ALTERATIONS IN PORCINE MUSCLE PROTEIN SOLUBILITY AND PROTEIN FUNCTIONAL GROUPS AS AFFECTED BY THE SMOKING PROCESS.

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ABSTRACT

ALTERATIONS IN PORCINE MUSCLE PROTEIN SOLUBILITY AND PROTEIN FUNCTIONAL GROUPS AS AFFECTED BY THE SMOKING PROCESS.

BY

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There have been few investigations on the effect that smoke has upon meat proteins. Although it is definitely known that heating affects protein properties, little information is available regarding the effect of smoke which often accompanies this heating process. The primary objective of this study was to determine if smoke *per se* had any effect upon meat proteins.

Pork *longissimus dorsi* muscles were sliced 1.1 cm thick, heated and heated smoked for 2.25 hr under one of two smokehouse conditions; the heated sample was used as a heated, non-smoked control. To obtain a cold smoked sample, the smokehouse condition was 32.2°C (90°F) and 45% relative humidity; to obtain a heated smoked sample, the smokehouse condition was 60°C (140°F) and 45% relative humidity.

To study the changes in protein solubility of pork samples, the nitrogen from these samples was fractionated into low ionic strength, sarcoplasmic and nonprotein nitrogen, total fibrillar protein nitrogen, soluble and denatured fibrillar protein nitrogen and stroma protein nitrogen fractions. Electrophoretic studies of the water, Weber-Edsall and meat-urea extracts were performed by starch gel, starch-urea gel and disc gel electrophoresis. Alterations in protein functional groups were determined on the heated and heated smoked pork and albumin samples as well

as on samples to which artificial smoke and phenolic compounds were added. In addition, an aqueous smoke solution was collected and subjected to thin layer chromatography for the detection of ninhydrin positive compounds.

Results indicated that considerable variation in protein composition existed between the untreated and treated pork loin samples. The solubility of the low ionic strength fraction decreased significantly ($P < 0.01$) during heating and heating smoking with the majority of this change being due to the loss in extractibility of the sarcoplasmic protein fraction. Starch gel electrophoresis of this fraction substantiated these results. The total fibrillar protein nitrogen fraction exhibited considerable changes with an increase being observed with the heated samples and a decrease with the heated smoked samples. Disc gel electrophoretograms of Weber-Edsall and meat-urea extracts gave similar results. A substantial increase in the stroma fraction was obtained with the heated smoked samples. These studies indicated that smoke definitely caused changes in protein solubility and electrophoretic behaviour of meat proteins.

Studies of protein functional groups demonstrated that heating and heating smoking caused changes in the pH, free sulfhydryl groups and amino nitrogen content of pork and albumin samples. The addition of artifical smoke and phenolic solutions also affected the free sulfhydryl and amino groups. Acid phosphatase activity was also affected during heating and heating smoking of pork samples. These results indicated that smoke constituents probably interact with protein functional groups.

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An interesting observation was the increase in the total fibrillar protein nitrogen fraction, pH and free sulfhydryl groups of the heated samples and the decrease of these values with the heated smoked samples.

The presence of ninhydrin positive compounds was not detected in a sample of aqueous smoke solution.

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INTRODUCTION

Wood smoke, used as a meat curing agent, may be considered as the effluent produced during the heat destruction of wood, generally under conditions of partial or incomplete combustion. Physically, wood smoke is an aerosol composed of a particulate phase suspended in a vapor phase. Most of the compounds responsible for smoke flavor deposition come from the vapor phase, and the smoking process is essentially a vapor scrubbing process. Chemically, wood smoke is composed of over two hundred compounds of which three main classes of compounds are considered to be of importance in smoked foods; phenols, acids, and aldehyde- and ketone-like organic compounds (carbonyls). The polycyclic hydrocarbons, an undesirable class of chemicals, have also been detected in wood smoke and smoked foods.

Meat smoking has been practiced for a long time, probably since prehistoric times. Without doubt, one of the primary purposes of smoking in the years gone by was to aid in the preservation of meat products. The preservation was due mainly to the bacteriostatic, antioxidant and dehydration properties imparted to the food during the smoking process. Today, smoking is done primarily to impart a characteristic desirable flavor and odor and to develop a desirable finish or gloss on the skin and/or flesh side of the smoked product. As practiced in the conventional smoking systems used, other processes may be accomplished simultaneously or complementary to the smoking. Heating is necessary for the development and fixation of cure color of lean portions of meat; cooking is required for pasteurization (bacterial destruction) and/or to produce a particular style of product. Also, the smokehouse is used for controlled drying or yield control. A great deal of biochemical research on muscle proteins in the raw or native state has been carried out. However, the chemical changes of muscle proteins occurring during the heating process have been studied infrequently. The heating of meat causes certain physical and chemical changes in meat proteins which affect the quality of cooked meats and meat products. Changes in protein solubility, ATPase activity of myosin, the contractibility of the muscle fibers, the hydration of muscle proteins, and changes in the sulfhydryl and disulfide groups in muscle proteins have been used to determine the extent of denaturation of muscle protein during heating. There have been very few investigations on the effect that smoke has upon meat proteins; the available data on the role of different smoking components affecting flavor, aroma and appearance of smoked products are scarce. The research in this area has dealt with changes in protein solubilities, changes in free amino acid content during smoking, surface color development of smoked food products as well as the interaction of smoke components with meat constituents.

The primary objective of this study was to determine whether smoke *per se* had any effect upon meat constituents, particularly meat proteins. The more detailed objectives were as follows:

1. To determine the effect that smoke has upon the protein solubility and electrophoretic patterns of pork samples.
2. To determine if smoke caused alteration in protein functional groups of smoked albumin and pork.

3. To determine if individual phenolic compounds effect the pH, the amino nitrogen and free sulfhydryl groups of albumin and pork.

4. To determine if any ninhydrin positive compounds were present in wood smoke.

REVIEW OF LITERATURE

Properties Acquired During Smoking.

Historically, and until relatively recent years, the smoking process was carried out to preserve meat or other food products over prolonged periods. Even today, a large part of the world's production of smoked meats; including fish, is used for the purpose of preservation. However, the mildly cured, lightly smoked-flavored meat products found in this country are only slightly less perishable than fresh meats. With the development of other methods of preservation, foods are now smoked mainly for their sensory qualities (Fiddler et al., 1966).

The smoking of meats today is almost always combined with heating in the smokehouse. The following effects may be listed as resulting from the deposition of smoke constituents and the effects of temperature (Brissey, 1959; Draudt, 1963; Tilgner, 1967) : (1) development and fixation of color of lean portions, (2) the imparting of a desirable finish or gloss on the skin and/or flesh side of the meat pieces, (3) the imparting of desirable organoleptic properties to foods, (4) the impregnation of the outer portions of the meat with smoke constituents that serve as effective antioxidant, bacteriostatic and bactericidal agents (preservation), (5) a tendering action from increased activity of autolytic enzymes of meat due to elevated temperatures, (6) a reduction of the micro-organism level present in the meat, (7) dehydration which results in a protective film on the surface of the smoked product, and (8) yield control.

Application and Factors Affecting the Smoking Process.

The smoke for food processing is produced commercially in the United States by three main methods; by burning dampened sawdust, by burning dry sawdust and by friction (Draudt, 1963). The most common method currently used is burning dampened sawdust in a batch operation; in a few cases, smoke is still produced by burning a pile of sawdust under the smokehouse (Table 1). Liquid smoke materials prepared by burning hardwood or hardwood sawdust are also available; however, they play a minor rôle in overall usage (Howard *et al*., 1966).

Table 1. Examples of smoked foodstuffs and methods used for smoking these products. ‡

Products	Method Used for Smoking
Frankfurters, bologna, hot sausage, smoked hams and picnics, smoked pork loin, bacon, smoked chicken and turkey, smoked salmon.	Hickory or hardwood sawdust damp-burned in atmospheric furnace and smoke forced-draft, pumped or circulated by mechanical convection in smokehouse.
Lebanon bologna, smoked fish, Smithfield-type smoked ham, Provolone cheese.	Hardwood logs, sawdust, hickory wood burned on floor of smokehouse.
Smoked pastrami, kippered cod.	Hardwood sawdust burned next to smoke-house; smoke gravity fed to smokehouse.
Smoked whitefish, smoked chub.	Charcoal burned in smoke oven under product.
Smoked cheese, barbecue sauce.	Aqueous soluble hickory smoke (liquid smoke).

‡From Malanoski *et al*. (1968).

Although Table 1 is not a complete representation, examples of smoked foodstuffs and methods used for smoking in this country are presented. There are two distinct types of smoking processes, cold and hot smoking, depending on the amount of heat to which the foodstuff is subjected. In cold smoking, the flesh temperature is taken up to 30°C (Foster, 1959), and in hot smoking, a flesh temperature of 90°C may be attained. Woskresienskij (1962) explained that with cold smoking a chemical rather than a thermal denaturation occurred and he found that a cold smoked product possessed more intense smoked flavor, odor and color than the hot smoked product.

The composition of curing smoke is believed to be affected by many factors including type of wood, type of generator, moisture content of the wood, temperature of combustion, and air supply (Draudt, 1963). Additional factors which might affect the rate of deposition and composition of smoke constituents include the method of application, the wetness of the product surface and the smoke temperature (Howard _et al._, 1966).

Although sawdust of hardwoods is preferred to those of soft, coniferous woods in this country, various workers (Rusz, 1960; Spanyár _et al._, 1960; Tilgner and Wierzbicka, 1959) have reported no consistent variations in the smoke produced by hard and soft woods. Although Rusz (1960) and Spanyár _et al._ (1960) indicated that variations in water content of the sawdust did not alter the chemical composition of smoke, Jahnsen (1961) and Tilgner _et al._ (1962c) noted a dilution effect in the phenolic content of smoke with increased moisture content of sawdust.

In comparing different types of smoke, it was found that smoke produced by a friction generator is much more concentrated in acids, carbonyls and phenols than smoke produced by smouldering sawdust (Husaini and Cooper, 1957; Rusz, 1960; Tilgner _et al._, 1962a); thus resulting in higher smoke flavor intensity. Electrostatic smoke deposition has been examined (Foster, 1959; Rusz, 1968; Woskresienskij, 1962) but generally, in respect to color, flavor and odor, foodstuffs smoked conventionally are preferred to those smoked electrostatically.

It has been shown that generation temperature influences the composition of the resulting smoke (Porter et al., 1965; Rusz, 1960; Simon et al., 1966; Tilgner et al., 1962b) with a range of 260°C to 350°C being the most applicable for smoke generation. The rate of air flow also influences smoke composition (Pettet and Lane, 1940; Tilgner et al., 1962b).

Simon et al. (1966) demonstrated with water filled casings that the temperature and relative humidity in the smokehouse influenced appreciably the total absorbed smoke which increased with increased smokehouse temperature and decreased with increased per cent relative humidity. However, Dolezal (1959) found that the best quality smoked cured meats were obtained by processing in smoke of increased per cent relative humidity. The absorption of smoke vapors is also enhanced by increased water content of foods and increased smoke velocity in the smoke chamber (Foster and Simpson, 1961).

The Composition of Wood Smoke.

Goos (1952) revealed the complexity of wood as a chemical substance by listing over two hundred separate compounds that have been identified in the products of its destructive distillation. However, as was mentioned by Draudt (1963), these same compounds may not all exist in smoke since the products of heating wood depend considerably on the conditions under which it is heated.

The combustion of wood for the smoking of foods involves two stages. First of all, a thermal, destructive breakdown of the wood particles (pyrolysis) occurs and the resultant breakdown products are then oxidized (Tilgner, 1967). The resultant smoke is composed of two phases, a particulate phase and a vapor phase (Foster, 1959). The particulate phase makes up the dense visible part of smoke and contains tars, wood resins, high-boiling compounds of the phenolic type and variable amounts of lower-boiling compounds. Most of the substances responsible for smoke flavor deposition are in the vapor phase (Foster and Simpson, 1961), and the smoking process is essentially a vapor scrubbing process.

Wood smoke, as used for the processing of foodstuffs, is a complex mixture of many classes of compounds. Separating the smoke condensate into a steam-distillable and a non-steam-distillable fraction provides a useful means of classifying smoke components since Husaini and Cooper (1957), Porter et al. (1965), and Tilgner (1957) showed that the steam-distillable fraction contained most of the flavoring materials of smoke, including phenols, acids, alcohols and carbonyls. The non-steam-distillable fraction is made up largely of water-insoluble tars and water-soluble wood resins. Certain undesirable components of smoke, the polycyclic hydrocarbons, have been isolated from wood smoke (Rhee and Bratzler, 1968) and are generally associated with the tars and resins. A summary of the many constituents that have been identified in wood smoke is presented in Table 2.

The phenols, acids, alcohols, carbonyl compounds and hydrocarbons have been regarded as the most important constituents in the smoking of meats and fish (Spanyár et al., 1966; Tilgner, 1957). Although it is generally conceded that the phenolic compounds are of great importance in smoke flavor (Bratzler et al., 1969; Husaini and Cooper, 1957; Tilgner et al., 1962b) other volatile components such as acids, alcohols and carbonyls undoubtedly play an important but secondary rôle in the development of the characteristic aroma (Doerr et al., 1966). Tilgner (1967) has indicated that a number of easily volatile, low boiling point compounds

Table 2. Constituents identified in wood smoke. ‡

Phenolic Compounds	Carbonyl Compounds
Catechol*	Acetaldehyde*
3-methoxycatechol	Butyraldehyde
m-cresol	Isobutyraldehyde
o-cresol	Crotonaldehyde
p-cresol	Formaldehyde*
Guaiacol*	Methacryaldehyde
4-allylguaiacol	Tiglicaldehyde
4-ethylguaiacol	Valeraldehyde
4-methylguaiacol	Isovaleraldehyde
4-propylguaiacol	α-methyl valeraldehyde
4-vinylguaiacol	Acetol (1-hydroxy-2-propanone)
Hydroquinone	Acetone
1-naphthol	Butanol
2-naphthol	2-butanone
Phenol	3-methyl-2-butanone
2,6-dimethylphenol	Diacetyl (Butandione)
3,4-dimethylphenol	Furan
3,5-dimethylphenol	2-methylfuran
2,6-dimethoxyphenol*	Furfural
2,6-dimethoxy-4-allylphenol	5-methyl furfural
2,6-dimethoxy-4-ethylphenol	Furfuryl alcohol
2,6-dimethoxy-4-methylphenol	Methyl glyoxal
2,6-dimethoxy-4-propylphenol	2-hexanone
Phloroglucinol	3-hexanone
Pyrogallol	Methylvinylketone
Resorcinol	Pentanol
Thymol	2-pentanone
Vanillin	4-methyl-3-pentanone
Veratrole	Pinacolone
	Propanol
	Propenal (Acrolein)
	2-propen-1-ol (allylalcohol)
	Octanal (n-caprylaldehyde)
	2-octanone

Table 2. (cont'd)

Acids and Other Compounds	Polycyclic Hydrocarbon Compounds
Acetic acid*	Acenaphthene
Butyric acid*	Anthracene
Isobutyric acid	1,2-benzanthracene
Isocaproic acid	1,2-benzopyrene
n-caproic acid	3,4-benzopyrene*
Caprylic acid	Carbazole
Formic acid*	Chrysene
Heptylic acid	Fluoranthene
Nonylic acid	Fluorene
Propionic acid*	Naphthalene
Isovaleric acid	Phenanthrene
n-valeric acid	Pyrene
Benzene	
Carbon dioxide	(In Smoked Foods)
Carbon monoxide	Alkyl-benzanthracene
Ethanol	Benzo(b)chrysene
Methanol	Benzo(g,h,i)perylene
Methane	Coronene
Toluene	Dibenz(a,h)anthracene
	Perylene

*Those present most frequently.
‡From : Doerr et al. (1966), Fiddler et al. (1966), Hamid and Saffle (1965), Harper (1967), Huff and Kapsalopoulou (1964), Lijinsky and Shubik (1964 and 1965), Love and Bratzler (1966), Porter et al. (1965), Rhee and Bratzler (1968), Spanyár et al. (1966).

are responsible for the aroma; particularly some phenol compounds, dicarbonyl compounds, vanillin, diazethyl and some acids, all in small quantities. It was reported by Kurko (1959) that the phenols are the effective antioxidants, whereas other classes, including neutral compounds (alcohols and carbonyls), organic bases and organic acids are ineffective. The typical golden-yellow to blackish-brown color of the surface of a smoked product is mainly due to the phenol and carbonyl compounds (Tilgner, 1967) with phenols having only a secondary significance in the characteristic coloring of smoked food surfaces (Kurko and Kelman, 1962). Although wood smoke imparts bactericidal or germicidal properties to foodstuffs (Gibbons et al., 1954; Kochanowski, 1962; Wolkowskaja and Lapszin, 1962), Kochanowski (1962) could not determine whether phenols or aldehydes had the predominate action. Ircze (1965) found that phenols play little part in the bacteriostatic effect of smoke.

Nitrogenous Compounds in Wood and Smoke.

A comprehensive review of the literature available on the occurrence and distribution of nitrogen in wood has been presented by Cowling and Merrill (1966). The nitrogen content of most woods is approximately 0.2% of the dry weight of wood (Browning, 1967) and is generally assumed to be proteinaceous in nature (Laidlaw and Smith, 1965). In studying the amino acids of softwoods and hardwoods, Fukuda (1963) and Merrill and Cowling (1966) each detected 12 common protein amino acids and Laidlaw and Smith (1965) detected 19 amino acids in hydrolysates of Scots pine. In addition to proteins and amino acids, Cowling and Merrill (1966) suggest that other nitrogenous compounds such as peptides, nucleic acids, indole compounds, inorganic nitrogen compounds as well as alkaloids exist in wood.

In a study of tobacco smoke, Izawa and Kobashi (1957) identified ammonia, methylamine, ethylamine, pyridine and nicotine in the low boiling point nitrogenous compound fraction. In a later study, Izawa *et al.* (1959) and Izawa and Taki (1959) detected 14 amino acids in cigarette smoke. Ziemba (1957) stated that free ammonia is present in curing smoke and it can condense with formaldehyde to form urotropin; a reaction which occurs in the curing smoke after the smoke generation.

Heat Effects Upon Meat Constituents, Particularly Proteins.

Although numerous reports appear in the literature concerning the effects of heating and cooking on the tenderness and juiciness of cooked meat, outside of work related to the histological studies on the alteration of meat structure during heating, very little has been reported about the chemical changes of meat proteins during heating. Hamm (1966) stated that the most drastic changes in meat during heating are those that involve the muscle proteins. The shrinkage of tissue and the release of juice are caused by changes in the fibrillar proteins; the discoloration of muscle and the loss of many muscle enzymes are the result of denaturation of the sarcoplasmic proteins (Hamm, 1966).

Proteins of muscle can be separated by solubility into various fractions and it has been demonstrated that these fractions may be affected in different ways by heating. Usborne et al. (1968) found significant changes in all protein components except total nitrogen and collagen from the raw to the cooked state.

The water-soluble globular proteins of the sarcoplasm which include many of the enzymes of muscle are changed by heating of muscle. With increasing temperature, the solubility of the sarcoplasmic proteins decreases (Cohen, 1966; Hamm and Deatherage, 1960; Paul et al., 1966); at 60°C only 20 to 23% of the proteins soluble at 20°C were extracted (Bol'shakov et al., 1968; Hamm and Deatherage, 1960); whereas at 80°C they all become insoluble (Hashimoto and Yusui, 1957; Usborne et al., 1968). Electrophoretic studies of sarcoplasmic proteins indicate that the cathodic proteins are more thermostable than the anodic proteins and the fastest migrating proteins (anodic and cathodic) are denatured most quickly (Grau and Lee, 1963; Kakō, 1968b). Lee and Grau (1966) observed with chromatographic studies of sarcoplasmic proteins that the number, size and maxima of peaks were affected by heating.

The soluble fibrillar proteins (actin, myosin, actomyosin) show a marked decrease in solubility between 40°C to 60°C and beyond 60°C, the structural proteins become almost insoluble (Bol'shakov et al., 1968; Hamm and Deatherage, 1960; Paul et al., 1966; Usborne et al., 1968). This decrease in the myofibrillar fraction was also demonstrated by Kakō (1968a) in chromatographic and electrophoretic studies. Paul et al. (1966) and Usborne et al. (1968) observed large increases in the denatured myofibrillar fraction during heating. Lawrie (1968) is of the opinion that the shrinkage and most of the loss of water holding capacity in cooked meats are due to the changes in the myofibrillar proteins.

The connective tissue proteins (collagen and elastin) have generally been studied from the histological aspect. However, Usborne et al. (1968) demonstrated that heat denatured some of the residual connective tissue proteins and agreed with Paul et al. (1966) that the stroma fraction remains relatively constant.

Although Usborne et al. (1968) found that the concentration of individual amino acids changed significantly with heating, these workers and Krol (1966) observed no significant difference between total free amino acid content of raw and cooked pork. Usborne et al. (1968) obtained a significant decrease in the nonprotein nitrogen fraction with heating, Paul et al. (1966) found little change, and Hamm and Deatherage (1960) noticed a slight increase in the nonprotein nitrogen fraction with increased temperature.

The pH of muscle tissue of various meats and poultry increased during heating (Hamm and Deatherage, 1960; Kauffman et al., 1964; Paul et al., 1966; Rogers et al., 1967) more rapidly and to a higher level with increased temperature. Using cured hams, Cohen (1966) and Karmas and Thompson (1964) observed a similar increase in pH upon heating. Wierbicki et al. (1957) and Hamm and Deatherage (1960) pointed out that changes in the pH of muscle, caused by heating, depend on the initial pH of the muscle. It has been proposed (Hamm, 1966) that these pH changes may be caused by charge changes or hydrogen bonding or both within the myofibrillar proteins.

This heat denaturation of meat caused an unfolding of the peptide chains and the release of reactive sulfhydryl groups (Hamm and Hofmann, 1965). Hydrogen sulfide is formed during the heating of meat (Fraczak and Pajdowski, 1955; Mecchi _et al._, 1964) and originates from the destruction of sulfhydryl groups in the structural proteins and not from the water soluble substances present in the sarcoplasm (Hamm and Hofmann, 1965). However, Lawrie (1968) stated that excessive heating may cause breakdown of amino acids and the release of hydrogen sulfide and ammonia.

The changes which occur in the proteins of meat during cooking depend on the temperature reached inside the meat and are a function not of time or of temperature alone, but of the time-temperature complex (Paul _et al._ 1966). Most denaturation of the muscle proteins occurs between 40°C and 80°C (Hamm, 1966) with only minor changes occurring above and below this range.

Smoke Effects Upon Meat Constituents, Particularly Proteins.

The nutritive value of smoked food products has been examined by several workers. Munro and Morrison (1965) found that salting and smoking had no effect on the biological value of cod protein as indicated by the protein efficiency ratio, gross protein values, total lysine and methionine content, available lysine values, and plasma-free lysine and methionine levels in human subjects. However, other work has indicated a decrease in available lysine in smoked foods (Dvôrák and Vognarova, 1965; Inagami and Horii, 1966), especially when the smoking process proceeded for several hours. Rice _et al._ (1947), in studying thiamine, riboflavin and niacin content of hams, found that a cooked, cured and smoked ham retained as much of its initial vitamin content as a cooked fresh ham.

Using fish, Cutting (1962) observed no destruction of vitamin A and no appreciable loss of the B vitamins during mild smoking.

Studying sausage, de Abreu and Correia (1962) and Nicora et al. (1966) found that the amino acid content increased from slaughter to the end of the manufacturing process, especially during smoke drying. During smoking, and particularly with prolonged smoking, Kihara (1962) indicated that an enhancement of the free amino acid content of chicken breast and leg muscles occurred. The content of extractable amino acids of smoked herring was found to be twice that of raw fish (Kida and Tamoto, 1967) with the neutral amino acids predominating over the acidic and basic amino acids.

Various functional groups of meat proteins have been indicated as being involved in the surface color formation as well as being involved in organeolytic properties of smoked products. Krylova et al. (1962) stated that protein functional groups such as the amino, sulfhydryl, hydroxyl and phenol groups are able to interact with smoke components.

Ziemba (1962) was of the opinion that considerable color formation involved phenol and protein interaction, whereas Draudt (1963) indicated that reactions between aldehydes and amino groups could be taking place in color formation. Recently, Ziemba (1967) concluded that the typical "smoked" color formation is primarily due to carbonyl-amino reactions which agreed with Ruiter (1968) who stated that the formation of the brown color upon smoking is due to the Maillard reaction.

To some extent, the flavor of smoked products depends partly on the reactions between the components of smoke and the functional groups of meat proteins (Lawrie, 1968). Krylova and Bazarova (1960) indicated that the chemical activity of functional groups (amino, hydroxyl and sulfhydryl) increased as proteins became denatured during the smoking process. Thus, phenols and polyphenols react with sulfhydryl groups and carbonyls with amino groups (Krylova _et al._, 1962) and interactions occur between various phenol components and individual amino acids (Kurko, 1967).

The effect that smoke has upon protein solubility and electrophoretic patterns of meat proteins have been examined in Japanese studies. Kihara (1962) showed that the total protein fraction extracted with low ionic strength buffer, the sarcoplasmic protein fraction, and the total myofibrillar protein fraction decreased during the smoking of chicken and pork muscles. Using electrophoretic analysis, he also observed that the smoking process caused alterations of the water-soluble protein patterns. Many changes were observed in the starch gel electrophoretic patterns of a meat-urea extract containing sarcoplasmic proteins, myosins, and undefined components after the smoking of beef, pork and chicken samples (Kakō, 1968b). Similar results were obtained by the use of DEAE cellulose column chromatography.

Acid Phosphatase and Ham Processing Temperatures.

In this country and in some European laboratories, coagulation tests are being used to determine the maximum temperature attained in heat processing of hams and picnics. This test depends on heating a solution prepared by extracting the meat product with 0.9% NaCl and observing the temperature at which a flocculent precipitate is formed (Cohen, 1966); within limits, this temperature corresponds to the highest temperature reached internally when the product is processed. However, these tests present difficulties in interpretations and often give false results. Olsman (1968), using a temperature range of 64.1°C (147.4°F) to 67.1°C (152.8°F)-the approximate temperature range of "fully cooked" hams, concluded that the coagulation test was completely inadequate for checking the adequacy of heat treatment which is in agreement with Lind (1965b) who found that the coagulation test often gave results which were 10°C lower than the actual temperature attained. A reliable test is necessary to ensure that imported products have been processed at high enough temperatures to meet requirements; with domestic products, there are regulations which require heating the product to, or above, specified temperatures (Cohen, 1966).

For a number of years, phosphatase activity has been used to indicate the extent of pasteurization of dairy products. Based on the earlier work of Körmendy and Gantner (1960), Lind (1965a,b) successfully used meat acid phosphatase activity as an indication of the heating rate of hams in a temperature range of 65°C (149°F) to 70°C (158°F). Both Cohen (1966) and Gantner and Körmendy (1968) were of the opinion that by the determination of residual acid phosphatase activity, the adequacy of heat treatment of meat products can be estimated. Although Gantner and Körmendy (1968) and Olsman (1968) stated that the phosphatase test is a valuable tool for controlling the pasteurization of hams and shoulders, Olsman (1968) found that there was a 5% chance of condemning a ham which had attained the correct internal temperature. It has also been shown that a high salt content (Lind, 1965b), the presence of polyphosphates (Körmendy and Gantner, 1967) and the lowering of pH values (Körmendy and Gantner, 1960) inhibit the heat inactivation of this enzyme. In disagreement with other workers, Suvakov _et al_. (1967) came to the conclusion that acid phosphatase activity was not a reliable method for determining the maximum temperature attained in hams.

EXPERIMENTAL PROCEDURES

Samples and Sample Preparations.

Pork loin samples from the right and left longissimus dorsi muscle were used in this study. The samples were either from the 11th to the last rib section or from the last rib to the last lumbar vertebra section. The samples were deboned and if not used immediately, they were packaged in Cry-O-Vac bags which were sealed under vacuum and then frozen and stored at -20°C. Before use, one loin section was thawed, most of the external fat removed, and sliced on a meat slicer resulting in slices 1.1 cm thick. In order to have control, heated, and heated smoked samples, the slices were divided randomly into three equal groups.

The slices to be heated or heated and smoked were treated similarly in the smoke chamber with the exception of smoke being added to the latter samples; the heated samples were used as a heated, non-smoked control. Prior to using the smokehouse, it was operated for 1 hr to remove any residual smoke particles. The samples were placed on a 0.6 cm wire mesh screen on a rack in the smoking chamber; this arrangement was used to provide uniform distribution of smoke or heat on all sides of each slice. The samples were in the smoking chamber for 2.25 hr for either treatment and two different sets of conditions were used. To obtain a heated smoked sample, the temperature of the smoke chamber was 60°C (140°F) and the relative humidity was 45% which resulted in an internal temperature of the meat of 58.8°C (138°F). To obtain a cold smoked sample, the temperature of the smoke chamber was 32.2°C (90°F) and the relative humidity was 45% which resulted in an internal temperature of the meat of 32.0°C (89.6°F). The smoke was generated from smoldering dampened hardwood sawdust. The heated and heated smoked samples were weighed before being placed in the smoking chamber and after removal from the chamber to obtain the weight loss.

All separable fat was removed from the loin slices before grinding. The samples were ground three times through a 1 cm plate and three times through a 2 mm plate and then stored in sealed jars at 4°C until analyzed.

Lard and albumin samples were also heated or heated and smoked using a smoke chamber temperature of 60°C (140°F) and a relative humidity of 45%. The albumin was either used in the powdered form or as a 5% solution containing 10% sucrose. When used as the powder, it was spread on ten thicknesses of cheesecloth and then laid on a wire mesh screen on a rack in the smoking chamber. The 5% albumin solutions were placed in 100 x 15 mm petri dishes in 50 ml portions as were the lard samples.

<u>Addition of Pure Phenols to Samples</u>.

Fresh pork samples were obtained and ground as outlined previously. To 10 g meat was added 1 ml of an alcohol solution of each phenol (1.0 M) used and then the meat sample was thoroughly mixed. The samples were heated in a water bath shaker for 2 hr at 60°C after which they were stored at 4°C for subsequent analyses.

Similarly, 1 ml of phenol solution was added to 10 ml of a 5% albumin solution containing 10% sucrose. After heating, the pH was obtained and then the precipitated material was removed by centrifugation at 27,000 x G for 10 min before further analyses were performed.

Addition of Artificial Smoke to Samples.

The artificial smoke solutions used were aqueous solutions of natural wood smoke flavors and were from three sources : Solu-Smoke (Stange Co.), Charsol (Red Arrow) and Natural Smoke Flavor SF - 12 (Griffith Laboratories Inc.). The recommended amount of artificial smoke solution to be applied is 1 oz per 100 lb of meat (Griffith Laboratories Inc.). This was accomplished by diluting 1.3 ml of the concentrated smoke solution to 100 ml with water and then adding 1 ml of this latter solution to 20 g meat (equiv. 0.013 ml artificial smoke/20 g meat). Using 5% albumin solutions, 1 ml of diluted smoke solution was added to 20 ml albumin solution. The samples were thoroughly mixed and then heated in a water bath shaker for 2 hr at 60°C. They were then stored at 4°C until analyzed.

Moisture and Fat Determination.

Moisture was determined according to the method described in A.O.A.C. (1965). Five g samples were placed in disposable aluminum dishes and dried to a constant weight for 16 to 18 hr at 100°C in an air oven. Ether extract was determined from the same samples used in moisture analysis. The fat was extracted with anhydrous ether for 3.5 hr in a Goldfisch Fat Extractor. All samples were weighed to the nearest 0.001 g.

Nitrogen by Micro-Kjeldahl Analysis.

This method is a combination of that described by A.O.A.C. (1965) and the American Instrument Company (1961). The basic equipment for the determination was built by the American Instrument Company, Inc. and consisted of a twelve-flask rotary digestion unit, 100 ml digestion flasks with expansion bulbs and ground glass joints, and compatible steam distillation and condensation equipment.

For meat samples, 0.5 g of meat sample were weighed on nitrogen-free parchment paper squares and placed in a digestion flask. When protein solutions were used, 15 ml of the protein extract were pipetted into the digestion flask. To each flask were added 1 g Na_2SO_4, 1 ml 10% $CuSO_4$, 7 ml concentrated H_2SO_4 plus one glass bead. The contents of the flasks were boiled with occasional swirling until the solution cleared, usually 3 to 4 hr. The samples were cooled, 25 ml water added, cooled again, and then the flasks were connected to the distillation apparatus. Sufficient 40% NaOH was added to make the sample strongly alkaline and then steam-distilled into 10 ml 2% boric acid solution containing 2 drops bromcresol green indicator for a period of 6 min. The samples were then titrated to a bromcresol green endpoint with 0.1 N H_2SO_4.

Nitrogen contents were reported as mg of nitrogen per ml of solution or per g of sample.

pH Measurements.

The samples for pH measurements were prepared by homogenizing 10 g of the meat sample in 100 ml of distilled water for 1 min; the pH of albumin was either measured directly on the albumin solution or a 5% solution was prepared with the powdered albumin. All pH measurements were performed with a Corning, Model 12, expanding scale pH meter.

Statistical Analysis.

Analysis of variance, standard deviations and standard errors were calculated. The data which indicated a significant difference by analysis of variance were further analyzed by ranking and comparing means by Duncan's Multiple Range Test (Steel and Torrie, 1960). Levels of significance were used as indicated by Bliss (1967).

Estimation of Total Phenols.

The colorimetric method of Tucker (1942) was modified to provide an estimate of the total quantity of phenols in each sample. The basis for his procedure was the indophenol test which was described by Gibbs (1927) and involves the condensation of phenols with quinonechlorimide compounds to produce the blue-colored indophenol dyes.

For meat samples, 12.5 g of the meat were blended for 5 min with 50 ml of 50% ethanol. The resultant solution was filtered through S & S #560 filter paper. After 12 hr storage at 4°C, the filtrate was refiltered through Whatman #2 filter paper at this same temperature. The filtrate from the smoked samples was diluted by 1 to 5, the heated and fresh samples were not diluted. Using albumin solutions, the same procedure was followed except that 20 ml of the albumin solution were blended with 50 ml of 50% ethanol. The lard samples were treated in the same manner as the meat samples.

The colorimetric procedure was carried out on the sample solution as follows. A 5 ml aliquot of the sample was pipetted into a 15 x 180 mm test tube, and this was followed in order by the addition of 5 ml of 0.5% sodium borate solution and 1 ml of the indophenol reagent (Appendix C). The tube was then stoppered and the contents thoroughly mixed by shaking; next, the tube was placed in a controlled-temperature cabinet at 38°C for 1 hr to permit completion of the color reaction. Following this, the indophenol dye was extracted from the aqueous solution with 15 ml of n-butanol in a small separatory funnel; the butanol-dye layer was transferred into a 25 ml graduated test tube and the volume increased to 23 ml with n-butanol and mixed by gentle shaking. The optical density was read against a reagent blank at 635 mμ on a Bausch and Lomb Spectronic 20 spectrophotometer.

Similarly, deionized distilled water solutions of standard phenol in concentrations of 0.0 (reagent blank), 0.25, 0.50, 0.75, and 1.0 mg per 100 ml were used to derive a standard curve. The estimate of total phenols (mg/100 g) was then obtained by comparing the optical density of the sample with the standard curve and taking into account the dilution made.

Protein Fractionation.

The protein fractionation procedures were adapted from those used by Hegarty *et al*. (1963) and Weiner (1967). All fractionation procedures were carried out at 4°C with cold extracting solutions. Details of these procedures are outlined in Figures 1 and 2.

In the scheme for the quantitative determination of meat protein nitrogen fractions (Figure 1), a 5 g sample of meat was placed in a microblender jar containing 50 ml of 0.05 M phosphate buffer (pH 7.6). This was homogenized for 1 min at a speed of 8000 rpm. The meat slurry was transferred to a 125 ml erlenmeyer flask and gently stirred by means of a magnetic stirrer for 30 min. The blender jar was then rinsed with 50 ml of the extracting solution, which was used for the second extraction. The meat slurry was centrifuged in a Sorvall Model RC2-B centrifuge at 6000 x G for 20 min at 0°C. The supernatant was retained. The residue was resuspended in 50 ml of the above rinse solution, stirred and centrifuged as described. The volume of the combined supernatants was recorded and designated as solution A (nitrogen solution soluble at low ionic strength). This solution was filtered through eight layers of cheesecloth to remove fat particles. A 15 ml aliquot of solution A was mixed with 5 ml of 10% trichloroacetic acid (TCA) solution. After 30 min, the material was centrifuged at 10,000 x G and the resulting filtrate was

5 g Meat Sample.

Extract 2x with PO_4 buffer.
Mix 30 min.
Centrifuge 30 min at 6000 x G.

Residue.

Extract 2x with 0.1 M NaOH.
Mix 1 hr.
Centrifuge 20 min at 10,000 x G.

Residue (Discarded).

Solution C
(total fibrillar protein nitrogen fraction).

Solution A
(nitrogen solution soluble at low ionic strength).

Treat aliquot with 10% TCA.
Centrifuge 10 min at 10,000 x G.

Residue (Discarded).

Solution B
(nonprotein nitrogen fraction).

Figure 1. Scheme for the quantitative determination of meat protein nitrogen fractions.

5 g Meat Sample.

Extract 2x with PO_4 buffer.
Mix 30 min.
Centrifuge 30 min at 6000 x G.

Residue.

Solution A
(See Figure 1).

Extract 2x with KCl-PO_4 buffer.
Mix 30 min.
Centrifuge 20 min at 10,000 x G.

Residue.

Solution D.
(soluble fibrillar protein nitrogen fraction).

Extract 2x with 0.1 M NaOH.
Mix 1 hr.
Centrifuge 20 min at 10,000 x G.

Residue (Discarded).

Solution E.
(denatured fibrillar protein nitrogen fraction).

Figure 2. Scheme for the quantitative determination of the soluble and denatured fibrillar protein nitrogen fractions.

designated as solution B (NPN - nonprotein nitrogen fraction). The sarcoplasmic protein nitrogen was estimated by subtracting the NPN value from the nitrogen value obtained for solution A.

The residue remaining from the 0.05 M phosphate buffer extraction was suspended in 50 ml of 0.1 M NaOH (Figure 1). The mixture was stirred gently for 1 hr on a magnetic stirrer and then centrifuged at 10,000 x G for 20 min. The extraction and centrifugation were repeated. The volume of the combined supernatants was recorded and designated as solution C (total fibrillar protein nitrogen fraction). In some studies, the total fibrillar protein nitrogen value was obtained by combining the values obtained for the soluble and denatured fractions. The scheme for the quantitative determination of the soluble and denatured fibrillar protein nitrogen fractions is presented in Figure 2. The residue remaining from the 0.05 M phosphate buffer extraction was suspended in 50 ml of a mixture of phosphate buffer (pH 7.5) in 0.4 M KCl (total ionic strength 0.55). The mixture was stirred gently for 30 min and then centrifuged at 10,000 x G for 20 min. The extraction and centrifugation were repeated. The volume of the combined supernatants was recorded and designated as solution D (soluble fibrillar protein nitrogen fraction). The residue remaining from the KCl-PO_4 buffer extraction was suspended in 50 ml of 0.1 M NaOH, stirred gently for 1 hr, and then centrifuged at 10,000 x G for 20 min. Extraction and centrifugation were repeated. The volume of the combined supernatants was recorded and designated as solution E (denatured fibrillar protein nitrogen fraction).

Solutions A, B, C, D and E were analyzed for nitrogen, and the results were designated as A^n, B^n, etc. The total nitrogen content of the meat sample was denoted F^n. These symbols (nitrogen contents) represent the following fractions:

A^n = nitrogen extractable at low ionic strength.

B^n = nonprotein nitrogen.

$A^n - B^n$ = sarcoplasmic protein nitrogen.

D^n = soluble fibrillar protein nitrogen.

E^n = denatured fibrillar protein nitrogen.

$C^n = D^n + E^n$ = total fibrillar protein nitrogen.

$F^n - (A^n + C^n)$ = connective tissue protein nitrogen.

Sample Preparation for Electrophoresis.

The pork samples for electrophoretic studies were heated or heated and smoked using smokehouse conditions of 60°C (140°F) and 45% relative humidity.

(a) Starch gel electrophoresis.

The samples for starch gel electrophoresis were obtained at the same time as the samples for protein fractionation. In preliminary trials, two fractionation procedures were examined. The first procedure utilized the extraction of the sample with an equal volume of 0.9% NaCl (Cohen, 1966) and allowing the slurry to stand at room temperature for a 30 min extraction before centrifugation. The second procedure utilized the extraction of the sample with two volumes of water and mixing the slurry with a magnetic stirrer at 4°C for a 30 min extraction before centrifugation (Scopes, 1964). Each slurry was centrifuged at 35,000 x G for 30 min at 0°C and each supernatant was filtered through eight layers of cheesecloth to remove fat particles.

Before electrophoretic analyses were performed, the pH and the concentration of the filtrate obtained by the second procedure were adjusted. The pH was adjusted to 8.6 with a 1 M Tris buffer; in subsequent analysis, this step was discontinued since no improvement of the protein patterns was obtained. The filtrate was then dialyzed for 12 hr against a 0.01 M Tris - 0.001 M citric acid buffer containing 0.5 M sucrose. The resulting concentrated solution was now similar in concentration to the 0.9% NaCl extract. The solutions were now ready to be placed on the starch gels.

(b) Disc gel electrophoresis.

The extraction procedure for obtaining the myofibrillar protein solution for electrophoresis was an adaptation of that used by Rampton (1969).

The samples were extracted in 12 volumes of a pH 7.6 buffer (0.25 M sucrose, 1 mM EDTA, 0.05 M Tris). The meat slurry was transferred to a 125 ml erlenmeyer flask and gently mixed with a magnetic stirrer for 30 min, then centrifuged 15 min at 15,000 x G. The supernatant was discarded and the residue was resuspended in 12 volumes of the above buffer, stirred and centrifuged as described. The supernatant was again discarded and the residue suspended in 6 volumes of Weber-Edsall solution (0.6 M KCl, 0.04 M $KHCO_3$, 0.01 M K_2CO_3, pH 9.2; Perry 1953) and gently mixed with a magnetic stirrer for 24 hr. The mixture was then centrifuged 1 hr at 25,000 x G. The supernatant was designated as the Weber-Edsall extract and was dialyzed for 12 hr against 15 volumes of 8 M urea. The myofibrillar protein solution was now ready to be placed on the disc gels.

(c) Meat-urea extracts for electrophoresis.

The extraction procedure used to obtain the meat-urea extracts was an adaptation of that used by Kakō (1968a). The samples were extracted in 9 volumes of 7.7 M urea-containing 0.055 M Tris - HCl buffer (pH 8.6) for 5 min using a Virtis Hi-Speed "45" Homogenizer and an ice bath. The homogenate was allowed to stand for 1 hr at $4^{\circ}C$ and was then centrifuged 20 min at 12,000 x G. The supernatant was filtered through eight layers of cheesecloth to remove fat particles. For disc gel electrophoresis, 0.025 ml of extract was applied to the gels.

Starch Gel Electrophoresis (SGE).

The apparatus used was similar to that described by Smithies (1959a) but with two modifications. The trough-ends of the gel former were not used; the starch solution was poured into the main body of the former with removable Perspex blocks preventing the starch from flowing into the ends. Platinum wire electrodes were used in place of Ag/AgCl electrodes.

Horizontal starch gel electrophoresis was carried out in a discontinuous buffer system. The starch gels were prepared in a slightly modified form of Kristjansson's (1960, 1963) methods and consisted of 26 g of hydrolyzed starch (Connaught Medical Research Laboratories, Toronto) in 250 ml of a 0.19 M Tris - 0.2 M HCl buffer solution (pH 8.5). In order to provide uniform gel preparation conditions, 190 ml of buffer were heated to $95^{\circ}C$ and added rapidly to the starch suspended in 60 ml of buffer at room temperature. The resulting viscous mass was shaken vigorously for about 15 sec and poured into the gel form; the de-aeration step was omitted.

After cooling, the gels were cut across their width parallel to and 5.8 cm from one end to form the insertion line. The smaller portion of the gel was carefully pushed back and pieces of Whatman # 3 chromatography paper (1 cm x 0.6 cm) containing 0.2 ml of the sarcoplasmic extract to be analyzed were placed against the exposed cut surface of the larger portion of the gel. The opened cut was then carefully closed against the line of inserts and a piece of Saran Wrap was used to cover the gel.

The Saran Wrap was folded back to expose 2.2 cm of the gel at the insert end and 2.0 cm at the other end to provide contact surfaces for the filter paper wicks used to connect the gel to the electrode chambers.

Gels were connected to the electrode chambers with three thicknesses of Whatman # 3 chromatographic paper. The electrode chambers contained a 0.6 M boric acid - 0.2 N NaOH solution (pH 8.6). An initial voltage of 165 volts was applied for 20 min, then the voltage was increased to 350 volts for the remainder of the electrophoresis. All starch gel electrophoretic runs were performed at 4°C.

The brown borate boundary which was observed to migrate in this discontinuous starch gel system was allowed to migrate 12 cm from the insert line. The gel was carefully removed from the gel former and sliced in the manner described by Smithies (1959a) using a dermatome-knife blade.

<u>Starch-Urea Gel Electrophoresis</u>.

The apparatus used was the same as that described in the previous section. Gels consisted of 32 g of hydrolyzed starch plus 72 g of urea added to 200 ml of buffer (Neelin and Rose, 1964). The buffer (pH 8.5) was of the following composition : 0.076 M Tris - 0.005 M citric acid. A slot former was used instead of the paper inserts. The conditions of electrophoresis were similar to those described; the only exception was that the voltage was maintained at 350 volts for the entire run.

<u>Identification of Acid Phosphatase and Protein Activity</u>.

Acid phosphatase activity on the starch gels was determined by the following method. The substrate, sodium α-naphthyl acid phosphate, was dissolved in 0.05 M acetate buffer (pH 5.0) at a concentration of 1 mg per ml. The coupling dye, Fast Garnet GBC (4-amino-3:1 dimethyl azobenzene) was dissolved in the same buffer at the same concentration.

The two solutions were mixed and then poured over the gel. The gel was incubated in this solution for 1 hr at 37°C (Allen et al. 1963).

Protein activity was detected on the starch and the starch-urea gels by staining the sliced gel with a solution of 1% Amido Black 10B and 0.5% Nigrosine in methanol-acetic acid-water (5:1:4) for 1 min. The unbound dye was removed by washing the gel in several changes of the above solvent.

A benzidine test was applied to the starch gels for the detection of hemoproteins. The reagent used consisted of benzidine (0.2 g), 30% hydrogen peroxide (0.2 ml), glacial acetic acid (0.5 ml) and 100 ml distilled water (Smithies, 1959b).

Disc Gel Electrophoresis.

The technique of disc electrophoresis used was similar to that described by Davis (1964) but with the following modifications. Cyanogum (E.C. Apparatus Co.) was used in making the gels instead of acrylamide and N,N′-Methylenebisacrylamide, ammonium persulfate solution was not used in preparation of the gels, and a sample gel was not used. The stock solutions and working solutions for the preparation of the gels differ somewhat from those used by Davis (1964) and this information is presented in Appendix B. A 6.5% running gel and 5.0% spacer gel were used and the concentration of urea in each gel was 7 M. The quantity of sample solution applied per tube was 0.05 ml unless stated otherwise. During electrophoresis and destaining a current of 2 ma per tube was applied. After electrophoresis, the gels were removed from the tubes and stained with a solution of Amido Black 10B for 20 min. The composition of the tank buffer, staining and destaining solutions are given in Appendix B. After destaining, the gels were stored in a 7% acetic acid solution.

The stained disc gels were subjected to densitometric readings in a Photovolt Densicord Model 542 Recording Electrophoresis Densitometer at a response setting of L, using a red filter. The densitometer was equipped with a recorder and integraph attachment (Integraph Model 49 Automatic Integrator). The integral, i.e., the area under the curve, was automatically recorded with the curve.

Amino Nitrogen Content.

The Sørensen method as outlined in A.O.A.C. (1965) was used. A 10 g sample of meat was homogenized in 100 ml of distilled water for 1 min. To 20 ml of the slurry were added 10 ml of freshly prepared phenolphthalin-formol mixture (Appendix C). The mixture was titrated with 0.2 N $Ba(OH)_2$ until a distinct red appeared, then it was back-titrated to neutrality with 0.2 N HCl. Similarly, a blank titration was obtained by using 20 ml water in lieu of the meat slurry. From the quantity of 0.2 N $Ba(OH)_2$ required to neutralize the mixture, corrected for the quantity used in the blank titration, the quantity of amino nitrogen present was calculated (1 ml 0.2 N $Ba(OH)_2$ solution = 2.8 mg amino nitrogen).

Total Ninhydrin Positive Material (NPM).

This determination was used as an estimate of the total free amino acids in the sample and is an adaptation of that used by McCain *et al*. (1968). The samples were prepared by the method of Tallon *et al*. (1954).

A 10 g sample of meat (free of external fat) was homogenized for 2.5 min with 10 volumes of 1% picric acid solution (30 ml glacial acetic acid diluted to 1 liter with 1% picric acid). The picric acid precipitate was removed by centrifuging at 15,000 x G for 20 min. The excess picric acid was removed by passing 100 ml of the supernatant through a Dowex 2-X8 column, then eluting with 50 ml 0.02 N HCl. For analysis, the eluted samples were diluted by one half with distilled water.

From the diluted sample, a 0.3 ml aliquot was pipetted into a screw cap test tube and 3 ml of the ninhydrin reagent (Appendix C) were added. The tubes were stoppered, placed in boiling water for 2 min, diluted to 10 ml with 50% ethanol, vibrated for 3 min and the optical density read at 570 mμ on a Bausch and Lomb Spectronic 20 spectrophotometer. A standard curve was plotted using alanine solutions at concentrations of 0.0 (blank), 0.50, 0.75, 1.00, 1.25, 1.50 and 1.75 mM.

Ninhydrin Colorimetric Method (NCM).

This determination was used as an estimate of the free amino groups in albumin samples and was an adaptation of that used by Moore and Stein (1948). It was quite similar to the method described in the previous section. The 5% albumin solutions were diluted 1 to 10 before analysis. To test tubes (18 x 150 mm) containing 0.1 ml sample was added 1 ml ninhydrin reagent (Appendix C); the test tubes were capped with aluminum foil and the contents mixed. The tubes were heated in boiling water for 20 min, cooled and rapidly mixed with 5 ml of 50% aqueous isopropanol and read at 570 mμ within 15 min of removal from the water bath. A standard curve was plotted using leucine solutions at concentrations of 0.0 (blank), 0.50, 0.75. 1.00, 1.25, 1.50, and 1.75 mM.

Free Sulfhydryl Groups.

Ellman's reagent (1959), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), a water soluble disulfide for the determination of sulfhydryls, was adapted for use. The DTNB reagent was prepared by adding 39.6 mg DTNB to 10 ml of 95% ethanol.

A 2.5 g meat sample was homogenized with 25 ml of 8 M urea for 1 min. The slurry was centrifuged at 25,000 x G for 10 min at 0°C.

For analysis, this supernatant was diluted 1 to 5 with phosphate buffer (pH 8.0) and filtered through Whatman #2 filter paper.

Using albumin, 0.8655 g were dissolved in 25 ml distilled water. Five ml of distilled water and 2 ml of phosphate buffer (pH 8.0) were added to 3 ml of this albumin solution for analysis.

The colorimetric procedure was carried out on the sample solution as follows. A 3 ml aliquot of sample solution was mixed with 0.02 ml of DTNB color reagent in a Beckman 1-cm cell. The color was allowed to develop for 20 min at room temperature (23°C to 25°C) and the optical density was measured at 412 mμ (E = 12,000; Flavin, 1962) with a Beckman DU spectrophotometer equipped with a Gilford, Model 220, absorbance indicator. A 3 ml sample of solution with no added DTNB was used as a reference solution.

Acid Phosphatase Activity.

Andersch and Szckzypinski's (1947) method for the determination of serum acid phosphatase was adapted for the determination of acid phosphatase in meat. A 10 g sample of meat was homogenized in 20 ml distilled water or 0.05 M phosphate buffer (pH 7.6) for 1 min. The resulting slurry was then centrifuged at 25,000 x G for 30 min. The supernatant from the fresh sample was diluted 1 to 5 for analysis; the supernatant from the heated and smoked samples was not diluted.

The substrate used in this procedure was prepared just prior to use and consisted of equal parts of reagent A (M/10 citrate - HCl buffer, pH 4.8) and reagent B (0.4% solution of disodium-p-nitrophenyl phosphate in 0.001 N HCl).

The colorimetric procedure was carried out on the sample solution as follows. One ml of substrate was pipetted into a 15 x 100 mm test tube which was placed in a water bath at 38°C. To this was added a 0.2 ml aliquot of the sample and the tubes heated at 38°C for 30 min. Then, 3.0 ml of 0.1 N NaOH were added to develop the color and the contents thoroughly mixed by shaking. The optical density was read at 400 mμ on a Bausch and Lomb Spectronic 20 spectrophotometer. From this reading, a substrate blank (1 ml substrate plus 3.2 ml 0.1 N NaOH) and a sample blank (0.2 ml sample plus 4.0 ml 0.1 N NaOH) were subtracted.

Similarly, standard solutions containing 0.2, 0.4, 0.6, 0.8 and 1.0 mM of p-nitrophenol were prepared. To 0.2 ml of each standard measured into test tubes were added 4.0 ml of 0.1 N NaOH. The optical density was read at 400 mμ and from these values a standard curve was prepared. The estimate of acid phosphatase activity (mμ moles substrate hydrolyzed/ min/mg N_2) was then obtained by comparing the optical density of the sample with the standard curve and taking into account the dilution made initially.

A second method (Lind, 1965a), used in Europe as an indicator of the heat processing of hams, was adapted to determine acid phosphatase activity. Into each of three test tubes was weighed 2.5 g sample, with the third being used as the control sample. To each test tube, 10 ml citrate buffer (pH 6.5) were added and, in addition, 5 ml 20% TCA were added to the control sample. The samples were mixed, placed in a water bath at 37°C and 5 ml disodiumphenyl phosphate solution (436 mg disodiumphenyl phosphate in 200 ml water) were added to each sample. The contents were thoroughly mixed and after 60 min, the reaction was stopped by the addition of 5 ml 20% TCA to the sample tubes. After mixing, the samples were transferred to centrifuge tubes and centrifuged at 25,000 x G for 10 min.

For analysis, the supernatant of the untreated samples was diluted 1 to 5 with distilled water; the filtrate from the heated and heated smoked samples was not diluted. A 3 ml aliquot of the filtrate was pipetted into a test tube, this was followed in order by the addition of 3 ml 0.5 M $NaCO_3$ solution and 0.1 ml of 2,6 dibromoquinone-chlorimide reagent (40.0 mg 2,6 dibromoquinone-chlorimide in 10 ml absolute alcohol). The contents of the tubes were thoroughly mixed by shaking; then placed in a controlled-temperature cabinet at 38°C for 30 min to permit completion of the color reaction. The optical density of the unknowns and the standards was read against a water blank at 610 mμ on a Bausch and Lomb Spectronic 20 spectrophotometer.

To prepare a standard curve, duplicate aliquots of 0.0 ml, 0.5 ml, 1.0 ml, 1.5 ml and 2 ml of the stock solution (Appendix C) were pipetted into test tubes and 5 ml, 4.5 ml, 4.0 ml, 3.5 ml and 3.0 ml, respectively, of 5% TCA were added to each solution. Then, 5 ml 0.5 M Na_2CO_3 solution were added to each tube, the contents were mixed, and 0.1 ml 2,6 dibromoquinone-chlorimide reagent were added to each test tube. The color was allowed to develop for 30 min at 38°C, then the optical density was read.

Collection of Smoke.

Whole smoke was collected from a commercial air-conditioned smokehouse equipped with a Mepaco smoke generator. The smoke collection apparatus consisted of a 4 L flask containing 1.5 L water and 1.2 L ether, six washing bottles containing water connected in series (Izawa et al., 1959) plus a trap packed with glass wool (to remove tar). This latter flask was connected to a vacuum line and the vacuum was so adjusted that there was a constant air flow. The 4 L flask was connected directly to the smokehouse and was cooled with a freezing mixture of dry ice and ethanol. The collection period was 48 hr; approximately 1.2 L of aqueous smoke solution was obtained.

All glassware used was washed first with acetone, then with a hot detergent solution and finally rinsed with deionized distilled water.

Preparation of Sample Solution.

The water collected smoke sample was prepared as outlined by Izawa and Taki (1959). The sample was first filtered through Whatman #1 filter paper to remove any suspended material. Next, it was washed with ether three times, saving the residual aqueous solution. To this solution was added 15 g $Ba(OH)_2$, then it was washed with ether five times. The aqueous layer was neutralized to pH 5 with 1 N H_2SO_4; the precipitated $BaSO_4$ was removed by centrifugation. The filtrate was concentrated to almost dryness in vacuo. The concentrated solution was dissolved in a small amount of water and developed and eluted on a Dowex 50W-X4 column with 2 N NH_4OH. After the elute was evaporated to almost dryness to remove the ammonia, the residue was dissolved in a small amount of water and filtered. The filtrate was developed and eluted on a Dowex 2-X8 column with 0.2 N HCl. After the elute was concentrated in vacuo to remove the hydrochloric acid, it was dissolved in a small amount of water and filtered. The filtrate was subjected to one more respective treatment with Dowex 50W-X4 and Dowex 2-X8. The concentrated solution was stored in dark bottles until used. The concentration steps utilized a Calab Model C Evaporator with the sample in a boiling flask submerged in a water bath at 50°C. A sample of water was subjected to these same steps to obtain a control sample.

Thin Layer Chromatography (TLC).

The thin layer plates were prepared as follows. A basic absorbant was prepared by slurrying 15 g of cellulose powder MN 300 (Macherey, Nagel & Co.) with a mixture containing 70 ml water and 10 ml ethanol for 1 min by using the Virtis Hi-Speed "45" Homogenizer at full speed; five 20 x 20 cm glass plates were immediately layered with a 300 μ thickness of the slurry by use of a Desaga adjustable applicator and mounting board (von Ark and Neher, 1963). When the surfaces of the thin layers became dull (20 min), the plates were transferred to a drying rack and stored overnight at room temperature (23°C to 25°C) for equilibration with atmospheric moisture.

Two solvent systems were used for developing the plates: (1) 2-propanol: formic acid: water (40:2:10; von Ark and Neher, 1963) and (2) chloroform: methanol : 17% NH_4OH (2:2:1; Bailey, 1967). Development was carried out in a Desaga development tank with a ground, fitted lid. The tank contained 100 ml of the developing solvent and was sealed with a small amount of joint sealer.

The smoke and control samples dissolved in water were spotted on the plate with a Pasteur capillary disposable pipette guided by a Camag spotting guide. The plate was then placed in the developing tank until the solvent had migrated 15 cm above the point of sample application. The development occurred at room temperature and required approximately 3 hr. Following development, the plates were removed from the tank and the solvent blown off with warm air. They were then sprayed with a ninhydrin spray reagent (Appendix C; Jones and Heathcote, 1966) and held in warm air and heated until colored spots appeared.

RESULTS AND DISCUSSION

General Chemical Composition.

The weight losses observed in pork loin slices heated or heated and smoked under two smokehouse conditions are presented in Tables 3 and 4. The weight losses were increased with increased temperature which is in agreement with the work of Gibbons et al. (1954). Only minor differences were observed between the heated and heated smoked samples within a given temperature-humidity setting. The weight losses were probably due to the loss of drip and fat during the heating process.

In Tables 3 and 4, the differences in the composition of pork loin samples exposed to two temperature variables are given. At 32.2°C (90°F), only minor differences were observed in the moisture and fat content of the untreated, heated and heated smoked pork samples (Table 4). However, at 60°C (140°F), the heated and heated smoked samples contained noticeably less moisture than the untreated samples (Table 3). This decrease in moisture was partially compensated for by a higher fat content and may be related to the increased weight losses observed at this temperature. There were little if any differences observed in the total nitrogen content of the untreated, heated and heated smoked pork samples using either of the smokehouse conditions.

Phenol content of the various samples was determined since phenols are a good indicator of the amount of smoke deposition and also of smoke penetration and these values are presented in Table 5. There were no

Table 3. The effect of heating and heating and smoking on the composition of pork samples.[1]

Variables	State of Muscle Untreated	Heated	Heated Smoked
% weight loss	-	27.28	29.00
% moisture	68.91	58.31	57.70
% ether extract	7.05	9.68	10.42
% nitrogen[a]	14.82	14.74	14.49

[1]Smokehouse condition : 60°C (140°F), 45% R.H.
[a]Of muscle on a dry, fat-free basis.

Table 4. The effect of heating and heating and smoking on the composition of pork samples.[1]

Variables	State of Muscle Untreated	Heated	Heated Smoked
% weight loss	-	14.06	11.98
% moisture	63.67	64.17	63.86
% ether extract	9.78	9.91	9.78
% nitrogen[a]	14.70	14.83	14.52

[1]Smokehouse condition : 32.2°C (90°F), 45% R.H.
[a]Of muscle on a dry, fat-free basis.

noticeable differences in the phenol content of the smoked pork samples obtained by the use of two temperature conditions, indicating that the deposition of smoke is not a function of the smokehouse temperature. Lard and albumin samples were also smoked and contained appreciable amounts of phenols; thus indicating that both fat and protein constituents of meat absorb smoke components, especially phenols. This is in agreement with Tucker (1942) who detected phenols in the fat and lean tissue of smoked hams. The phenol values obtained for the pork samples

Table 5. Estimate of total phenols in smoked products.

Smoked Product	Total Phenols (as phenols)[a]
Pork loin slice[b]	1.98
Pork loin slice[c]	1.81
Powdered albumin[b]	3.63
Albumin solution[b]	5.13
Lard[b]	1.27

[a]Calculated as mg/100 g product.
[b]Smokehouse condition : 60°C (140°F), 45% R.H.
[c]Smokehouse condition : 32.2°C (90°F), 45% R.H.

are in agreement with values attained for other heavily smoked meats. Gabrielyants (1962) obtained values of 2.13 and 3.25 mg phenol respectively per g of "Leningrad" sausage and "Lyubitelskaya" sausage and Bratzler et al. (1969) obtained values of 3.70, 2.04, 1.41 and 1.02 mg phenol per 100 g respectively from the A, B, C and D layers (each layer 1.5 mm thick) of bologna. Thus, if smoke *per se* has any effect upon the properties of meat proteins, it should be evident in studying the heavily smoked products obtained in this present study.

Protein Fractionation.

Tables 6 and 7 show the averages for the nitrogen composition of the different protein fractions from untreated, heated, and heated smoked pork samples subjected to a high and a low smokehouse temperature. The values obtained for the various fractions of the untreated samples were similar to those obtained by McLoughlin (1968), Sayre et al. (1966) and Usborne et al. (1968) using porcine *longissimus dorsi* muscle.

There was an appreciable change in the solubility of the protein nitrogen solutions extracted with 0.05 M phosphate buffer. These changes

Table 6. Distribution of nitrogen in various protein fractions of untreated, heated, and heated smoked pork samples.[1,2]

Variables	State of Muscle		
	Untreated	Heated	Heated Smoked
% total nitrogen[a]	14.82	14.74	14.49
Protein nitrogen solution extracted at low ionic strength[b]	30.17	17.48	13.92**
Sarcoplasmic protein nitrogen[b]	18.09	5.59	1.99**
Nonprotein nitrogen[b]	11.89	12.08	11.93
Soluble fibrillar protein nitrogen[b]	7.99	5.76	4.32**
Total fibrillar protein nitrogen[b]	47.57	60.33	35.79**
Connective tissue protein nitrogen[b] (Insoluble in any solution used)	22.26	22.19	50.32**

[1]Smokehouse condition : 60°C (140°F), 45% R.H.
[2]The means underlined by the same line do not differ significantly.
[a]Of muscle on a dry, fat-free basis.
[b]Calculated as % of total nitrogen.
**$P < 0.01$

were more noticeable with samples subjected to increased temperature and with heating smoking. The continued decrease observed with increased temperature is in agreement with Paul et al. (1966) and the additional decrease observed with heated smoked samples is in accord with the work of Kihara (1962). This low ionic strength fraction was divided into a nonprotein nitrogen fraction and a sarcoplasmic protein nitrogen fraction to determine in which fraction the changes occurred. The nonprotein nitrogen values were uniform throughout for the 60°C samples (Table 6) whereas noticeable decreases occurred in the sarcoplasmic protein nitrogen fractions of the heated and the heated smoked samples with a greater decrease of solubility in the heated smoked sample. Thus, it appears that the low ionic strength fraction of the heated and heated

Table 7. Distribution of nitrogen in various protein fractions of untreated, heated, and heated smoked pork samples.[1,2]

Variables	Heated	Untreated	Heated Smoked
	State of Muscle		
% total nitrogen[a]	14.83	14.70	14.52
Protein nitrogen solution extracted at low ionic strength[b]	24.63	29.88	21.34**
Sarcoplasmic protein nitrogen[b]	13.51	17.16	8.88**
Nonprotein nitrogen[b]	11.12*	12.72	12.46
Total fibrillar protein nitrogen[b]	61.57	52.88	46.66**
Connective tissue protein nitrogen[b] (Insoluble in any solution used)	13.82	17.24	32.00**

[1]Smoke chamber condition : 32.2°C (90°F), 45% R.H.
[2]The means underlined by the same line do not differ significantly.
[a]Of muscle on a dry, fat-free basis.
[b]Calculated as % of total nitrogen.
*$P < 0.05$; **$P < 0.01$

smoked samples subjected to a smokehouse temperature of 60°C consisted primarily of nonprotein nitrogen. Outside of the significant decrease in the nonprotein nitrogen fraction of the heated sample, a similar trend of results was obtained with the samples heated and heated smoked at 32.2°C, (Table 7).

There was a definite increase in the amount of total fibrillar protein nitrogen extracted from the heated samples and a definite decrease in this fraction from the heated smoked samples with these changes being more noticeable at 60°C. Similar results were obtained by Usborne <u>et</u> <u>al</u>. (1968) with heated samples and by Kihara (1962) with heated smoked samples. The soluble fibrillar protein fraction was examined in the samples heated and heated smoked at 60°C (Table 6) and a significant decrease in solubility was obtained with both samples. The loss in solubility observed with both the sarcoplasmic and soluble fibrillar protein nitrogen fractions of the heated smoked samples was therefore not entirely due to heating, the smoke ingredients are also involved in this decrease in solubility.

The connective tissue protein nitrogen fraction, or more correctly, the fraction insoluble in any of the solutions used, exhibited no significant difference between the heated and untreated samples. However, a significant increase was observed in the heated smoked sample with this increase being greater at 60°C (Table 6). Although this fraction contains the connective tissue proteins, the increase observed was probably due to the insolubilization of some of the other protein constituents. In order to obtain complete data, some type of connective tissue analysis would have been desirable as this fraction was determined by difference and reflects all the errors incurred in the other analyses.

In general, heating alone caused definite changes in the solubilities of the various nitrogen fractions obtained with these changes being more noticeable at 60°C than at the lower temperature of 32.2°C. With the heated smoked samples (Table 6), noticeable changes were observed in the amounts of nitrogen containing compounds extracted in either of the salt buffers (0.05 M phosphate buffer and 0.55 μ KCl-PO_4 buffer) and in the total fibrillar and connective tissue protein nitrogen fractions. The results indicate that smoke ingredients, in addition to heat, cause additional changes in the solubility of meat protein components. However, the changes in the solubility of the various protein nitrogen fractions of the heated smoked samples were not entirely due to smoke ingredients as indicated by Kihara (1962). The results obtained with the cold (32.2°C) smoked samples (Table 7) supply further proof of the action of smoke on meat protein solubilities.

Electrophoretic Studies.

a). Starch gel electrophoresis of water extracts.

In the study of the sarcoplasmic proteins, two extraction procedures, a 0.9% NaCl extract and a water extract, were examined. However, after several preliminary trials, the extraction with 0.9% NaCl was abandoned in favor of the water extraction procedure. Very poor resolution was obtained with the 0.9% NaCl extract and the resulting zones were somewhat distorted in shape.

The starch gel electrophoretic patterns of sarcoplasmic proteins of pork muscle extracted with water are shown schematically in Figure 3. The pattern of the untreated sample revealed that a large proportion of the proteins were anionic whereas a smaller proportion were cationic; a total of 22 stained protein bands were observed. Giles (1962) and Scopes (1968) have demonstrated by starch gel electrophoresis the variety of proteins present in sarcoplasmic extracts of many species with the majority of the proteins being anionic in mobility.

Very distinguishable differences are present in the patterns of the treated and untreated samples (Figure 3). Protein bands are either totally absent or very decreased in stainability, with these changes being greater in the heated smoked sample than in the heated sample. The cationic proteins are more thermostable than the anionic ones with only the two fastest moving bands of the cationic proteins being lost during heating which is in agreement with Lee and Grau (1966). Over half of the components moving toward the anode are lost in the treated samples with

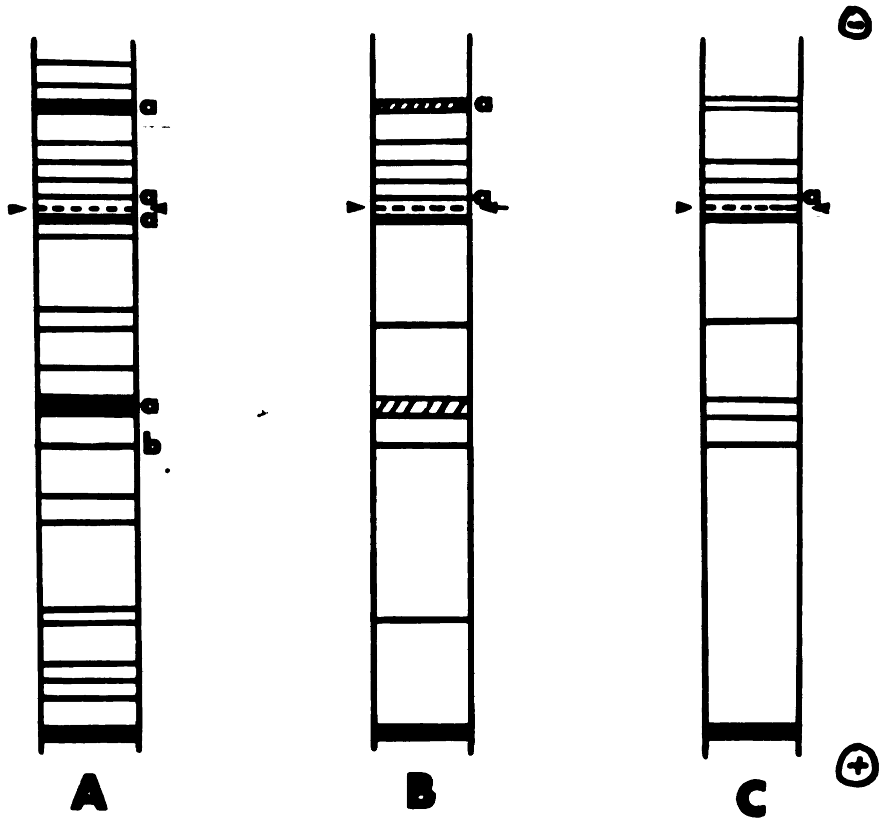


Figure 3. A comparison of protein patterns of the water-soluble extract of pork loin samples.
The arrows indicate point of sample application, a = bands displaying acid phosphatase activity and b = the band displaying activity to benzidine test.

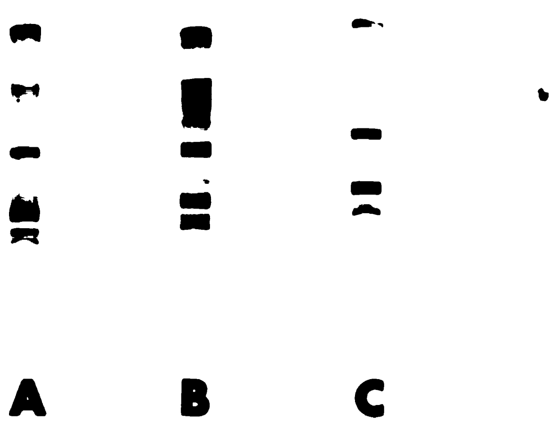


Figure 4. A comparison of the protein patterns of the Weber-Edsall extracts of pork loin samples.

N.B. In Figures 3, 4, 5, 6 A = untreated sample, B = heated sample, C = heated smoked sample.

all remaining components exhibiting less color intensity, especially with the extract of the heated smoked sample. Using free-boundary electrophoresis, Kihara (1962) observed the disappearance and decrease of peaks in studying the water soluble proteins of smoked chicken muscle. The electrophoretic separation of the sarcoplasmic proteins obtained with the pork samples substantiate the data obtained for the sarcoplasmic protein fractions in the solubility studies.

Although none of the zones were positively identified, a benzidine test was applied and band b in the untreated sample exhibited activity, possibly identifying it as a hemoprotein. None of the bands in the treated samples demonstrated any activity, probably because of protein denaturation. The bands marked "a" exhibited acid phosphatase activity; however, since these bands appeared dark orange against an orange background, it was impossible to obtain positive confirmation of phosphatase activity.

b). Disc gel electrophoresis of Weber-Edsall extracts.

The Weber-Edsall extract contains the majority of the salt soluble proteins, or the myofibrillar protein fraction. The electrophoretic behaviour of the myofibrillar proteins of untreated, heated and heated smoked pork samples is shown in Figure 4. The electrophoretograms of the untreated and heated samples were very similar with little differences being observed in the intensity of staining of the six fastest moving bands; however, the color intensities of the 10 to 12 slower moving bands are more distinct in the heated sample. With the exception of the fastest moving bands, changes are observed in all bands of the heated smoked sample with the majority of the bands disappearing. These results probably reflect changes in the solubilization of the various components of the myofibrillar protein fraction.

c). Electrophoresis of meat-urea extracts.

It has been suggested by Kakō (1968a) that a 7.7 M urea-containing 0.05 M Tris-HCl buffer (pH 8.6) is the most suitable solvent to solubilize meat proteins before and after heat-coagulation. Thus, it was decided to examine this extraction method of solubilizing meat proteins.

Figure 5 is a starch-urea electrophoretogram of the protein patterns of meat-urea extracts of untreated, heated and heated smoked pork samples. Poor resolution was obtained with both the cationic and anionic proteins with much of the protein remaining near the sample slots, probably indicating denatured protein. From these patterns, though there may be some differences in details, the mobility of the components shown as bands and zones are similar throughout the gel. With the cationic proteins, all bands are present but decreased protein activity was noted in the heated and the heated smoked samples. Although poor resolution was obtained with the anionic proteins, the group of fastest moving components may represent the myofibrillar protein fraction. The intensity of staining was strongest in the heated samples, intermediate in the untreated and weakest in the heated smoked samples. This pattern of activity is similar to the results obtained with the total myofibrillar protein fraction in solubility studies. However, to positively identify these components, individual protein fractions would have to be analyzed. Because of the poor resolution obtained, the meat-urea extracts were subjected to disc gel electrophoresis.

The disc gel electrophoretic patterns of the meat-urea extracts are presented in Figure 6. The resolution was considerably improved over that obtained with the starch-urea electrophoretograms with 16 to 18 bands

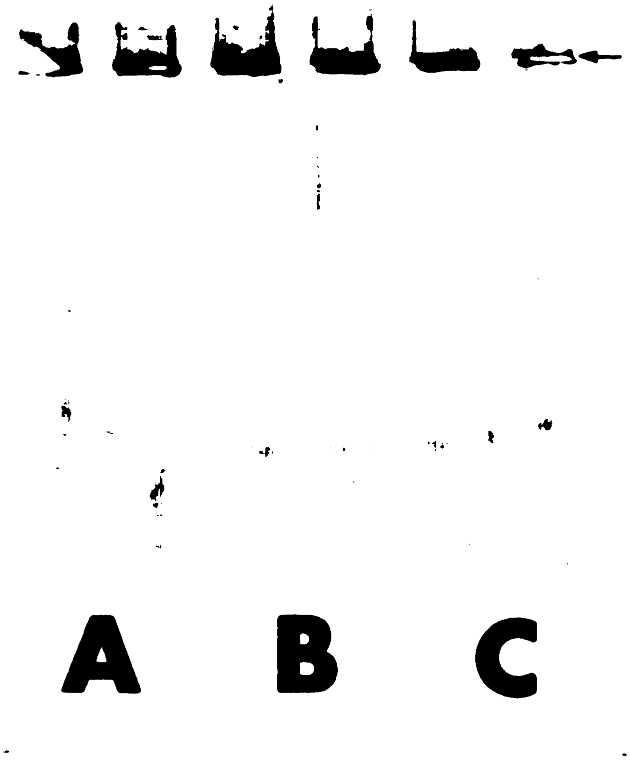


Figure 5. Starch-urea gel electrophoretograms of meat-urea extracts of pork loin samples.

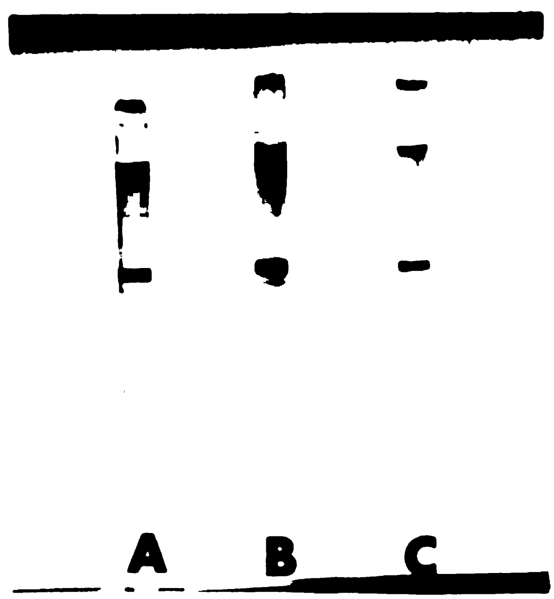


Figure 6. Disc gel electrophoretograms of meat-urea extracts of pork loin samples.

being observed. The mobility of the bands was similar for all extracts, however, the color intensity of the protein pattern of the heated smoked sample was considerably decreased with the possibility of some bands disappearing (Figure 6). In comparing the protein patterns of the untreated and heated samples, very little differences were observed. However, the densitometric tracings revealed that the peak heights were greater for the heated samples. With all three samples, there appears to be a large amount of protein which did not migrate down into the gel, which may indicate denaturation during the extraction process.

The results obtained with the electrophoretic studies of the water extracts, Weber-Edsall extracts and meat-urea extracts substantiate the results obtained with the protein solubility studies. Obvious changes were shown in the electrophoretic patterns of the heated smoked samples which indicate that smoke causes changes in the electrophoretic behavior of meat proteins.

Alterations in Protein Functional Groups.

pH measurements.

Mean values for pH of untreated, heated and heated smoked pork and albumin samples are listed in Tables 8, 9, and 10. It was only with pork samples obtained under the smokehouse conditions of 60°C and 45% relative humidity that a significant difference was obtained between all samples. However, a similar pH pattern was displayed throughout, i.e., the heated samples had the highest pH values and the heated smoked samples gave the lowest pH values. The small sample numbers were probably a factor in the non-significance obtained with the pork samples obtained at 32.2°C. A slight increase in pH values with heating was observed by Cohen (1966),

Table 8. The effect of heating and heating smoking on the pH, free sulfhydryl groups, amino nitrogen content and ninhydrin positive material of pork samples.[1,2]

Variables	State of Muscle		
	Untreated	Heated	Heated Smoked
pH	5.31	5.48	4.95**
Free sulfhydryl groups (µmoles/g protein)	91.87	120.37	69.81**
Amino nitrogen (mg/g protein)	9.048**	7.059	6.566
Ninhydrin positive material, (µmoles/g protein), (NPM)	526.67	559.67	540.30
NPM (µmoles/g pork)	179.90*	259.70	229.90

[1]Smokehouse condition : 60°C (140°F), 45% R.H.
[2]The means underlined by the same line do not differ significantly.
*P < 0.05; **P < 0.01

Table 9. The effect of heating and heating smoking on the pH, free sulfhydryl groups and amino nitrogen content of pork samples.[1,2]

Variables	State of Muscle		
	Untreated	Heated	Heated Smoked
pH	5.28	5.31	4.90
Free sulfhydryl groups (µmoles/g protein)	91.41	100.23	71.70**
Amino nitrogen[a] (mg/g protein)	8.985	8.450	8.659

[1]Smokehouse condition : 32.2°C (90°F), 45% R.H.
[2]The means underlined by the same line do not differ significantly.
[a]Statistical analyses were not performed.
**P < 0.01

Table 10. The effect of heating and heating smoking on the pH, free sulfhydryl groups and amino nitrogen content of albumin.[1]

Variables	State of Muscle		
	Heated	Untreated	Heated Smoked
pH	5.18*	4.91	4.88
Free sulfhydryl groups (μmoles/g protein)	6.03	4.95	2.23**
Amino nitrogen (mmoles/g material)	0.315	0.380	0.269*

[1]Smokehouse condition : 60°C (140°F), 45% R.H.
*$P < 0.05$; **$P < 0.01$

Hamm and Deatherage (1960), Kauffman et al. (1964) and Paul et al. (1966) with ham, beef, pork and rabbit muscles respectively. Hamm (1966) has suggested that the pH changes occurring during heating of meat may be caused by charge changes, or hydrogen bonding, or both, within the myofibrillar proteins. Krylova et al. (1962) observed at pH drop of 0.33 units with a meat extract which was exposed to smoke at 20°C for 2 hr and Yuditskaya (1962) observed a similar decrease in smoked fish. The smoking of beef, pork and chicken sausages at 25°C for 5 hr resulted in a pH drop of 0.37 and 0.29 units respectively with beef and chicken sausages and only a 0.06 pH unit decrease in pork sausage (Kakō, 1968b). The changes observed in the heated smoked samples are probably caused by the penetration of smoke components, such as organic acids, into the food products.

Free sulfhydryl groups.

The data in Tables 8, 9, and 10 show that there were appreciable differences in the free sulfhydryl groups of untreated, heated and heated smoked pork and albumin samples subjected to smokehouse conditions of 60°C and 45% relative humidity. The free sulfhydryl content of untreated samples correspond to values obtained by Hamm and Hofmann (1965) for meat and

Ellman (1959) for albumin. The slight increase in sulfhydryl groups of heated pork samples at 32.2°C and the significant increase at 60°C are in agreement with an earlier study by Hamm and Hofmann (1965). These workers observed a steady increase within the temperature range of 30°C to 70°C and attributed this increase to the unfolding of peptide chains, especially those of actomyosin. With the conditions utilized in this study, a loss of 22 to 24% of the free sulfhydryl groups occurred in the heated smoked pork samples; studying beef, Krylova et al. (1962) observed a 60% decrease in the free sulfhydryl groups of smoked samples. The loss of free sulfhydryl groups in the heated smoked samples could be attributed to several factors. Hydrogen sulfide may be formed and volatilized; however, Hamm (1966) stated that hydrogen sulfide is not liberated until the muscle has been heated to 80°C or greater. During the smoking process, a loss of drip and fat occurs; Dzinleski et al. (1969) obtained appreciable amounts of free sulfhydryl groups in drip as did Pepper and Pearson (1969) in adipose tissue. However, a similar loss of drip and fat occurred with the heated samples and these samples displayed a significant increase in their sulfhydryl content. Therefore, the decrease of free sulfhydryl groups in the heated smoked samples was probably caused by the formation of complexes between smoke constituents and free sulfhydryl groups. Studying the effect that various smoke condensate fractions had upon the free sulfhydryl groups of various compounds, Krylova et al. (1962) noted that the phenolic fractions exerted the greatest effect.

Amino nitrogen.

There was an appreciable change in the amino nitrogen content of the heated and heated smoked pork samples (Table 8) with the majority of the decrease being due to heat effects. Bautista et al. (1961) observed a similar decrease in amino nitrogen content upon heating beef longissimus dorsi muscle to 65°C. With albumin samples (Table 10), heating caused a 17% decrease and heating smoking caused an additional 12% decrease of the free amino nitrogen content. Little if any changes were observed with the cold (32.2°C) smoked pork samples (Table 9) whereas Krylova et al. (1962) observed a 20 to 25% decrease in the amino nitrogen content of cold smoked beef samples. However these workers obtained a very slight decrease in the amino nitrogen content with the addition of smoke condensate fractions to a meat-water extract. Kihara (1962) obtained a slight increase in the amino nitrogen content (mg/g meat) of smoked poultry and pork as did Kida and Tamoto (1967) with smoked herring but this increase was probably a reflection of the weight lost during smoking rather than an actual increase in the amino nitrogen content.

McCain et al. (1968) stated that the determination of the total ninhydrin positive material may be used as an estimation of the total free amino acids in the sample. By this determination, no appreciable changes were observed between any of the samples (Table 8) when calculated as µmoles per g protein. Usborne et al. (1968) observed a significant increase of total free amino acids during heating, de Abreu and Correia (1962) had a similar increase during smoke drying of sausage and Kida and Tamoto (1967) found a 50% increase of total extractable amino acids of smoked herring based upon the wet weight of the sample. By calculating the ninhydrin positive material (Table 8) on a wet weight basis, a significant increase was observed in the present study between the treated and the untreated samples.

Again, as was observed with protein solubility and electrophoretic studies, heating caused definite changes in various protein properties of pork samples. However, the effects of smoke were definitely displayed, indicating that there are chemical interactions of smoke components with meat constituents, with proteins in particular.

Since Dzinleski et al. (1969) observed considerable amounts of free sulfhydryl groups in the drip of beef muscle and Usborne et al. (1968) obtained appreciable amounts of free amino acids in the drip of cooked pork, it would have been of interest to have collected the drip in this present study and analyzed this fraction. Then, it would have been possible to observe if the drip from the heated and heated smoked samples reacted in a similar manner as the meat samples.

In comparing the results of the protein solubility studies and those of the protein functional group studies, there appears to be a relationship existing between the pH, free sulfhydryl content and the total fibrillar protein nitrogen fraction. With the heated pork samples, an increase was observed in all three values whereas a decrease was obtained in these values with the heated smoked samples. The increases observed with the heated samples are in agreement with the studies of Hamm (1966) who is of the opinion that changes in pH and free sulfhydryl groups are related to the unfolding of the peptide chains of myofibrillar proteins. The decreases observed with the heated smoked samples are probably due to smoke constituent interactions with the various reactive groups within the proteins.

Since liquid smoke is being used to some extent and samples were available; liquid smoke solutions were applied to pork and albumin samples to observe the effects that they exerted upon pH, free sulfhydryl groups and amino nitrogen values (Table 11). Although the pH was affected very little, similar effects as noted previously, were obtained for the free sulfhydryl groups and amino nitrogen values. These changes are probably

Table 11. The effect of the addition of artifical smoke solutions on pH, free sulfhydryl groups and amino nitrogen content of pork and albumin samples.[1]

	pH		Sulfhydryl groups (μmoles/g)		Amino nitrogen (mg/g)	(mmoles/g)
	Pork	Albumin	Pork	Albumin	Pork	Albumin
Untreated	5.57	6.90	20.75	4.19	1.82	.364
Heated	5.70	7.11	-	4.36	1.36	.359
Natural Smoke Flavor SF-12	5.65	6.77	15.50	1.73	1.09	.328
Charsol	5.69	6.84	13.88	2.96	1.12	.305
Solu-Smoke	5.68	6.84	14.50	2.07	1.01	.305

[1]All calculations are on a wet weight basis.

due to interactions of liquid smoke constituents with sulfhydryl and amino groups and again indicate that smoke constituents affect meat protein properties.

Acid Phosphatase Activity.

It has been suggested by several workers (Cohen, 1966; Lind, 1965a,b; Gantner and Körmendy, 1968; Olsman, 1968) that acid phosphatase activity may be used as a criterion for the heat treatment of hams and picnics. Although salt content, polyphosphates and pH have been shown to effect the phosphatase activity; the effect that smoke has upon acid phosphatase activity is apparently unknown. Since Cohen (1966) had detected acid phosphatase activity on an electrophoretogram of a 0.9% NaCl extract from heated ham; a similar extract, later replaced by a water extract, was obtained from the untreated, heated and heated smoked samples and subjected to starch gel electrophoresis (Figure 3). Although the bands marked "a" appeared to exhibit acid phosphatase activity, it was impossible to obtain positive confirmation of acid phosphatase activity by this method. Further analysis for acid phosphatase activity was performed by chemical methods and these results are presented in Table 12. With either method, there was a significant decrease in the acid phosphatase activity of the heated and the heated smoked samples. Although heat caused a definite decrease in acid phosphatase activity and this decrease may be correlated to heating temperatures (Lind, 1965b, Olsman, 1968), it would appear from the above results, that if smoke accompanies this heating, the effect that smoke exerts upon acid phosphatase activity would also have to be taken into consideration.

Table 12. The effect of heating and heating smoking on the acid phosphatase activity of pork samples.[1]

	State of Muscle		
	Untreated	Heated	Heated Smoked
mμ moles substrate hydrolyzed/min/mg N_2 (Andersch and Szckzypinski,1947)	7.12	0.56	0.24**
μmoles phenol/g sample (Lind, 1965a)	7.75	1.19	0.39**

[1]Smokehouse condition : 60°C (140°F), 45% R.H.
**$P < 0.01$

Addition of Phenolic Compounds to Pork and Albumin Samples.

Many phenolic compounds have been detected in wood smoke and the phenolic fraction is the fraction most often associated with the desirable qualities attributable to smoke. Krylova et al. (1962) observed that the phenolic fraction of a smoke condensate had a noticeable effect upon the sulfhydryl groups of meats but little if any effect upon the amino nitrogen content. Dividing this phenolic fraction into narrower fractions, these workers observed that the amino groups of meat react with "fractions 2 and 5", and "fraction 8" reacted very actively with the sulfhydryl groups. In this present study, it was decided that it would be of interest to observe the effects that individual phenolic fractions had upon the pH, free sulfhydryl groups and amino nitrogen content of meat and albumin samples. Although very inconclusive, the results of this study are presented in Table 13. Very inconsistent results were obtained and about the only statement which can be made regarding their activity is that different phenols react differently with the functional groups of proteins. The majority of the phenolic compounds caused an increase in pH and a decrease in the availability of the free sulfhydryl and amino groups. It was of interest to note the differences which were obtained in the color of albumin solutions treated with the various phenols, probably indicating that phenols may have a rôle in the color development during smoking.

The effect of the addition of o-cresol, α-naphthol and vanillin to a 5% albumin solution upon the pH, free sulfhydryl groups and amino nitrogen content of albumin over a definite time period is shown in Figure 7. Outside of the initial change, the length of time of heating at 60°C appears to have little effect on the pH and the amino nitrogen values of the treated albumin samples in comparison with the changes occurring in the untreated albumin sample. However, very obvious differences were observed in the free sulfhydryl groups. Whereas there was a constant decrease of sulfhydryl content of untreated albumin, little if any change occurred with α-naphthol and vanillin treated albumin with an increase occurring with the o-cresol treated albumin.

It would appear that before a study of this type can be performed, the approximate concentrations of the individual phenols in smoke would have to be known. Then, the effects of individual phenols upon model

Table 13. The effect of individual phenolic compounds on the color, pH, free sulfhydryl groups and amino nitrogen content of pork and albumin samples.

	pH		Sulfhydryl groups (μmoles/g material)		Amino nitrogen (mg/g)	Amino nitrogen (mmoles/g)	Color of Albumin Solution
	Pork	Albumin	Pork	Albumin	Pork	Albumin	
Untreated	5.28	6.85	26.62	5.37	1.78	.332	-
Heated	5.44	6.82	26.50	6.28	1.54	.350	-
Phenol	5.57	7.18	25.25	5.32	1.17	.096	cream
o-cresol	5.57	7.25	23.12	5.58	0.96	.022	white
m-cresol	5.58	7.28	23.50	4.78	1.05	.022	white
p-cresol	5.47	7.18	20.75	4.51	1.07	.022	white
Pyrocatechol	5.52	6.84	20.75	0.81	1.42	.066	pink
Hydroquinone	5.51	7.02	12.75	1.66	1.26	.158	orange
Resorcinol	5.50	7.08	24.50	4.08	0.98	.090	white
Pyrogallol	5.47	6.68	19.75	-	1.30	.110	salmon
Phloroglucinol	5.48	6.72	25.75	5.69	1.49	.162	white
Guaiacol	5.53	6.96	20.75	3.28	1.05	.086	white
4-methylguaiacol	5.59	7.26	18.75	3.33	0.77	.080	white
4-ethylguaiacol	5.61	7.28	22.00	3.92	1.16	.078	white
4-allylguaiacol	5.56	7.28	21.00	2.20	0.91	.084	orange
3-methoxycatechol	5.54	6.72	13.00	-	1.19	.125	purple
Vanillin	5.46	6.19	21.00	1.45	1.25	.110	yellow
2,6-dimethylphenol	5.51	7.30	25.62	5.69	0.98	.062	white
3,4-dimethylphenol	5.63	7.34	24.00	5.26	1.02	.102	cream
3,5-dimethylphenol	5.56	7.30	23.25	3.22	1.12	.119	white
Thymol	5.56	7.40	26.00	5.26	1.26	.100	white
2,6-dimethoxyphenol	5.51	7.16	22.25	0.86	1.35	.078	white
2,6-dimethoxy-4-allylphenol	5.58	7.22	17.50	0.54	1.20	.098	salmon
2,6-dimethoxy-4-ethylphenol	5.48	6.96	12.50	0.75	1.00	.092	brown
2,6-dimethoxy-4-methylphenol	5.57	7.22	20.50	0.86	1.07	.102	white
α-naphthol	5.56	7.44	19.00	3.44	1.54	.120	purple

Figure 7. The effect of heating at 60°C for a 150 min time period on pH, free sulfhydryl groups and amino nitrogen content of o-cresol-, α-naphthol- and vanillin-treated and untreated albumin solutions.

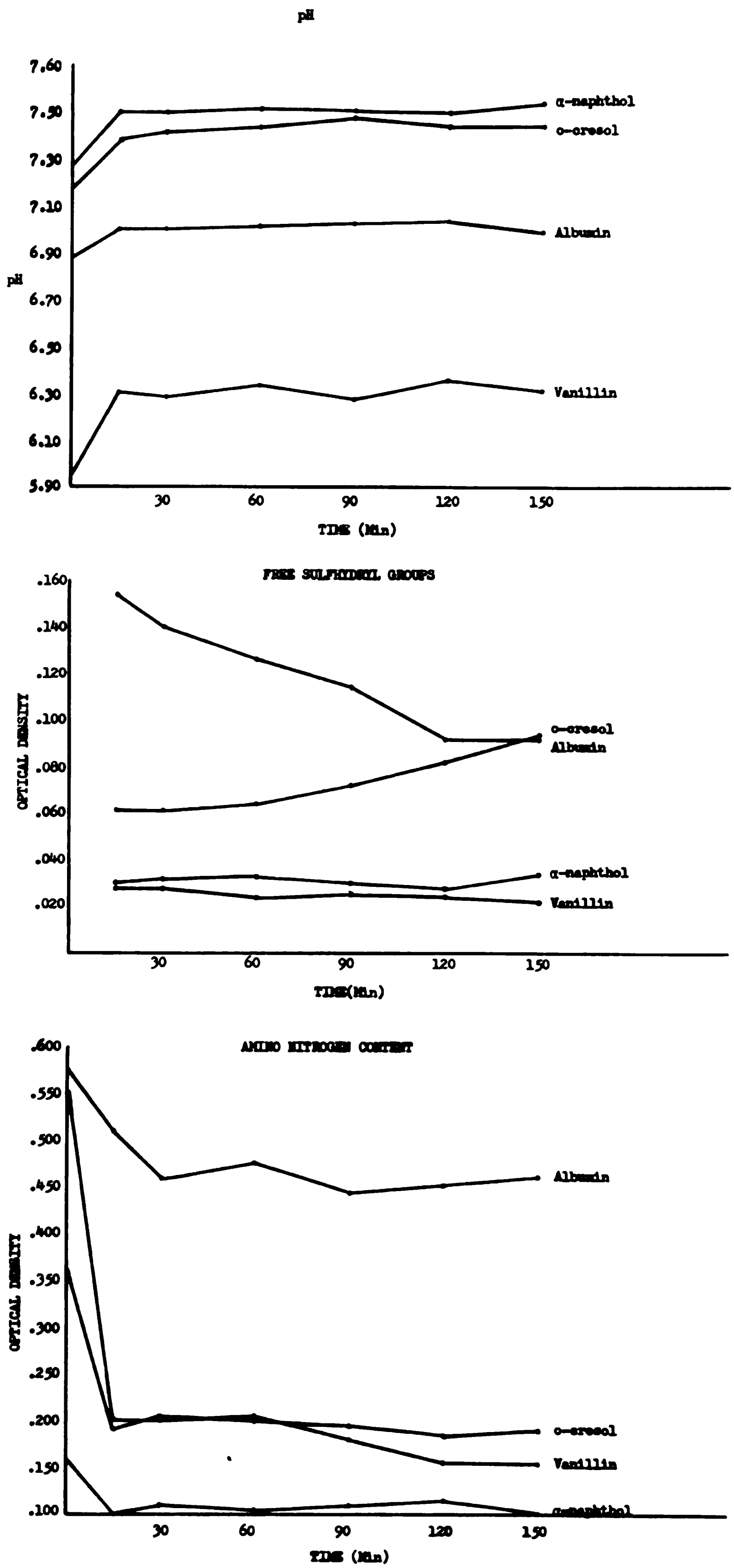

pH
7.60
7.50
7.30
7.10
6.90
6.70
6.50
6.30
6.10
5.90
pH
α-naphthol
o-cresol
Albumin
Vanillin
30
60
90
120
150
TIME (Min)
FREE SULFHYDRYL GROUPS
.160
.140
.120
.100
.080
.060
.040
.020
OPTICAL DENSITY
o-cresol
Albumin
α-naphthol
Vanillin
30
60
90
120
150
TIME(Min)
AMINO NITROGEN CONTENT
.600
.550
.500
.450
.400
.350
.300
.250
.200
.150
.100
OPTICAL DENSITY
Albumin
o-cresol
Vanillin
α-naphthol
30
60
90
120
150
TIME (Min)

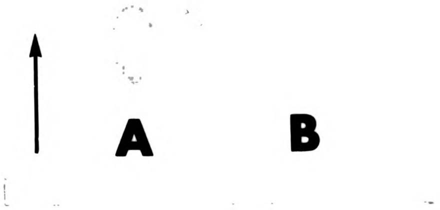
Figure 7.

protein systems should be studied before attempting to study their affects upon meat.

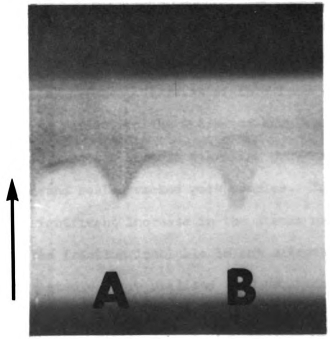
Ninhydrin Positive Substances in Wood Smoke.

Although there was no observed increase in the total amino acid content of the heated smoked samples (Table 8), it became of interest to investigate if the presence of some naturally occurring nitrogen compounds in wood smoke existed. It has been demonstrated that various nitrogenous compounds are present in cigarette smoke; ammonia, ethylamine, methylamine, nicotine, pyridine (Izawa and Kobashi, 1957), amino acids and other ninhydrin positive compounds (Buyske et al., 1956; Izawa et al., 1959; Izawa and Taki, 1959). Various workers (Fukuda, 1963; Laidlaw and Smith, 1965; Merrill and Cowling, 1966) have established the presence of amino acids in wood.

An aqueous smoke solution was collected and the thin-layer chromatograms obtained by utilizing two solvent systems and spraying the plates for ninhydrin positive substances are presented in Figure 8. It is obvious that the separation is incomplete and that little if any differences exist between the control and the sample solutions. Thus, it appears from this study that ninhydrin positive compounds are not present in the aqueous wood smoke solution collected.



Solvent : 2-propanol: formic acid: water (40:2:10).



Solvent:: chloroform: methanol: 17% NH_4OH (2:2:1).

Figure 8. TLC chromatograms of an aqueous smoke solution sprayed for ninhydrin positive compounds (A = sample; B = control).

SUMMARY

The effects of heating and heating smoking on the chemical properties of porcine muscle proteins and albumin were investigated. The effect of the addition of artificial smoke and phenolic compounds was also studied.

Changes in protein solubility of untreated, heated and heated smoked pork longissimus dorsi muscle samples were examined. Results showed that the total nitrogen content remained constant in all samples. Appreciable changes were observed in the solubility of the low ionic strength fraction (0.05 M phosphate extract) of the heated and heated smoked samples with this decrease being primarily due to the loss in solubility of the sarcoplasmic protein nitrogen fraction. There was a definite increase in the total fibrillar protein nitrogen fraction of the heated samples and a significant decrease of this fraction with the heated smoked samples when compared with the untreated samples. A noticeable decrease was obtained with the soluble fibrillar protein nitrogen fraction with both the heated and heated smoked pork samples. The heated smoked sample displayed a significant increase in the stroma nitrogen fraction, or more correctly, the fraction insoluble in any extraction solution.

Electrophoretic studies were utilized to substantiate the solubility studies. Starch gel electrophoresis of water extracts (the sarcoplasmic fraction) demonstrated the loss of numerous protein components in the heated and heated smoked samples with these changes being greater in the latter samples. Small differences were observed in the electrophoretic

patterns of the Weber-Edsall extract (myofibrillar protein fraction) and the meat-urea extract of the untreated and heated samples except for sharper resolution of the protein bands in the heated sample. However, electrophoretic studies of these extracts from the heated smoked samples showed protein patterns in which most components were either lost or lower in color intensity.

The results of the solubility and electrophoretic studies indicated that although heating caused definite changes in the protein fractions of untreated and treated pork muscle samples, smoke constituents caused additional changes in the various protein fractions.

A consistent difference in pH values was observed throughout, i.e., the heated samples displayed the highest pH values and the heated smoked samples the lowest values. Appreciable changes were obtained for the free sulfhydryl groups of the untreated, heated and heated smoked pork and albumin samples. A noticeable increase in the free sulfhydryl content was obtained with heated samples and a significant decrease was observed with the heated smoked samples. The amino nitrogen content was decreased in the heated and heated smoked pork and albumin samples. The addition of artificial smoke solutions to pork and albumin samples resulted in a decrease of both the free sulfhydryl and amino groups. The results of these studies of the functional groups of proteins indicated that smoke constituents react with functional groups of proteins.

The acid phosphatase activity was determined on pork samples obtained under a smokehouse condition of 60°C and 45% relative humidity. A significant decrease was observed in the acid phosphatase activity of the heated and heated smoked samples with the decrease being greater with the latter samples.

The addition of phenolic compounds to meat and albumin solutions resulted in very inconsistent results; however, most phenols appeared to cause a decrease in the availability of the free sulfhydryl and amino groups. Various color changes were also observed in the albumin solutions.

An aqueous wood smoke solution was collected and subjected to thin layer chromatography for the detection of ninhydrin positive substances. By comparison with a water blank, ninhydrin positive substances were not detected.

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APPENDIX

Appendix A. Composition of solutions used for protein fractionation studies and sample preparations for electrophoretic studies (deionized distilled water used in all cases).

1. 0.05 M phosphate buffer pH 7.6.

   2.710 g K_2HPO_4, 0.475 g KH_2PO_4. Make to 1 L.

2. KCl-phosphate buffer $\mu = 0.55$, pH 7.5.

   29.842 g KCl, 8.186 g K_2HPO_4, 1.225 g KH_2PO_4. Make to 1 L.

3. Tris-citric acid buffer containing sucrose pH 8.6.

   1.211 g Tris, 0.210 g Citric acid, 170.15 g Sucrose. Make to 1 L.

4. Tris-EDTA buffer containing sucrose pH 7.6.

   6.055 g Tris, 0.292 g EDTA, 85.07 g Sucrose. Make to 1 L.

5. Weber-Edsall solution.

   44.70 g KCl, 3.60 g $KHCO_3$, 1.38 g K_2CO_3. Make to 1 L.

6. 0.055 M Tris-HCl buffer containing 7.7 M urea pH 8.6.

   25 ml 0.2 M Tris, 12.5 ml 0.1 N HCl, 46.245 g Urea. Make to 100 ml.

Appendix B. Composition of solutions used for electrophoresis (deionized distilled water used in all cases).

1. Tris-HCl buffer for starch gels.

   27.5 ml Tris (0.19 M) + 6 ml HCl (0.2 N). Make to 250 ml.

2. Solutions for disc gels.

   Solution A (Running gel) : 5 ml 2 N HCl, 7.62 g Tris, 0.10 ml TMED (N, N, N^1, N^1-Tetramethylethylenediamine), 81.25 ml 10 M Urea. Make to 100 ml.

   Solution A (Spacer gel) : 5 ml 2 N HCl, 1.25 g Tris, 0.075 ml TMED, 81.25 ml 10 M Urea. Make to 100 ml.

   Solution B (Running gel) : 43.3 g Cyanogum, 25 ml 10 M Urea. Make to 100 ml.

   Solution B (Spacer gel) : 33.3 g Cyanogum, 25 ml 10 M Urea. Make to 100 ml.

   Solution C (Running and Spacer gel) : 1 mg Riboflavin, 35 ml 10 M Urea. Make to 50 ml.

3. Preparations of disc gels (for 8 tubes).

	Solution A	Solution B	Solution C
Running gel	6.4 ml	1.6	2.67
Spacer gel	1.6 ml	0.4	0.67

4. Disc gel electrophoresis.

   Tank buffer : 6.0 g Tris, 28.8 g Glycine. Make to 1 L. Dilute 100 ml to 1 L before use.

   Staining solution : 250 ml Water, 250 ml Methanol, 50 ml Glacial acetic acid, 2 g Amido Black 10B.

   Destaining solution : 1 L Water, 1 L Methanol, 200 ml Glacial acetic acid, 200 ml Glycerol.

Appendix C. Composition of reagents used in chemical analyses.

1. Bromcresol green indicator (Micro-Kjeldahl).

   0.1 g Bromcresol green, 14.3 ml 0.01 N NaOH. Make to 250 ml with water.

2. Indophenol reagent (Phenols).

   Stock solution : 0.25 g 2,6-dichloroquinonechlorimide in 30 ml absolute alcohol.

   Working solution : 1 ml of stock solution diluted 1 to 15 with deionized distilled water.

3. Phenolphthlin-formol mixture (Amino nitrogen).

   50 ml 40% HCHO solution containing 1 ml 0.5% phthln solution in 50% alcohol, exactly neutralized with 0.2 N $Ba(OH)_2$.

4. Ninhydrin Reagent (NPM & NCM).

   Citrate buffer - 4.3 g citric acid + 8.7 g sodium citrate in 250 ml distilled water, adjust to pH 5.0 with NaOH. Add 400 mg $SnCL_2$-$2H_2O$ to the citrate buffer. Add to 250 ml methyl cellosolve (ethylene glycol monoethyl ether) containing 10 g ninhydrin.

5. Phosphate buffer $\mu = 0.1$ pH 8.0 (Sulfhydryl groups).

   4.648 g Na_2HPO_4, 0.245 g $NaH_2PO_4.H_2O$. Make to 1 L.

6. Reagent A (Acid phosphatase).

   M/10 Citrate-HCl acid buffer pH 4.8. To 21.008 g citric acid in water in a liter flask add 200 ml 1 N NaOH. Dilute to volume. To 900 ml of this solution add 100 ml N/10 HCl.

7. Citrate buffer pH 6.5 (Acid phosphatase).

   13.83 g sodium citrate + 0.588 g citric acid in 1 L distilled water.

8. Stock solution (Acid phosphatase).

   1.0 g phenol dissolved in 1 L distilled water; 5 ml phenol solution pipetted into 1 L volumetric flask. Then, 200 ml water plus 100 ml 50% TCA are added, mixed, then made to volume with distilled water.

Appendix C. (cont'd)

9. Ninhydrin spray reagent (TLC).

0.3 g Ninhydrin, 2 ml Glacial acetic acid, 5 ml Collidine (2,4,6-trimethyl pyridine). Make to 100 ml with ethanol.