The amino acid and sulfhydryl contents of both diffusates were also similar. Since both diffusates demonstrated these similarities, detailed analyses were conducted only on diffusate II because of its greater concentration.

Creatine phosphate, creatine and creatinine were identified by comparing the R_f values of the unknown to standard compounds, and by the orange color developed on spraying both the unknowns and standards with picric acid and sodium hydroxide solutions. The R_f values for standard samples of creatine phosphate, creatine and creatinine were 0.08, 0.23 and 0.56, respectively (Figure 10). The R_f values for the unknown orange spots were 0.09, 0.24 and 0.56, thus, establishing the presence of creatine phosphate, creatine and creatinine, respectively, in the adipose tissue diffusate. Although peaks 1-3 contained creatine and its derivatives, the heaviest concentration of these components was found in peak 2.

Lactic acid was not detected by employing either the Silica Gel G or the Cellulose MN 300 plates with their respective solvent systems. Carbonyl compounds could not be detected when the chromatograms were sprayed with 2,4-dinitrophenylhydrazine solution. When aliquots of all the peaks were spotted on Silica Gel plates and sprayed for phosphate, all peaks except 4 and 5 gave positive reactions.

Peaks 1 and 2 streaked extensively when cellulose plates were used. Peak 3 possessed one u.v. absorbing spot which was also present in peak 4. Peak 3 contained no components that fluoresced at 254 nm, while fluorescing substances were just barely visible at 366 nm. Peaks 4-8 contained components that fluoresced at both 254 and 366 nm, however, peaks 7 and 8 demonstrated the most intense fluorescence. Upon developing the cellulose plates with water, peaks 4-8 possessed yellow fluorescent material in the region of the origin, whereas, the yellow fluorescent material of these same peaks appeared at the solvent front when the cellulose plates were developed with the methanol-HCl-water solvent system. All fluorescing components, except those that fluoresced yellow, demonstrated 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 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).

It also appears likely that all fractions collected after the elution of peak 3 contained these fluorescent substances. Compounds absorbing u.v. light are responsible for the peaks. If these fluorescent compounds are continually eluted, there would be no noticeable baseline fluctuations. These compounds could be non-enzymatic browning products, since Spark (1969) reported that browning products fluoresce under u.v. light. Also, chemical analyses revealed the presence of both aldoses and free amino groups, which are necessary for non-enzymatic browning.

The browning reaction probably accounts for the dark yellow color of the concentrate from diffusate I. To concentrate this diffusate, the

solution was held at an elevated temperature (40°C) for a prolonged period. These conditions would greatly enhance the browning reaction. However, the concentration of diffusate II required no heating, except that required for sublimation, thus, the browning reaction would probably be largely inhibited. The lack of browning probably accounted for the light yellow appearance of diffusate II. However, it is not possible to competely prevent browning from occurring when the solution contains aldoses and free amino acids.

Figure 11 shows the thin layer chromatogram of various standard compounds and peaks 4, 5, 6, 7 and 8 upon development with water. Figure 12 is a chromatogram, which is identical to the chromatogram in Figure 11 except that it was developed with a methanol-HCl-water solvent system. Table 3 gives the R_f values for the standard and unknown compounds in the above chromatograms.

Since peaks 6, 7 and 8 contained components that were phosphate positive, they were next subjected to thin layer chromatographic systems designed for nucleotide separation. Cellulose MN300 plates (laboratory prepared) were spotted with peak 6, 7 and 8 and developed with a tert.amyl alcohol-formic acid-water solvent system (Figure 13). Peak 6 was 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 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 u.v. light very faintly and gave a fairly intense positive phosphate reaction.

Table 3. R_f values for standard and unknown compounds in diffusate II. Cellulose MN300 plates (Laboratory prepared).

Compound	R_f[1]	R_f[2]	Peak	R_f[1]
Adenine	0.41	0.48	4	0.80, 0.75[a], 0.64[a], 0.51[a], 0.03[b]
Adenosine	0.63	0.57	5	0.64[a], 0.03[b]
Cytidine	0.77	0.65	6	0.60, 0.05[b]
Cytosine	0.70	0.62	7	0.95, 0.90[a], 0.82[a], 0.69[a], 0.38[a], 0.13[b]
Guanidine	0.00	streak	8	0.93, 0.31, 0.08[b]
Guanosine	0.64	0.47		
Hypoxanthine	0.66	0.45	Peak	R_f[2]
Inosine	0.84	0.59	4	0.99[b], 0.81[a], 0.73, 0.65, 0.62[a]
Thymidine	0.87	0.88	5	0.99[b], 0.73, 0.65, 0.62[a]
Thymine	0.81	0.81	6	0.99[b], 0.82, 0.36
Uracil	0.80	0.74	7	0.99[b], 0.83, 0.68[a], 0.61[a], 0.53[a], 0.43[a], 0.35[a]
Uridine	0.88	0.76	8	0.99[b], 0.84, 0.67[a], 0.54[a], 0.44[a], 0.34[a]

[1]Solvent-water.
[2]Solvent-CH_3OH, HCl, H_2O (65:17:18).
[a]Indicates light blue fluorescence (254 and 366 nm).
[b]Indicates yellow fluorescence (254 and 366 nm).

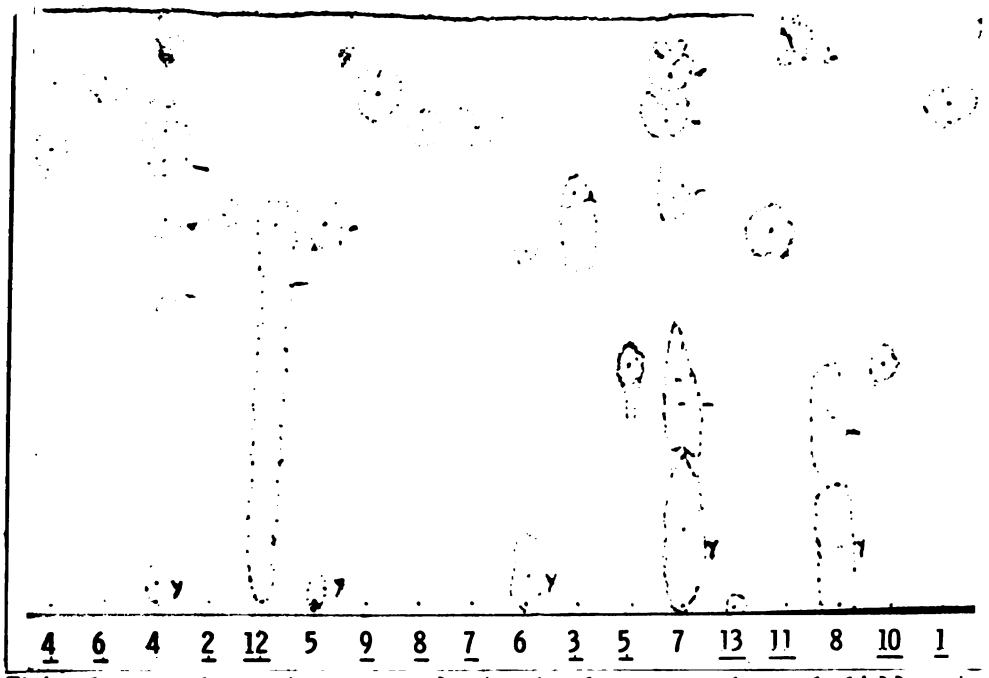


Figure 11. Thin layer chromatograms of standard compounds and diffusate II components. Cellulose MN300 plate (laboratory prepared); Solvent-water. 1-inosine, 2-hypoxanthine, 3-cytosine, 4-cytidine, 5-adenine, 6-uridine, 7-uracil, 8-thymine, 9-thymidine, 10-adenine, 11-adenosine, 12-guanosine, 13-guanidine, 4-peak 4, 5-peak 5, 6-peak 6, 7-peak 7, 8-peak 8.

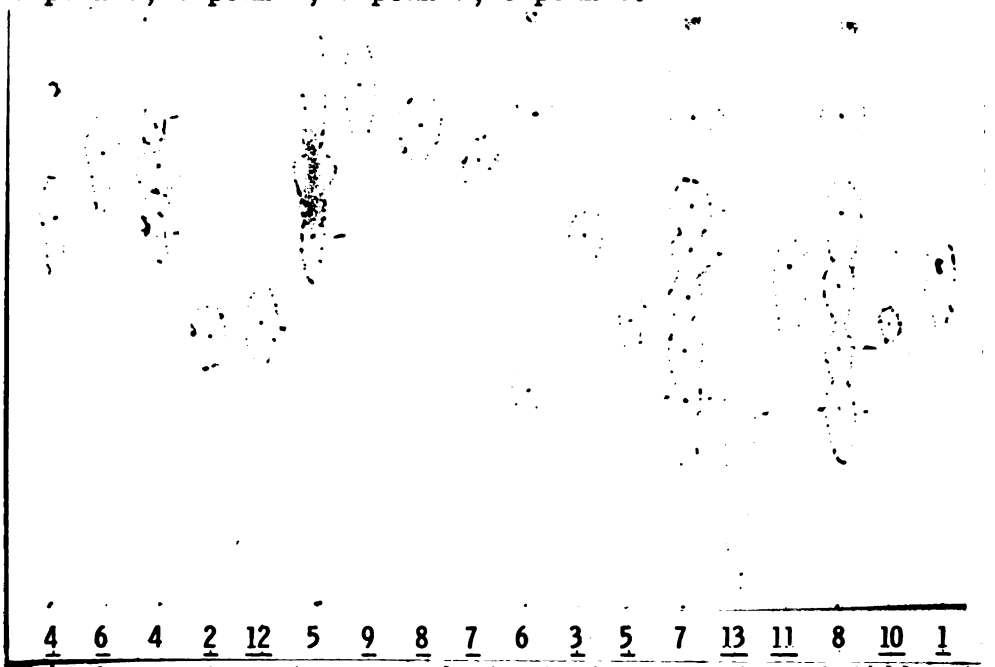


Figure 12. Thin layer chromatograms of standard compounds and diffusate II components. Cellulose MN300 plate (laboratory prepared); Solvent-CH_3OH, HCl, H_2O (65:17:18). (See Figure 11 for sample identification.)

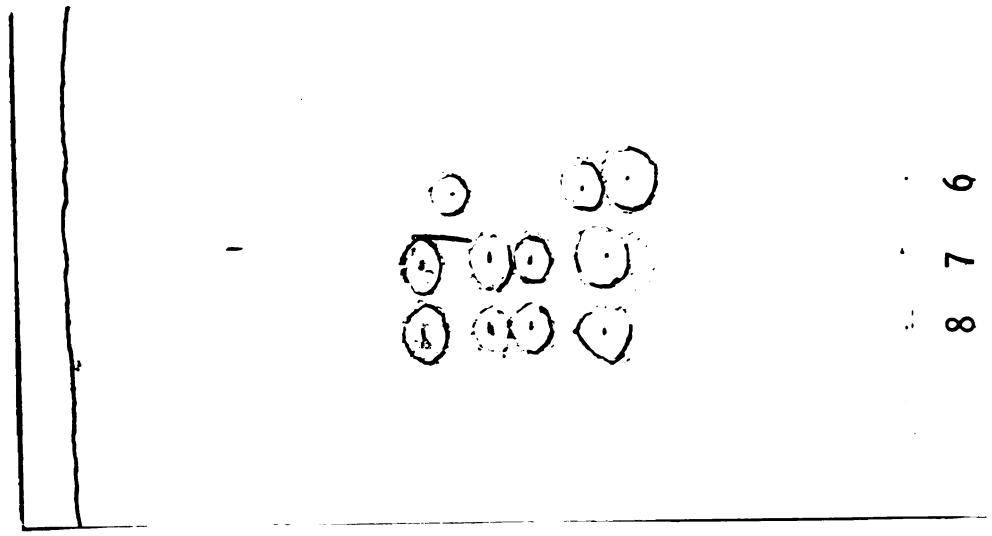


Figure 13. Thin layer chromatogram of diffusate II components. Cellulose MN300 plate (laboratory prepared); solvent-tert. amyl alcohol, formic acid, water (3:2:1)(1-r. Diffusate II, 8-peak 8, 7-peak 7, 6-peak 6.)

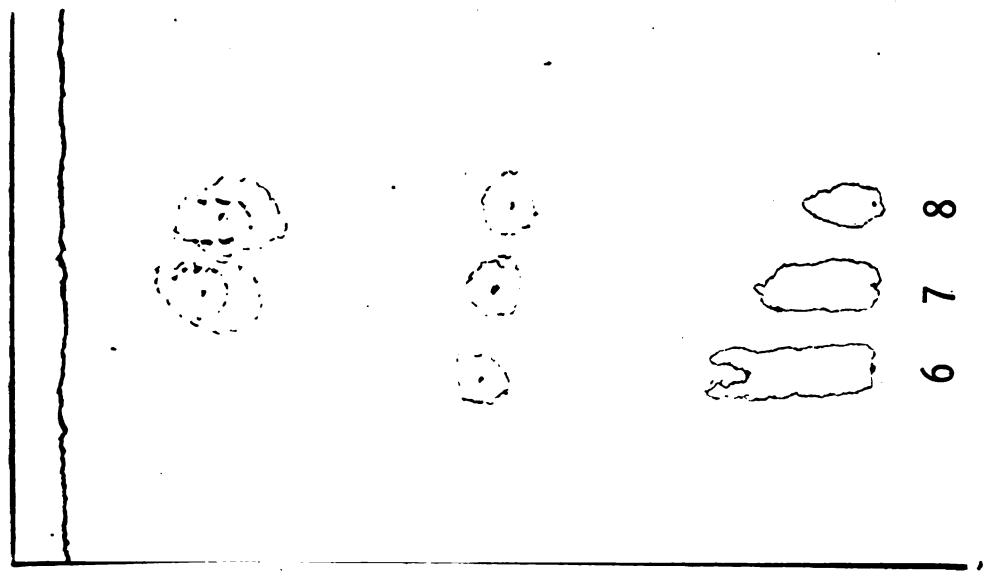


Figure 14. Thin layer chromatogram of diffusate II components. ECTEOLA plate (laboratory prepared); solvent-0.15 M NaCl (1-r. Diffusate II, 6-peak 6, 7-peak 7 and 8-peak 8.)

To determine whether spot 4 (Figure 13) was a nucleotide, peaks 7 and 8 were subjected to anion exchange thin layer chromatography. ECTEOLA plates were prepared according to Randerath (1966) using 0.15 M sodium chloride as the developing solvent. After the solvent was allowed to travel 10 cm, both peaks 7 and 8 were separated into three components. Peaks 7 and 8 both contained fractions that streaked from the origin to a height of 1.5 and 1.0 cm, respectively. The R_f values for spots 2 and 3 of peak 7 were 0.47 and 0.83, respectively, while peak 8 revealed two spots with R_f values of 0.44 and 0.81 (Figure 14).

Peak 6 was also applied to ECTEOLA plates to confirm the absence of nucleotides. Peak 6 was separated into two components. The first component streaked from the origin to a height of 2.0 cm, while the second component had a R_f value of 0.48. All spots were phosphate negative in peaks 6, 7 and 8. Peaks 7 and 8 were similar, since both revealed blue streaks from above their second components (R_f = 0.47 and 0.44, respectively) to the solvent front. Peak 6 demonstrated blue streaks from component 1 (0-2.0 cm streak) to the solvent front. However, none of the components in peaks 6, 7 and 8 contained any phosphate positive material. Therefore, nucleotides were absent in the diffusate. The components of peaks 6, 7 and 8 that streaked from the origin to 2.0, 1.5 and 1.0 cm, respectively, gave strong white fluorescence under u.v. light. However, after spraying the chromatograms with 2,7-dichlorofluorescein, the components that streaked at the origin fluoresced giving a brilliant purple color under u.v. light.

All thin layer chromatographic systems except those designed for lactic acid, creatine and its derivatives and 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 Analysis of Dialyzable and Non-Dialyzable Components

The protein and sulfhydryl content of the water soluble protein fraction of adipose is given in Table 4. Free amino group, aldose and sulfhydryl analyses of diffusates I and II are also listed in Table 4.

Table 4. Chemical analyses of water soluble dialyzable and non-dialyzable adipose tissue components.

Treatment	Protein mg/ml	Sulfhydryl μm/g protein	Free amino groups μm/g powder[7]	Aldoses μm/g powder[7]
N_2[1]	9.81	9.05		
O_2[2]	9.30	8.17		
N_2S[3]	12.16	16.00		
O_2S[4]	11.61	15.79		
I[5]		Trace	273.20	40.23
II[6]		Trace	48.24	3.76

[1]Adipose tissue stored under nitrogen
[2]Adipose tissue stored under air
[3]Adipose tissue with 3% NaCl stored under nitrogen
[4]Adipose tissue with 3% NaCl stored under air
[5]Combined diffusates from N_2 and O_2 treatments
[6]Combined diffusates from N_2S and O_2S treatments
[7]Lyophilized extract

On studying the precursors of hydrogen sulfide in the water soluble fraction of adipose tissue, the effect of different atmospheres (nitrogen and air) on the protein sulfhydryl groups was determined. Since sausage products contain high fat levels and approximately 3% sodium chloride, the influence of air or nitrogen atmospheres on adipose tissue is of special interest. It seemed probable that air would reduce the sulfhydryl content due to oxidation. As sodium chloride has been shown to accelerate autoxidation of adipose tissue (Ellis et al., 1968; Gaddis, 1952; Chang and Watts, 1950) and because autoxidation continues during freezer storage (Ellis et al., 1968; Gaddis, 1952), it was anticipated that the sulfhydryl content of the salt treated samples would decrease to a greater extent than that of the salt-free samples.

Results suggest that there was a slight atmospheric effect on the sulfhydryl content in both the salt and the salt-free treatments. In both treatments, the sulfhydryl content was slightly lower in air than in the nitrogen atmosphere. However, the most significant and unexpected finding was the difference in sulfhydryl content between the salt and salt-free treatments. Under either nitrogen or air, the sulfhydryl content of the salt treatments was almost twice that of the salt-free treatments. The greater sulfhydryl content in the salt treatments was also verified by disc gel electrophoresis (Figure 8) as shown by a larger number of sulfhydryl bands.

Ellis et al. (1968) reported that the fat immediately adjacent to the lean streaks in bacon had higher peroxide and monocarbonyl values. They found an increase in the lean to fat ratio greatly enhanced autoxidation

during freezer storage in lean and fat mixtures containing 4% sodium chloride. It was also noted that the C_6 alkanal was the primary oxidation product, while there appeared to be a selective oxidation of linoleate. They suggested that the lean in the sodium chloride-cured bacon exerted a special effect on tissue oxidation, and that some component(s) in the lean was/were activated by sodium chloride, which changed the oxidation characteristics of pork adipose tissue. They finally concluded that a characteristic sodium chloride-accelerated oxidation occurred during freezer storage of pork. They stated that the meat pigments were relatively inactive in the salt-free samples during freezer storage of pork, although they acknowledged the non-specific autoxidation effect of hematin. They then speculated that heme pigments may specifically decompose linoleate hydroperoxide to form monocarbonyls. They further stated that nothing is known about the separate catalytic effects of heme pigments.

Lea (1937) reported the presence of a lipoxidase in pork. However, Tappel (1952) was unable to demonstrate the presence of lipoxidase in animal tissues. Ellis et al. (1968) acknowledged the absence of lipoxidase, but stated that knowledge of the independent oxidative influence of sodium chloride and lipoxidase would serve to clarify the mechanisms involved.

In the present study, disc gel electrophoresis separated the water soluble extract of adipose tissue into a number of bands. The sulfhydryl content of these bands was greater in the salt treatments (Figure 8). It is possible that sodium chloride could bring about an increase in the number of sulfhydryl bands in two ways. First, salt could stimulate reduction of disulfides, or secondly, it could stabilize the sulfhydryls

present by preventing their oxidation. The stabilizing action of salt seems to be more reasonable than the reduction of disulfides, yet the mechanism for sulfhydryl stabilization is not understood.

The sulfhydryl content of both diffusates I and II could not be determined. There was a very slight increase in the yellow color of the samples, but the amount of chromophore developed was too small to be accurately measured.

Both aldoses and free amino groups were present in the diffusate, therefore, a system conducive to development of browning existed. On heating adipose tissue to 100°C, browning occurred. Thus, the carbonyl compounds and free amines from adipose tissue may, in part, be responsible for browning of meat. Tonsbeek et al. (1969) ether extracted the aqueous fraction of a heated beef-water slurry, which possessed a typical meaty odor, and detected two furanone derivatives. They stated that the derivatives resulted from the browning reaction and could contribute to the aroma of beef broth, thus, indicating that browning products may be involved in the production of meat aroma. Therefore, the aldoses and amine compounds of the diffusate may participate in production of meat aroma. Since amino acids have been found in the diffusates, they could be responsible for some of the free amino groups.

Peaks 7 and 8 of diffusate II were very tightly held by the anion exchange resin. Thus, it is possible that they may contain sulfate groups. White et al. (1964) have previously reported that sulfate groups are present in many biological systems, such as blood, urine, lipids, mucopolysaccharides and others. Therefore, the diffusate fractions were subjected

to the barium chloride test for sulfate. However, the amount of precipitate was so minute that the test was inconclusive.

Development of a Method to Determine Sulfhydryl and Disulfide Groups

The analysis of sulfhydryl and disulfide groups was based on the formation of a S-nitroso derivative with ultimate formation of an azo dye. The first step involved disulfide reduction with tetramethylammonium borohydride (Equation 1).

$$RSSR \xrightarrow{[H]} 2\ RSH \qquad (1)$$

The excess reducing agent was destroyed with an excess of nitrous acid (Equation 2).

$$3\ (CH_3)_4\ NBH_4 + 8\ HONO \longrightarrow 3\ (CH_3)_4\ NH_2BO_3 + 4\ N_2 + 7\ H_2O \qquad (2)$$

The excess nitrous acid reacted with the thiol to form a S-nitroso derivative (Equation 3).

$$RSH + HONO \longrightarrow RSNO + H_2O \qquad (3)$$

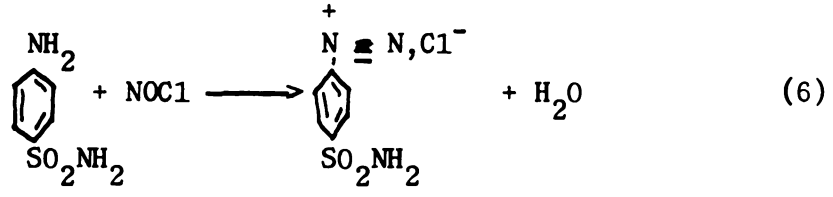
The remaining nitrous acid was destroyed with ammonium sulfamate (Equation 4).

$$HONO + NH_4SO_3NH_2 \longrightarrow NH_4HSO_4 + N_2 + H_2O \qquad (4)$$

The S-nitroso derivative was cleaved with mercuric chloride and nitrosyl chloride was generated (Equation 5).

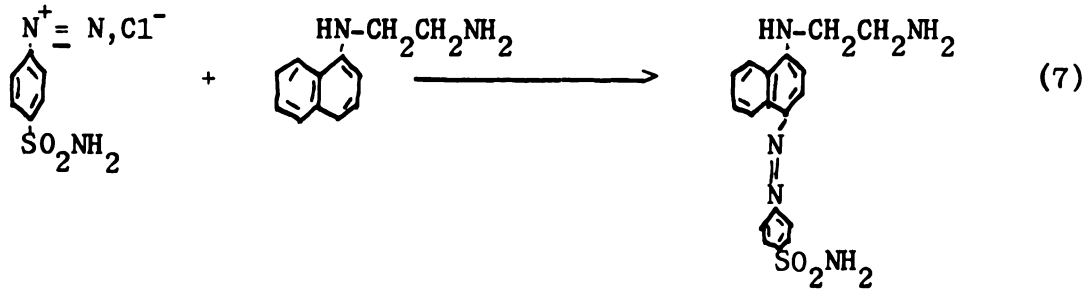
$$RSNO + HgCl_2 \longrightarrow (RS)_2\ Hg + 2\ NOCl \qquad (5)$$

The nitrosyl chloride combined with sulfanilamide to form a diazonium salt (Equation 6).



(6)

The diazonium salt was coupled with N-1-naphthylethylenediamine to form an azo dye (Equation 7). The dye gives maximum absorbance at 540 nm and exhibits a 1 to 1 stoichiometric relationship with the thiols, i.e., one mole of dye is formed per mole of thiol.



(7)

Because urea destroys nitrous acid, it was not suitable as a protein denaturating agent. In the present study, sodium lauryl sulfate (1%) in 0.1 M phosphate buffer (pH 8.0) was successfully used in studying the reduction of DTNB. Since sodium lauryl sulfate (0.9%) has been used by Damjanovich and Kleppe (1967) as a denaturating agent for exposing the sulfhydryl groups in proteins, it would appear to be applicable to the present method. In the present study, a period of 5 min was allowed for the destruction of nitrous acid by ammonium sulfamate. The concentration of mercuric chloride was kept to a minimum to prevent formation of a mercuric chloride-diazonium slat complex, which gives a precipitate.

Since Kobayashi (1966) demonstrated that the diazonium salt decomposed with increasing temperatures, it is suggested that the method be modified by reducing the temperature of the reaction mixture to 5°C after destruction of nitrous acid with ammonium sulfamate. The mercuric chloride-sulfanilamide solution should also be cooled to 5°C to insure that diazonium salt decomposition is held at a minimum. In modifying the method, it is also believed that cooling of the N-1-naphthylethylenediamine solution to 5°C would increase the stability of the diazonium salt prior to coupling, even though Kobayashi (1966) reported that coupling did not occur below 14°C. Since temperatures higher than necessary are to be avoided, it is believed that coupling would occur at the minimum critical temperature by allowing the reaction mixture to warm up gradually at room temperature. Sodium lauryl sulfate caused foaming on agitation of the reaction mixture, so acetone was added to the N-1-naphthylethylenediamine solution as an antifoaming agent. The acetone, however, changed the maximum absorbance from 540 to 550 nm.

When using DTNB as the potential source of sulfhydryl groups, 5% potassium cyanide in 1.0 N ammonium hydroxide was used for reducing the disulfides. The method was very sensitive and reproducible. However, DTNB possesses no α-amino group, so formation of an iminothiazolidine ring was not possible as is the case for cystine. Since potassium cyanide could not be used as a reducing agent for cystine residues in proteins, a suitable reducing agent was sought. The reducing agent must be stable in aqueous solutions, quantitatively reduce disulfides in an alkaline solution at a pH $<$ 9.0, be completely destroyed by nitrous acid and produce no

decomposition products detrimental to the reaction. Tetramethylammonium borohydride appears to be a suitable reducing agent and is presently under investigation to determine whether it is compatible with protein solutions.

In order to allow addition of 0.5 ml aliquots of each reagent to 0.5 ml of the sample, the following concentrations of reagents are necessary:

Thiol - Dissolve 59.5 mg DTNB in 100 ml 95% ethanol. Dilute 50 to 1 with ethanol.

Reducing agent - Dissolve 22.5 mg tetramethylammonium borohydride in 10 ml water.

Solution A - Dissolve 0.5000 g sodium nitrite in 100 ml 1.0 N HCl.

Solution B - Dissolve 0.6000 g ammonium sulfamate in 100 ml water.

Solution C - Make a solution containing 0.0490 g mercuric chloride in 100 ml 0.4 N HCl. Add 0.2580 g sulfanilamide to 50 ml of the solution.

Solution D - Dissolve 0.1950 g N-1-naphthylethylenediamine dehydrochloride in 10 ml 0.4 N HCl and make to a final volume of 25 ml with acetone.

To 0.5 ml of disulfide, add 0.5 ml reducing agent (tetramethylammonium borohydride) and allow the necessary time for complete disulfide reduction. To the reduced solution, add 0.5 ml solution A (nitrous acid) and allow 1 to 2 min for the destruction of the reducing agent and the formation of the S-nitroso derivative. Add 0.5 ml of solution B (ammonium sulfamate) and allow 5 min for complete destruction of excess nitrous acid. Cool the mixture to 5°C in an ice bath and add 0.5 ml solution C (sulfanilamide-mercuric chloride) at 5°C, allow 1 to 2 min for the formation of the

mercuric mercaptide and the diazonium salt. Add 0.5 ml of solution D (N-1-naphthylethylenediamine), at 5°C, to the cool reaction mixture and allow time for maximum color development. The dye has been reported to be stable for at least 3 hrs by Kobayashi (1966).

Elemental analysis of the synthesized N-1-acenaphthylethylenediamine has not been carried out to date. It has, however, undergone coupling in an acid medium with a diazonium salt, which indicates that it is an aromatic amine. The dye formed is similar in color to the azo dye of N-1-naphthyl-ethylenediamine.

The method is rapid for both sulfhydryl and disulfide groups in low molecular weight disulfides. After protein denaturation and exposure of the disulfide groups, however, the method should also be rapid and efficient. The time required for protein denaturation is dependent on the nature of the protein or proteins being studied. The actual time to complete color development from thiol formation is approximately 20 min for triplicate samples. The method is specific for thiols and none of the reagents absorb in the region of the dye. However, the method cannot be used if the material under investigation contains substances that will destroy nitrous acid or diazonium salts. Fortunately, substances of this nature are not normally present in proteins, so the method should be useful for disulfide and sulfhydryl analyses of proteins.

SUMMARY

Beef adipose tissue was separated into the water soluble, salt soluble and insoluble (water-salt-ether) fractions, and the amount of hydrogen sulfide evolved upon heating each of the fractions was determined. The yield of hydrogen sulfide per 100 g of adipose tissue was 17.6, 1.7 and 5.5 μM for the water soluble, salt soluble and involuble fractions, respectively. Thus, the water soluble fraction contributed 71% of the hydrogen sulfide, while the corresponding contributions from the salt soluble and insoluble fractions amounted to approximately 7 and 22%.

The water soluble fraction of adipose tissue was subjected to four treatments (air and nitrogen atmospheres and 3% sodium chloride under each atmosphere). The sulfhydryl groups and other possible flavor precursors were determined. The aqueous extract from each treatment was divided into dialyzable and non-dialyzable fractions. The dialysates were concentrated by pervaporation and dialysis against 1 M sucrose. The diffusates were concentrated by either flash evaporation or lyophilization.

The dialysates were successfully separated by disc gel electrophoresis, and demonstrated the presence of heme proteins and the absence of glycoproteins, nucleoproteins and lipoproteins. The sulfhydryl contents of the nitrogen, air, nitrogen with 3% salt and air with 3% salt treatments were 9.05, 8.17, 16.00 and 15.79 μM per g of protein. Thus, there was a slight atmospheric effect on the sulfhydryl contents, since the air treatments for both salt and salt-free treatments were lower than those under nitrogen.

The salt treatments under either air or nitrogen contained almost twice the sulfhydryl content of the salt-free treatments. The increase in sulfhydryls in the salt treatments were also reflected in the disc gel electrophoretic patterns, which contained a greater number of sulfhydryl containing bands.

Amino acid analysis of both dialysates and diffusates demonstrated the presence of lysine, histidine, arginine, aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, cystine, valine, methionine, isoleucine, leucine, tyrosine and phenylalanine. Glycine, alanine and glutamic acid comprised approximately 25 and 50% of the amino acid residues in the dialysates and diffusates, respectively.

The diffusates were separated into a number of fractions employing a Dowex 1 anion exchange resin (formate form). These fractions were subjected to thin layer chromatography and contained creatine, creatine phosphate, creatinine, uracil, cytosine, some unidentified phosphate containing compounds and a number of fluorescent compounds. Purine bases, nucleosides, nucleotides and lactic acid were absent.

A quantitative method for determination of sulfhydryl and disulfide groups in proteins based upon formation of an azo dye was investigated. After reduction of the disulfides, the sulfhydryl groups were nitrosated. The S-nitroso derivatives were cleaved with mercuric chloride. The resulting nitrosyl chloride was combined with sulfanilamide to form a diazonium salt. The diazonium salt was coupled with N-1-naphthylethylenediamine to give an azo dye. The dye absorbs maximally at 540 nm. The thiol:dye exists in a 1:1 stoichiometric relationship.

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