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ABSTRACTS:
IN THIS ISSUE

BACTERIA-INDUCED BIOCHEMICAL CHANGES IN CHICKEN SKIN STORED AT 5°C. M. ADAMCIC & D. S. CLARK. *J. Food Sci.* 35, 103–106 (1970)–The effect of psychrotolerant bacteria on the water-holding capacity, as measured by the extract release volume (ERV), pH and protein degradation in chicken skin stored at 5°C was determined using strains of *Achromobacter* and pigmented and nonpigmented *Pseudomonas*. All organisms caused a rapid decrease in ERV to about 50% of the original value during the early log phase of growth before development of off-odor. Changes in pH and content of extractable nitrogenous materials occurred during bacterial growth, but were not pronounced until after the count was greater than 10^8 / cells/g of skin and a faint off-odor was detectable. The content of extractable materials decreased during the early log phase, corresponding to the period of rapid increase in pH; later, during the late log or stationary phases of growth, it rapidly or gradually increased, depending on the organism.

PHYSICAL AND CHEMICAL PROPERTIES OF EPIMYSIAL ACID-SOLUBLE COLLAGEN FROM MEATS OF VARYING TENDERNESS. W. G. KRUGGEL, R. A. FIELD & G. J. MILLER. *J. Food Sci.* 35, 106–110 (1970)–Components of the molecular structure of epimysial acid-soluble collagen (ASC) from bovine longissimus dorsi muscles of varying tenderness were studied. Results of sedimentation analyses and viscosity measurements suggested that epimysial ASC from tender meat contains a lesser degree of cross-links. Amino acid analyses indicated that lysine was significantly higher in epimysial ASC from tough meat. Chemical estimation of aldehydic-type cross-links in epimysial ASC samples showed that epimysial ASC of tough meat contained significantly more aldehyde than that of tender meat.

LOCALIZATION OF MYOGLOBIN IN PIG MUSCLE. S. MORITA, R. G. CASSENS, E. J. BRISKEY, R. G. KAUFFMAN & L. L. KASTENSCHMIDT. *J. Food Sci.* 35, 111–112 (1970)–Myoglobin is localized in specific fibers in pig muscle. A number of muscle fibers are essentially myoglobin negative in pig longissimus muscle. The correlation between myoglobin positive fibers and color score was low but the hypothesis was offered that the differential concentration of myoglobin in various fibers may be related to the PSE condition.

THERMAL BEHAVIOR OF PORCINE COLLAGEN AS RELATED TO POST-MORTEM TIME. R. A. FIELD, A. M. PEARSON, D. E. KOCH & R. A. MERKEL. *J. Food Sci.* 35, 113–116 (1970)–Differential thermal analyses of epimysial connective tissue from normal and low quality porcine muscle were compared at 0 and 24 hr post-mortem. In addition, the melting characteristics of intramuscular collagen were determined at 0 hr post-mortem. In all tissues studied, collagen from low-quality muscle consistently gave slightly lower peak melting points than that from normal muscle. Epimysial collagen had a significantly lower peak melting point at 24 hr than at 0 hr post-mortem. Peak melting temperatures were slightly higher for intramuscular connective tissue than for epimysial tissue.

ENZYMIC OXIDATION OF SIMPLE DIPHENOLS AND FLAVONOIDS BY ORANGE JUICE EXTRACTS. J. H. BRUEMMER & B ROE. *J. Food Sci.* 35, 116–119 (1970)–Extracts of orange juice vesicles oxidized p-hydroquinone (HQ) and o-dihydroxyphenylalanine (DOPA) with O_2. Most of the oxidase activity was associated with a particulate fraction that sedimented at 100,000 × g. Sonic disruption of the particulates followed by chromatography on DEAE-cellulose increased specific activities with both substrates. The partially purified enzyme oxidized numerous naturally occurring o-diphenols and o-methoxyphenols. Acidic media below pH 4 and heating above 70°C destroyed most of the oxidase activity. KCN, diethyldithiocarbamate and 8-hydroxyquinoline but not EDTA inhibited the oxidase. The partially purified enzyme reduced benzoquinone and oxidized the reduced form of nicotinamide-dinucleotide (NADH). Ubiquinone, a benzoquinone derivative, but not menadione or vitamin K_1, naphthoquinone derivatives, could replace benzoquinone in the oxidation of NADH. Ubiquinone, benzoquinone and similar p-quinones may function in the orange as the oxidation-reduction couple between NADH-quinone reductase and diphenol oxidase.

A METHOD FOR ESTIMATING THE FUNCTIONAL CAPILLARY SYSTEM IN SKELETAL MUSCLE. R. DALRYMPLE, R. G. CASSENS & E. J. BRISKEY. *J. Food Sci.* 35, 120–122 (1970)–The functional capillary system was investigated by injecting trypan blue dye into 8 market-weight porcine animals followed by extracting the dye from various muscles. The trapezius contained more dye than the biceps femoris, with the gluteus medius and longissimus dorsi being equal in dye content but containing less dye than the trapezius and biceps femoris muscles. This rank compared well with the fiber type distribution, with the trapezius being high in red fiber content. The method presents possibilities for study of capillary function but some problems were encountered with the injection procedure.

EFFECT OF TEMPERATURE ON VISCOSITY OF FRUIT JUICES AND PUREES. G. D. SARAVACOS. *J. Food Sci.* 35, 122–125 (1970)–The viscosities of selected fruit products were measured with a coaxial-cylinder viscometer at temperatures 20–70°C. Clear apple and grape juices were Newtonian fluids and their viscosity decreased considerably at higher temperatures. Cloudy apple and orange juices changed from Newtonian to pseudoplastic fluids at higher concentrations and the effect of temperature was smaller than on clear juices. Fruit purees were pseudoplastic and their apparent viscosities decreased slightly at higher temperatures. The activation energy for flow increased with the juice concentration and decreased with the presence of suspended particles in the product. The viscometric data can be utilized in the design and operation of efficient processing equipment.

EFFECT OF PROCESSING AND STORAGE CONDITIONS ON THE MICROFLORA OF Clostridium perfringens–INOCULATED FRANKFURTERS. M. SOLBERG & B. ELKIND. *J. Food Sci.* 35, 126–129 (1970)–The growth support potential of frankfurters for *Clostridium perfringens* was determined. Inoculated emulsions were stuffed into glass tubes and processed so that they reached an internal temperature of 68–69°C in 30–48 min. The tubes were sealed with agar plugs and rubber stoppers to simulate vacuum-packaging conditions. Total aerobic and anaerobic bacterial counts in uninoculated raw emulsion were 1 to 3 log cycles higher when petri dishes were incubated at 23°C as opposed to 37°C. The low-temperature-growing mesophiles and psychrotrophs were destroyed during processing. The 37°C counts were reduced 1 log cycle and the 23°C counts, 2 to 3 log cycles. *Clostridium perfringens* reproduced rapidly in frankfurters at 37 and 23°C, more slowly at 15 and 12°C, and not at all during 2 wk of storage at 10°C or 4 wk at 0–5°C. *Clostridium perfringens* made up 2.5–10% of the total anaerobic count at 0 time but became the dominant organism during storage at all temperatures permitting growth.

PRODUCTION AND PROPERTIES OF Penicillium roqueforti LIPASE. R. R. EITENMILLER, J. R. VAKIL & K. M. SHAHANI. *J. Food Sci.* 35, 130–133 (1970)–A *Penicillium roqueforti* strain produced maximal amounts of lipase when grown in 0.5% casitone–1% Proflo broth, pH 5.5, at 27°C. Addition of butteroil, corn oil or olive oil to the growth medium inhibited lipase production. Under pH stasis the partially purified lipase of *P. roqueforti* had an optimum pH of 8.0 and an optimum temperature of 37°C. Maximum lipolytic activity occurred with 5% butteroil emulsion as the substrate. Manganese chloride and magnesium chloride stimulated the enzyme activity. Calcium, sodium and potassium salts had no appreciable effect on lipolysis; silver, mercury and zinc salts were inhibitory. The lipase was thermolabile, being inactivated completely within 10 min at 50°C. The lipase hydrolyzed tributyrin, tricaprylin, tricaprin, tripropionin and triolein in decreasing order.

PYROGLUTAMYL DIPEPTIDES IN MUSHROOM, Agaricus campestris. M. R. ALTAMURA, R. E. ANDREOTTI, M. L. BASINET & L. LONG JR. *J. Food Sci.* 35, 134–139 (1970)–A fraction was isolated from the edible mushroom, *Agaricus campestris*. Upon hydrolysis, it liberated much glutamic acid, small quantities of other amino acids and a hexosamine. The hydrolytic products were separated, quantified and identified by an amino acid analyzer. Besides proline and pyroglutamic acid, evidence was obtained for the presence in the unhydrolyzed fraction of the pyroglutamyl dipeptides of threonine, aspartic acid, valine, leucine, citrulline, phenylalanine, glycine, alanine, glutamic acid and proline and the amino acid sugar, N-pyroglutamylglucosamine. Pyroglutamylcitrulline and N-pyroglutamylglucosamine were tentatively identified. The first 6 dipeptides are reported for the first time. A mechanism is proposed for the enzymic biosynthesis of the pyroglutamyl dipeptides in mushroom.

POLYPHENOLS OF CASHEW KERNEL TESTA. A. G. MATHEW & H. A. B. PARPIA. *J. Food Sci.* 35, 140–143 (1970)–The major polyphenols of cashew kernel testa have been identified as (+) catechin, (−) epicatechin and polymeric proanthocyanidins, making use of paper chromatography and reactions with specific reagents. Two monomeric leucopelargonidins and two monomeric leucocyanidins have been noticed as minor components. The darkening occurring during the processing of nuts has been shown to be caused by interaction of polyphenols with iron.

POST-MORTEM ISOMETRIC TENSION CHANGES AND SHORTENING IN TURKEY MUSCLE STRIPS HELD AT VARIOUS TEMPERATURES. R. A. JUNGK & W. W. MARION. *J. Food Sci.* 35, 143–145 (1970)–A pattern of tension development and gradual relaxation has been demonstrated to occur post-mortem in strips of turkey breast muscle held isometrically. The time to maximum tension development occurs in 3.85 ± 0.19 hr and is not linearly related ($P < .05$) to temperature. The amount of maximum tension developed averaged 25 g/cm^2 and was significantly ($P < .05$) related to temperature. Relaxation to about 50% of maximum occurs in 18 hr. The amount of shortening that occurs post-mortem is linearly related ($P < .01$) to temperature. No "cold shortening" of turkey breast muscle was evident.

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BENZO(A)PYRENE IN SMOKED MEAT PRODUCTS. K. S. RHEE & L. J. BRATZLER. *J. Food Sci.* 35, 146–149 (1970)–A procedure was developed for determining benzo(a)pyrene (BaP) in smoked meat products. Artificial cellulose casings markedly reduced the BaP content of bologna smoked during processing. Penetration of BaP into the bologna was minimal (1.4–1.6 mm). More than half the amount of BaP in smoked bacon was found in the fat drippings following cooking.

SEROLOGIC IDENTIFICATION OF SPECIES OF ORIGIN OF SAUSAGE MEATS. A. B. KARPAS, W. L. MYERS & D. SEGRE. *J. Food Sci.* 35, 150–155 (1970)–A partially purified immunoglobulin G (IgG) solution prepared from the serum of a species to be tested was heated to the specifications for sausages. The resulting supernatant fluid was decanted and the precipitate washed with saline and used to immunize rabbits. The supernatant fluid was used to sensitize tanned sheep red blood cells. The immune serum was rendered monospecific by absorptions with heterologous, heated IgG precipitates. A sample of monospecific immune serum was absorbed with a washed homogenate of sausage. Aliquots of the monospecific immune serum, both untreated and sausage absorbed, were tested with cells sensitized with the homologous heated IgG supernatant fluid. A significant reduction of titer by sausage absorption indicated that the sausages contained the meat homologous to the immune serum.

COMPARATIVE GROWTH OF *Clostridium perfringens* IN CARBON DIOXIDE AND NITROGEN ATMOSPHERES. K. G. PAREKH & M. SOLBERG. *J. Food Sci.* 35, 156–159 (1970)–Relative growth rates of 8 food poisoning strains of *Clostridium perfringens* in a 100% nitrogen atmosphere and in a 100% carbon dioxide atmosphere at 760-mm pressure were determined. Generation time in nitrogen atmosphere was from 12.9–16.9 min; in carbon dioxide, 12.9–17.2 min. When comparing individual strains in carbon dioxide vs. nitrogen, no significant difference was noticed. Significant differences were noticed in growth rates in both media when strains were compared with one another.

BIOCHEMISTRY OF TEA FERMENTATION: CONVERSION OF AMINO ACIDS TO BLACK TEA AROMA CONSTITUENTS. H. CO & G. W. SANDERSON. *J. Food Sci.* 35, 160–164 (1970)–It was shown that leucine, isoleucine, valine and phenylalanine were partially converted to aldehydes via what appears to be a Strecker degradation during the course of tea fermentation in a tea-leaf homogenate. In contrast, aspartic acid, glutamic acid, glutamine, arginine, threonine, serine and theanine gave rise to no detectable volatile compounds under the same conditions. Identical results were obtained in a model tea fermentation system containing crude soluble tea enzymes, epigallocatechin gallate and ^{14}C-amino acids. Ascorbic acid inhibited formation of aldehydes from amino acids, whereas dehydroascorbic acid caused the conversion of amino acids into aldehydes.

VARIATIONS OF RELATIVE RETENTION TIMES IN OPEN-TUBULAR GAS-CHROMATOGRAPHIC COLUMNS: RELEVANCY TO FRUIT VOLATILES. T. H. SCHULTZ, T. R. MON & R. R. FORREY. *J. Food Sci.* 35, 165–169 (1970)–Open-tubular gas chromatographic columns made from stainless steel tubing, coated with methyl silicone oil, give variable retention times for individual alcohols, dependent on the size of sample. This difficulty is largely overcome by using a small proportion of adsorption-reducing material with the methyl silicone. There are cases of reversal of peak order, caused by differences in column temperature or minor differences in the coating. However, with care, relative retention times are nearly constant and are helpful as an aid in identification of compounds. A procedure for precise comparison of the retention times of unknown fruit volatiles with known compounds is described. Relative retention times of representative compounds are given for twelve different stationary liquids.

EFFECTS IN RATS OF PARTIAL REPLACEMENT OF COW'S MILK PROTEIN BY SUPPLEMENTARY NITROGEN. V. R. YOUNG & A. VILLARREAL. *J. Food Sci.* 35, 170–174 (1970)–Studies were made on growth of weanling rats and urinary nitrogen excretion in young adult rats when a mixture of nonessential L-amino acids or a mixture of diammonium citrate and glycine (DAC-Gly) replaced cow's milk protein to varying degrees. In weanling rats, 20% replacement caused a significant reduction in growth. This reduction was not entirely prevented by supplementation with the sulfur amino acids and other essential amino acids. Changes in urinary nitrogen excretion in young adult rats also revealed an alteration in nitrogen utilization as a result of protein replacement with DAC-Gly. The reduced dietary nitrogen utilization appeared to be due to decreased utilization of the sulfur amino acids and possibly all essential amino acids in the diet.

LOW-TEMPERATURE, LONG-TIME HEATING OF BOVINE MUSCLE. 1. Changes in Tenderness, Water Binding Capacity, pH and Amount of Water-Soluble Components. E. LAAKKONEN, G. H. WELLINGTON & J. W. SHERBON. *J. Food Sci.* 35, 175–177 (1970)–Relationships between the tenderness of very slowly cooked meat and its water-holding capacity, pH and the amount of water-soluble components were studied. Muscle samples were heated under fixed temperature programs. Samples were analyzed at 1-hr intervals between the 3rd and 10th hr of heating. During the first 4 hr of heating there were only minor changes in tenderness. The major decrease in shear values occurred between the 4th and 6th hr, when the meat was warming from 50–60°C. After 6 hr of heating to 60°C there were still uncoagulated water-soluble proteins. The shrinkage of collagen in long-time, low-temperature cooking is considered.

LOW-TEMPERATURE, LONG-TIME HEATING OF BOVINE MUSCLE. 2. Changes in Electrophoretic Patterns. E. LAAKKONEN, J. W. SHERBON & G. H. WELLINGTON. *J. Food Sci.* 35, 178–180 (1970)–Polyacrylamide gel electrophoresis was used to follow changes in the nature of the water-soluble proteins and juices of bovine muscle during low-temperature heating. The slowest-moving anodic proteins were coagulated first. The myoglobins and myoalbumins were altered significantly only by holding the meat at 60°C. The largest changes in tenderness and amount of water-soluble material in the meat occurred at the same temperatures under which the slow-moving proteins were denatured. The most heat-sensitive proteins detected were denatured before there were significant changes in tenderness or water-soluble substance content.

LOW-TEMPERATURE, LONG-TIME HEATING OF BOVINE MUSCLE. 3. Collagenolytic Activity. E. LAAKKONEN, J. W. SHERBON & G. H. WELLINGTON. *J. Food Sci.* 35, 181–183 (1970)–Naturally occurring collagenolytic activity was found in the water-soluble fraction of bovine muscle. General proteolytic activity determined with Azocoll indicated that this total activity was much greater than the collagenase activity specifically determined according to the method of Wünsch and Heidrich. The collagenase fraction was concentrated by polyacrylamide gel electrophoresis and the activity of the enzyme was studied under various pH and temperature conditions. This collagenase could remain active in the meat at cooking temperatures experienced in long-time, low-temperature cooking, $< 60°C$. With faster heating and higher internal temperatures, $> 70–80°C$, the collagenase observed in this study is inactivated.

POULTRY PRODUCT QUALITY. 1. Compositional Changes During Cooking of Turkey Roasts. J. H. MacNEIL & P. S. DIMICK. *J. Food Sci.* 35, 184–186 (1970)–White and dark turkey roasts were made with meat taken from selected samples of the various entries in the Pennsylvania Turkey Random Sample Meat Test. Additional roasts were made from sample birds from the University research flock. Cooking was carried out in a Telkes oven and all roasts were cooked to an internal temperature of 170°F. There were no sex differences in cooking losses except when skin was examined separately. Cooking loss differences did exist between Bronze and White for breast meat but not thigh meat; cooking losses were higher for breast meat than for thigh meat. There was an indication that size of bird was not a significant factor in determining percentage cooking losses of roasts made from breast and thigh meat. When fat drippings from cooked skin were analyzed for carbonyl content high skin yielding males were characterized by the high concentration of the 2-enals in relation to the methyl ketones. Low skin yield groups consisted mainly of methyl ketones.

POULTRY PRODUCT QUALITY. 2. Storage Time-Temperature Effects on Carbonyl Composition of Cooked Turkey and Chicken Skin Fractions. P. S. DIMICK & J. H. MacNEIL. *J. Food Sci.* 35, 186–190 (1970)–The carbonyl compounds in cooked turkey and chicken skin fractions after storage were isolated as their 2,4-dinitrophenylhydrazones. The monocarbonyl class was separated into methyl ketones, 2-enals and 2,4-dienals and measured spectrophotometrically. The turkey skin residue fraction contained higher concentrations of carbonyls than did the chicken samples. The oil extract from the skin of both groups was similar in carbonyl concentration. Lower storage temperature dramatically lowered the development of carbonyls. Phospholipid phosphorus determinations indicated the residue contained high levels of polar lipid; whereas, negligible amounts were in the oil. Thin-layer chromatography of the carbonyl classes from the skin residue indicated mainly C_7-C_9 2-enals and C_8, C_9 2,4-dienals in the unsaturated aldehyde fractions. Changes in fatty acid composition of the residue polar lipids during storage suggested linoleic and arachidonic acids as the probable substrates in autoxidative deterioration.

POULTRY PRODUCT QUALITY. 3. Organoleptic Evaluation of Cooked Chicken and Turkey Skin Fractions as Affected by Storage Time and Temperature. J. H. MacNEIL & P. S. DIMICK. *J. Food Sci.* 35, 191–194 (1970)–Cooked turkey and chicken skin residue and separated drippings or oil were stored at various temperatures then presented to a trained taste panel for flavor evaluation. Panel members were able to discriminate between a control (unstored) and a sample of turkey residue after 3 wk of storage at 40°F. They were not able to differentiate between control and stored oil even after 7 wk of storage. When chicken skin residue and oil were evaluated after storage at 40°F the panel members could detect differences between the residue samples at 3 wk, but unlike the turkey oil stored at the same temperature they indicated discriminatory ability after 1 wk of storage. When both cooked chicken and turkey skin fractions were presented to the panel at the same time without a reference control (unstored) they were able to identify differences but could not indicate a clear preference for either one.

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BACTERIA-INDUCED BIOCHEMICAL CHANGES IN CHICKEN SKIN STORED AT 5°C

SUMMARY—The effect of psychrotolerant bacteria on the water-holding capacity as measured by the extract release volume (ERV), pH and protein degradation in chicken skin stored at 5°C was determined using strains of Achromobacter *and pigmented and nonpigmented* Pseudomonas. *All organisms caused a rapid decrease in ERV to about 50% of the original value during the early log phase of growth before development of off-odor. Changes in pH and content of extractable nitrogenous materials occurred during bacterial growth but were not pronounced until after the count was greater than 10^8 cells/g of skin and a faint off-odor was detectable. The content of extractable materials decreased during the early log phase, corresponding to the period of rapid increase in pH; later, during the late log or stationary phases of growth, it rapidly or gradually increased, depending on the organism.*

INTRODUCTION

AT ABOVE-FREEZING temperatures the shelf-life of whole chicken is often limited by the activity of psychrotolerant strains of *Pseudomonas* and *Achromobacter* growing on the surface of the skin (Lochhead et al., 1935; Ziegler et al., 1954). The activity involves the production of off-odor and off-flavor compounds, presumably resulting from the breakdown of free amino acids and possibly skin proteins. Biochemical changes leading to the production of off-odor and off-flavor in poultry meat stored aseptically have been studied (van den Berg et al., 1963, 1964; Khan et al., 1964; Khan, 1965), but little is known about the biochemical changes that occur in the skin or muscle resulting from bacterial growth.

This paper reports results of a study to determine biochemical changes in skin proteins caused by psychrotolerant strains of *Pseudomonas* and *Achromobacter*. This included measurement of changes in the extractable protein and nonprotein nitrogenous materials as well as in the water-holding capacity, as measured by Extract Release Volume (ERV) according to Jay (1964a; Jay, 1964b), and pH of skin stored at 5°C. The extent and rates of the changes for 9- and 24-week-old birds were compared. The two ages of skin were studied to determine whether possible differences in composition known to occur in mammalian skin (Carmichael et al., 1967) would affect biochemical changes induced by psychrotolerant bacteria. The work is part of a larger project dealing with biochemical and quality changes in poultry meat during storage.

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MATERIALS & METHODS

SKIN WAS OBTAINED from 9- and 24-week-old broiler-type chickens (Ottawa Meat Control Strain) raised and processed in the laboratory. The birds were slaughtered by cutting the jugular vein and carotid arteries, scalded (59°C) for 1 min, plucked by hand and rubbed free of loose pieces of the epidermal layer of skin and parts of feathers with a sterile damp cloth. The skin was removed, cooled to about 5°C, stretched on wooden blocks and the subcutaneous fat removed with a sharp knife. The defatted skin was then cut into small pieces and frozen by immersion in liquid nitrogen. The frozen skin was ground to a coarse powder by passing it twice through a hand-operated meat grinder continually cooled with liquid nitrogen. The resulting slurry was placed in a large beaker and stirred thoroughly to provide a homogeneous mixture of skin from the different birds. After removal of the nitrogen by evaporation the skin was stored in plastic bags at −100°C until used. For each age, skin from enough birds to provide the material required for all subsequent tests was processed at one time.

Tests were made with pure cultures of psychrotolerant bacteria isolated from the skin of commercially processed poultry (Clark et al., 1969). Included were one strain each of pigmented *Pseudomonas* (strain 1), nonpigmented *Pseudomonas* (strain 47) and *Achromobacter* (strain 89), and a mixed inoculum of 10 strains of *Achromobacter*, 5 strains of nonpigmented *Pseudomonas* and 2 strains of pigmented *Pseudomonas*. The single cultures were judged to be typical of the three types that cause deterioration of refrigerated poultry meat (Ayres et al., 1950; Thornley et al., 1960; Barnes et al., 1968). The relative proportion of each type in the mixed inoculum was the same as that found on commercially processed poultry in Canada (Clark et al., 1969). 24-hr-old cells grown on Standard Methods agar (SM agar, Difco) at 20°C and prepared as described previously (Clark, 1968) were used to make the inoculum in all tests.

In most tests, ground freshly thawed skin was irradiated (500,000 rads), spread thinly over the bottoms of Petri plates and inoculated by the spray-chamber method (Clark, 1963) to give about 200,000 cells/g. Preliminary work showed that a 500,000-rad dosage was sufficient to destroy all psychrotolerant bacteria in the ground skin. The inoculated skin from all plates was pooled, mixed thoroughly to spread the inoculum uniformly and then distributed in 6-g samples into Petri plates for incubation. One sample for each inoculum was analyzed immediately after inoculation; the others were incubated at 5°C in a water-saturated atmosphere together with controls consisting of irradiated but uninoculated skin. Incubated samples were removed for analysis at regular intervals over 21 days.

In a few tests, analyses were also made with samples irradiated but not inoculated or incubated. This was done to determine initial pH and composition differences between the two ages of skin and to provide a basis for comparison with inoculated skin.

All samples were analyzed for bacterial count, extract release volume, pH and content of total extractable nitrogen. The extractable nitrogen fraction was further analyzed for its content of nonprotein nitrogen and ninhydrin- and phenol-reagent-positive materials. To determine bacterial count, 0.5 g of skin was comminuted with 100 ml of 0.1% peptone in a Waring Blendor and the resulting suspension plated on SM agar using the surface method (Clark, 1967). For determination of ERV, 5 g of skin was ground with 20 ml of glass-distilled water in a Sorval Omni Mixer for 2 min (50-ml capacity cup, 5,000 rpm); the resultant mixture was filtered through Whatman No. 1 filter paper and the volume of filtrate collected during 15 min of filtration, as described by Jay (1964a), was measured. pH changes in the skin were measured electrometrically on the ERV extracts.

To determine changes in the content of extractable nitrogenous materials, 5 g of skin was extracted with 35 ml of 0.2 M NaCl at 2–3°C (Jackson et al., 1958) for 24 hr on a shaker. The suspension was centrifuged at 10,000 rpm at 2–3°C for 20 min and the residue washed with 10 ml of extractant and recentrifuged. The supernatant and washing were combined, made up to 50 ml and filtered (No. 1 Whatman). Preliminary tests comparing 4 extractants, 0.3 M sodium phosphate buffer (Orekhovich et al., 1948), citrate buffer (Orekhovich, 1948), KCl-borate buffer (Khan, 1962) and 0.2 M NaCl (Jackson, 1958), showed that extraction of soluble protein and nonprotein nitrogen materials was highest with 0.2 M NaCl. The total nitrogen content of the extract and the nonprotein nitrogen content of the protein-free extract (treated with trichloracetic acid, 5% w/v final concentration) were determined by a micro-Kjeldahl method. The contents of ninhydrin-positive materials and phenol-reagent-positive materials in TCA-treated extracts were mea-

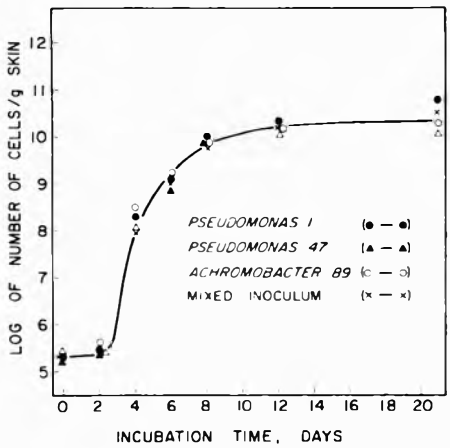

Fig. 1—Growth of psychrotolerant bacteria in comminuted skin of 9-week-old chickens.

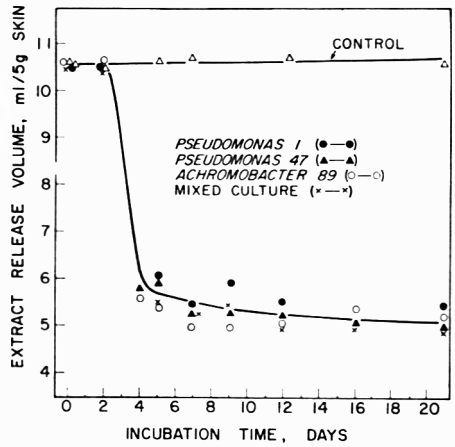

Fig. 2—Effect of bacteria on extract release volume of skin of 9-week-old chickens.

sured by the methods of Rosen (1957) and Folin et al. (1927), respectively, and were expressed as tyrosine equivalents.

RESULTS & DISCUSSION

RESULTS OF analysis of uninoculated samples showed that skin from 9-week-old birds contained more extractable nitrogenous material (29–39% more, depending on the specific material measured) and had lower ERV and pH values (8–10% lower) than skin from 25-week-old birds (Table 1). The differences in extractability were probably related to the effect of age on the solubility of neutral-salt-soluble collagenous proteins. Evidence obtained for bovine skin has shown that the solubility of these proteins in a sodium chloride solution decreases with age (Carmichael et al., 1967). The higher ERV value for older skin also indicates that solubility of skin collagen decreased with bird age. With decreased solubility the amount of inter- and intramolecular cross-linking among the α chains of the collagen molecule increases (Banga, 1966; Carmichael et al., 1967); as a result, sites capable of binding water are reduced. The effect of age on pH is not understood but the change was similar in extent to that reported for human skin; the skin of children is about 0.5 of a pH unit lower than that of adults (Behrendt et al., 1955; Behrendt et al., 1964).

The effect of bird age on the chemical composition of the skin, however, had no effect on the rate of bacterial growth (Clark, 1968) nor on the pattern of bacteria-induced biochemical changes measured. Therefore, only the results for skin of 9-week-old birds (broiler age) are presented in detail. Rates of growth of the individual test organisms and of the mixed culture in the ground skin were about the same (Fig. 1) and led to off-odor after about 4 days of incubation, coinciding with a total count of about 10^8 cells per gram of skin.

Results of tests with inoculated skin showed that all inocula caused a rapid decrease in the extract release volume during the early log phase of growth (Fig. 2). The decrease reached a maximum of about 50% of the original value after 4 days, corresponding to a bacterial count of about 10^8 per g. The test gave consistent and similar results for the relatively small sample used [5 g compared to 25 g used by Jay (1964a)] and for all types of bacteria studied. Therefore, the results show that the ERV test indicates the bacterial quality of whole poultry as it has been shown to do for ground beef (Jay, 1964b). It is noteworthy that with beef the ERV decreased with incubation time to zero (Jay, 1964a). This difference in extent of decrease is no doubt a reflection of the types and relative amounts of the proteins involved. Bacteria probably increase the water-holding capacity of tissues by enzymatically breaking the interor intramolecular linkages of proteins, thereby increasing the content of free end-groups, which are known to bind water (Ling, 1965).

Figure 3 shows that pH changes began to occur in inoculated skin after the log period of growth, but that these changes were not pronounced until after the count had reached 10^8 per g and a faint but distinct off-odor was noticeable. The pH then rose rapidly for all organisms, particularly for *Pseudomonas* 47 and *Achromobacter* 89. This period of increase corresponded to a rapid increase in the extent of off-odor, presumably caused mostly by ammonia and other decomposition products. The relatively slower rate of pH increase for the mixed inoculum compared to that for *Pseudomonas* 47 and *Achromobacter* 89 would appear to indicate a dominant effect of the pigmented pseudomonads in the mixture even though their proportion initially was low (about 12%). The small pH change obtained before off-odor development (0.1–0.2 of a pH unit), similar to that reported for skin of poultry contaminated naturally during processing (Fromm et al., 1965), indicates that a pH test on skin is of doubtful value for assessment of quality of whole poultry.

Figures 4 and 5 show that the bacteria caused changes in the nitrogenous constituents of the skin but, as with the pH test, the changes were not large until after 4 days of incubation and development of off-odor. All three types of bacteria caused a decrease in the contents of total extractable nitrogen, nonprotein nitrogen and phenol-reagent-positive- and ninhydrin-positive materials during the log

Table 1—Effect of age on pH, ERV and content of extractable nitrogenous material of uninoculated chicken skin.

Material	Skin from 9-week-old birds (14 samples)		Skin from 25-week-old birds (10 samples)	
	Avg	Range	Avg	Range
Total extractable nitrogen (mg/g)	2.8	2.5–3.0	1.8	1.7–2.0
Nonprotein nitrogen (mg/g)	0.85	0.78–0.95	.57	0.35–0.75
Ninhydrin-positive material (mg tyrosine/g)	6.8	5.9–8.2	4.8	3.6–6.5
Phenol-reagent-positive material (μg tyrosine/g)	172	160–185	109	70–180
ERV (ml/5 g)	10.8	9.4–11.6	11.4	9.9–12.0
pH of ERV extract	6.6	6.5–6.7	7.2	7.1–7.5

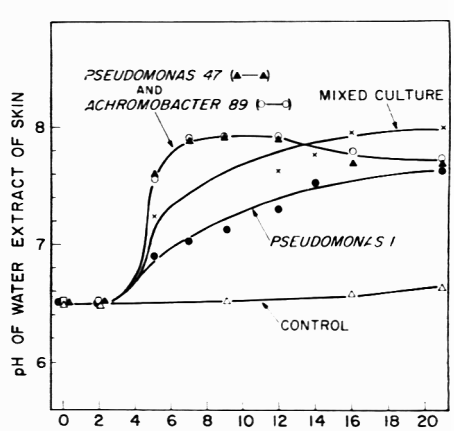

Fig. 3—Effect of bacteria on pH of skin of 9-week-old chickens.

phase, particularly between the 4th and 6th day of incubation, corresponding to the period of rapid increase in pH. After this period, values for *Pseudomonas* 47 and *Achromobacter* 89 remained stationary or increased gradually to the original values; those for *Pseudomonas* 1 increased rapidly, giving maximum values after 12-16 days. These results suggest that the organisms utilize the low-molecular-weight nitrogenous compounds during the period of rapid growth and subsequently replace such compounds through breakdown of proteins, rapidly or gradually, depending on the organism. Further studies are presently under way to determine the compounds affected.

The changes in pH in inoculated skin during incubation appeared not to affect markedly the results of the various tests. Figures 2 and 3 show that most of the changes in ERV values occurred before a measurable change in pH. Preliminary work in which skin samples were extracted (24 hr at 5°C) with buffered 0.2 M NaCl at various pH values between 6.5 and 8.0 showed that over this range the total extractable nitrogen content varied less than ± 4% from the average. Other workers have also shown with other tissues that pH between the values 6.5 and 8.0 has little effect on the solubility of protein and nonprotein nitrogenous materials in NaCl solutions (Dyer et al., 1950; Yasui et al., 1964).

Since cells of the test organisms could not be separated from the skin samples before extraction and centrifugation, part of the observed changes in composition could have resulted from the extractable metabolic end-products and autolyzed bacterial cells present. However, preliminary tests in which 1-g quantities of cells were extracted under the conditions used in the tests with skin, showed that less than 2% of the nonprotein nitrogen and total extractable nitrogen in the tests with inoculated skin could be accredited to the bacterial cells.

CONCLUSIONS

THE RESULTS showed that psychrotolerant spoilage bacteria cause a large increase in the water-holding capacity of chicken skin before development of off-odor. This change appears to be independent of the type of organism and of their relative ability to degrade proteins. Bacteria-induced changes in the pH and content of nitrogenous materials in skin begin as the organisms start to grow, but are not large until the cell count is above 10^8 per g and off-odor is produced. Therefore, ERV appears to be the most promising test among those studied for assessment of the microbial quality of whole poultry (skin intact). Since psychrotolerant bacteria grow and produce off-odor more quickly on the skin than on other poultry tissues (Lochhead et al., 1935; Ziegler et al., 1954), a bacteriological quality test involving the skin of whole birds would in most circumstances apply to the whole carcass. Further work is required to determine whether similar results for the ERV test are obtained for leg and breast muscle exposed in cut-up poultry.

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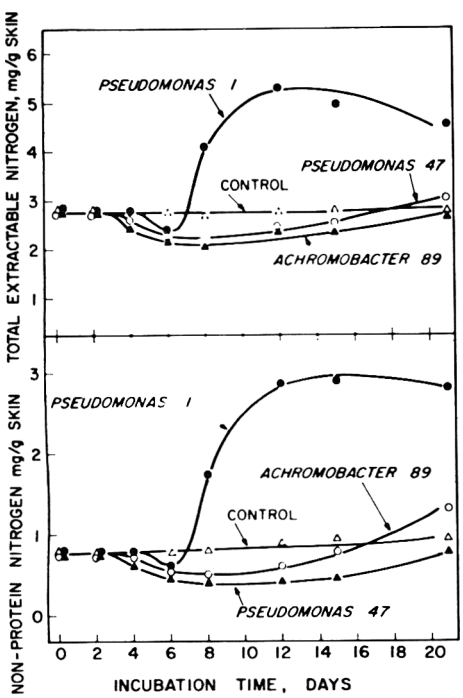

Fig. 4–Effect of bacteria on the content of extractable nitrogenous materials in skin of 9-week-old chickens.

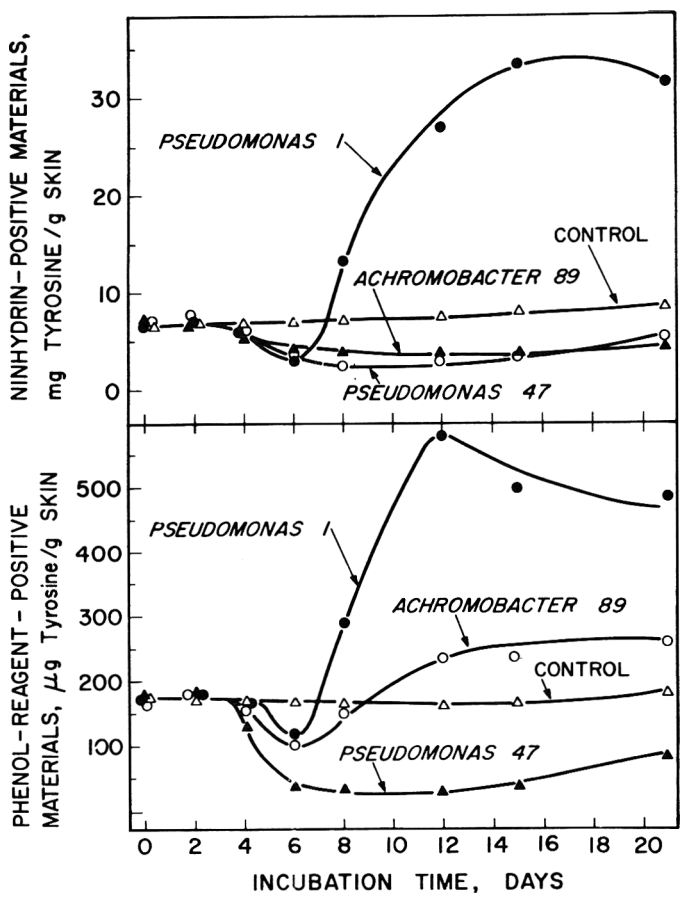

Fig. 5–Bacteria-induced changes in the content of phenol-reagent-positive- and ninhydrin-positive materials in skin of 9-week-old chickens.

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PHYSICAL AND CHEMICAL PROPERTIES OF EPIMYSIAL ACID-SOLUBLE COLLAGEN FROM MEATS OF VARYING TENDERNESS

SUMMARY—Components of the molecular structure of epimysial acid-soluble collagen (ASC) from meats of varying tenderness were studied by several methods. The ASC was studied since it contains an appreciable amount of intramolecular cross-links but is still soluble. Though the amount of total collagen in epimysial tissue was found to have no correlation with meat tenderness, the molecular studies indicated some correlation of the type of epimysial ASC with meat tenderness. Sucrose density-gradient ultracentrifugation analyses of denatured epimysial ASC suggested that this type of collagen contains a lesser degree of cross-links when obtained from tender meat samples. Viscosity measurements were found to be correlated to tenderness of meat in a manner similar to the results obtained by ultracentrifugation, in that the intrinsic viscosity of epimysial ASC from tender meat was lower than that from tough meat, indicating differences among the relative sizes of the collagen molecules from the different samples. Partial amino acid analyses of the epimysial ASC samples via gas chromatography showed no differences in the levels of aspartic, serine or hydroxyproline, amino acids which may be involved in the ester type of cross-links. Results of chemical estimation of the ester type of cross-links indicated that the proportions of esters in the various ASC samples were similar. The amount of lysine was found to be significantly higher ($P < 0.05$) in epimysial ASC from tough meat compared to tender meat, suggesting an increased potential of the aldehydic-type of cross-link, which is formed via an aldol condensation of two aldehydes derived biosynthetically from lysine. This was strengthened by the results obtained in the chemical estimation of the aldehydic-type of cross-link, in that the epimysial ASC of tough meat contained significantly more aldehyde than did that of tender meat ($P<0.05$).

INTRODUCTION

AS THE INQUIRY into the causes of tenderness or toughness of meat continues, investigators have concluded that this is a complex problem and cannot be simply resolved by the study of a single moiety, since many factors contribute to variations in beef tenderness. These factors may be divided into three broad classifications: ante-mortem, post-mortem and structural factors. Ante-mortem factors include physiological factors, such as age and inheritance, and feeding and management practices; whereas, post-mortem factors include temperature and length of storage time after slaughter, methods of trimming and cutting and cooking methods. The structural factors of tenderness are now being investigated more thoroughly and this study is centered upon some of the molecular implications of the structural factors. The structural factors may be said to include the molecular structure of the connective tissue protein, collagen, and how it influences the degree of tenderness of a muscle.

A few researchers have deduced from their investigations that tenderness or toughness is concerned with molecular cross-links in the collagen of connective tissue. Goll et al. (1962) studied the rate of connective tissue solubilization brought about by the action of collagenase in the biceps femoris muscle obtained from aged cow, cow, steer and veal age groups. The study indicated the occurrence of more frequent or stronger cross-links within and among the tropocollagen molecules of collagen from the older age group, since a decreasing rate of solubilization occurred as age increased. Herring et al. (1967) found results similar to those of Goll et al. (1962), collagen solubility decreasing significantly with advancing maturity of beef even though the collagen content was not different.

Cover et al. (1962) suggested that a tightening of the protein structure during heating (denaturation) occurs as new stable intermolecular cross-links are formed between the peptide chains of collagen. An alternate way that this may occur is for the tightening effect to occur intramolecularly along the peptide chains of collagen, thereby making the chains more resistant to cleavage.

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Goll et al. (1964a) suggested that stronger or more extensive cross-links in the collagen from older animals occur, and that the number and strength of these cross-links play an important role in meat tenderness (Goll et al., 1964b). Hill (1966) found strong presumptive evidence to confirm the earlier work of Goll et al. (1964a), and stated that increased toughness of bovine meat may be explained by an increase in cross-links. Carmichael et al. (1967) found an increased formation of dimer or higher aggregates of the α-chains of collagen with an increase in animal age. It was indicated that there was a marked increase in the proportion of components in bovine collagen having intramolecular cross-links between the 5th and 9th month of gestation. In more mature animals, there was some indication of an enhanced degree of intermolecular cross-links. Therefore, the molecular structure of the collagen present may be of some importance. The purpose of this investigation was to study components of the molecular structure of collagen to discover if there were variations in such components among beef muscles of varying tenderness.

EXPERIMENTAL

Chemicals and solutions

The amino acid standards used in gas-liquid chromatographic analyses were purchased from Mann Research Laboratories. Trifluoroacetic anhydride was an Eastman organic chemical purchased from Distillation Products Industries. Anhydrous hydrogen chloride gas was obtained from Matheson Scientific, Inc. and hydrogen peroxide (30%) from Matheson, Coleman and Bell. The ρ-dimethylaminobenzaldehyde was purchased from Brinkmann Instruments Inc., and Tris (hydroxymethyl) aminomethane from Fisher Scientific Company. All other chemicals were "Baker Analyzed" reagents and were used without further purification, except the methanol, which was dried according to the procedure of Vogel (1951).

The 2.5 N hydrogen chloride in dry methanol was prepared by bubbling anhydrous hydrogen chloride gas into the methanol with vigorous stirring. The end point of addition was determined by titration of 1-ml aliquots from the methanolic solution against standard sodium hydroxide to a phenolphthalein end point. The 2 N hydrogen chloride in butanol was prepared in a similar manner.

Instruments

Gas-liquid chromatographic analyses were carried out in an Aerograph 200 Dual Column Gas Chromatograph with flame ionization detectors. Ultracentrifugations were performed in an International Model B-60 ultracentrifuge using the SB-283 rotor. A Hitachi Model 139 Spectrophotometer was used for all colorimetric analyses, and the Beckman DU Spectrophotometer was used for the Δ 215–225 method of protein determination.

Selection and preparation of tissue

Steaks obtained from the ninth rib of the longissimus dorsi muscle 2 days after slaughter of the animals were trimmed of the epimysial layer and cooked to an internal temperature of 71°C. Warner-Bratzler shear-force values were obtained on 1-in. cores. 7 animals of varying Warner-Bratzler shear-force values were divided into 3 groups: Group I, the most tender animals, consisting of 1 steer and 2 bulls; Group II, animals of intermediate tenderness, 1 bull and 1 steer; Group III, the toughest animals, 1 cow and 1 steer. The epimysial layer of uncooked steaks from these animals served as sources of collagen for molecular studies.

After the fat and adhering meat were dissected from the epimysial layer, the samples were sonicated for 10 sec and loosened particles gently scraped away. Each sample was then sliced in the cold into strips approximately 1 in. by 1/4 in.

Total collagen determination

The extraction of total collagen from the epimysial samples was carried out according to the procedure of Neuman et al. (1950), except that 10-mg samples in 12 ml demineralized water were autoclaved for 12 hr, instead of placing the samples into 4 ml of water and autoclaving for 2, 3-hr periods. The extracted collagen was hydrolyzed in 6 N HCl for 7 hr at 104°C. After neutralization with 6 N sodium hydroxide, the samples were filtered into 50-ml volumetric flasks and made up to volume with demineralized water. A solution of a standard hydroxyproline sample (0.3 mg/ml) was treated similarly.

Hydroxyproline in each of the samples was determined according to the modified procedure of Dahl et al. (1963), using hydrogen peroxide for the oxidation of hydroxyproline to pyrrole. After color development the o.d. was measured at 550 mμ. Values obtained for hydroxyproline content were used in determining the total amount of collagen in each sample according to the calculations of Neuman et al. (1950).

Extraction of acid-soluble collagen (ASC)

The epimysial strips were extracted 3 times with 0.45 M sodium chloride for 24 hr at 2°C (Gross, 1958) and the ASC extracted from the remaining residue and purified according to the procedure of Rubin et al. (1965). After precipitation and lyophilization, the epimysial ASC samples were stored at −20°C.

The epimysial ASC was chosen for the following analyses, since it contains appreciable amounts of cross-links but is still soluble.

Density-gradient ultracentrifugation of epimysial ASC

Sedimentation analyses of ASC solutions were performed by centrifugation in 12-ml linear gradients of 4–17% (w/v) sucrose buffered with 0.15 M Tris buffer, pH 8.0. The gradients were allowed to stand after preparation for 24 hr at 2°C before being used for ultracentrifugation. Solutions of epimysial ASC were prepared by dissolving 1 mg of collagen in 0.2 ml of 0.15 M Tris buffer, pH 8.0. After denaturation at 45°C and centrifugation at 2,600 rpm, the solutions were carefully applied to the top of the sucrose gradients and centrifuged at 9°C for 18 hr at 30,000 rpm (105,000 × g) in the ultracentrifuge. Each of the ultracentrifuged epimysial ASC samples was fractionated into 5 drop fractions collected in test tubes. After addition of 0.5 ml demineralized water to each tube, the optical densities were read at 215 and 225 mμ. A plot of tube number vs. the difference in o.d. at 215 and 225 mμ was made (Chaykin, 1966). The difference gives a measure of the peptide bonds present in the solution. Thus, the larger the difference in absorbance between the 2 wavelengths, the greater the protein concentration. The accuracy of the method was checked by the Lowry method of protein determination (Lowry et al., 1951). The $s_{w,20}$ (sedimentation) values of the peaks obtained by the Δ 215–225 mμ method were determined by the tables of McEwen (1967).

Determination of viscosities

Solutions for viscometry measurements were clarified by centrifugation at 30,000 rpm (105,000 × g) for 90 min at 20°C in the ultracentrifuge. Viscosities of epimysial ASC (0.015–0.530 mg protein /ml 0.2 N acetic acid) were measured at 20.0 ± 0.1°C in an Ostwald viscometer with a flow time for water of 112 sec. The intrinsic viscosity of each of the samples was determined from a plot of reduced viscosity vs. concentration (Mathews et al., 1954).

Preparation of samples for gas-liquid chromatographic analyses

1-mg samples of epimysial ASC were hydrolyzed in sealed tubes under nitrogen in 5 ml 6 N HCl for 24 hr at 110°C. After hydrolysis the hydrolysates were concentrated to about 2 ml on a rotatory evaporator, about 3 ml of demineralized water added, and again concentrated. After a second addition of demineralized water, the samples were taken to dryness.

Methyl and butyl esters of the hydrolysates were prepared according to the procedure of Gehrke et al. (1965), except that the methyl esters were made by heating in capped tubes at 105°C for 2 hr and the butyl esters by heating in capped tubes at 150°C for 4 hr. Acylation of the samples with trifluoroacetic anhydride at 150°C to form the *n*-butyl N-trifluoroacetyl derivatives was carried out according to the sealed-tube acylation method of Stalling et al. (1966). Dichloromethane was added to each sample so as to have a concentration of 2 mg/ml. A standard mixture of amino acids similar in amount to those present in collagen was subjected to the same conditions of hydrolysis and derivative formation to decrease preparation error. After preparation of the *n*-butyl N-trifluoroacetyl esters, the derivatives were subjected immediately to gas-liquid chromatographic analyses.

Gas-liquid chromatography of amino acid derivatives

Amino acid analyses of the *n*-butyl N-trifluoroacetyl esters were carried out by gas-liquid chromatography employing the following operating conditions: Column temperature, initial 100°C, final 260°C. Program rate, 4°/min after 8 min initial delay. Detector temperature, 230°C. Injector temperature, 140°C. Carrier flow, N_2, 30 ml/min. Air flow to detectors, 175 ml/min. Hydrogen flow to detectors, 15–20 ml/min. Columns: 10 ft by 1/8 in. i.d. Pyrex columns packed with 3% OV-17 on 100/120 mesh Gas-Chrom Q.

Unit detector response (peak height × retention time) for each amino acid was determined from the standard amino acid preparation. Reproducibility of this method of determination of the amount of each amino acid was found to be 95%. The standard response of each amino acid was then used to determine the quantity of each amino acid in the epimysial ASC samples.

Ester content of epimysial ASC samples

Hydroxamates were prepared according to the procedure of Blumenfeld et al. (1962) and

Table 1—Summary of percent total collagen of epimysial tissue from the longissimus dorsi muscle of beef animals of different age, sex and shear-force values.

Animal[1]	Age (months)	Sex	Warner-Bratzler shear force values (lb)[2]	Total collagen (%)[3]
A	13	Steer	11.89	72
B	13	Bull	12.69	48
C	13	Bull	12.97	57
E	13	Bull	16.47	45
F	13	Steer	18.36	55
G	13	Steer	22.75	56
H	48	Cow	29.86	56

[1] Animals A, B, C were considered tender; E, F intermediate; G, H tough.
[2] Increasing values indicate a decrease in tenderness.
[3] Represents average of 4 trials.

Table 2—Percentage of the components obtained upon sucrose density-gradient ultracentrifugations of ASC samples from steaks of varying tenderness.[1]

Animal[2]	3S (α)	9S (β)	15S (γ)	19S
A (♂)	69	*	18	13
B (♂)	85	*	7	8
C (♂)	67	*	15	18
E (♂)	53	25	8	14
F (♂)	50	22	17	11
G (♂)	59	25	9	7
H (♀)	43	24	16	17

* Shoulder at 9S.
[1] Values are percent of total peak area.
[2] A, B, C tender samples; E, F intermediate; G, H tough.

assayed by the colorimetric method of Lipmann et al. (1945), except that o.d. was determined at 505 mμ.

Aldehyde content of epimysial ASC samples

Phenylhydrazones were prepared according to the procedure of Levene (1962). The absorption of each phenylhydrazone solution was measured at 390 mμ.

All of the preceding analyses were duplicated; where appropriate, values obtained were subjected to analysis of variance according to the methods of Steel et al. (1960).

RESULTS

PERCENTAGES of total collagen in the epimysial tissues were found to be about equal in value, except for 1 animal that had an extremely high content of collagen, and 2 that had low values for total collagen (Table 1). No positive relationship was found between percent total collagen in the epimysial tissue samples and Warner-Bratzler shear-force values. Cover et al. (1956); Parrish et al. (1962); Ritchey et al. (1963) and Kim et al. (1967) in working with biceps femoris and longissimus dorsi muscles found no positive relationship between amount of total collagen and tenderness.

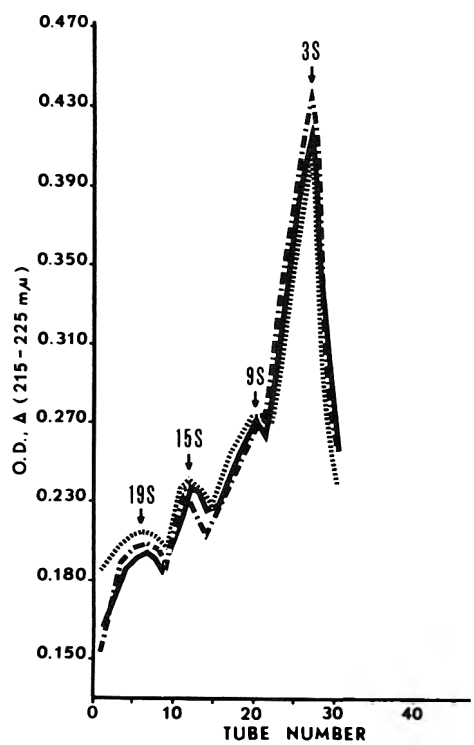

Fig. 1—Representative sucrose density-gradient ulracentrifugation patterns obtained from denatured epimysial ASC from meat of varying tenderness. Broken line, tender sample (C); solid line, intermediate sample (F); dotted line, tough sample (H).

It has long been felt that connective tissue plays a very distinctive role in tenderness of beef, and since collagen is a structural element of the tissue this protein has been studied with relation to tenderness. Only recently has it been suggested that the toughness-tenderness factor may be influenced by the number and strength of cross-links in the collagen molecule. The degree of cross-linking in epimysial collagen was investigated in this research by the physical measurements of sedimentation and viscosity and by the chemical approximation of cross-links.

Figure 1 is an illustration of the sucrose density-gradient ultracentrifugation patterns obtained with denatured epimysial ASC from meat with varying degrees of tenderness. Upon calculation of sedimentation coefficients, values of 3, 9, 15 and 19S were obtained. All of the tender samples studied exhibited a shoulder at 9S, whereas, intermediate and tough samples studied showed a definite peak at 9S. Even though sucrose density-gradient ultracentrifugation of denatured collagens has not been reported in the literature, ultracentrifugations at about 60,000 rpm of ASC solutions in sodium formate buffer have been performed (Piez et al., 1961; Piez et al., 1963; Lewis et al., 1964a; 1964b; Cooper et al., 1965). On the basis of the data from these papers the peaks obtained in the sucrose density-gradient ultracentrifugations have been assigned to the various chain-components of the collagen molecule. The peak at 3S has been attributed to the α-component, that at 9S to the β-component and that at 15S to the γ-component. The peak appearing at 19S is apparently attributable to a more highly cross-linked collagen molecule.

The percentage of each peak from the ultracentrifuged epimysial ASC samples is shown in Table 2. From this table it can be seen that the α-component makes up the majority of the total in all cases. However, it is very evident that the ASC of epimysial tissue from tender meat contains more α-component and less β-component than does that from intermediate or tough meat. Actually, the β-component in that from tender meat was so small that the peak area could not be calculated, but this component comprised approximately 25% of the total in that from intermediate and tough meat. The percentage of the γ-component and 19S component was very variable and there were no apparent differences among samples. These results suggest that ASC of epimysial tissue from tough meat contains a higher proportion of cross-links than does that from tender meat.

Viscosities of epimysial ASC solutions were found to be correlated to the tenderness or toughness of meat in a manner similar to the sedimentation coefficients (Table 3). As meat tenderness decreased, the intrinsic viscosities of the epimysial ASC increased. These differences in intrinsic viscosity may be reflecting differences among the relative sizes of the collagen molecules from the various samples.

The partial amino acid compositions of the various epimysial ASC samples are shown in Table 4. Due to the small percentage of several amino acids present in collagen (histidine, hydroxylysine, isoleucine, methionine and tyrosine), the amount of each could not be adequately determined. In addition, threonine and leucine could not be resolved under the conditions employed. Aspartic acid, an acidic amino acid, has been implicated in

Table 3—Intrinsic viscosities of epimysial ASC samples as a function of decreasing tenderness of meat.

Animal[1]	ASC $[\eta]$, dl/g
A (♂)	8.5
B (♂)	12.0
E (♂)	21.0
F (♂)	21.5
G (♂)	24.5
H (♀)	23.0

[1] A, B tender samples; E, F intermediate; G, H tough.

Table 4—Partial amino acid composition of the various epimysial ASC samples.[1]

Amino acid	Standard mixture	Samples[2] A (♂)	B (♂)	C (♂)	E (♂)	F (♂)	G (♂)	H (♀)
Ser	0.8	1.4	2.1	2.1	2.1	0.9	2.0	1.5
Asp	0.9	1.6	1.5	1.9	2.3	2.0	1.6	3.1
Hpr	2.2	3.7	2.9	4.6	3.7	4.1	4.3	8.5
Glu	1.5	1.8	1.9	1.7	1.9	2.0	2.4	2.3
Lys	0.5	0.4	0.5	0.6	1.3	1.1	1.7	1.1
Arg	0.8	1.1	1.9	2.0	3.1	2.5	2.0	2.0
Gly	3.2	3.5	3.3	3.4	3.4	3.4	3.4	3.6
Pro	3.1	4.8	3.7	6.1	5.1	5.0	5.4	9.1
Ala	3.3	4.8	4.2	4.6	5.8	4.0	5.1	4.7
Val	0.7	1.2	1.1	0.9	2.0	0.9	1.1	1.0
Phe	0.3	0.5	0.4	0.5	1.1	0.7	0.4	1.0

[1] Values are 10^{-9} moles/mg.
[2] A, B, C tender samples; E, F intermediate; G, H tough.

cross-links via an esterlike link (Blumenfeld et al., 1962), with the hydroxyamino acids, hydroxyproline, serine and threonine (Gallop et al., 1959). As can be seen in Table 4, the amounts of serine, aspartic acid, hydroxyproline and glutamic acid were not significantly different among the various ASC samples ($P < 0.05$). Results of chemical estimation of esterlike cross-links in the epimysial ASC samples via ester content are shown in Table 5. There were no apparent differences in the ester content among the various samples.

Lysine is thought to be involved in cross-links via an aldehyde derivative. This involvement appears to be through aldol condensation of 2 aldehydes derived biosynthetically from 2 lysine residues in collagen (Rojkind et al., 1968). Results obtained by gas chromatographic analyses indicate a significantly higher level ($P < 0.05$) of this amino acid in epimysial ASC from tough meat samples (Table 4), and this suggests a potential for more of the aldehydic type of cross-link. Furthermore, results of the chemical estimation of aldehydes present in epimysial ASC samples tended to confirm this suggestion (Table 5). A significant difference was found in the amount of aldehyde present in the tender, intermediate and tough samples ($P < 0.05$). This indicates that epimysial ASC of tough meat contained more aldehyde than did that from tender meat, which implies that as the meat becomes tougher, the epimysial ASC has a higher level of aldehyde taking part in the formation of intramolecular cross-links.

DISCUSSION

AS EXPECTED, the amount of total collagen in epimysial tissue has no correlation with meat tenderness. However, results obtained by molecular studies have indicated that the type of epimysial ASC present is of more importance than the quantity. Evidence was obtained indicating that an increase in the number of cross-links in epimysial ASC accompanies a decrease in meat tenderness. Hill (1966) has suggested that an increase in toughness may be explained by an increase in cross-links, and the current investigation appears to lend credence to this point of view. The sedimentation studies clearly indicated that the α-component is higher and the β-component lower in epimysial ASC from tender meat. This can be explained by a higher proportion of macromolecules with lower amounts of intramolecular cross-links. Results of the viscosity studies point to the same conclusion, since intrinsic viscosities were lower in epimysial ASC of tender meat, indicating smaller molecules. Both of these measurements together suggest a difference in the amount of intramolecular cross-links rather than any differences in intermolecular cross-links. However, since the degree of intermolecular cross-links was not assayed, it cannot be stated whether there is an association of tenderness with such cross-links.

The partial amino acid analyses of the epimysial ASC samples do not indicate a definite trend in regard to levels of the various amino acids and the tenderness of meat. The levels of 3 of the amino acids reported as being involved in cross-links via esterlike links, serine, aspartic acid and hydroxyproline, were not significantly different among the various ASC samples. Even though glutamic acid has not been reported as playing a role in cross-links via an esterlike link similar to that of aspartic acid, there is a possibility that it could be involved. However, the levels of glutamic acid in the ASC samples were not significantly different. Results of chemical estimation of ester in the epimysial ASC support the amino acid data, since the proportions of esters in the ASC were similar.

The level of lysine was significantly higher in epimysial ASC of tough meat samples compared to tender meat samples. Since this amino acid is suggested as being a precursor to aldehydes which can link peptide chains together via aldol condensation, it is possible that epimysial ASC of tough meat has more of the aldehyde type of cross-link. This possibility was strengthened by the fact that such epimysial ASC contained significantly more apparent aldehyde.

Table 5—Esterlike links and aldehyde content of epimysial ASC from meat of varying tenderness.

Animal[1]	Esterlike link[2]	Aldehyde content[3]
A (♂)	7.8	1.9
B (♂)	7.5	2.0
C (♂)	7.5	1.9
E (♂)	7.6	1.6
F (♂)	7.7	2.9
G (♂)	7.4	2.4
H (♀)	7.5	3.7

[1] Animals A, B, C are considered tender; E, F intermediate; G, H tough.
[2] Values represent μmoles hydroxamate/100 mg collagen.
[3] Values represent equivalents aldehyde/mole collagen.

It must be re-emphasized that the collagen studied was epimysial collagen, which is not that collagen interspersed in the muscular areas. Certainly, this collagen should have more direct influence upon tenderness than the epimysial collagen. However, all collagens in a tissue might be related, in that if a high degree of cross-linking occurred in one then it might also occur in the others. If this is true, then the amounts of cross-linking in the epimysial and intramuscular collagen may be related.

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A RESEARCH NOTE

LOCALIZATION OF MYOGLOBIN IN PIG MUSCLE

PALE, soft, exudative (PSE) muscle is a problem to the food industry (Briskey, 1964). Lawrie (1950) has shown that the concentration of myoglobin in muscle varies with age, species, anatomical location and activity. It might be assumed that the pale component of PSE muscle is closely associated with decreased concentrations of myoglobin. However, the situation is not clear and some disagreement exists in the literature. Briskey et al. (1959) reported that the decreased intensity of visual color (comparing normal and PSE muscle post-mortem) was not accompanied by significantly lower myoglobin concentrations. Henry et al. (1958), and Scopes et al. (1963) attribute the paleness largely to diminished myoglobin whereas Wismer-Pedersen (1959) and Goldspink et al. (1964) attribute it to denaturation of myoglobin.

We used a histochemical technique for myoglobin localization (James, 1968; Morita et al., 1969) and compared the cellular localization of myoglobin in muscle that ultimately became PSE to that in muscle which retained a normal color and gross morphology. The longissimus muscle from 14 market weight Poland China pigs and 15 market weight Chester White pigs was used. The histochemical procedure is given in detail elsewhere (Morita et al., 1969). The longissimus muscle was scored subjectively for color (Forrest et al., 1963) 24 hr post-mortem. Score 1 is considered extremely PSE and 5 is considered very dark, firm and dry. Scores of 2 and below are PSE.

Figure 1 illustrates typical histochemical results. Three staining intensities are distinguishable, and are indicated on the figure as positive, intermediate and negative. We made photographic enlargements of the sections and evaluated percent of total fiber area contributed by each of the three staining intensities with a Zeiss particle size analyzer. The quantitative results for myoglobin positive fibers (does not include intermediate) are shown in Figure 2. The results are expressed as a correlation for group 1 and as a correlation for groups 1 and 2 combined. Groups 1 (11 Poland China and 12 Chester White) and group 2 (3 Poland China and 3 Chester White) represent two separate experiments conducted approximately 6 weeks apart.

Results from group 1 gave a significant correlation ($r = 0.52$, $P < .05$) but when the second group was added the correlation ($r = 0.23$, $P < .05$) was greatly diminished. Previous work (Cooper et al., 1969) has implicated the metabolism of intermediate fibers in the development of the PSE condition, but we do not know the significance of the "intermediate" reaction for myoglobin. There was slightly more area for intermediate fibers in PSE muscle (score 2 and below) than in normal muscle. If the fibers intermediate in myoglobin reaction are intermediate with histochemical techniques for phosphorylase and ATPase, then they may behave as white fibers in terms of metabolism post-mortem (Cooper et al., 1969).

When only group 1 represented in Figure 2 is considered, then the muscle with low color score had a small area of myoglobin positive fibers. Our histochemical results show a distinct difference between fibers in respect to myoglobin concentration. Pig muscle has areas of myoglobin positive fibers surrounded by large areas of fibers negative for myoglobin. This is contrasted to beef muscle (Morita et al., 1970) which contains a larger fiber area of myoglobin positive fibers; the red fibers are distributed in a more random fashion about the bundle even though the red fiber content of beef muscle is not

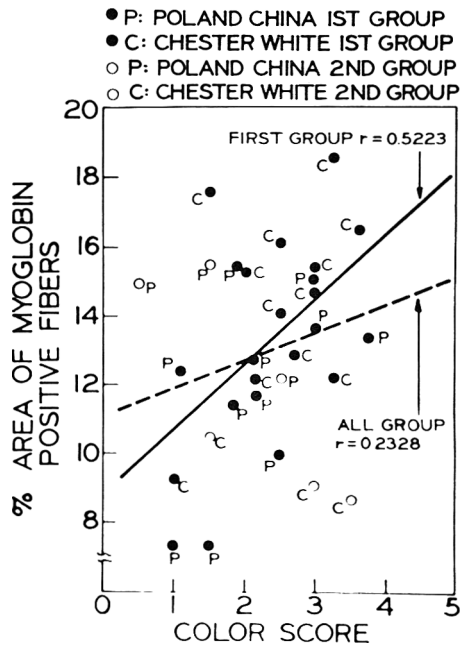

Fig. 2—Scatter plot and correlation coefficients for color score versus myoglobin positive fiber area.

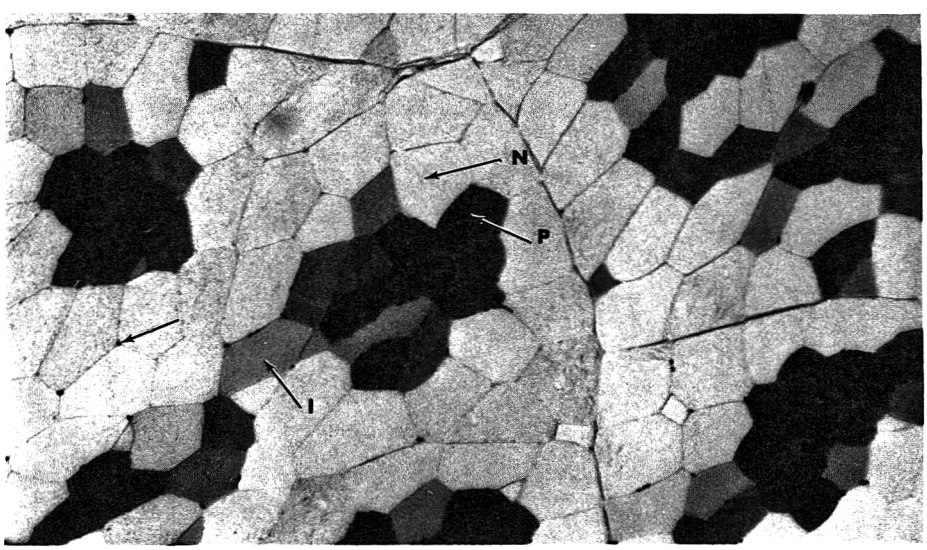

Fig. 1—Section of porcine longissimus muscle reacted for myoglobin. Three levels of reaction product intensity are indicated as positive (P), negative (N) and intermediate (I). The unlabelled arrow points out a positive reaction for hemoglobin given by a trapped erythrocyte. 30μ thick section. 84×.

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much greater than pig muscle. The rapid post-mortem glycolysis associated with the development of PSE muscle is not common in beef muscle. Therefore, the cellular concentration of myoglobin and the distribution of myoglobin positive fibers within the muscle bundle may be an important property of pig muscle which allows the great range in rate of post-mortem glycolysis.

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THERMAL BEHAVIOR OF PORCINE COLLAGEN AS RELATED TO POST-MORTEM TIME

SUMMARY—DTA (differential thermal analysis) thermograms of epimysial connective tissue from normal and low quality porcine muscle were compared at 0 and 24 hr post-mortem. In addition, the melting characteristics of intramuscular collagen were determined at 0 hr post-mortem. In all tissues studied, collagen from low quality muscle consistently gave slightly lower peak melting points than that from normal muscle. Epimysial collagen had a significantly ($P < .05$) lower peak melting point at 24 hr than at 0 hr post-mortem (64.24 vs. 65.77°C). Using epimysial collagen, a significantly ($P < .01$) greater proportion of the total melting range occurred at lower temperatures at 24 hr post-mortem as compared to 0 hr (41.1 vs. 30.9%). The thermal behavior of intramuscular collagen at 0 hr post-mortem was similar to that of epimysial collagen at 0 hr, but peak melting temperatures were slightly higher for intramuscular connective tissue.

INTRODUCTION

THE SHRINKAGE temperature (T_s) of collagen is defined by Rigby (1964) as the temperature at which an unconstrained sample in 0.9% NaCl solution undergoes an abrupt dimensional change. Another thermal transition occurs at the equilibrium melting temperature, T_m, the temperature of equilibrium between crystalline and amorphous phases in a partially melted sample. Shrinkage is due to the melting of the crystalline phase when both the intramolecular forces within the triple-stranded molecule and the crystallization energy are overcome (Harrington et al., 1961). For mammalian collagen in 0.9% saline, T_s is 59–60°C and T_m is about 51°C (Rigby, 1964).

In severe cases of pale, soft exudative (PSE) pork, muscles lose their connective tissue attachments. Briskey (1963) hypothesized that this condition was due to a high temperature (36–40°C) and a low pH (5.0–5.2) within the first few hours after death. He stated that thermal shrinkage or hydrolysis, or both may occur in connective tissue. Therefore, studying shrinkage characteristics of connective tissue may aid in better understanding the PSE syndrome, as well as in helping define the role of connective tissue in tenderness. The present study utilized differential thermal analysis (DTA) to eludicate changes in thermal shrinkage of collagen at 0 and 24 hr post-mortem. Differences in thermal shrinkage characteristics of collagen from normal and lower quality muscle at the two different post-mortem times were compared. In addition, the melting characteristics of intramuscular collagen at 0 hr post-mortem were compared to those of epimysial collagen at 0 hr post-mortem.

EXPERIMENTAL

INTRAMUSCULAR connective tissue and the epimysium from the longissimus muscle of 16 Yorkshire pigs approximately 5 months old furnished the collagen used in this study. Within 5 min after exsanguination, and before dehairing or evisceration: (1) the longissimus samples from the lumbar region of the carcass were removed; (2) the epimysial tissues were scraped free of fat and muscle; and (3) both muscle and cleaned epimysium were frozen in liquid nitrogen. These tissues are hereafter referred to as 0-hr post-mortem tissues. Adjacent longissimus samples anterior to the lumbar region were removed and frozen in liquid nitrogen 45 min post-exsanguination. Muscle and cleaned epimysial tissues were removed from the 7th to 8th rib region of the longissimus at approximately 24 hr post-mortem, and frozen in liquid nitrogen.

The frozen muscle samples were removed from liquid nitrogen and stored up to 72 hr at −20°C, then powdered as described by Berchert et al. (1965). The fine intramuscular connective tissue, which collected on the screen as the 0-hr muscle samples were being shaken through a 40-mesh screen at −20°C, was removed and saved for subsequent DTA determinations. Intramuscular connective tissue could not be separated by this method for muscle samples removed from the longissimus of the carcasses at 24 hr post-mortem. However, McClain (1969) has only recently reported the isolation of intramuscular connective tissues, using techniques similar to those reported herein.

Although the low quality carcasses were selected as being pale, soft and exudative (PSE) on the basis of appearance, quality scores and transmission values, the carcasses did not show high temperatures and low pH within a few minutes after death (Table 1) as is characteristic of PSE muscle (Briskey, 1964). Thus, these carcasses were called low quality instead of PSE. From among the 16 pigs on which the above procedures were followed, 10 were selected for this study. Quality parameters used in selecting 5 lower quality and 5 normal carcasses included transmission values at 24 hr post-mortem (Hart, 1962) and subjective scores for firmness and color at 24 hr post-mortem (Forrest et al., 1963).

DTA is a technique for studying the thermal behavior of materials as they undergo physical and chemical changes during heating or cooling. The DTA method has been reported to have excellent sensitivity and reproducibility when used to study hydrothermal shrinkage of collagen (McClain et al., 1968). DTA thermograms from the DuPont 900 Differential Thermal

Table 1—Comparison of means and standard deviations for characteristics of longissimus muscles from 5 normal and 5 low quality pigs.

Muscle variable	Normal		Low quality	
	Mean	SD	Mean	SD
Number	5		5	
Subjective score[1]				
Color	4.30[2]	0.45	2.60[3]	0.42
Firmness	4.10[2]	0.82	2.60[3]	0.82
Transmission value, 24 hr	16.30[2]	14.28	69.60[3]	14.89
Postmortem pH				
0 hr	6.16	0.06	6.16	0.06
45 min	6.05	0.15	5.96	0.19
24 hr	5.20	0.05	5.19	0.07
Postmortem temperature, °C				
0 hr	39.39	0.52	40.00	0.15
45 min	39.45	0.65	40.15	0.30

[1]Subjective scores: 1 = pale, soft and exudative; 5 = dark, firm and dry.
[2,3]Means on horizontal lines bearing different superscripts differ significantly ($P < .05$).

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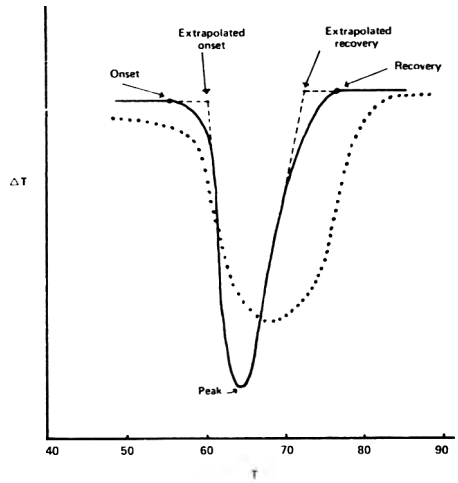

Fig. 1.—Effect of sample preparation on shape of thermogram for epimysial collagen (18-month-old steer). T scale setting (T) = 10° /2.54 cm; temperature differential between sample and reference (△ T) = 0.2° /2.54 cm; and heating rate of collagen = 10° /min. The sharp peak (solid line) is for epimysial collagen mixed in 0.9% NaCl and held for 1 hr. The broad peak (dotted line) is for collagen placed in 0.9% NaCl and run immediately.

Analyzer were obtained on epimysial connective tissue from the normal and low quality porcine muscle at 0 and 24 hr post-mortem. In addition, the melting characteristics of intramuscular collagen were determined at 0 hr post-mortem. Approximately 30-mg samples of epimysial or intramuscular connective tissue were placed in glass tubes 25 mm in length and 4 mm in diameter. They were mixed with 50λ of 0.9% NaCl in distilled water and allowed to stand at room temperature 1 hr, with intermittent mixing with a straightened paper clip. Thermocouples were placed into the center of the 4-mm tubes containing the collagenous fibers. Shrinkage temperatures of the samples were recorded with the DTA apparatus. The T Scale was set at 10°/2.54 cm, the △ T Scale at 0.2° /2.54 cm and the heating rate at 10° /min in an air atmosphere. Duplicate determinations were made on all samples.

RESULTS & DISCUSSION

THERMOGRAMS of epimysial tissue are shown in Figure 1. The thermograms trate the type of curves obtained with porcine connective tissue and define the location of the transition temperatures reported in this paper as follows:

Onset: The temperature at which the thermogram starts to depart from the base line.

Extrapolated onset: The temperature corresponding to the intersection of extrapolation of the base line and the longest straight-line section of the low-temperature side of the peak.

Peak: The temperature of reversal.

Extrapolated recovery: Same as extrapolated onset, except it is on the high-temperature side of the peak.

Recovery: The temperature at which the thermogram returns to either the same or a different base line.

Table 2—Means from thermograms of epimysial and intramuscular collagen from normal and low quality muscle.[1]

	Epimysial				Intramuscular	
	0 hr post-mortem		24 hr post-mortem		0 hr post-mortem	
	Low quality	Normal	Low quality	Normal	Low quality	Normal
Number	5	5	5	5	5	5
Onset, °C	59.48	59.28	55.08	54.18	59.58	60.92
Extrapolated onset, °C	61.20	61.56	58.58	59.06	62.58	63.38
Peak, °C	65.66	65.88	63.96	64.52	66.28	67.28
Extrapolated recovery, °C	75.82	75.42	72.08	71.96	71.68	72.78
Recovery, °C	80.28	80.92	75.92	76.22	75.98	76.08

[1] See Figure 1 for a definition of the thermogram points.

Differences in the shape of the thermograms in Figure 1 are due to differences in sample preparation. The sharper thermogram represented by the solid line is connective tissue which was removed from the freezer, mixed in 0.9% NaCl and allowed to stand 1 hr as previously described (Experimental). The thermogram represented by the broader melting peak is from connective tissue removed from the freezer, placed in 0.9% NaCl and analyzed immediately without wetting or mixing. Onset and extrapolated onset were similar in both cases. However, the sharper peak was chosen since it gave melting points for collagen that were closer to the values reported in the literature. In addition, the sharper peak was more reproducible. Mechanical working of leather in water prior to determining DTA peak temperatures was also the method employed by Witnauer et al., (1964). Each curve in Figure 1 represents an average of 7 thermograms. Average peak temperatures and standard deviations were 64.08 and 0.63, respectively, for epimysial samples held 1 hr in 0.9% NaCl, compared to 68.26 and 1.02 for those run immediately. The difference in peak temperatures is probably due to wetting of the collagen fibers by mixing them in 0.9% NaCl. The fibers became more dispersed (less tightly bound or less concentrated) when compared to those which were not mixed and air was removed from the sample in the DTA tubes. This supports the work of Lennox (1949), who stated that the shrinkage temperature of collagen increases with increasing concentration and decreasing moisture content. It also seems probable that the samples allowed to stand for 1 hr in 0.9% NaCl lost some of their ground substance into solution (Lowther et al., 1967; McClain, 1968), which would also make the fibers less concentrated or more disperse. Saline solutions have commonly been used by researchers to prevent changes such as swelling in collagenous fibers (Gustavson, 1956). The saline solution per se would not be expected to cause changes in the thermal characteristics of the fibers during a 1-hr period.

Means from thermograms of longissimus epimysial and intramuscular collagen for the normal and low quality pigs are given in Table 2. Collagen from low quality muscles consistently gave slightly lower peak melting points than that from normal muscle. This was also true for thermal transitions occurring at the extrapolated onset and the recovery. Onset temperatures for the epimysial collagen at 0 hr post-mortem did not follow this pattern. However, onset temperatures are open to some subjective interpretation and they show more variation in multiple runs on the same tissue than the other temperature parameters. Differences in means between the normal and low quality tissues shown in Table 2 are not significant ($P < .05$). Nevertheless, the differences in peak melting points between low quality and normal muscle collagen of 0.56°C at the 24-hr post-mortem period are similar to the highly significant ($P < .01$) difference of 0.63°C reported by McClain (1968). The difference in statistical significance between this study and the previous one may be due to smaller quality differences in this study. Collagen from the low quality muscle at 0 hr post-mortem also melted at lower temperatures than that from the normal tissues.

Although the small differences in shrinkage temperatures between collagen from low quality and normal muscle are not statistically significant, it is possible that they are real. However, little has been reported on the relationship of connective tissue characteristics to quality of muscle substantiating this. If connective tissue differences at 0 hr post-mortem do exist, these differences may be extremely meaningful in further elucidating the PSE condition.

Means showing different points on thermograms of the epimysium at 0 hr

Table 3–Means from thermograms of the epimysium at different times post-mortem.

	Epimysial collagen	
	0 hr Post-mortem	24 hr Post-mortem
Number[1]	10	10
Onset, °C	59.38[2]	54.63[3]
Extrapolated onset, °C	61.38[2]	58.82[3]
Peak, °C	65.77[2]	64.24[3]
Extrapolated recovery, °C	75.62[2]	72.02[3]
Recovery, °C	80.60[2]	76.07[3]
Actual melting range, °C	21.22[2]	21.44[2]
Extrapolated melting range, °C	14.24[2]	13.20[2]
Onset to peak/actual range, %	30.42[2]	44.61[3]
Extrapolated onset to peak/extrapolated range, %	30.87[2]	41.07[3]

[1]Tissue from the same 10 pigs was used at 0 and 24 hr post-mortem.
[2,3]Means on horizontal lines bearing different superscripts differ significantly ($P < .01$).

and 24 hr post-mortem are shown in Table 3. Epimysial collagen had significantly ($P < .01$) lower melting points at 24 hr than at 0 hr post-mortem. However, actual and extrapolated melting ranges for epimysium at 0 and 24 hr post-mortem were not significantly different. At 24 hr a greater ($P < .01$) proportion of the total melting range occurred at lower temperatures than at 0 hr. Calculated proportions of the melting ranges were based upon the actual range (onset to recovery) and the extrapolated range (extrapolated onset to extrapolated recovery) for comparative purposes. As mentioned earlier, onset temperatures involve subjective interpretation and show more variation in multiple runs on the same tissue than extrapolated onset temperatures. However, similar conclusions can be drawn regardless of the temperature range utilized in calculating different portions of the curve.

The data in Table 3 clearly show that changes take place in hydrothermal stability of epimysial tissue between 0 hr and 24 hr post-mortem. Since any pH change occurring in muscle during this period is still within the range of maximum hydrothermal stability for collagen (Gustavson, 1956), other factors must be responsible for the lower melting temperatures. Accumulation of lactic acid may be involved, since it averages 0.9% of the total weight for muscle after rigor mortis (Lawrie, 1966). According to Verzar (1964), weak acids, such as 0.1% acetic acid, dissolve collagen. Hence, the concentration of lactic acid in the muscle rather than the hydrogen ion concentration could be responsible for the decrease in hydrothermal stability. Gustavson (1956) reported that the shrinkage temperature of an acetic acid–pretreated pelt in the neutralized state was 12°C lower than that of the original. However, Banga (1957) stated that lactic acid had no influence on breakability and elasticity of collagen fibers. Lennox (1949) reported that a 1 M solution of lactic acid actually elevated the T_s of skin.

Another possible reason for the lowered melting point of collagen at 24 hr post-mortem (when compared to 0 hr) deals with physical stress which may occur in connective tissue during rigor. Rigby (1964) has shown that the T_m and T_s of tendons decrease if they are mechanically strained. Mechanical working of connective tissue during rigor may lower thermal shrinkage temperatures. In a microscopic characterization of perimysial tissues from stretched and control muscle samples, Buck et al. (1968) found a greater degree of tissue granulation in the stretched samples after cooking. This would tend to substantiate the fact that connective tissues subjected to stress have a lower thermal shrinkage temperature, which may be reflected in a greater degree of granulation during cooking and finally in more tender meat (Buck et al., 1968).

Onset and peak temperatures from thermograms of epimysial and intramuscular connective tissue at 0 hr post-mortem were not significantly different ($P < .05$), but peak melting temperatures were slightly higher for intramuscular connective tissue. Means and standard deviations were 65.77 and 1.09°C, respectively, for epimysial tissue, compared to 66.78 and 1.65°C for intramuscular connective tissue. The differences in peak melting points were investigated further by solubility studies reported in another study (Field et al., 1970). Determination of hydroxyproline (Woessner, 1961) in samples of epimysial tissue used for DTA determinations indicated that 66.9% of the tissue on a dry weight basis was collagen. Intramuscular samples contained only 14.1% collagen on a dry weight basis. The difference between the collagen content of epimysial and intramuscular tissues was thought to be due to elastic tissue from veins and arteries and bits of muscle that could not be separated from the intramuscular tissue without altering its physical structure. According to the DuPont 900 DTA instruction manual, differences in impurities may affect the shape of the thermogram, i.e., the onset and recovery temperatures, but should not affect the peak melting point. Therefore, it was not logical to conclude that degree of purity accounted for the differences in peak melting points.

When the percent of heat-soluble collagen in the epimysium was compared to percent of heat-soluble collagen in intramuscular connective tissue, much larger amounts of heat-labile collagen were found in the epimysium (Field et al., 1970). Percent heat-labile collagen is probably not directly correlated with DTA melting point. Nevertheless, large differences between intramuscular and epimysial tissue in amount of heat-labile collagen indicate that the small differences in peak melting points may be real.

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ENZYMIC OXIDATION OF SIMPLE DIPHENOLS AND FLAVONOIDS BY ORANGE JUICE EXTRACTS

SUMMARY—Extracts of orange juice vesicles oxidized p-hydroquinone (HQ) and o-dihydroxyphenylalanine (DOPA) with O_2. Most of the oxidase activity was associated with a particulate fraction that sedimented at 100,000 × g. Sonic disruption of the particulates followed by chromatography on DEAE-cellulose increased specific activities with both substrates. The partially purified enzyme oxidized numerous naturally occurring o-diphenols and o-methoxyphenols. Acidic media below pH 4 and heating above 70°C destroyed most of the oxidase activity. KCN, diethyldithiocarbamate and 8-hydroxyquinoline but not EDTA inhibited the oxidase. The partially purified enzyme reduced benzoquinone and oxidized the reduced form of nicotinamide-dinucleotide (NADH). Ubiquinone, a benzoquinone derivative, but not menadione or vitamin K_1, naphthoquinone derivatives, could replace benzoquinone in the oxidation of NADH. Ubiquinone, benzoquinone and similar p-quinones may function in the orange as the oxidation-reduction couple between NADH-quinone reductase and diphenol oxidase.

INTRODUCTION

DIPHENOL oxidases (EC No. 1.10.3.1 and EC No. 1.10.3.2), ascorbic acid oxidase (EC No. 1.10.3.3) and cytochrome oxidase (EC No. 1.9.3.1) have been considered as terminal oxidases in plant respiration, but most evidence supports cytochrome oxidase as the O_2-activating agent (Beevers, 1961). In citrus fruit, however, the evidence is very weak. The cytochrome system was implicated in orange fruit respiration by Hussein (1944). He showed that CO inhibited 20% of O_2-uptake by orange flavedo slices and that light completely reversed the inhibition. (CO inhibition of diphenol oxidase and ascorbic acid oxidase is not reversed by light.) However, Hussein neither identified the cytochromes nor demonstrated their function in respiration.

Biale (1961) questioned whether cytochrome oxidase solely could account for respiration in citrus. Cytochrome oxidase is saturated at relatively low partial pressures of O_2 and therefore its activity is practically independent of O_2-tension. Biale found that respiration in orange slices was a function of O_2-tension in the range of 0–8% O_2. He showed that CO_2 production by oranges decreased rapidly at subatmospheric levels of O_2. Below 2.5% O_2 the respiratory quotient (R.Q.) rose rapidly, indicating that anaerobic reactions dominated. These results suggested that phenol oxidase and ascorbic acid oxidase might be involved in respiration because they require more O_2 for saturation than does cytochrome oxidase. However, ascorbic acid oxidase was not found in mature oranges (Baker et al., 1968) and diphenol oxidase has not been reported in citrus.

To determine whether diphenol oxidase participates in citrus respiration, orange juice cells were examined for oxidation of *o*- and *p*-diphenols. This paper describes the partial purification of diphenol oxidase and its characterization as a potential terminal oxidase in the reoxidation of NADH.

[a]Paper presented at the 29th Annual Meeting of the Institute of Food Technologists in Chicago.

MATERIALS & METHODS

CHEMICALS were obtained as follows: DL-3, 4-dihydroxyphenylalanine (DOPA), nicotinamide-adenine dinucleotide, reduced (NADH), nicotinamide-adenine dinucleotide, phosphate, reduced (NADPH), 8-hydroxyquinoline and rutin from Sigma Chemical Company; tris-(hydroxymethyl)aminomethane (Tris), ethylenediaminetetraacetic acid (EDTA), L-ascorbic acid, potassium cyanide, p-benzoquinone, resorcinol, p-phenylenediamine, 2-mercaptobenzothiazole, hydroquinone, sodium diethyldithiocarbamate, ammonium sulfate, pyrogallol and sulfosalicylic acid from Fisher Scientific Company; catechol and L-tyrosine from Nutritional Biochemicals Company; o-catechin, chlorogenic acid and ferulic acid from K & K Laboratories; ubiquinone, DEAE cellulose, homovanillic acid, vitamin K_1, menadione, quercetin, 3-methoxy, 4-hydroxyphenylethylamine, hesperidin, hesperetin, quercetrin, naringin, naringenin and eriodictyol from Calbiochem Company. Feruloylputrescine was provided by Dr. Adair Wheaton, Florida Citrus Experiment Station, Lake Alfred.

Preparation and fractionation of juice cell extract

Peeled, mature oranges var. Valencia were quick-frozen in liquid nitrogen and the frozen vesicles separated from seeds and membranous tissues. The vesicles were mixed in sufficient 1 M Tris in frozen-bead form to neutralize the fruit acid, then powdered in a precooled blender. This powder was thawed and strained through four layers of cheese cloth. The neutralized orange juice was then centrifuged at 1,000 × g for 10 min in a Lourdes Betafuge at 5°C. The supernatant separated from the residue was designated Juice Cell Extract (JCE).

The JCE was fractionated into 2 particulate fractions, R_{10} and R_{100}, and 1 soluble fraction by centrifugation. R_{10} was obtained by centrifuging the extract at 10,000 × g for 10 min.

R_{100} was obtained from the supernatant of R_{10} by centrifuging it at 100,000 × g for 60 min. The supernatant from R_{100} was designated as the soluble fraction. Both R_{10} and R_{100} were resuspended in 0.05 M Tris, pH 7.0, to give a protein concentration of 1 mg/ml.

Purification of diphenol oxidase

The JCE was saturated to 90% with ammonium sulfate, adjusted to pH 7.0, equilibrated for 1 hr at 0°C and centrifuged at 5,000 × g for 10 min at 0°C. The clear supernatant was decanted and the residue resuspended in a minimum of 0.05 M Tris, pH 7.0, and sonicated for 30 sec at 0°C in a Sonifier Cell Disruptor. The sonicate was dialyzed for 16 hr against 0.005 M Tris, pH 8.0, then centrifuged at 5,000 × g for 10 min. The clear supernatant was diluted with the buffer to 1 mg protein per milliliter and 40 ml of it was applied to a 5°C-precooled DEAE cellulose column (25 by 500 mm) equilibrated with 0.005 M Tris, pH 8. The column was eluted with a parabolic NaCl gradient as described by Moore et al. (1960). 3, 10-ml fractions were collected per hour. The column, eluting solution and eluates were maintained at 5°C.

Assay of oxidase

Oxidase activities were assayed by the following methods:

1. Oxygen uptake (Gregory et al., 1966). 2. Formation of chromogen product, DOPAchrome (Constantinides et al., 1967). 3. Oxidation of ascorbate (El-Boyoumi et al., 1957).

1. **O_2-uptake** was measured at 30°C with the YSI Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Company, Yellow Springs, Ohio). The reaction vessel contained the substrate in 0.1 M potassium phosphate buffer, pH 7.0, saturated with air and the enzyme preparation in a total volume of 4 ml. Rate of O_2-uptake was recorded on a Sargent recorder, Model-SR, calibrated in microliters of O_2. Specific activities of preparations were expressed as μl O_2/min/mg protein.

2. **DOPAchrome formation** was measured at 30°C by recording the rate of increase in absorbance at 475 mμ in a Gilford Spectrophotometer, Model 222. The sample and reference cuvettes contained 0.1 mmole of DOPA in 2.5 ml 0.1 M potassium phosphate buffer, pH 7.0. The reaction was initiated by adding the enzyme preparation and 0.1 M buffer to the sample cuvette and 0.1 M buffer to the reference cuvette for a total volume in each of 3 ml. Specific activities of preparations were calculated from the change in absorbance and expressed as Δ A/min/mg protein of sample.

3. **Oxidation of ascorbate** was measured at 30°C by recording the rate of decrease in absorbance at 265 mμ in the Gilford spectrophotometer. Ascorbate is oxidized nonenzymically by the oxidized substrate and is used here as the redox indicator of the rate of oxidation of the substrate. Reference and sample cuvette contained 0.1 μmole of ascorbate, 0.1 μmole of EDTA to prevent O_2-metallic oxidation of ascorbate and 0.6 μmole of substrate in 2.5 ml of 0.1 M potassium phosphate buffer. The reaction was initiated by adding the enzyme preparation and 0.1 M phosphate buffer to the sample cuvette and 0.1 M phosphate buffer to the reference cuvette for a total volume in each of 3 ml. Specific activities of the preparations were calculated from change in absorbance and expressed as Δ A/min/mg protein of sample. This value was converted to μliters O_2 by using the molar absorption coefficient, 1.5×10^4, and 11.2 μliters O_2 per μmole ascorbate.

Protein determination

Protein of samples was determined by the sulfosalicylic acid method as described by Layne (1957).

Quinone reductase

Reductase activity was measured by recording the rate of NADH oxidation at 340 mμ and also by recording the rate of reduction of 1,4-benzoquinone at 290 mμ. Reference and sample cuvette contained 0.45 μmole of benzoquinone and 0.5 μmole of NADH in 2.5 ml of 0.1 M potassium phosphate buffer, pH 6.5. Reaction was initiated by adding the enzyme preparation and 0.1 M buffer to the sample cuvette and 0.1 M buffer to the reference cuvette for a total volume in each of 3 ml. Activity of the preparation was calculated from the change in absorbance using 6.22×10^3 as molar extinction coefficient. 1 unit of activity was defined as oxidation of 1 μmole NADH/min/mg protein.

RESULTS

Distribution of hydroquinone oxidase in juice cell extract (JCE)

JCE oxidized hydroquinone (HQ) and dihydroxyphenylalanine (DOPA), respectively, at rates of 0.2 and 0.3 μliter O_2/min/mg protein. The cell extract contained suspended particles salted out by saturating the extract to 30% maximum with ammonium sulfate. All the oxidase activity followed the suspended particles into the residue. The data in Table 1 show that most of the HQ oxidase activity is concentrated in the fine particle fraction that can be sedimented between 10,000 and 100,000 × g. Most of the ascorbate-reduced cytochrome c oxidase and succinoxidase activities were associated with the fraction that sedimented at 10,000 × g.

Purification of HQ and DOPA oxidases

Specific activities of both HQ and DOPA oxidases increased about 20-fold after sonication and chromatography on DEAE cellulose. Figure 1 shows that HQ and DOPA oxidases and quinone reductase (QR) eluted into the same fractions. Attempts were made to purify HQ and DOPA oxidases with acetone fractionation, temperature and pH selectivity. Both oxidase activities were lost on exposure to acetone, and temperature- and pH-stability did not differentiate the active enzyme from other protein.

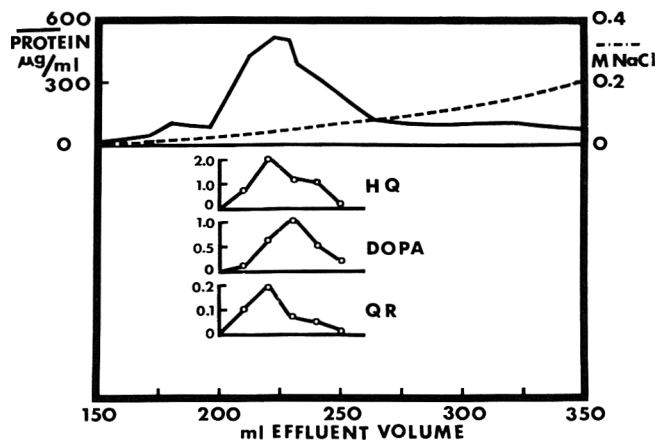

Fig. 1—Localization of enzyme activities. Activities of HQ, hydroquinone, and DOPA, dihydroxylphenylalaine, are in μliters O_2/min/ml, and QR, quinone reductase, in μmoles NADH/min/ml.

Table 1—Distribution of hydroquinone and cytochrome c oxidases in juice cell extracts.

	μliters O_2 /min/mg protein[2]		
Fraction[1]	Hydroquinone	Ascorbate plus cytochrome c	Succinate plus cytochrome c
R_{10}	0.2	7.1	3.4
R_{100}	2.3	1.3	0
Soluble	0.4	0	0

[1] R_{10} designates residue after centrifuging extract at 10,000 × g for 10 min. R_{100} designates residue after centrifuging supernatant from R_{10} at 100,000 × g for 60 min.

[2] All substrates were 10^{-2} M.

Table 2—pH and temperature stability of DOPA orange oxidase.[1]

pH stability relative to pH 7		Temperature stability relative to 0°C	
pH	DOPA oxidase (% activity)	Temp	DOPA oxidase (% activity)
9	94	0	100
8	100	30	94
7	100	50	55
6	94	70	6
5	87	75	0
4	25		
3	12		

[1]Partially purified enzyme, 1 mg protein/ml, was exposed to the test pH or temperature for 5 min. Then pH was adjusted to 7, temperature to 30°C and activity determined by DOPAchrome assay.

Table 3—Inhibition of orange DOPA oxidase.[1]

	M conc	% Inhibition
EDTA	1.3×10^{-3}	0
Resorcinol	3.3×10^{-4}	40
8-Hydroxyquinoline	1.3×10^{-3}	60
Potassium cyanide	3.3×10^{-4}	100
Sodium diethyldithiocarbamate	1.7×10^{-3}	100

[1]Substances were added directly to DOPA-enzyme reaction mixture in the DOPAchrome assay.

pH and temperature stability

Table 2 shows that pH stability drops off rapidly below pH 5. Only 25% of the activity remained at pH 4. A substantial drop in activity occurred as the temperature increased from 50 to 70°C. Only 6% of the activity remained after heating to 70°C for 5 min.

Inhibitors

EDTA had no effect on DOPA oxidase and was used to suppress autoxidation of ascorbic acid in the ascorbic acid assay for DOPA oxidase. Table 3 lists a number of inhibitors of DOPA oxidation. Resorcinol, the meta-hydroquinone, and 8-hydroxyquinoline did not inhibit completely at 5×10^{-2} M.

Substrate specificity

The partially purified oxidase showed specificity for o- and p-diphenolics and o-methoxyphenolics. Table 4 lists a number of simple diphenolic and flavonoid compounds that supported O_2 uptake with the enzyme preparation. All of the flavonoids and many of the simple diphenolics have been reported in citrus (Horowitz, 1961). Feruloylputrescine was recently isolated from the orange (Wheaton et al., 1965). Rates for the o- and p-diphenolics were about equal and were higher than for the o-methoxyphenolics. Rates for the o-methoxyphenolic compounds decreased in order as the substituted group changed from acid to amine to α, β-unsaturated acid. Quercetrin and hesperidin supported the same rate of O_2-uptake as did the aglycones, quercetin and hesperetin. Tyrosine, resorcinol and naringenin (4′,5,7-trihydroxyflavanone) were inactive; they do not have the requisite o- or p-dihydroxy group. Vitamin K_5 (2-methyl-4-amino-1-naphthol) and p-phenylenediamine also were inactive.

Quinone reductase

The purified enzyme preparation oxidized NADH with p-benzoquinone; p-hydroquinone was formed in the reaction. The rate of oxidation of NADPH was about 25% of the rate with NADH. Table 5 shows that QR activity is specific for benzoquinone. The 2-methyl derivative of 1,4-naphthoquinone (menadione) and the phytyl derivative, vitamin K_1, were inactive. NADH was also oxidized by the enzyme preparation when benzoquinone was replaced by HQ, or by the o-diphenols, DOPA and catechol. Homovanillic acid or other methoxyphenolic compounds could not replace the diphenolics. Rate of oxidation of NADH with benzoquinone was higher than with the diphenol forms.

Table 4—Substrate specificity of orange diphenol oxidase.

Substrate		μliters O_2 /min/mg protein
	o-Dihydroxyl	
DOPA	3-(3,4-Dihydroxyphenyl)alanine	7.0
Pyrogallol[1]	1,2,3-Trihydroxybenzene	4.6
Catechol[2]	1,2-Dihydroxybenzene	4.0
	p-Dihydroxyl	
p-Hydroquinone	1,4-Dihydroxybenzene	4.5
	o-Hydroxyl-methoxy	
Homovanillic Acid	4-Hydroxy-3-Methoxyphenylacetic Acid	1.4
3-Methoxytyramine	4-Hydroxy-3-Methoxyphenylethylamine	0.34
Ferulic Acid[3]	4-Hydroxy-3-Methoxycinnamic Acid	0.14
Feruloylputrescine[3]	N-(4-Aminobutyl)-4-hydroxy-3-methoxycinnamide	0.21
Hesperetin	3′, 5, 7-Trihydroxy-4-methoxyflavanone	0.56
Hesperidin	Hesperitin-7 rutinoside	0.56
	o-Dihydroxyl	
Catechin	3,5,7, 3′, 4′-Pentahydroxyflavan	2.7
Chlorogenic Acid	3-(3,4-Dihydroxycinnamoyl)quinic acid	0.81
Eriodictyol	3′, 4′, 5, 7-Tetrahydroxyflavanone	1.1
Rutin	Quercetin-3-rutinoside	0.56
Quercetin	5, 7, 3′, 4′ -Tetrahydroxyflavonol	1.3
Quercetrin	Quercetin-3-rhamnoside	1.3
	m-Dihydroxyl	
Resorcinol	1,3-Dihydroxybenzene	0
	Mono-hydroxyl	
L-Tyrosine	3-(4-Hydroxyphenyl)alanine	0
Naringenin	4′,5,7-Trihydroxyflavanone	0
Naringin	Naringenin-7-neohesperidoside	0
	p-Diamino	
p-Phenylenediamine	1,4-Diaminobenzene	0
	p-Amino-hydroxyl	
Vitamin K_5	4-Amino-2-methyl-1-naphthol	0

[1]Assayed at pH 5.6 instead of pH 7.
[2]Catechol 1×10^{-8} M with ascorbate 1×10^{-4} M and EDTA 1×10^{-4} M.
[3]Ferulic acid or feruloylputrescene 3×10^{-5}, ascorbate 1×10^{-4} M and EDTA 1×10^{-4} M.

Table 5—Substrate specificity of orange quinone reductase.

	μmoles NADH /min/mg protein
Benzoquinone	1.6
Ubiquinone	0.6
Menadione	0.0
Vitamin K_1	0.0

DISCUSSION

THE DIPHENOL oxidase in oranges as in apples (Harel et al., 1966) is concentrated in the particulate fraction. Diphenol oxidase is also particulate-bound in tea (Gregory et al., 1966), lettuce seedlings (Mayer, 1961), tobacco (McClendon, 1953) and sugar beet (Mayer et al., 1960). Using cytochrome c oxidase as the enzyme marker for mitochondria, the data on distribution can be considered evidence for the concentration of orange diphenol oxidase in a particle smaller than the mitochondria. This particulate fraction has not been examined for other enzyme functions, but it corresponds in sedimentation rate to microsomal fraction of plant and animal cells.

In contrast to the 5,000-fold purification of diphenol oxidase from tea (Gregory et al., 1966), orange diphenol oxidase was purified only about 20-fold. The specific activity of the tea enzyme is 100 times higher than the orange enzyme. Even with limited purity, the response to inhibitors, pH and temperature stability and substrate specificity suggests that orange diphenol oxidase is similar to the enzymes purified from tea (Gregory et al., 1966), tobacco (Clayton, 1959) and sweet potato (Eiger et al., 1949).

The orange enzyme is different from these oxidases, in that methoxy but not amine can substitute for one of the hydroxyl groups on the substrate. This capacity of the orange enzyme to oxidize o-methoxyphenolic compounds may be metabolically significant. However, the unknown oxidation product of the o-methoxyphenolic compounds did not support the oxidation of NADH in the QR assay. Therefore, the methoxy derivatives do not function as intermediates in the reoxidation of NADH.

Demonstration that o- and p-diphenolic compounds support the oxidation of NADH with O_2 uptake by the purified preparation is evidence that orange juice cells contain components of a NADH-linked terminal oxidase. The lower rate of oxidation with the diphenolics compared to benzoquinone indicates that the diphenol oxidase is rate-limiting in the over-all reaction. Contribution of this oxidase system to the total respiratory O_2 uptake must be determined to assess its significance to citrus metabolism.

The poor stability of diphenol oxidase below pH 4 indicates why diphenol oxidase has not hitherto been detected in orange juice. This inactivation in acidic solution might explain why enzymic browning is not a problem in citrus processing and why ascorbic acid is stable in the presence of the many phenolic compounds in citrus juices.

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References in this paper to specific commercial products do not constitute endorsement.

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A METHOD FOR ESTIMATING THE FUNCTIONAL CAPILLARY SYSTEM IN SKELETAL MUSCLE

SUMMARY—The functional capillary system was investigated by injecting trypan blue dye into 8 market-weight porcine animals followed by extracting the dye from various muscles. The trapezius contained more dye than the biceps femoris, with the gluteus medius and longissimus dorsi being equal in dye content but containing less dye than the trapezius and biceps femoris muscles. This rank compared well with the fiber type distribution, with the trapezius being high in red fiber content. The method presents possibilities for study of capillary function but some problems were encountered with the injection procedure.

INTRODUCTION

THE IMPORTANCE of the capillary network in skeletal muscles is in the control of metabolic functions within the living animal. The capillary function may also play a significant role in setting the stage for post-mortem conversion of muscle to meat.

A method for estimating the capillary function of skeletal muscle, expressed as permeability and extent of vascularization, has been developed by modifying the procedures of Judah et al. (1962) and Hawk (1968). Cecil et al. (1966) and Cooper et al. (1968) have used a similar trypan blue dye method to quantitate the capillary function of uterus tissue of rats and ewes as it is affected by endocrine and other physiological factors. With this in mind, we developed a procedure for using trypan blue dye to quantitate capillary function in porcine skeletal muscle. Trypan blue is an azo dye compound; it has relatively low toxicity when injected into the circulatory system of living animals. The particulate dye is ingested completely by the fixed macrophages contained in the connective tissue that is a structural part of the capillary wall (Bloom et al., 1968).

MATERIALS & METHODS

A SOLUTION of 8% trypan blue and 0.5% NaCl approximately of body temperature (39°C) was injected into the jugular vein of 8 heavy market-weight (100–120 kg) Chester White and Poland China pigs anesthetized with 50/50 dial with urethane and nembutal. The dye solution was injected at the level of 3 ml per kg of body weight. The trypan blue dye was allowed to circulate for 30 min, whereupon the pigs were exsanguinated by severing the anterior vena cava. (This process allows the blood to flow from the tissues.) The biceps femoris, gluteus medius, longissimus and trapezius muscles were excised and trimmed of exterior fat and connective tissue. Large samples (50-g) were obtained from standardized locations (medial and both distal ends) of the entire longissimus muscle so as to be representative of the complete muscle. The trapezius was used in its entirety. These samples were frozen in liquid nitrogen and pulverized in a Waring Blendor to obtain a fine muscle powder, then stored for analysis. Samples of frozen muscle powder weighing 200–400 mg were weighed into 15-ml centrifuge tubes. The following procedure was used to extract the bound trypan blue from the samples: The muscle samples were mixed with 5 ml of cold 5% TCA and allowed to stand in the cold for a minimum of 5 min. After centrifuging at 1,500 × g (0°C) for 10 min the supernatants were discarded and the interiors of the tubes wiped with absorbent paper wrapped around a glass rod. This procedure was found to be helpful in preventing turbidity in the final extract. To remove lipid material, the precipitate was extracted with 5 ml of absolute ethanol at room temperature for 5 min, and, after centrifugation at 1,500 × g for 5 min, re-extracted twice with absolute ethanol at 70°C for 5 min. Following centrifugation (5 min at 1,500 × g) the inside walls of the centrifuge tubes were again wiped with absorbent paper. The trypan blue was either immediately extracted from the precipitate or the dye protein complex was stored in a freezer for subsequent analysis. Extraction of trypan blue from the protein complex was accomplished by adding 4 ml of aqueous 25% pyridine to each tube and allowing them to stand covered for 4 hr at room temperature accompanied by frequent mixing. A more rapid method of extraction involved the use of a 70°C water bath for 2 hr, also with frequent mixing of the covered tubes. The samples were centrifuged for 10 min at 1,500 × g and then brought to a volume of 5 ml. The concentration of trypan blue in each supernatant was then determined colorimetrically by reading at 610 mμ in a B & L Spectronic 20. The values obtained were compared with standards of 0, 5, 10, 20, 30 and 40 μg of trypan blue in 5 ml of aqueous 25% pyridine.

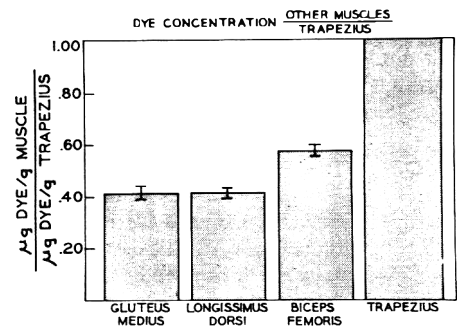


Fig. 1—Relationship of trypan blue concentration in the 4 muscles examined, expressed as ratio of dye per gram muscle to dye per gram trapezius.

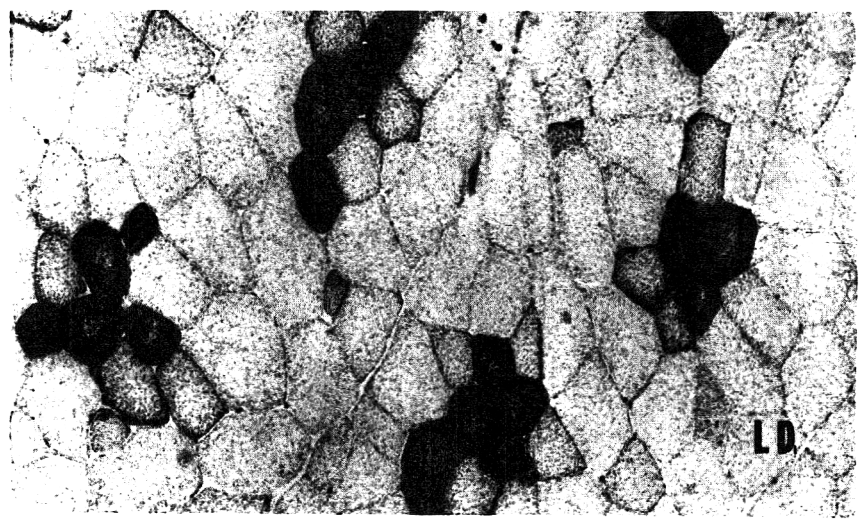
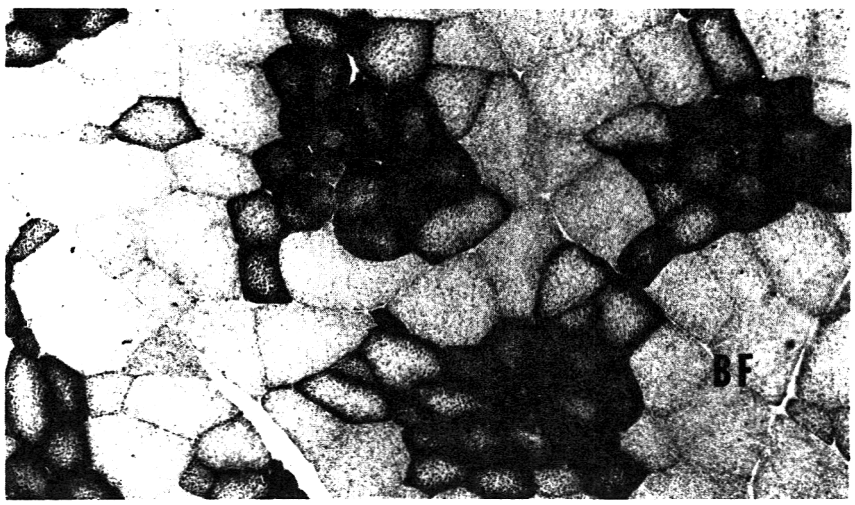


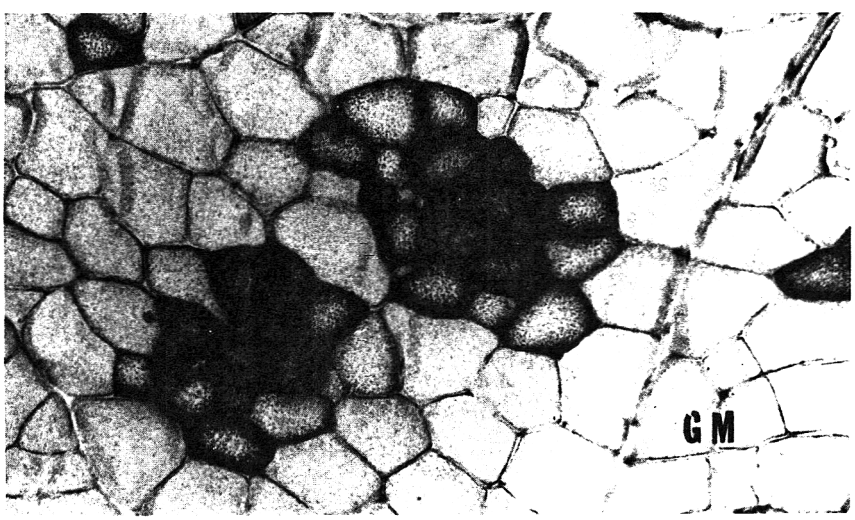
Fig. 2.—Fresh frozen sections (magnification 50 ×) representing the 4 muscles examined in the dye experiment. Each section was reacted for DPNH-TR so Type I fibers stained positive and appear dark, whereas Type II fibers stain only slightly and appear light.

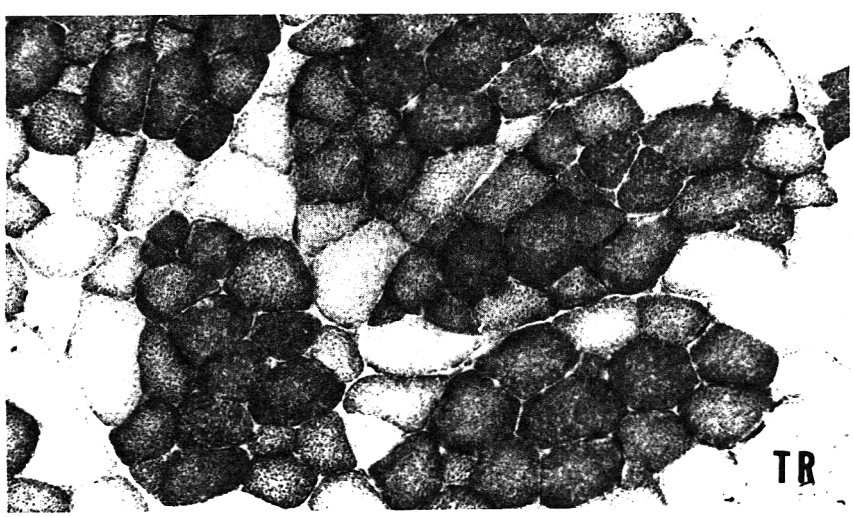
RESULTS & DISCUSSION

FIGURE 1 shows the trypan blue concentration in the 4 muscles examined. Mean values for the 8 pigs shown in this figure are expressed as the ratio of μg dye/g muscle to μg dye/g trapezius. Through the use of this trypan blue method the longissimus and gluteus medius were found to have similar relative dye values, indicating similar vascular capabilities. The standard deviation of the mean was slightly greater for the gluteus medius than for the longissimus, indicating slightly more variation between animals in the capillary function of this muscle. As seen in Figure 1, the biceps femoris had a greater vascularization than the previous 2 muscles, but the relative value was only slightly higher than half that of the trapezius. According to data presented in this figure the trapezius was a highly vascularized muscle, as it had the highest dye concentration of all muscles examined. The standard deviation of the mean was quite small for all these relative values, indicating that this relationship between vascularization and dye binding was basically the same for all animals used, regardless of breed.

Fig. 2–Concluded







It has been shown by Romanul (1965) that the extent of vascularization in skeletal muscle is related to muscle fiber types. Type I or red fibers are aerobic and have been shown histologically to have a greater capillary to muscle fiber ratio than do Type II or white fibers which are anaerobic. It appears that at a muscle with a greater number of red fibers should have taken up a greater concentration of trypan blue. To see if this relationship could be shown by our procedure, we made histochemical sections of the same 4 muscles in a control pig of similar age and weight. Figure 2 shows the sections stained with diphosphopyridine nucleotide tetrazolium reductase (DPNH-TR). The fibers classified as Type I (or red) stain positive are the dark fibers. Type II (or white) fibers stain only lightly; thus appear light in these sections. The trapezius consists mainly of red fibers and thus its circulation should be high. This supports the high trypan blue content found in the trapezius muscle as seen in Figure 1. The biceps femoris contained fewer red fibers than the trapezius and this was also supported by the trypan blue data (Fig. 1). The longissimus and gluteus medius are seen in Figure 2 to be white muscles, due to their low content or red fibers. This indicates that their circulation and trypan blue content should also be low. This was definitely indicated from our trypan blue data in Figure 1. It can generally be concluded that the quantitative relationship of fiber types within a muscle is associated with the vascular capacity of that muscle; the trypan blue dye method can be used to determine these vascular capacities.

An important aspect should be considered before the dye injection method is applied to projects to estimate capillary function. When it is desired to compare the concentration of a specific muscle from animal to animal, it is critical that the dye volume be constant per body weight. The controlled injection of large standard quantities of dye is a major difficulty in the application of this technique.

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EFFECT OF TEMPERATURE ON VISCOSITY OF FRUIT JUICES AND PUREES

SUMMARY—The viscosities of selected fruit juices and purees were measured with a coaxial-cylinder viscometer in the temperature range 20–70°C. Depectinized apple juice and Concord grape juice were Newtonian fluids at all concentrations and their viscosity decreased considerably at higher temperatures. Cloudy apple and orange juices changed from Newtonian to pseudoplastic at concentrations higher than 50 and 20° Brix, respectively. Temperature had a smaller effect on viscosity of cloudy juices than of clear juices. The apparent viscosity of fruit purees (pseudoplastic fluids) decreased slightly at higher temperatures. The activation energy for flow increased with the juice concentration and decreased with the presence of suspended particles in the fruit product. The data and conclusions are useful in the design and operation of efficient food-processing equipment.

INTRODUCTION

VISCOSITY is usually considered an important physical property related to the quality of liquid food products. Viscometric data are also essential for the design and evaluation of food-processing equipment such as pumps, piping, heat exchangers, evaporators, sterilizers, filters and mixers.

Literature values of viscosity for many liquid foods have been reported at a single temperature and frequently no mention is made of whether the material is a Newtonian or a non-Newtonian fluid. Such data are of limited usefulness in food processing, where temperatures and shear rates may be quite different from the conditions of a single viscosity measurement.

Viscosities of water and sucrose solutions at various temperatures have been published (Perry, 1963). Harper (1960), and Saravacos et al. (1967) reported viscometric constants of fruit purees at 2 temperatures. The effect of temperature on the apparent viscosity of pear puree was investigated by Harper et al. (1962) and the viscometry of tomato concentrates reported by Harper et al. (1965).

Fruit juices and purees vary widely in their viscometric behavior and it is necessary that each product be studied separately. The purpose of this work was to obtain the needed viscometric data on various fruit products and to relate the effect of temperature on the composition of each.

EXPERIMENTAL

Fruit products

Purees of apple, pear and peach were prepared as described by Saravacos et al. (1967). The purees were stored in glass jars at 32°F for 12 to 20 months before measurements were made. No visible changes of consistency were noticed due to storage. Concord grape juice (64° Brix) was prepared commercially from New York State grapes and stored at 0°F for 10 months before being used. Most of the tartrates had been removed during concentration of the grape juice. Commercial orange juice concentrate (44° Brix) from Florida was also kept frozen before it was used. Juices of lower concentrations were prepared from the concentrates by dilution with distilled water.

Two types of juice were prepared in the pilot plant from New York State apples. Mixed varieties were ground in a hammermill and the juice extracted in a continuous screwpress. Cloudy juice was prepared by filtration through a rotary vacuum filter, operated with a Celite precoat. Clear, depectinized juice was prepared by treating the extracted juice with a commercial pectic enzyme preparation and filtering through the precoat rotary filter.

Both the clear and the cloudy apple juices were concentrated under vacuum at 100°F in a pilot falling-film evaporator. Samples of juices at intermediate concentrations were obtained for viscometric measurements. The highest concentrations obtained were 75° Brix for the depectinized apple juice and 65.5° Brix for the cloudy juice. Viscometric measurements of the apple juices were made immediately after preparation.

Viscometric measurements

All measurements reported here were made with a coaxial-cylinder viscometer, the Contraves Rheomat 15, manufactured by Contraves AG, Zurich, Switzerland. It is the same instrument as the Drage viscometer described by van Wazer et al. (1963).

Each liquid sample was placed in the appropriate measuring system and brought to the desired temperature (20–70°C) using a constant-temperature water bath. Speeds of the rotating inner cylinder varied 5.6–352.0 rpm. The instrument can be operated at 15 different speeds, which are changed stepwise with a selector switch. Readings of the instruments were converted to either viscosity (centipoise) or shear stress, using tables supplied by the manufacturer. The same tables contain the Newtonian shear rates, which are a function of the rotational speed and the dimensions of each measuring system.

Fruit purees and some juices are considered pseudoplastic fluids, obeying the power-law equation $\tau = K\gamma^n$, where τ is the shear stress, γ is the shear rate, K is the fluid consistency coefficient, and n is the flow behavior index.

The index n was obtained as the slope of the straight line resulting when log τ was plotted against log N, where N is the rotational speed (rpm). The shear rates (γ) of pseudoplastic fluids were determined by dividing the tabulated Newtonian shear rate by the compensation factor C, calculated from the following equation (Contraves, 1966):

$$C = \frac{n\left[1 - \left(\frac{Ri}{Ra}\right)^{2/n}\right]}{1 - \left(\frac{Ri}{Ra}\right)^{2}}$$

where Ri is the radius of the rotating cylinder and Ra is the radius of the measuring cup.

The fluid consistency coefficient K was obtained graphically from a plot of log τ vs. log γ.

The shear stress–shear rate diagram of each product was constructed by taking readings in the following order: Starting with the lowest speed, the ascending line was obtained first by increasing stepwise the speed of rotation. When the maximum speed was reached, the speed was decreased gradually and the descending line obtained. In most products (Newtonian and pseudoplastic) both lines coincided. In thixotropic fluids, the descending line fell below the corresponding ascending line.

RESULTS & DISCUSSION

THE VISCOMETRIC constants of the various fruit products obtained with the coaxial-cylinder instrument were in general agreement with those obtained with the tube viscometer at a single temperature (27°C) by Saravacos (1968).

Apple juices

Depectinized (clear) apple juice behaved as a simple Newtonian fluid at all concentrations within the temperature range 20–70°C. For this type of liquid, a single point measurement is sufficient to obtain the viscosity at the desired temperature. The viscosity of apple juices increased rapidly with concentration, particularly above 50° Brix (Fig. 1).

Cloudy apple juice, which contained significant amounts of suspended and colloid particles, was more viscous than the depectinized juice. This type of juice was Newtonian below 50° Brix but at higher concentrations it behaved as a pseudoplastic fluid. Its flow behavior index (n) ranged from 0.85 at 50° Brix to 0.65 at 65.5° Brix. The apparent viscosities of cloudy apple juice, shown in Figures 1 and 3, were calculated for a shear rate of 100 sec^{-1}. This shear rate was chosen as a moderate value for comparison of viscosities among various fruit products. It should be noted that the apparent viscosity of a pseudoplastic fluid increases at lower shear rates (rotational speeds).

Viscosities of depectinized apple juices decreased considerably as the temperature was increased from 20 to 70°C (Fig. 2). An exponential relationship between temperature and viscosity is indicated by the straight lines obtained when the data are plotted on semilogarithmic paper. The effect of temperature was more pronounced at higher juice concentrations. These results are very similar to the viscosities of sucrose solutions at various temperatures (Perry, 1963).

Temperature had a less pronounced effect on the apparent vicosities of cloudy apple juices (Fig. 3). The same trend was noticed with concentration, i.e., the more concentrated the juice, the steeper the decrease of viscosity upon heating.

At temperatures lower than 50°C, the concentrated cloudy apple juice (65.5° Brix) exhibited definite thixotropic properties. Thixotropy was indicated by a progressive decrease in the instrument reading at a given rotational speed and by formation of a thixotropic loop when viscometric data of ascending and descending speeds were plotted. For purposes of analysis, thixotropic products were considered time-dependent pseudoplastic fluids and their viscometric constants estimated by taking readings at ascending speeds, 30 sec after the instrument was brought to a particular speed.

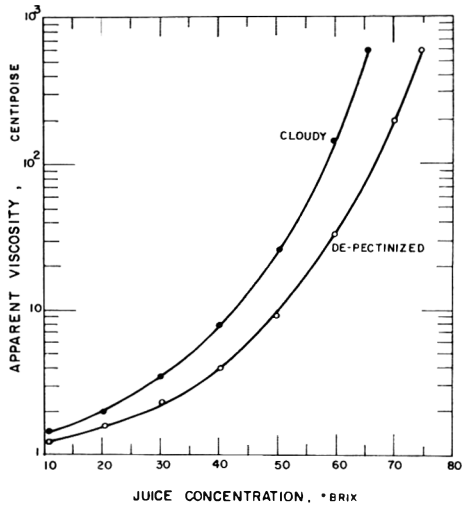

Fig. 1–Viscosity of apple juices (30°C).

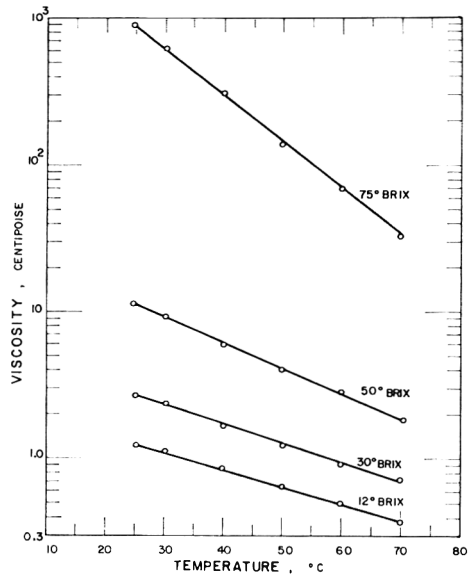

Fig. 2–Effect of temperature on viscosity of depectinized apple juices.

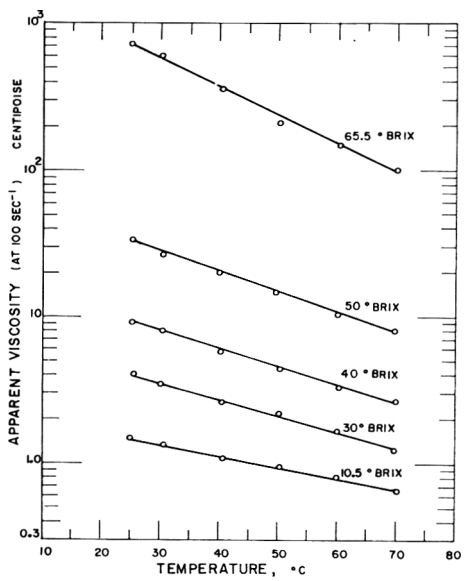

Fig. 3–Effect of temperature on viscosity of cloudy apple juices.

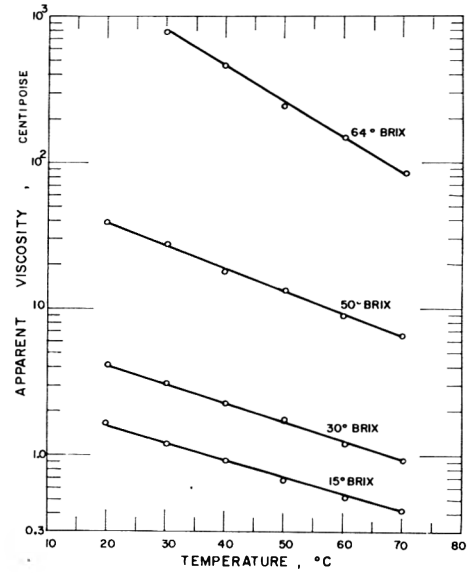

Fig. 4–Effect of temperature on viscosity of Concord grape juices.

Grape and orange juices

Figure 4 shows the effect of temperature on the viscosity of Concord grape juice. This juice was a Newtonian fluid at concentrations lower than 55° Brix and the effect of temperature was similar to that of depectinized apple juice. The grape juice contained some suspended solids (mainly pectins and tartrates) which made the 64° Brix concentrate slightly pseudoplastic (n = 0.90).

The commercial orange juice concentrate used in our experiments contained significant amounts of suspended pulp of various sizes. The 44 and 30° Brix concentrates were pseudoplastic and their flow behavior index (n) was 0.65 and 0.85, respectively.

Diluted orange juices of concentrations lower than 20° Brix were Newtonian. However, the determination of their viscosity with the available narrow-gap

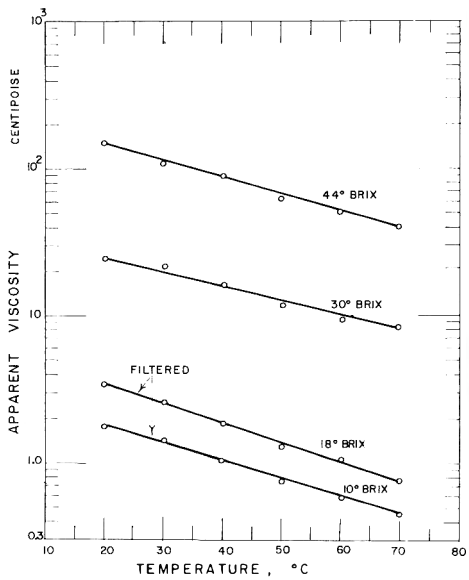

Fig. 5—Effect of temperature on viscosity of orange juices.

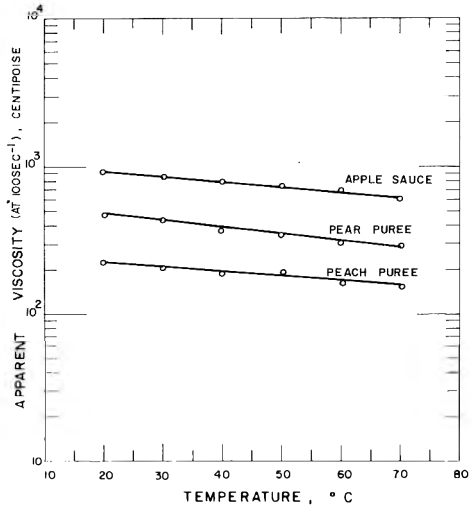

Fig. 6—Effect of temperature on viscosity of fruit purees.

measuring system (MS-O) was inconsistent, because of the presence of large pulp particles which tended to settle and interfere with the rotating inner cylinder. At higher concentrations (° Brix), these particles remained in suspension and, since the gap of the measuring system was larger, no serious interference with the rotating cylinder was noticed.

Filtered orange juices of 18 and 10° Brix were prepared by removing the suspended pulp with a No. 1 Whatman filter paper. Figure 5 shows the effect of temperature on the viscosity of orange juice concentrates and diluted filtered juices. The apparent viscosities of the concentrates (at 100 sec^{-1}) decreased only moderately with increasing temperature, presumably because of the presence of considerable amounts of suspended pulp. The filtered orange juices behaved more like clear apple juice and sucrose solutions and their viscosity decreased more rapidly with temperature than did that of the unfiltered concentrates.

Fruit purees

In contrast to various fruit juices, temperature had a rather small effect on the apparent viscosity of fruit purees (Fig. 6). The fluid consistency coefficient (K) decreased slightly at higher temperatures, while the flow behavior index (n) remained practically constant. For all three purees, the value of n was approximately equal to 0.30. The apparent viscosities shown in Figure 6 were estimated at a shear rate of 100 sec^{-1}, and a similar effect of temperature can be expected at any other shear rate, i.e., rotational speed or flow rate. Apple sauce was thixotropic, due to the larger size of suspended particles, and its apparent viscosity showed the least dependence on temperature.

In fruit purees and pulps, viscosity depends primarily on the concentration, size and shape of the suspended particles. The contribution of the serum to viscosity is relatively small, except in concentrated purees. Temperature affects the flow properties of suspended solids only slightly and, therefore, the over-all effect of temperature is rather small. The effect of temperature becomes more significant when the viscosity of the serum is relatively high, as in concentrated purees, or when sugar has been added to the puree.

Activation energies

When the logarithm of the viscosity of a Newtonian fluid (log μ) is plotted vs. the reciprocal of absolute temperature (1/T, where, T is °Kelvin), a straight line is usually obtained, according to the Arrhenius equation

$$\log \mu = \frac{\Delta E}{2.3RT} + B$$

where, ΔE is the activation energy for viscous flow, R is the gas constant, and B is a constant.

Activation energies (ΔE) of various fruit products were calculated from the slopes of the plots of our experimental data (see Table 1). For comparison, the activation energy of water between 20 and 70°C is 3.56 kcal/g mole (van Wazer et al., 1963). The activation energies of depectinized apple juice and Concord grape juice are very close to those of sucrose and glucose solutions of the same concentration. As a rule, the higher the juice concentration, the higher the activation energy.

The activation energy of flow (ΔE) has been related to some fundamental thermodynamic properties of the Newtonian fluids. For example, ΔE has been found to be approximately equal to 1/3 or 1/4 the heat of vaporization, depending on the shape and bonding of liquid molecules. Empirical equations have been suggested for the estimation of the activation energy as a function of the viscosity and the temperature of various classes of liquids (van Wazer et al., 1963).

Activation energy decreased significantly when suspended particles were present in the product, as in cloudy juices and fruit purees. The highest activation energy (14.2 kcal/g mole) was obtained with the 75° Brix clear apple juice and the lowest (1.2 kcal/g mole) with the apple sauce. In pseudoplastic fruit products, the activation energy was directly proportional to the flow behavior index

Table 1—Activation energies for viscous flow of fruit juices and purees.

Product	°Brix	Flow behavior index, n	Activation energy ΔE, kcal/g mole
Depectinized apple juice	75	1.00	14.2
	50	1.00	8.4
	30	1.00	6.3
	15	1.00	5.3
Cloudy apple juice	65.5	0.65	9.1
	50	0.85	6.1
	40	1.00	5.8
	30	1.00	5.1
	10.5	1.00	3.5
Concord grape juice	64	0.90	11.2
	50	1.00	6.9
	30	1.00	6.2
	15	1.00	5.3
Orange juice	44	0.65	5.4
	30	0.85	4.2
Filtered orange juice	18	1.00	5.8
	10	1.00	5.3
Apple sauce	11.0	0.30	1.2
Peach puree	11.7	0.30	1.7
Pear puree	16.0	0.30	1.9

(n), i.e., the more pseudoplastic the product, the less the effect of temperature on its apparent viscosity.

Activation energies of the pseudoplastic fruit products reported on in Table 1 were obtained at a constant shear rate (100 sec^{-1}). Different activation energies would be obtained when the effect of temperature is considered at a constant shear stress.

The low activation energies of fruit purees were similar to those obtained from the data of Harper et al. (1962) on pear puree and Harper et al. (1965) on tomato concentrates. The data on tomato concentrates were obtained at a shear rate of 500 sec^{-1} and the absolute temperature was expressed in ° Rankine. The resulting slope of the lines

$$\frac{\triangle E}{R},$$

called viscosity-temperature coefficient, was equal to 1,710. The corresponding activation energy ($\triangle E$) in metric units is 1.9 kcal/g mole, which agrees closely with the values of Table 1 for the fruit purees.

Processing considerations

The results and conclusions on the viscosity of fruit products have direct application to several food-processing operations involving fluid flow, heat transfer and mixing. The effects of temperature and shear rate on the apparent viscosity of a product are of primary importance and depend largely on its composition and concentration.

The fact that temperature has a strong effect on the viscosity of clear, concentrated juices can be utilized by performing more efficient heat exchange operations at high temperatures. For example, evaporation of clear fruit purees will be more efficient at high temperatures, provided the quality of the particular juice is not damaged. For the same reasons, high-temperature–short-time sterilization is indicated for clear juices.

High shear rates, achieved by agitation or high flow rate, are effective in reducing the apparent viscosity of fruit purees and cloudy concentrated juices. For such products, agitated heat exchanges, for example, wiped film evaporators (Harper, 1960) and scraped vessels (Saravacos et al., 1967), are very efficient. Improved heat transfer can also be achieved when pseudoplastic products are pumped through small-diameter tubes at high flow rates. In film evaporators, high flow rates are desirable for improved heat transfer. With pseudoplastic fluids, low flow rates without agitation may result in fouling of the heating surface.

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EFFECT OF PROCESSING AND STORAGE CONDITIONS ON THE MICROFLORA OF CLOSTRIDIUM PERFRINGENS–INOCULATED FRANKFURTERS

SUMMARY—The growth support potential of frankfurters for Clostridium perfringens *was determined. Inoculated emulsions were stuffed into glass tubes and processed so that they reached an internal temperature of 68–69°C in 30–48 min. The tubes were sealed with agar plugs and rubber stoppers to simulate vacuum-packaging conditions. Total aerobic and anaerobic bacterial counts in uninoculated raw emulsion were 1 to 3 log cycles higher when petri dishes were incubated at 23°C as opposed to 37°C. The low-temperature-growing mesophiles and psychrotrophs were destroyed during processing. The 37°C counts were reduced 1 log cycle and the 23°C counts, 2 to 3 log cycles.* Clostridium perfringens *reproduced rapidly in frankfurters at 37 and 23°C, more slowly at 15 and 12°C, and not at all during 2 wk of storage at 10° or 4 wk at 0–5°C.* Clostridium perfringens *made up 2.5–10% of the total anaerobic count at 0 time but became the dominant organism during the storage at all temperatures permitting growth.*

INTRODUCTION

CLOSTRIDIUM perfringens was responsible for more food poisoning cases than any microorganism with the exception of Salmonella in 1968 (Anon., 1969). Several food poisoning strains have been isolated from cured meat products. Strain 1362 was isolated from salami and strain S–88 from cold cuts (Hall et al., 1963). With the introduction and rapid increase of vacuum-packaged meat products in the market, the shelf life of many items has been extended considerably and the previously available indicators of product deterioration, mold growth and discoloration are no longer valid. The potential public health hazard may have been increased by the new packaging procedure in which anaerobes or facultative anaerobes become the predominant microorganisms in the microflora (Shank et al., 1963; Ogilvy et al., 1953; Allen et al., 1960). *Clostridium perfringens* has a reported minimal growth temperature of 15°C (Breed et al., 1957). Therefore, vacuum-packaged cured meat products would have to be poorly handled to result in establishment of a *Clostridium perfringens* food poisoning condition, since refrigeration equipment is expected to operate between 5 and 10°C. Many home refrigerators are operated at temperatures approaching 15°C. Vacuum-packaged luncheon meats left out of refrigeration for several hours or even days may show no color change even after significant microbial growth has occurred.

Frankfurters are typical of many luncheon meats, since they are cooked to a ready-to-eat form. They are often manufactured using meat mixtures and spice mixtures identical to those used in bologna manufacture. Modern frankfurter manufacturing procedures have shortened the heating cycle to 30 to 50 min followed by rapid cooling.

The effect of reduced process time on the microbial population of frankfurters has not been reported in the scientific literature. Conventional processing cycles requiring 60–120 min produced frankfurters of very low microbial counts and frankfurter spoilage by microorganisms has usually been the result of post-processing contamination during peeling and packaging operations.

This study was designed to evaluate the effect of 30–48 min heating cycles upon the total microbiological population of frankfurters and to determine the growth characteristics of *Clostridium perfringens* in a cured meat environment.

EXPERIMENTAL

SPORES of *Clostridium perfringens* (strain 1362–heat sensitive and S–80–heat resistant, obtained from Dr. H. E. Hall, Taft Sanitary Engineering Center, Ohio) were harvested following the procedure described by Duncan et al. (1968). Washed spores and vegetative cells were stored in distilled water at 4–5°C.

Heat shocking of mixed vegetative cell and spore suspensions was carried out at 80°C for 20 min followed by rapid cooling in an iced water bath.

SPS agar (Angelotti et al., 1962) was used for all platings of *Clostridium perfringens*.

Frankfurters were prepared using 6 lb of frozen boneless cow meat, 4 lb of pork jowls, 1 lb chilled water, ¼-lb salt, 34 g spice mix (BHSS-WP Presco Foods, Flemington, N.J.) and 10 ml of cure solution made by dilution of 7 g of sodium nitrite in 100 ml of sterile distilled water. Cow meat, water, salt, spice mix and cure were mixed together and chopped in a silent cutter (Hobart Model T215 GA) for 7 min. Pork was added followed by an additional 5-min chopping. Inoculum from the stored vegetative cell and spore suspension was added in a carrier of 100 ml of water and chopping continued to a final emulsion temperature of 13°C. All of the frankfurter studies were inoculated with *Clostridium perfringens* strain 1362. The emulsion was then spread in a 0.5–1-in. layer in a tray, covered with Saran, and stored at 5°C 1–2 hr. The emulsion was then transferred to 1,000 ml tall form beakers which were kept in an ice bath. A 26-mm (i.d.) glass tube was then filled with emulsion by drawing approximately 50 g of meat up to the tube from the beaker with a syringe. The tubes were then plugged at the bottom with a Saran-coated rubber plug and the top covered with a plastic foam plug.

Filled tubes were placed in a water bath at 53°C and the bath adjusted to increase in temperature at rates varying 0.3–0.6°C per minute. Frankfurters were processed until they reached an internal temperature of 68–69°C, after which they were rapidly cooled in tap water at approximately 10°C until an internal temperature of 21°C was reached.

The frankfurters, in tubes, were then covered with 1 in. of 1.5% agar (Difco) and placed in storage at various temperatures. Sample storage was at 4, 10, 12, 15, 23 and 37°C. Samples were drawn regularly for microbial analysis.

Total aerobic and total anaerobic plate counts were obtained using Tryptone Glucose Extract (TGE) Agar. Anaerobic conditions were obtained in Case Anaerobic Jars evacuated and

Table 1 –Stability of Clostridium perfringens spores and vegetative cells during storage in distilled water at 5°C.

Strain	Storage time (days)	Vegetative cells (organisms/ml)	Spores (organisms/ml)
1362	7	1.3×10^8	3.7×10^6
	18	1.6×10^8	7.1×10^6
	62	8.2×10^7	—
	119	7.4×10^7	—
	169	8.4×10^7	4.5×10^6
S–80	3	2.6×10^7	5.5×10^4
	7	1.3×10^4	4.7×10^4
	13	1.5×10^4	4.9×10^4
	83	3.0×10^4	3.3×10^4

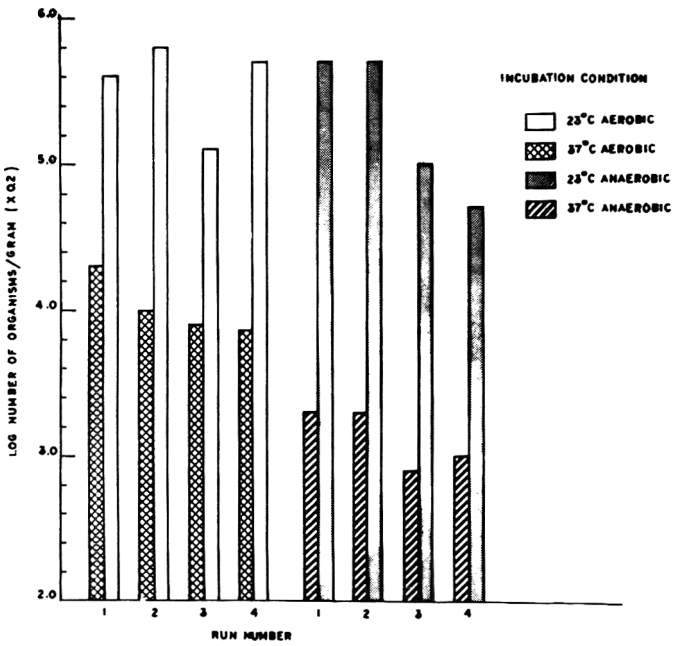

Fig. 1—Total plate counts for raw frankfurter emulsion evaluated in Tryptone Glucose Extract Agar incubated at 37 and 23°C.

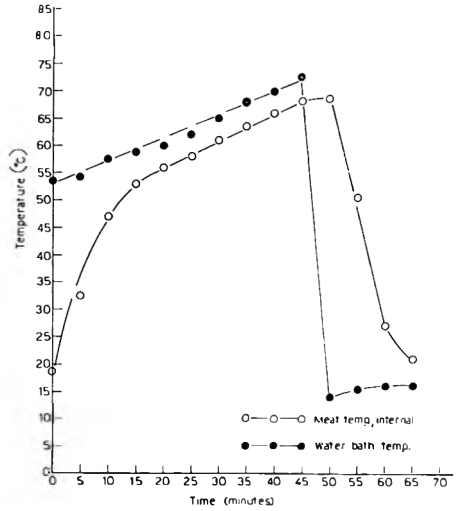

Fig. 2 —Heating and cooling profile for rapid-process frankfurters prepared under experimental conditions.

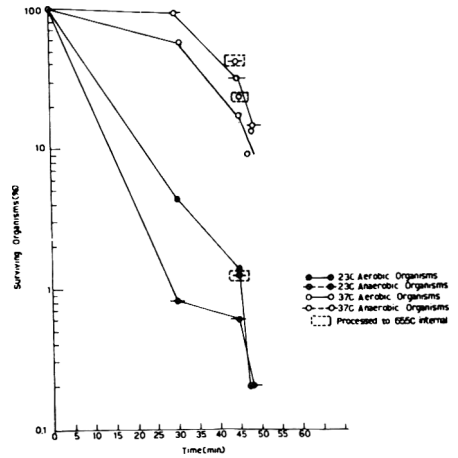

Fig. 3 —Survival of aerobes and anaerobes in frankfurters processed to reach an internal temperature of 68°C at various rates. Total plate counts were made in Tryptone Glucose Extract Agar incubated at 37 or 23°C.

flushed with nitrogen 4 times. Petri dish incubation was at 37 or 23°C.

Frankfurters being sampled were pushed from the glass tubes into a stainless steel Waring Blendor cup and 200 ml of 0.1% peptone water added. The mixture was blended at high speed for 3 min. Serial dilutions were prepared and appropriate petri dishes poured using TGE Agar or SPS-Agar.

Petri dishes incubated at 37°C were counted after 36–48 hr. Petri dishes incubated at 23°C were counted after 60–72 hr.

Aseptic technique was used throughout the experiments with all glassware, utensils, media and diluents sterilized and all equipment thoroughly sanitized before use.

RESULTS & DISCUSSION

THE STABILITY of harvested spores and the accompanying vegetative cells was evaluated over long periods of time. Results shown in Table 1 indicated that spore counts remained essentially constant throughout extended storage at refrigerated temperature. Vegetative cells appeared to decrease during the first 1 or 2 wk of storage after which they too remained constant throughout several months.

The raw emulsions were sampled prior to inoculation; data obtained for total aerobic and total anaerobic plate counts at 23 and 37°C are shown (Fig. 1). Total aerobic counts were 15 to 65 times greater on plates incubated at 23°C; total anaerobic counts were 40 to 275 times greater at 23°C.

The higher aerobic and anaerobic total plate counts at 23°C may be due to the facultative pseudomonad population of fresh meat. Many pseudomonads will grow at 23 but not at 37°C.

A typical frankfurter heat-processing profile used in these experiments is presented (Fig. 2). Such a process profile is commercially attainable using continuous-processing equipment available to the industry today. The formulation of the frankfurters prepared was designed for a minimal shrink such as would be expected from a process within a glass tube. The total water added was approximately 10% of the meat. There was some slight fat separation in some of the experiments. The frankfurters reached an internal temperature of 49°C after 10–15 min and then increased from 0.5–1.0°C per minute until an internal temperature of 68°C was reached. The frankfurters were then rapidly cooled to 21°C during a 15–20-min period. One experiment was terminated at an internal temperature of 65.5°C.

The effect of the heating rate upon survival of total aerobes and anaerobes is presented in Figure 3; data for survival of *Clostridium perfringens* spores and vegetative cells are in Table 2. The shortest process cycle, which raised the frankfurters to 68°C in 30 min, destroyed only 10% of the anaerobic organisms and only 43% of the aerobic organisms growing at 37°C. The destruction of the more psychrotrophic organisms which grew at 23°C was 99.2% of the anaerobes and 95.6% of the aerobes.

The organisms which grew at 37°C were much more heat resistant than those which grew at 23°C. The effect of decreasing the rate of heating was to significantly reduce the total plate counts. The outer surfaces of the frankfurter were exposed to heat treatment for longer times during the slower heating processes, with the resulting destructive effect being greater.

The continually changing slopes evident in Figure 2 may be due to different microorganisms within the mixed flora being inactivated at different time-temperature relationships. It appears that the survival was diminishing rapidly in the post-45-min period, indicating that considerable advantage would be gained either from continued heating to higher temperatures or short holding periods at 68–69°C before cooling. The experimental data from the frankfurters processed to 65.5°C internal showed slightly higher counts than an equal time process to 68°C. These points are enclosed in dashed squares in Figure 3.

Table 2 —Effect of frankfurter processing rate to a final internal temperature of 68°C upon survival and recovery of spores and vegetative cells of Clostridium perfringens, strain 1362.

Process time	Inoculum (spores/g)	Post-processing recovery (organisms/g)	Inoculum (vegetative cells/g)	Raw emulsion recovery (organisms/g)
30	2.8×10^2	4.6×10^3	3.3×10^3	6.6×10^3
35	2.8×10^2	1.9×10^3	8.0×10^3	5.0×10^3
45	3.0×10^2	1.6×10^3	4.9×10^3	4.5×10^3
48	3.0×10^2	1.7×10^3	5.5×10^3	7.0×10^3

The vegetative cells of *Clorstridium perfringens* added to the emulsion were recoverable after mixing as demonstrated in Table 2. The process time affected the *Clostridium perfringens* recovery level with the longer process times resulting in greater destruction. The post-processing survival of vegetative cells was of such magnitude that the spore inoculum represented less than 20% of the total cells. Therefore, no statement can be made relating to the efficiency of the heating process as a spore-germinating mechanism.

The similarity of total plate counts at 37 and 23°C indicated in Figure 4 further demonstrated the susceptibility of the psychrotrophic organisms to the heating process. The lower-temperature, normal-time treatment (Expt. 1, Fig. 4) allowed the spread between 23 and 37°C plate counts to remain noticeable, whereas the short-time, normal-temperature process (Expt. 4, Fig. 4) reduced the psychrotroph-mesophile differential. The *Clostridium perfringens* plate counts were equivalent when the SPS Agar plates were incubated at 37°C for 24 hr or 23°C for 72 hr. The stationary phase data presented in Figure 6 offered additional support to this finding, since the 23 and 37°C storage temperatures yielded equivalent concentration maxima in frankfurters.

It is also apparent from Figure 4 that the total number of anaerobes present immediately after processing was generally greater than the number of *Clostridium perfringens.*

The effect of frankfurter storage temperature upon the exponential growth phase of *Clostridium perfringens* organisms is illustrated in Figure 5. The growth rate diminished as the temperature was reduced from 37 to 12°C. At 10 and 5°C no growth of *Clostridium perfringens* was observed after 14 days' storage, although the aerobic counts at 10°C had increased approximately 1 log cycle after 14 days and the total aerobic count at 4°C had increased almost 2 log cycles at the end of 21 days' storage. Growth of *Clostridium perfringens* at 12°C has not been reported in the literature. Many household refrigerators and supermarket refrigerated cases operate at temperatures of approximately 12°C although they should be kept below 10°C. Short-term exposures to room temperature could result in significant increases in *Clostridium perfringens* populations which might not be detectable on a basis of gas production in the semirigid type of vacuum package now used for cured meat products. The growth rate at poor refrigeration temperatures as represented by 15 and 12°C was quite rapid with counts increasing from 10^3 to 10^6 in 3 days at 15°C and in 5 days at 12°C. Subsequent growth proceeded at a slower rate until a maximum cell concentration was reached between 7 and 14 days. Figure 6 shows the maximum cell concentration reached by *Clostridium perfringens,* at the various frankfurter storage temperatures. No explanation can be offered for the low maximum count at 15°C. The result was reproduced in 4 different experiments.

The maximum cell level reached in the stationary phase by *Clostridium perfringens* as compared to that attained by total anaerobes is illustrated in Figure 7. In the stationary growth phase there was no apparent difference between total *Clostridium perfringens* and total anaerobes at any of the storage temperatures or at any of the petri dish incubation temperatures. Although *Clostridium perfringens* made

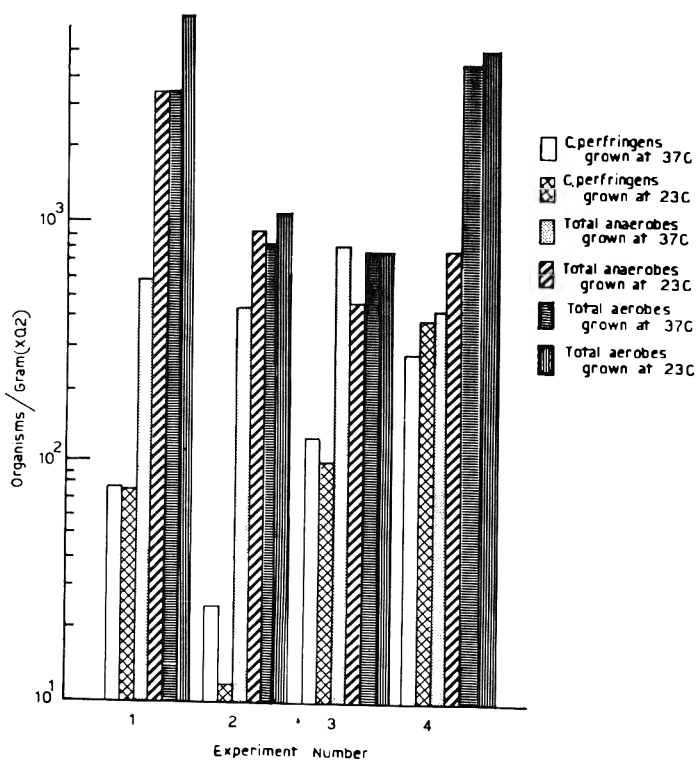

Fig. 4 —The post-processing microbial contamination of frankfurters as a function of process time and temperature. Process conditions: Expt. 1: 66.5°C internal, 45 min. Expt. 2: 68°C internal, 48 min. Expt. 3: 66.5° internal, 47 min. Expt. 4: 68°C internal, 30 min. Clostridium perfringens cultivated on SPS Agar. Total plate counts on TGE Agar.

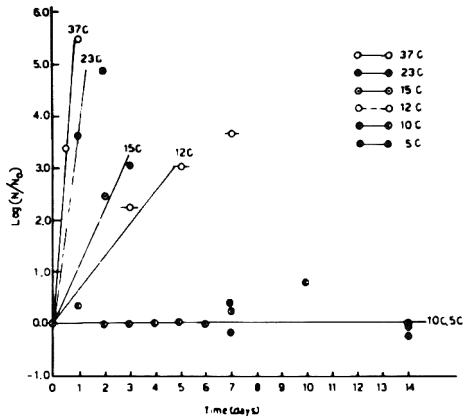

Fig. 5 —Exponential growth phase for Clostridium perfringens in frankfurters stored at various temperatures. Plate counts were made in SPS Agar incubated at 37°C.

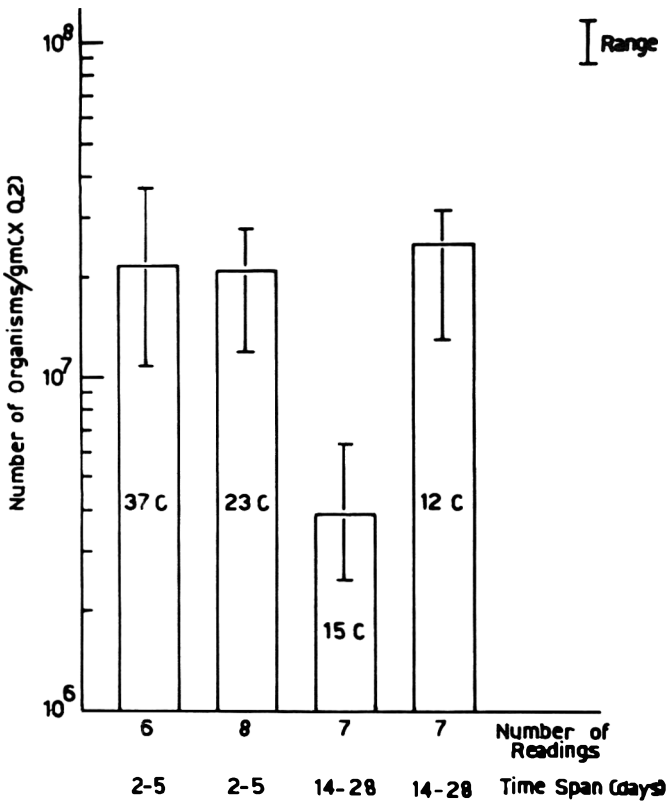

Fig. 6 —Maximum cell concentration reached and maintained by Clostridium perfringens in frankfurters stored at various temperatures.

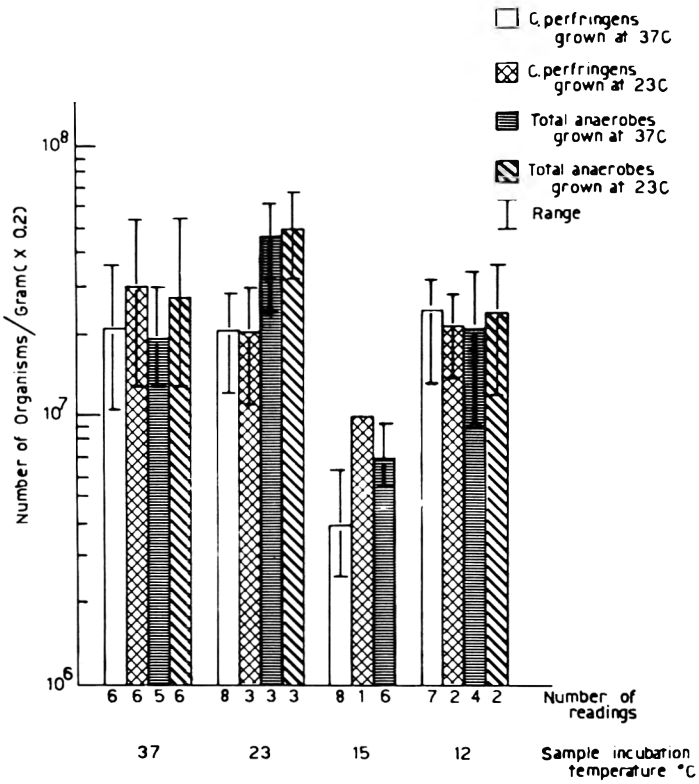

Fig. 7 —A comparison of the vaible cell population of Clostridium perfringens on SPS Agar with the total anaerobic plate count on TGE Agar during the stationary growth phase. (2–5 days at 37 or 23°C; 14–28 days at 15 or 12°C.)

up 10% or less of the total anaerobic population immediately after processing in 3 of the 4 experiments plotted, comparison of Figure 7 with Figure 4 indicates that the large differences disappeared during storage at all temperatures permitting the growth of *Clostridium perfringens*. Therefore, *Clostridium perfringens* must have become the dominant anaerobe in the system. Further proof was gained by overlaying the TGE agar with SPS agar after counting the colonies. The overlaid plates were reincubated for 24 hr and black colonies were counted; all of the colonies were black.

CONCLUSIONS

THE SURVIVAL of microorganisms in frankfurters subjected to a simulated high-speed continuous process was significant. The development of such systems must be accompanied by microbiological studies to establish the necessary time-temperature relationships to assure shelf life and public health safety.

A heat-sensitive strain of *Clostridium perfringens* inoculated into frankfurter emulsion at concentrations of 3,000 to 8,000 organisms per gram survived processing to the extent of 20–50%. Frankfurter storage at temperatures 12–37°C supported *Clostridium perfringens* growth. Therefore, cured meat products which may be represented by frankfurters will not serve as a vector for the transmission of *Clostridium perfringens* food poisoning if stored at temperatures below 10°C, but could become a vector as a result of storage at temperatures as low as 12°C.

Clostridium perfringens became the dominant anaerobe in processed frankfurters stored between 12 and 37°C even when the initial post-processing population represented only 2.5% of the total anaerobes.

Therefore, any cooked, cured, comminuted meat product of the frankfurter type subjected to improper storage conditions could become a vector of *Clostridium perfringens* food poisoning if contaminated with a significant number of *Clostridium perfringens* organisms before or during processing.

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PRODUCTION AND PROPERTIES OF PENCILLIUM ROQUEFORTI LIPASE

SUMMARY—A Penicillium roqueforti *strain produced maximal amounts of lipase when grown in 0.5% casitone–1% Proflo broth, pH 5.5, at 27°C. Addition of butteroil, corn oil or olive oil to the growth medium inhibited the lipase production. Under pH stasis the partially purified lipase of* P. roqueforti *had an optimum pH of 8.0 and an optimum temperature of 37°C. Maximum lipolytic activity occurred with 5% butteroil emulsion as the substrate. Manganese chloride and magnesium chloride stimulated the enzyme activity. Calcium, sodium and potassium salts had no appreciable effect on lipolysis; silver, mercury and zinc salts were inhibitory. The lipase was thermolabile, being inactivated completely within 10 min at 50°C. The lipase hydrolyzed tributyrin, tricaprylin, tricaprin, tripropionin and triolein in decreasing order.*

INTRODUCTION

LIPASES have been studied extensively in the dairy and food industry because they are responsible for the development of hydrolytic rancidity in various food products. Even though lipase action constitutes a serious economic problem of the food industry, many lipases play a major role in developing characteristic flavors in ripened cheeses. In this regard, the lipase of *Penicillium roqueforti* is of particular importance as it is largely responsible for the development of the characteristic flavor of Blue cheese. In addition, lipases from various sources are being used to develop, through controlled lipolysis, partially lipolyzed products for use in the food industry as flavor modifiers and enhancers.

The objectives of the present study on the lipase of *P. roqueforti* were to study factors that affect lipase production by the organism, to partially purify the lipase and to study factors that affect the activity of the enzyme. It was believed that such a study might provide information to control undesirable rancidity in dairy and food products. Also, completion of the objectives would appear to be a logical step toward adapting the lipase of *P. roqueforti* to the production of modified fat preparations for commercial use.

MATERIALS & METHODS

Cultures

The *P. roqueforti* strain used throughout this study was isolated from a commercial sample of Blue cheese. The organism was maintained with periodic transfers on Czapek solution agar slants.

Media

The following commercial media were used: Proflo (Traders Oil Mill Co.), Bacto-Casitone (Difco), Czapek Solution Agar (Difco) and Czapek Dox Broth (Difco). All media except those containing Proflo were autoclaved at 15 psi for 15 min. Proflo media were autoclaved at 15 psi for 20 min.

Culture methods

To examine the lipase-producing capabilities of the mold, the organism was grown by stationary and shake culture techniques. When stationary culture was used, 100–ml aliquots of broth in 500–ml Erlenmeyer flasks were inoculated with 1 loopful of spores collected by scraping the surface of a Czapek agar slant.

When shake culture was used, the broth was inoculated with pregerminated spore inoculum. Pregerminated inoculum was prepared by inoculating 50 ml of broth in 125–ml Erlenmeyer flasks with 1 ml of a previously standardized spore suspension containing approximately 2.0 × 10^7 spores per milliliter in 0.01% polyvinyl alcohol. The broth was incubated at 27°C for 24 hr on a New Brunswick rotary shaker set at 170 rpm. For lipase production studies, 5–ml aliquots of the pregerminated inoculum were transferred aseptically to 55 ml of broth in 125–ml Erlenmeyer flasks.

Lipase assay

Two lipase assay methods were used. Lipase assay of the crude broth was done by the silica gel chromatographic method of Harper et al. (1956). The enzyme assay of the partially purified lipase obtained by $(NH_4)_2SO_4$ fractionation of the broth was carried out by the pH-stat technique (Parry et al., 1966; San Clemente et al., 1967).

Partial purification of the lipase

After incubation, the broth was filtered through several layers of cheese cloth to remove the mycelia, then through Whatman No. 1 filter paper with the addition of celite filter-aid to remove any extraneous material. Solid $(NH_4)_2SO_4$ was added slowly with stirring to the clear filtrate at 4°C to give the desired saturation. Precipitates obtained after 30 min at the various saturation levels were collected by centrifugation at 17,000 × g for 30 min. The precipitates were dissolved in minimum cold distilled water and dialyzed for 12 hr at 4°C against 3 liters of distilled water charged twice during dialysis. The various fractions thus obtained were assayed immediately after dialysis and the active fractions frozen.

Protein determination

Protein concentration in the growth medium and in the $(NH_4)_2SO_4$ fractions was determined by the Folin Ciocalteau reagent method of Lowry et al. (1951).

RESULTS & DISCUSSION

Factors affecting lipase production by Penicillium roqueforti

Composition of the medium. Initially, the mold was grown as a stationary culture in several media to study the ability of each to support lipase production. In Czapek broth and a Czapek modified broth, which contained 1% lactose in place of saccharose as the carbon source, the mold did not produce any significant amounts of lipase. These results agree with the work of Thibodeau et al. (1942), who concluded that $NaNO_3$, the only nitrogen source in Czapek medium, was a very poor nitrogen source for growth and enzyme production by *P. roqueforti*. Essentially similar observations were made by Morris et al. (1953) who fortified Czapek's solution with peptone and butterfat for lipase production by *P. roqueforti* and by Alford et al. (1964) who used case peptone and yeast extract for the same purpose.

Other media tested in this study were 3% casitone broth, 1% Proflo broth and 2% casitone–1% Proflo combination broth. Casitone is hydrolyzed casein and Proflo is a high protein, partially defatted

Table 1—Effect of initial pH of the medium upon lipase production.

pH	Lipase activity[1]	mg Mycelium / ml Broth	Lipase activity per mg mycelium
5.5	85.8	1.6	54
6.0	78.8	1.5	53
6.5	74.6	1.4	53
7.0	58.2	1.1	53
7.5	54.6	1.0	55
8.0	45.6	0.9	51
8.5	44.6	0.9	50

[1] Lipase activity is expressed as μmoles of free fatty acids liberated in 1 hr by 1 ml of broth.

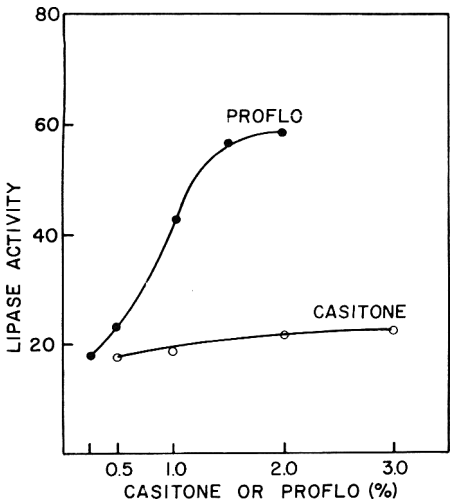

Fig. 1.—Lipase production in casitone or Proflo broth. Lipase activity is expressed as μmoles of free fatty acids liberated from the substrate in 1 hr by 1 ml of the broth as measured by the silica gel method.

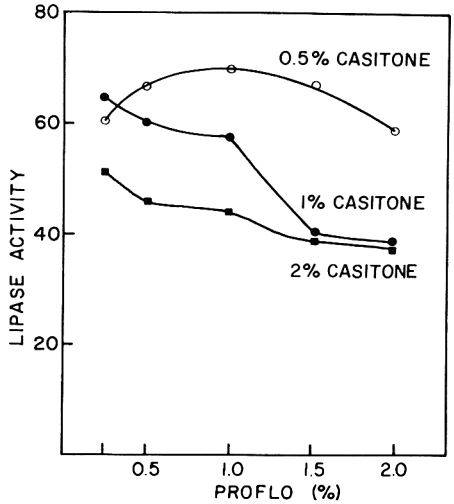

Fig. 2.—Lipase production in casitone: Proflo combination broth. Lipase activity is expressed as given in Figure 1.

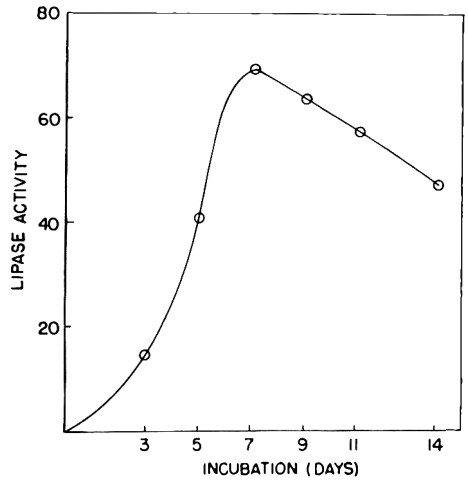

Fig. 3.—Effect of incubation time upon lipase production in stationary culture. Lipase activity is expressed as given in Figure 1.

cottonseed flour. To each of these media were added 0.1% Na_2HPO_4 and 0.01% KCl, and the pH was adjusted to 6.8. After 7 days of incubation at 27°C, high lipolytic activity was noted in the 3 media, with the highest lipase production being in the casitone-Proflo combination broth. These results indicated that *P. roqueforti* requires a complex organic nitrogen source for high lipase production.

Following the initial experiments, lipase production was determined after 7 days' incubation at 27°C in media containing varying concentrations of casitone and Proflo. As shown in Figure 1, in the 0.5, 1, 2 and 3% casitone broth, the lipase production was nearly the same. In the Proflo broth there was a sharp increase in lipase production as the Proflo concentration was increased from 0.25 to 1%. Above 1.5% Proflo, the enzyme production seemed to level off.

Figure 2 presents the lipase production in 0.5, 1 and 2% casitone broth in combination with varying concentrations of Proflo. The lipase production was highest in 0.5% casitone–1% Proflo combination broth. This medium, therefore, was used for all the additional studies.

Addition of oils to the medium. To study the effect of oils in the medium upon lipase production by *P. roqueforti*, 1% (v/v) sterilized butteroil, corn oil or olive oil was added to aliquots of the casitone-Proflo broth. After inoculation and incubation at 27°C for 7 days in stationary culture, lipase activity in the broths was determined. All of the oils added to the medium caused a decrease in lipase production. Olive oil inhibited lipase production by only 5%, but butteroil and corn oil inhibited lipase production of the mold by 21 and 29%, respectively.

Initial pH of the growth medium and incubation temperature. Table 1 presents data concerning the effect of initial pH of the growth medium, ranging between pH 5.5 and 8.5, upon the lipase production in stationary culture. Values below pH 5.5 were not checked, as the medium was not stable at lower pH levels. Highest lipase production occurred at pH 5.5 and, as the pH was increased the lipase production decreased. It was also observed that decreased lipase production at higher pH levels closely followed decreased growth of the mold. At all pH levels, the production of lipase per milligram dry weight mycelium remained nearly the same.

Data concerning the effect of incubation temperature upon the lipase production in stationary culture revealed that a broad temperature optimum for lipase production exists between 21 and 27°C (Table 2). At 16 and 30°C, the organism produced the lowest amounts of lipase. At 16°C, while only a small amount of growth occurred, a larger amount of lipase was produced per milligram dry weight mycelium. In contrast, at 30°C, while heavy growth occurred, only a small amount of lipase was produced per unit weight.

Effect of incubation time. Studies concerning the lipase production with respect to incubation time when the mold was grown by stationary culture in casitone-Proflo combination broth, pH 6.8, revealed that the maximum amount of lipase was produced at the end of 7 days, following which the lipase activity tended to decrease with time (Fig. 3).

Figure 4 shows lipase production by *P.*

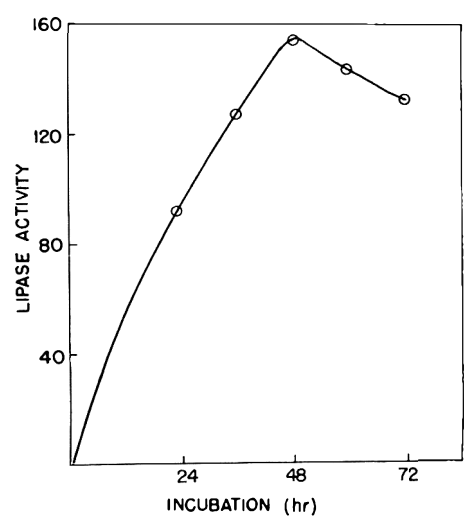

Fig. 4.—Effect of incubation time upon lipase production in shake culture. Lipase activity is expressed as given in Figure 1.

Table 2—Effect of incubation temperature upon lipase production.

Temp °C	Lipase activity[1]	mg Mycelium / ml Broth	Lipase activity per mg mycelium
16	16.6	0.26	65
21	66.2	1.3	51
25	66.8	1.4	48
27	68.4	1.3	53
30	31.0	1.3	24

[1] Lipase activity is expressed as μmoles of free fatty acids liberated in 1 hr by 1 ml of broth.

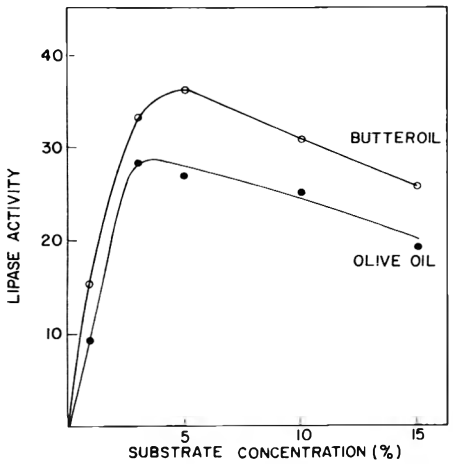

Fig. 5.—Effect of substrate concentration upon lipase activity. Lipase activity is expressed as μmoles of free fatty acids liberated from the substrate in 1 min by 1 ml of the enzyme as measured by the pH-stat method.

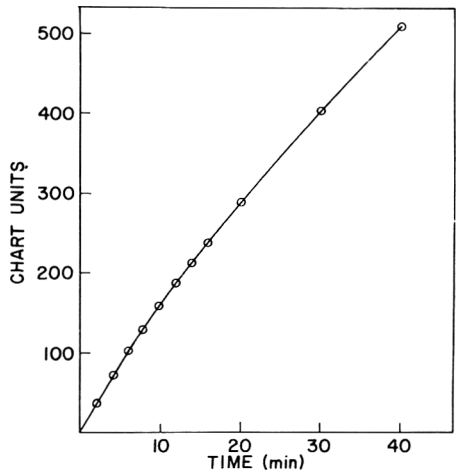

Fig. 6.—Effect of time upon reaction rate. 1 chart unit = 0.05 μmole of free fatty acid.

roqueforti grown by shake culture in casitone-Proflo combination broth at pH 5.5 inoculated with pregerminated spores. The shake culture method produced maximal amounts of lipase in 48 hr, yielding approximately 128% more lipase than when the mold was grown by stationary culture at pH 6.8.

In subsequent work, all lipase produced was obtained by inoculating 0.5% casitone–1% Proflo broth, pH 5.5, with pregerminated inoculum and incubating at 27°C on a New Brunswick rotary shaker operating at 170 rpm.

Partial purification of the lipase

For characterization of the lipase, a partially purified preparation was obtained by celite-aided filtration of the broth followed by $(NH_4)_2SO_4$ fractionation of the filtrate. The lipase was precipitated out over a broad range of ammonium sulfate saturation. However, a fraction with highest specific activity was obtained between 30 and 50% saturation. On a protein basis, this partially purified fraction had increased specific activity of about sevenfold over that of the original broth. This fraction was used in the following characterization studies.

Factors affecting lipase activity

pH and temperature. The effect of pH upon lipolysis of butteroil substrate was determined with the pH-stat method between pH 6.5 and 10. The lipase was quite active over a pH range of 7.5–9.0, with an optimum being at pH 8.0. This value agrees with the optimum pH value of *P. roqueforti* lipase reported by Fodor et al. (1949), but disagrees with the optimum pH values of 5.0–5.5 reported by Shipe (1951) and 6.5–6.8 reported by Morris et al. (1963). It is possible that the differences in the pH optimum observed in this study and in those of other workers may well be due to the use of different substrates, different assay conditions and different strains of *P. roqueforti*.

Although the lipase demonstrated high activity over a wide range, 25–45°C, it gave maximal lipolysis at 37°C, indicating its optimum temperature to be 37°C.

Substrate concentration. Using butteroil and olive oil as the substrate, the effect of the substrate concentration upon the lipase activity was studied. In general, butteroil was hydrolyzed more rapidly than olive oil at all the concentrations studied (Fig. 5). With butteroil as the substrate, lipolysis was maximum at the 5% substrate concentration, whereas with olive oil, maximal hydrolysis occurred at the 3% concentration. With both substrates, lipolytic activity decreased at concentrations above the maximal lipolysis point. A similar observation was made by Khan et al. (1967) with *Achromobacter lipolyticum* lipase which showed decreased activity at concentrations above 10% with synthetic glycerides.

Time of reaction. Figure 6 shows lipase activity in terms of chart units taken directly from the pH-stat recorder as a function of reaction time. One chart unit equals 0.05 μmole of fatty acid. The reaction rate remained linear for approximately 5 to 6 min, after which it tended to decrease slightly. San Clemente et al. (1967) reported that the reaction rate of a partially purified Staphylococcal lipase under pH stasis remained linear for 6–8 min and that lipase assay methods which utilize long incubation periods give values on the low side of the initial rate.

Heat stability. Because the susceptibility of enzymes to heat treatments is significant to the food industry, the susceptibility of the lipase to heat treatments was determined in an aqueous solution at pH 6.5. At 37°C, the enzyme lost 65% of its activity in 60 min, and at 40°C, 90% of its activity (Fig. 7). At 50°C, the lipase was inactivated completely within 10 min. However, the enzyme appeared to be fairly stable at low temperatures and could be held frozen for long periods without loss of activity.

The inactivation temperature of 50°C reported here compares well with inactivation temperatures reported for other fungal lipases. Fukumoto et al. (1963; 1964) reported that the lipases of *Aspergillus niger* and *Rhizopus delemar* were inactivated at 55 and 50°C, respectively. These lipases resemble milk and pancreatic lipases which are heat sensitive but differ markedly from some microbial lipases such as *Aspergillus lipolyticum* lip-

Table 3—Effect of salts on lipase activity.

Salt	Concentration (M)	Relative activity (%)	Stimulation or (inhibition) (%)
Control	–	100	–
$MnCl_2$	10^{-2}	135	35
	10^{-3}	100	–
$MgCl_2$	10^{-2}	112	12
	10^{-3}	100	–
NaCl	10^{-2}	103	3
	10^{-3}	99	1
KCl	10^{-2}	102	2
	10^{-3}	99	1
$CaCl_2$	10^{-2}	102	2
	10^{-3}	101	1
$ZnSO_4$	10^{-4}	65	(35)
$HgCl_2$	10^{-4}	31	(69)
$AgNO_3$	10^{-4}	24	(76)
$CoCl_2$	10^{-4}	101	1
$FeSO_4$	10^{-4}	102	2
$CuSO_4$	10^{-4}	98	2

ase, which is very heat stable (Khan et al., 1967).

Effect of salts. Since various salts generally present in most food products have been shown to affect lipase activity markedly, the effect of several salts on the activity of the Penicillium lipase was studied (Table 3). Rate of hydrolysis of butteroil substrate was stimulated by $MnCl_2$ and $MgCl_2$. However, calcium, sodium or potassium salts did not seem to have any appreciable effect. This observation differs from that of Shipe (1951), who reported that $CaCl_2$ accelerated the activity of *A. niger* and *P. roqueforti* lipases. Also, calcium salts have been shown to accelerate the activity of pancreatic lipases as well as that of several microbial lipases (Constantin et al., 1960; Iwai et al., 1964; Wills, 1961). The *P. roqueforti* lipase was inhibited by silver, mercury and zinc salts, but not by cobalt, iron and copper salts. Iwai (1965) found ferrous, ferric, silver, mercury, chromium, copper and tin ions to be inhibitory toward the action of crystalline *A. niger* lipase. These results show that the Penicillium lipase is affected by salts differently from other lipases.

Substrate specificity. To study the substrate specificity of the lipase, the activity of the enzyme against several triglycerides was determined. Results shown in Table 4 indicated that the Penicillium lipase was most specific for tributyrin. The lipase hydrolyzed tributyrin, tricaprylin, tricaprin, tripropionin and triolein in decreasing order. However, the difference between the rate of hydrolysis for tributyrin and tricaprylin was slight. Triolein and tripropionin were hydrolyzed at much lower rates than the other triglycerides studied. These observations are in harmony with those of Alford et al. (1961). Also, Wilcox et al. (1955) reported that *P. roqueforti* lipase preferentially hydrolyzed short-chain fatty acids from butter fat.

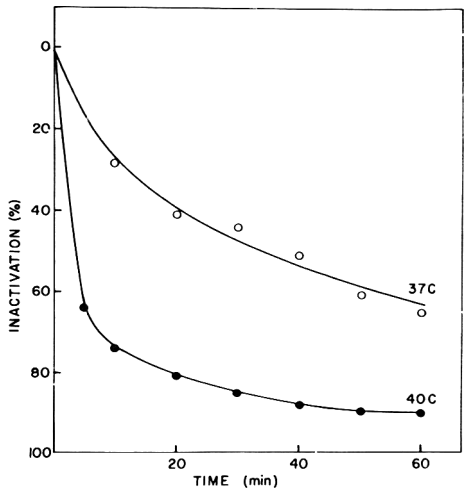

Fig. 7.—Heat inactivation of the lipase.

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Table 4—Effect of lipase upon various triglycerides.

Triglyceride	Lipase activity[1]	Relative hydrolysis[2]
Tripropionin	9.0	32.2
Tributyrin	28.0	100.0
Tricaprylin	26.3	93.2
Tricaprin	17.3	61.8
Triolein	5.7	20.0

[1] Lipase activity is expressed as μmoles of free fatty acids liberated from the substrate in 1 min by 1 ml of enzyme.

[2] Hydrolysis of various triglycerides is recorded on a relative basis, considering 100% for tributyrin.

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PYROGLUTAMYL DIPEPTIDES IN MUSHROOM, AGARICUS CAMPESTRIS

SUMMARY—A small fraction, containing evidence for the presence of pyroglutamyl dipeptides and an N-pyroglutamylhexosamine, was isolated from the edible, commercial mushroom, Agaricus campestris. *Upon hydrolysis, this isolate liberated a copious amount of glutamic acid, together with smaller quantities of other amino acids and a hexosamine. Analyses of the unhydrolyzed and hydrolyzed portions of the fraction by an automatic amino acid analyzer revealed the identities and quantities of the compounds released. Additional chemical and physical methods of analysis gave supporting evidence for the presence in the mushroom in addition to proline and pyroglutamic acid of the pyroglutamyl dipeptides of threonine, aspartic acid, valine, leucine, citrulline, phenylalanine, glycine, alanine, glutamic acid and proline and also of the amino acid sugar, N-pyroglutamylglucosamine. Pyroglutamylcitrulline and N-pyroglutamylglucosamine were tentatively identified. The first 6 dipeptides are reported for the first time. A mechanism is proposed for the enzymic biosynthesis of the pyroglutamyl dipeptides in the mushroom. Results of the present work shed additional light on the complex nature of the higher basidiomycetes.*

INTRODUCTION

IN GENERAL, early work on mushrooms was concerned with the isolation, separation and identification of the more common and essential amino acids, both free and bound, from simple extracts of mushrooms selected from different taxonomical families, and from those closely related to *Agaricus campestris,* such as *Agaricus hortensis* and *Boletus edulis.* Recently, the trend has been to obtain a deeper insight into the chemical composition of the higher fungi. Altamura et al. (1967) have reported a novel procedure which afforded the isolation of a number of the less common amino acids and other nitrogenous substances from *A. campestris.* Lately, 2 new amino acids, L-2-amino-3-hydroxymethyl-3-pentanoic acid and L-2-amino-3-formyl-3-pentanoic acid have been isolated (Doyle et al., 1968) from the mushroom, *Bankera fuligineoalba.*

Although almost all living tissues seem to contain quantities of low-molecular-weight peptides, the work reported in the literature regarding these substances in mushrooms is very meager. This is particularly true of the dipeptides. Touzé-Soulet (1961) detected 2 unidentified ninhydrin-active peptides in an extract from *B. edulis.* Two free dipeptides, carnosine (β-alanyl-L-histidine) and γ-L-glutamyl-S-methyl-L-cysteine, were identified (Altamura et al., 1967) in an isolate from *A. campestris.* Both of these dipeptides were positive to ninhydrin. With regard to the pyroglutamyl (Pyr; sometimes called pyroglutamoyl) dipeptides, however, no evidence was found in the literature of their presence in mushrooms. Conceivably, one reason for the failure to detect these substances can be attributed to the past practice of visualizing the chromatographically separated mushroom components with ninhydrin reagent. If pyroglutamyl dipeptides were present and did not possess an active amino group required for color formation (such as an α-amino group), they would not appear on the chromatograms and consequently would escape detection. Acid hydrolysis, followed by chromatography, paper or column (as in an amino acid analyzer), has provided an indirect method of establishing the presence of dipeptides (or higher peptides) regardless of whether or not they are colored by ninhydrin. For example, with ninhydrin-inactive pyroglutamyl dipeptides, a chromatographic spot or elution peak, as the case may be, would appear for glutamic acid after hydrolysis, due to the decyclization of liberated pyroglutamic acid (PCA, III). This indirect method has been reported by previous investigators of mushrooms (Touzé-Soulet, 1961) and marine alga (Dekker et al., 1949).

Although dipeptides (and higher peptides) containing the Pyr moiety in the structure have not previously been detected in mushrooms, a number of these compounds have been found, both free and bound, in other natural products. Three recorded examples are: Pyroglutamylglutaminyl-glutamine from the marine alga, *Pelvetia fastigiata* (Dekker et al., 1949), pyroglutamyl-asparaginyl-tryptophan, from snake's *(Agkistrodon halps blomhoffi)* venom (Kato et al., 1966) and the pyroglutamyl dipeptide, pyroglutamyl-glutamine, from the hydrolysate of the heavy chain of rabbit immunoglobin IgG (Wilkinson et al., 1966).

The presence of the amino acid sugar, N-pyroglutamylglucosamine (XVII) in mushrooms and other natural products has also escaped detection in the past. Related compounds, however, such as N-(L-β-aspartyl)-2-acetamido-2-deoxy-β-D-glucosamine, have been isolated (Marks et al., 1963) from a partial hydrolysate of hen's-egg albumen.

This paper presents results obtained from the characterization of a dipeptide-containing fraction, isolated from the fresh, white, edible and commercially cultivated mushroom, *Agaricus campestris* L. ex Fr. (Agaricaceae), sometimes referred to as *Agaricus bisporus* or *Psalliota campestris.* These results include evidence for the identity and general structure of the compounds present in the mushroom isolate and detected for the first time in mushrooms.

EXPERIMENTAL

Source of mushroom

The commercially grown fresh mushrooms, matured to a tight-button stage and harvested in July, were purchased from the Bay State Mushroom Co., Sudbury, Mass.

Source of model compounds and other materials

The authentic compounds and other materials were purchased as follows: trimethylsilyl (TMS)-L-proline (in hexane solution), TRI-SIL (silylation reagent), Silyl- 8 (GLC column conditioner) and PO-17 3% on chromosorb W (HP) 80/100 mesh (GLC stationary phase) from Pierce Chemical Co., Rockford, Ill.; L-pyroglutamic acid from Sigma Chemical Co., St. Louis, Mo.; L-pyroglutamyl-L-proline, L-pyroglutamyl-L-glutamic acid monohydrate, and γ-L-glutamyl-L-glutamic acid from Cyclo Chemical Corp., Los Angeles, Calif. The model compounds were used for comparative and identification purposes.

Isolation of mushroom fraction

The initial part of the isolation procedure, i.e., from the washing of the fresh mushrooms (3.2 kg) to the completion of the petroleum ether extraction step, is the same as that given in the previous paper (Altamura et al., 1967).

The petroleum ether insoluble solid (66.7 g) was shaken (10 × 200 ml) with wet ether (diethyl ether saturated with water) and the ether extract removed each time. The insoluble substance was shaken with absolute ethanol (150 ml), the solvent removed at 35–40° and under partial vacuum and the residue dried (35–40°/ 5 mm) to constant weight (65.8 g). The reddish-brown, resinous-like mass was extracted with dry pyridine (150, 4 × 50 ml), and the insoluble part was dried (room temperature/4 mm) to constant weight (63.5 g). The resultant powder was extracted with methanol (7 × 250 ml), the extracts combined, the solvent removed (Rinco/25–30°/ca. 34 mm) and the residue dried in vacuo (4 mm). This procedure gave a light-reddish-brown, resinous-like solid (39.1 g). For convenience, 20.8 g of the solid was stirred with water (416 ml).

The colored solution was filtered; the filtrate concentrated (Rinco) cautiously (foaming) at 15–20° and in vacuo (ca. 15 mm gradually reduced to 8 mm) to 166.4 ml. The concentrate was refrigerated (ca. 1°) overnight and then filtered cold. The filtrate was divided

into 3 equal parts, and each part stirred with acetone (1,550 ml) to give complete precipitation of a heavy, red oil and a white, fluffy solid. The mixture was refrigerated overnight to separate the phases. At room temperature, the supernatant liquid was removed and the bottom phase (oil and solid) stirred with 75% acetone in water until the white solid dissolved. Acetone was then added cautiously until the white solid just started to precipitate. At this point, the supernatant liquid was removed from the bottom oil, filtered and stirred with acetone (276 ml) for complete precipitation of the white solid. The 2 other portions of the above concentrate were treated in like manner, and the 3 mixtures pooled.

The combined mixture was refrigerated overnight and filtered cold. The solvents (acetone and water) were removed from the filtrate at 25–30° and under vacuum, so regulated as to distill first the acetone and then, very cautiously, the water. Occasional addition of ethanol (ca. 5 ml) retarded foaming and facilitated removal of the last traces of water. This afforded a dark, highly viscous, glass-like solid (1.2 g). The solid was then stirred with minimal (10 ml) methanol and the mixture refrigerated overnight. After filtration in the cold and removal of the solvent the residue was taken to constant weight (1.1 g) under the usual conditions. It was then shaken with ca. 7 ml of 40% methanol in isopropyl alcohol and subsequently refrigerated overnight and centrifuged at room temperature.

The supernatant liquid, after filtration and evaporation, gave 1.0 g of a colored material. Repetition of this step (minus refrigeration and centrifugation) with minimal (8 ml) absolute ethanol gave 753.2 mg of an ethanol soluble substance, which was subsequently decolorized (to pale yellow) by two separate treatments of its methanolic (15 ml) solution with 1.6 g of activated carbon (Darco, G–60) at boiling methanol. The light-colored, now odorless solid, was then stirred (5 ×) with 2 ml of 0.5% methanol in isopropyl alcohol; the mixture was centrifuged each time; the separated, supernatant liquids were combined and after completely removing the solvents, approximately 213.4 mg of a viscous product obtained. Final extractions (2 × 2 ml and 3 × 1 ml) with isopropanol, and following through as before (including drying to constant weight at room temperature/3 mm), yielded 211.7 mg of a clear, light-colored, odorless, viscous syrup. For 39.1 g of the above original solid, the estimated weight of this mushroom fraction was 398.0 mg (0.01% of the weight of fresh mushrooms used).

The mushroom fraction gave a) copious evolution of carbon dioxide with sodium bicarbonate (presence of free carboxyl group), b) positive (moderate intensity) Molisch test (presence of sugar moiety), c) positive (moderate intensity) ninhydrin test (presence of α-amino group), d) negative biuret test (absence of peptides with 2 or more peptide bonds), e) nitrogen content, 10.59% and f) solubility behavior as evident from the isolation procedure. The composition of the isolate is given in Table 1.

Table 1—Composition of mushroom isolate.

Component	Amount present (%)
Composite of free amino acids[1]	1.68
Pyroglutamylproline	3.50
Proline	5.77
Composite of pyroglutamyl dipeptides of amino acids[2] plus N-pyroglutamylglucosamine	11.90
Pyroglutamic Acid	25.72[3]
Pyroglutamylglutamic Acid	51.42[3]

[1] Minor amino acids.
[2] Bound amino acids.
[3] Approximate value.

Molecular weight measurement

The average molecular weight of the fraction was measured on a vapor pressure osmometer (Model 301A, Mechrolab, Inc., Mountainview, Calif.) using methanol and water as solvents.

Hydrolysis of the mushroom fraction

The isolate was hydrolyzed under conditions generally used for protein hydrolysis. The fraction (4 mg) and 6 NHCl (4 ml) were heated in a sealed tube (previously evacuated and flushed with nitrogen) at 110° for 20 hr. After hydrolysis, the contents of the tube were evaporated to dryness, the residue dissolved in buffer solution (4 ml) and an aliquot (1 ml) subjected to analysis on the amino acid analyzer.

Automatic amino acid analysis

The components, present in the unhydrolyzed and hydrolyzed portions of the mushroom fraction, were separated and quantified by ion-exchange column chromatography in the accelerated automatic amino acid analyzer (Model K–8000–C, Phoenix Precision Instrument Co., Philadelphia, Pa.). The method of analysis used with this instrument is essentially the one described by Spackman et al. (1958) as applied to protein hydrolysates in a 50° program, except that the analysis is carried out on an accelerated scale. The procedure is described in the operational manual, Liquid Chromatography Handbook, published ca. 1966, and provided with the instrument by the Phoenix organization.

Elution chromatograms were obtained for the acidic, neutral and basic amino acids. These were compared with the chromatograms from standard mixtures of amino acids, generally found in peptide and protein hydrolysates. The identity and concentration of each amino acid were determined by conventional procedures and the application of the prescribed method of calculation given in the instruction manual. Results are given in Table 2.

Infrared and ultraviolet measurements

The infrared spectra of the mushroom fraction (film between NaCl discs) and the reference compounds (in K Br pellets), PCA, pyroglutamylproline and pyroglutamylglutamic acid monohydrate, were recorded on an Infrared Spectrometer (Model 137, Perkin-Elmer, Norwood, Conn.).

The ultraviolet spectra of the above substances (in aqueous solutions) were taken on the Cary Recording Spectrophotometers (Models 11 and 11MS, Applied Physics Corp., Pasadena, Calif.) between 210–400 mμ.

Trimethylsilylation

For the analyses obtained from mass spec-

Table 2—Change in amino acid and glucosamine content of mushroom fraction due to hydrolysis.

Compound[1]	Amount[2] (before hydrolysis)	Amount[2] (after hydrolysis)	Amount[2] (released)
Glucosamine	0	0.011	0.011
Aspartic Acid (Asp)	Trace[3,4]	0.018	0.010
Threonine (Thr)	0.015	0.031[5]	0.016[5]
Glutamic Acid (Glu)	Trace	2.990	2.982
Proline (Pro)	0.500	0.580	0.080
Citrulline (Cit)[6]	Trace	0.056	0.048
Glycine (Gly)	0.010	0.041	0.031
Alanine (Ala)	0.049	0.091	0.042
Valine (Val)	0.035	0.055	0.020
Leucine (Leu)	0.032	0.041	0.009
Phenylalanine (Phe)	0.010	0.028	0.018

[1] Compound listed according to sequence of elution from columns.
[2] μMoles/mg fraction.
[3] Less than 0.008 μmole/mg fraction for a trace amount.
[4] For purposes of calculation, the presence of at most 0.008 μmole/mg fraction is assumed for a trace compound.
[5] Approximate value.
[6] For brevity the authors suggest "Cit" as a symbol for citrulline.

Table 3—Comparison of the abundance of the fragment ion 84.

TMS derivative of:	m/e 84 (% Abundance)
Pyroglutamic Acid	100.0[1]
Mushroom fraction	56.0
Pyroglutamylglutamic Acid	4.5
Pyroglutamylproline	4.2
Control (TRI-SIL)	0.1

[1]Mass peak 84 is the most intense peak (base peak) for TMS pyroglutamic acid.

Table 4—Comparison of the percent abundance of the fragment ions, 99, 127 and 140.

Mass ion (m/e)	TMS glutamyl-glutamic acid[1]	TMS pyroglutamyl-glutamic acid[2]	TMS mushroom fraction
99	6.9	0.8	3.3
127	3.5	0.8	0
140	19.8	0.4	2.5

[1]Alkyl-type, straight-chain dipeptide.
[2]Dipeptide containing a cyclic, pyroglutamyl moiety.

trometry (MS) and the combined gas-liquid chromatography (GLC) Time-of-Flight (TOF) mass spectrometry, the trimethylsilyl (TMS) derivatives of the mushroom isolate and the reference compounds, PCA, pyroglutamylproline, pyroglutamylglutamic acid monohydrate and glutamylglutamic acid were used. They were prepared essentially by the procedure of Sweeley et al. (1963), except that TRI-SIL (mixture of hexamethyldisilazane, trimethylchlorosilane and anhydrous pyridine) was employed as the silylation reagent. It was not necessary to isolate the TMS derivatives from the reaction mixtures (Sweeley et al., 1963). In each case the clear supernatant liquid, carefully removed from the reaction solids and containing the desired derivative, gave satisfactory results. The presence of excess silylation reagent did not interfere. The test liquid was either injected directly into the GLC column of the TOF-mass spectrometer or into the inlet system of the mass spectrometer, if the latter instrument was used alone.

Combined gas-liquid chromatography and time-of-flight mass spectrometry (GLC-TOF-MS)

For these analyses, a GLC coiled stainless steel column (1/8 in. by 10 ft) was attached to a modified Bendix Time-of-Flight mass spectrometer (Model 14), so that the effluent of the column was directed into the ionization source (Merritt et al., 1964). The column, packed with Chromosorb W (HP) 80/100 mesh support, coated with 3% PO-17 and conditioned with Silyl · 8, was operated at a wide temperature range program of 75–225°C (12°C/min), an injection temperature of 200°C and a carrier gas (helium) flow rate of 10 cc/min at atmospheric pressure.

Mass spectrometry

The spectral data for the TMS derivatives of the mushroom fraction and standard compounds were obtained on a Consolidated Electrodynamics Corp., Model 21-110B, mass spectrometer, operated at 70 e V and ca. 200°C. The test samples in the form of the supernatant liquids from the trimethylsilylation reactions were injected directly into the inlet system of the instrument with a direct introduction probe. The purpose of obtaining the data was to provide additional evidence for the presence of pyroglutamyl-type compounds in the mushroom fraction by a) selecting a specific fragment ion producible from the electron bombardment of the pyroglutamyl compounds, which would have sufficient stability for its detection and for the measurement of its intensity in the mass spectra. Accordingly, the fragment ion selected was the sufficiently stable, cyclic, γ-lactam, pyrrolidinonyl ion (V) of mass unit 84; b) determining from the spectra the abundance or deficiency of other significant mass ions which could be used to differentiate between alkyl-type, straight-chain, glutamyl compounds, e.g., glutamyl dipeptides, exemplified by glutamylglutamic acid (XVIII), and cyclic, pyroglutamyl-type compounds, e.g., pyroglutamyl dipeptides, such as pyroglutamylglutamic acid (XI). The 3 typical fragments selected have mass units 99, 127 and 140; c) furnishing the molecular ions (M+) or the molecular ions minus some logical fragment ions (M+-R) of the TMS pyroglutamyl compounds present in the TMS mushroom isolate.

Fragmentation patterns of the reference compounds were obtained to provide fragment-structure information and these were found most helpful for the interpretations required in evaluation of the mass spectra of the TMS mushroom fraction. The mass spectral data are presented in Tables 3, 4 and 5.

RESULTS & DISCUSSION

TABLE 1 lists the more important components of the mushroom isolate and their amounts. The 2 free, major amino acids are proline (I) and PCA; the free minor acids, present in above-trace quantities (Table 2), are Gly, Ala, Thr, Val, Leu and Phe. The quantities of PCA (the γ-lactam form of glutamic acid, and sometimes referred to as 5-oxo-proline, 5-oxo-2-pyrrolidine carboxylic acid and pyrrolidinone carboxylic acid, etc.), together with pyroglutamylglutamic acid (XI), pyroglutamyl dipeptides of the bound amino acids (VII–XVI) and N-pyroglutamylglucosamine were calculated from the corresponding amino acids and 2-glucosamine released by hydrolysis (Table 2). Approximately 2/3 of the mushroom fraction components are bound with PCA as dipeptides. The present finding and the previously reported presence of PCA in *Agaricus campestris* (Le Roux, 1961; 1962; Latché et al., 1968) and in other higher fungi (Touzé-Soulet et al., 1957) give support to the occurrence of dipeptides derived from this amino acid in the mushroom under consideration.

The probability that PCA is formed as an artifact from glutamic acid during the work-up of natural products has been disproved by a number of investigators (Niwaguchi et al., 1965; Wilkinson et al., 1966; Piquemal et al., 1967; Johnson et al., 1968). The very mild conditions used in the present work preclude the presence of PCA as an artifact. The rather high percentage of the pyroglutamyl dipeptides in the mushroom fraction indicates the efficacy of the isolation procedure to concentrate a fraction containing predominantly this class of compounds.

Results obtained from the hydrolysis of the mushroom fraction are shown in Table 2. Of particular interest is the copious amount of glutamic acid released in contrast to the quantity of the other amino acids. This is consistent with the large quantities of PCA and pyroglutamylglutamic acid present in the unhydrolyzed fraction (Table 1) which, per se, liberate solely glutamic acid upon hydrolysis. The other pyroglutamyl compounds also contribute some glutamic acid. The release of large amounts of glutamic acid upon the acid hydrolysis of pyroglutamyl peptides was also observed by Dekker et al. (1949). Presumably, hydrolysis not only splits the peptide linkage but also decyclizes the pyroglutamyl moiety (IV).

Table 5—Molecular ions and molecular ions minus fragment ions of some components in the TMS mushroom fraction.

Component	(M^+)	$(M^+ - R)$	Nature of R
TMS proline	187	–	–
TMS Pyroglutamic Acid	201	–	–
TMS Pyroglutamylproline	–	283 $(M^+ - 15)$	$CH_3 \cdot^+$
TMS Pyroglutamylglutamic Acid	–	256 $(M^+ - 146)$	$2\,(CH_3)_3Si \cdot^+$
TMS pyroglutamylglycine	258	–	–
TMS pyroglutamylalanine	272	–	–

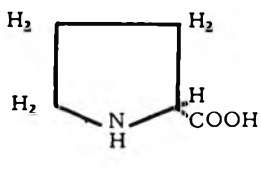
The hydrolytic products, which now have available active α-amino groups, color ninhydrin and become detectable on the elution chromatogram of the analyzer. However, the moderate ninhydrin reaction of the unhydrolyzed fraction was presumably due to the free proline and the free minor amino acids. PCA gives no color reaction with ninhydrin.

Besides glutamic acid, Table 2 also shows that aspartic acid and citrulline were practically all in the bound form before hydrolysis. The absence of free glucosamine in the unhydrolyzed fraction suggests that this amino sugar was bound to the Pyr moiety through the peptide linkage. This would then classify the amino acid sugar, N-pyroglutamylglucosamine, as an N-acyl type amino sugar. Hughes et al. (1958) reported the presence of glucosamine in an hydrolysate of a fraction derived from dried *A. campestris*. They considered that this sugar was originally bound to a protein. In contrast to the other amino acids, nearly all of the leucine was present as free leucine in the present unhydrolyzed fraction.

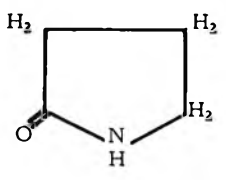
The average molecular weight (206.3) of the mushroom isolate indicates that the components present possess relatively low molecular weights. This value also suggests that the peptide components are not larger than dipeptides. This interpretation is consistent with the negative reaction of the isolate with biuret.

The infrared spectrum of the fraction was well defined. It exhibited absorption frequencies for the significant structures present in the components of the fraction, namely the γ-lactam structure, peptide link, un-ionized carboxyl, hydroxyl and hydrogen bonding. The high-frequency spectral region showed the general absorption characteristics of peptides, and also resembled that of the reference dipeptide, pyrolglutamylglutamic acid monohydrate. The fingerprint region, however, resembled PCA. Important maxima in the spectrum were a) broad, intense bands in the region 3330–3050 cm^{-1} (NH stretching vibration), 1670 cm^{-1} (C = O stretching mode, Amide I) and 1560 cm^{-1} (Amide II). The band at 3330–3050 cm^{-1} showed a split characteristic of dipeptides, b) an NH stretching band (partly overlapped) for the γ-lactam structure at 3170 cm^{-1} (the C = O stretching mode for this structure, however, was overlapped by the C = O frequency of the peptide group) and c) bands for un-ionized COOH at 3000–2500 cm^{-1} (OH stretching mode) partly overlapped at 1320–1210 cm^{-1} (C – O stretching, or OH deformation) and at 955 cm^{-1} (OH deformation out of plane). The C = O vibration mode for this group was also overlapped by the C = O stretching mode of the peptide group.

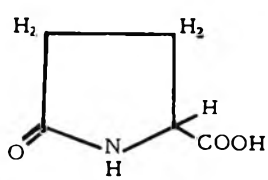
The mushroom fraction gave an ultraviolet spectrum which showed a smooth-rising absorption curve with 2 small inflections (near 260 mμ and 320 mμ) and no maxima. The 260 mμ inflection presumably arises from the benzyl chromophore of the minute amount of free phenylalanine present; the 320 mμ shoulder is probably due to the corresponding dipeptide, pyroglutamylphenylalanine. The mushroom spectrum compared favorably with the spectral profiles of the reference compounds, PCA, pyroglutamylproline and pyroglutamylglutamic acid, all of which gave featureless spectra. The spectrum of the isolate is consistent with the chemical composition of the isolate as revealed by the amino acid analyzer.



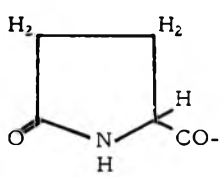
Proline
(I)



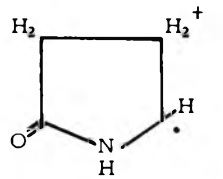
2-Pyrrolidinone
(II)



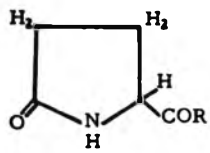
Pyroglutamic acid
(III)



Pyroglutamyl group
(IV)



Pyrrolidinonyl ion
(V)



R = amino acid moiety attached to (IV) through the peptide (amide) link. Only the structures of the more important moieties are indicated below.

Pyroglutamyl dipeptide

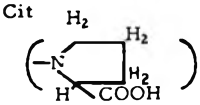
VI

(VII), R = Gly (XI), R = Glu, (-NH·CH(COOH)·CH_2·CH_2·COOH)

(VIII), R = Ala (XII), R = Val

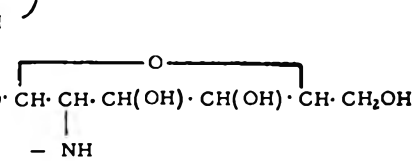
(IX), R = Thr (XIII), R = Leu

(X), R = Asp (XIV), R = Cit



(XV), R = Pro,

(XVI), R = Phe



(XVII), R = 2-Glucosamine, HO·CH·CH·CH(OH)·CH(OH)·CH·CH_2OH (O bridge, NH)

(XVIII), HOOC·$CH(NH_2)$·CH_2·CH_2·CO·NH·CH(COOH)·CH_2·CH_2·COOH

γ-Glutamylglutamic acid

The GLC-TOF-MS examination of the TMS mushroom fraction revealed the presence of 2 compounds whose elution characteristics and mass spectra matched those of the model compounds, TMS proline and TMS pyroglutamic acid. No other compounds were detected on the GLC chromatogram.

The detection of TMS pyroglutamic acid in the GLC-TOF-MS run confirms the presence of PCA in the mushroom under consideration.

The mass spectrum of the TMS mushroom fraction, like those of the reference compounds, was well defined. Table 3 gives a comparison of the percent abundance of the fragment ion of mass 84 (pyrrolidinonyl ion, V) for the TMS

mushroom fraction and its 3 major components. As expected from the structure and fragmentation pattern of standard PCA, the abundance of its 84 peak would be large. By comparison, the relatively high intensity of this fragment ion in the mushroom fraction resulting from electron bombardment indicates the presence of components containing the pyroglutamyl moiety or, if preferred, the cyclic, γ-lactam structure. The assignment of the m/e 84 to the pyrrolidinonyl ion is reported by Heyns et al. (1961), Biemann (1962) and Martin (1965).

Table 4 provides additional evidence for the presence of pyroglutamyl compounds in the mushroom fraction. It is evident that the abundances of the fragments of mass units 99, 127 and 140 are higher in the alkyl-type, straight-chain dipeptide, TMS glutamylglutamic acid, than in the TMS pyroglutamylglutamic acid. Values of the 3 mass ions for the TMS mushroom fraction lie between those of the 2 reference compounds, though closer to the TMS pyroglutamyl dipeptide.

The molecular ion (M+), or (M+–R), is listed in Table 5 for each of 6 components (4 major and 2 minor) present in the TMS mushroom fraction. Spectral correlations with reference compounds were good. The parent masses of TMS pyroglutamylcitrulline (469) and TMS pyroglutamylglucosamine (578) could not be detected. Values of the molecular ions given in Table 5 are in agreement with the calculated molecular weights for the TMS compounds. The excess silylation reagent, present in the test samples, did not interfere with the spectral measurements.

Reactions involved in the enzymatic biosynthesis of a pyroglutamyl dipeptide in mushrooms are unknown. However, plausible mechanism for the formation of this type of compound may be reasonably postulated by taking into account a) the presence of enzymes, free PCA and other amino acids in mushrooms, b) the formation of a peptide (amide) bond when appropriate energy supply is available and c) the analogy that exists in the biological systems of other living tissues (Niwaguchi et al., 1965). Accordingly, it is conceivable that the nucleotide, adenosine triphosphate (ATP), a high-energy compound present in *A. campestris* (Levenburg, 1962; Kritiskii et al., 1963) could activate (phosphorylate) the carboxyl group of PCA to give pyroglutamylphosphate. This anhydride, by virtue of its activated carboxyl group now being susceptible to nucleophilic attack, would react with an amino acid via the α-amino group to form the peptide link and liberate phosphoric acid (an acid present in higher fungi). If glutamic acid (an important and plentiful amino acid in mushroom) happens to be the participating amino acid, then pyroglutamylglutamic acid (XI) would be formed.

The other dipeptides disclosed in this paper could also be accounted for in the same manner. For N-pyroglutamylglucosamine, the coupling would have to occur between the activated carboxyl of PCA and the 2-amino group of glucosamine. The dipeptide-synthetase enzymes needed to catalyze the above phosphorylation and coupling reactions are presently unknown in mushrooms. Presumably they are present, since a) about 20, and possibly more, enzymes exhibiting a variety of catalytic activities have been detected in *A. campestris* and other mushrooms (Miller, 1929; Thoai, 1942; Voinovitch, 1950; Erickson et al., 1960; Latché, 1963) and in most cases identified, b) the existence of dipeptides has been reported (Altamura et al., 1967) in *A. campestris* and c) a dipeptide-synthetase enzyme, γ-glutamylcysteine synthetase, has been found (Snoke et al., 1952) in other tissue.

The proposed mechanism is exemplified as follows, using glycine as the participating amino acid:

1. Pyroglutamic acid + ATP $\underset{\longleftarrow}{\overset{\text{enzyme}}{\longrightarrow}}$ Pyroglutamylphosphate + ADP

2. Pyroglutamylphosphate + glycine $\underset{\longleftarrow}{\overset{\text{enzyme}}{\longrightarrow}}$ Pyroglutamylglycine (VII) + H_3PO_4

Another possible pathway for the enzymatic biosynthesis of a pyroglutamyl dipeptide would be via the γ-lactamization of an alkyl-type, straight-chain α-glutamyl dipeptide.

The wide distribution of PCA in nature (animals, plants, bacteria and fungi) suggests that it could act as a precursor in the biosynthesis of the pyroglutamyl dipeptides in mushroom. Some PCA, per se, may conceivably undergo enzymatic decarboxylation to 2-pyrrolidinone (II), followed by enzymatic hydrolysis of the latter compound to γ-aminobutyric acid, an amino acid, found in substantial quantity in *A. campestris*.

The physiological activities of the pyroglutamyl dipeptides present in mushrooms are as yet unknown. However, they may contribute in some way to the taste or flavor characteristics of the mushroom, possibly as flavor potentiators. From the nutritional viewpoint, the biochemical activity of a pyroglutamyl dipeptide may be altogether different from that of each of its consitituent amino acids, or it may reflect a suppressed activity of 1 or both of its components. For example, in pyroglutamylthreonine (IX) the growth function (McCoy et al., 1935) of the constituent, threonine (an essential amino acid), may be repressed or completely lost. Upon enzymic hydrolysis (in vivo), however, the released threonine would again exhibit its natural biological function. The rate of its release could, of course, influence the magnitude of its function. The liberated PCA would also become available for biochemical reactions.

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POLYPHENOLS OF CASHEW KERNEL TESTA

SUMMARY—The chemical nature of the polyphenols of cashew kernel testa has been determined by means of 2-dimensional paper chromatography and specific reactions. Presence of (+) catechin and (−) epicatechin as the major polyphenols has been confirmed by co-chromatography with authentic samples. Proanthocyanidins have been studied by treatment with hot acid and identification of the resultant anthocyanidins. Leucocyanidins and leucopelargonidins have been noticed as monomeric components, whereas polymeric proanthocyanidins contain in addition small quantities of leucodelphinidin. Individual components have been quantitatively estimated colorimetrically with Folin-Denis reagent, after separation by fractional extraction using solvents of increasing polarity and paper chromatography. The characteristic bluish-black discoloration noticed in cashewnuts has been identified as an iron-polyphenol complex formed during processing. The polypnenols were found to be derived from the testa.

INTRODUCTION

THE KERNEL of *Anacardium occidentale*, valued as cashewnut in trade, is covered with a thin reddish-brown skin, or testa. The testa has been reported to be a good source of the catechol type of tannins (Madhavan Pillai et al., 1963).

During the normal processing of cashewnut in the moisture-conditioning step before roasting, a small percentage of the nuts develop bluish-black patches on the surface. Such nuts do not find ready market, although the taste is unaffected. Prevention or removal of such darkening is bound to help the industry, particularly since India is facing a shortage of raw material for processing. Because of the close contact of the testa with the nut, the water-soluble constituents of the testa may reach into the nut. In this paper we present a systematic examination of the polyphenols of the testa and the influence of these on discoloration of the edible kernel.

EXPERIMENTAL

SAMPLES OF testa and nuts were collected from processing centers in Kerala State. The dried testa showed 42.5% total extractable solids and 32% total phenol by the Folin-Denis method (Swain et al., 1959) when extracted with 50% aqueous acetone.

Extraction and fractionation

Extraction of polyphenolic materials was carried out by 50% aqueous acetone under the conditions described in an earlier communication (Mathew et al., 1964). The extract on freeze-drying appeared as brownish powder and showed little change on chromatographic examination.

For fractionation of testa polyphenols, 0.2 g of the freeze-dried extractives was taken in 75 ml of water. This was extracted successively in a 150-ml separating funnel with 3 lots of 25 ml of ether. The residual aqueous phase was similarly extracted successively with ether-ethyl acetate (1:1), ethyl acetate-ethyl alcohol (4:1) to get different fractions such as 1) ether; 2) ether-ethyl acetate; 3) ethyl acetate; 4) ethyl acetate alcohol and 5) aqueous residue.

Chromatographic procedure

Identification of anthocyanidins formed was as described earlier (Lakshminarayana et al., 1967). The 2-dimensional paper chromatograms on No. 3 papers were developed using n-butanol-acetic acid-water (4:1:2.2 v/v) (BAW) as the first direction solvent and 2% aqueous acetic acid as the second. The chromatograms were sprayed with specific spray reagents (Roux et al., 1960; Govindarajan et al., 1963). The quantitative estimation of individual components was carried out after 2-dimensional separation on No. 3 Whatman paper, marking the areas from another paper sprayed with $FeCl_3$ + $K_3Fe(CN)_6$ and reacting with Folin-Denis reagent (Govindarajan et al., 1963; Mathew et al., 1969).

RESULTS

HOT EXTRACTION of the testa by water, 50% aqueous acetone and acetone showed that the best extraction, with respect to polyphenol content and maximum number of spots on chromatography was achieved by 50% aqueous acetone. All the extracts upon conversion into anthocyanidin (Govindarajan et al., 1965) showed a broad absorption peak at 545 mμ, suggesting that the leucoanthocyanidins are dominated by leucocyanidin and an inflection at 450 mμ, indicating a hydroxyl group at 5-position in the anthocyanidin formed (Jurd, 1962). No free anthocyanidin was noticed in the testa.

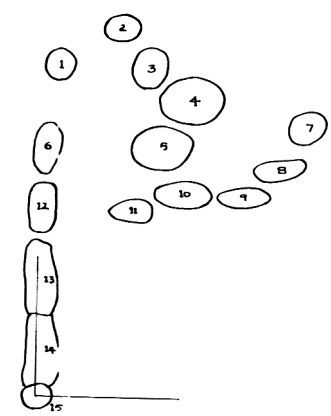

Fig. 1—Two-dimensional chromatogram of cashew kernal testa polyphenols.

↑ First direction BAW, → second direction 2% aqueous acetic acid. Material 0.001 g; spray — $K_3Fe(CN)_6$ + $FeCl_3$.
For details of the spots, see Table 2.

Table 1—Analysis of the anthocyanidin mixture from cashew kernel testa polyphenols.

Band No.	R_f Forestal solvent	R_f Formic Acid solvent	Color	Absorption peak mμ (Ethanol-HCl)	Shift in peak with $AlCl_3$ mμ	Phenolic Acid obtained on alkali fusion	Identity	Approx. % in the mixture
1	0.38	0.14	Violet-pink	560	+30	Gallic Acid	Delphinidin	3
2	0.50	0.24	Pink	540	+20	Protocatechuic Acid	Cyanidin	77
3	0.68	0.37	Red-pink	530	Nil	p-Hydroxy Benzoic Acid	Pelargonidin	15
4	0.81	0.42	Pink	545	+25	–	n-Butyl cyanidin	4
5	0.91	0.50	Red-pink	530	Nil	–	n-Butyl pelargonidin	1

Identification of polyphenols

The anthocyanidin mixture obtained on treatment with hot n-butanol-HCl separated into 5 bands. From the R_f values, color, spectral characteristics and alkaline degradation products their identity could be established as given in Table 1. From the known facts about the formation of additional anthocyanidin, bands 4 and 5 can be taken as n-butyl derivatives of cyanidin and pelargonidin, respectively (Mathew, 1969).

Figure 1 shows the 2-dimensional chromatographic separation of the total cashew kernel testa polyphenols. There were 15 spots or areas, whose properties and identity are presented in Table 2. The ether-extractable spots 3 and 4 were the major mobile components. These were confirmed as (+) catechin and (−) epicatechin, respectively, by co-chromatography with authentic samples from *Acacia catechu* and arecanut (Govindarajan et al., 1963). Spots 1 and 6 showed no mobility in 2% aqueous acetic acid, indicating the planar nature of the molecules (Harborne, 1960). From the color reactions and extractability with ether, these compounds appeared to be flavonol derivatives.

The proanthocyanidin spots 8 to 11 showed good mobility on paper and solubility in ethyl acetate, indicating their monomeric leucoanthocyanidin nature. Since spots 8 and 9 yielded pelargonidin on acid treatment, they were identified as two optical isomers of monomeric leucopelargonidin. Spots 10 and 11 gave cyanidin and hence identified as monomeric leucocyanidins.

Spots 12 to 15 yielded anthocyanidins on treatment with hot acid. They represent proanthocyanidins of increasing polymeric stages as shown by decreasing mobility on paper. Spot 12 appeared to be a simple oligomer from its relatively higher mobility and extractability both with moist ethyl acetate and with ethyl acetate containing alcohol. It was composed mainly of leucocyanidin and minor amounts of leucopelargonidin. Spots 13 and 14 were predominantly leucocyanidin with spot 13 showing traces of leucopelargonidin and spot 14 traces of leucodelphinidin. Spot 15, probably a highly polymerized proanthocyanidin, showed no mobility in either direction. This was composed of leucocyanidin and small amounts of leucodelphinidin. But for the differences in their proanthocyanidin makeup, spots 13, 14 and 15 could have been taken as a single spot.

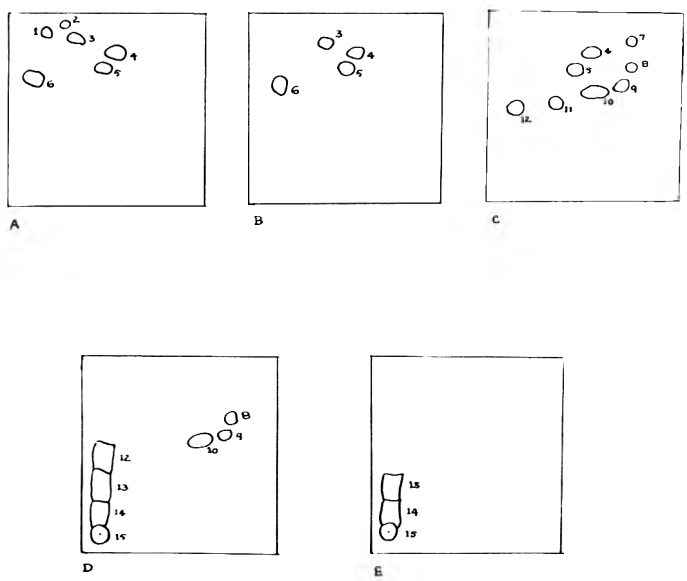

Fig. 2—Chromatograms of cashew kernel testa polyphenols after solvent fractionation.
A—Ether, B—ether-ethyl acetate, C—ethyl acetate, D—ethyl acetate-ethyl alcohol, and E—aqueous residue.
↑First direction—BAW, → second direction—2% aqueous acetic acid.
Estimation as in Table 3.

Table 2—Characteristics of different polyphenolic spots in cashew kernel testa.

Spot No. (Fig. 1)	Intensity with $K_3Fe(CN)_6$ + $FeCl_3$	R_f in BAW	R_f in 2% Acetic acid	Vanillin-HCl test	Leucoanthocyanidin test	Identity
1	Faint	0.80	0.04	Neg.	Neg.	Flavonol
2	Faint	0.89	0.16	Neg.	Neg.	Non-flavalphenol
3	Intense	0.81	0.23	Neg.	Neg.	Non-flavanphenol
4	Very intense	0.72	0.42	Pos.	Neg.	(+) Catechin
5	Very intense	0.62	0.37	Pos.	Neg.	(−) Epicatechin
6	Intense	0.64	0.00	Neg.	Neg.	Flavonol
7	Faint	0.73	0.67	Faintly pos.	Neg.	Flavan
8	Intense	0.58	0.62	Pos.	Pos.	Leucopelargonidin
9	Intense	0.48	0.54	Pos.	Pos.	Leucopelargonidin
10	Intense	0.54	0.41	Pos.	Pos.	Leucocyanidin
11	Faint	0.41	0.27	Pos.	Pos.	Leucocyanidin
12	Intense	0.41	0.0	Pos.	Pos.	Low polymeric proanthocyanidin
13	Intense	0.22–0.34	0.0 Slight streaking	Pos.	Pos.	Polymeric proanthocyanidin
14	Intense	0.0–0.22	0.0	Pos.	Pos.	Polymeric proanthocyanidin
15	Very intense	0.0	0.0	Pos.	Pos.	Polymeric proanthocyanidin

All spots reacted with *bis*-diazotized benzidine and Folin-Denis reagent. Pos. = positive, Neg. = negative.

Quantitative estimation of individual components

The chromatograms of the different fractions obtained after fractionation with solvents of increasing polarity, i.e., ether, ether-ethyl acetate, ethyl acetate, ethyl acetate-ethyl alcohol and aqueous residue, are represented in Figure 2. The individual spots in each of the fractions were then estimated by Folin-Denis reagent and calculated in terms of the total phenol in that fraction (Table 3). Values for similar spots in different fractions were pooled together for calculation of the percentage in the total. It can be seen that by this method the estimation was more accurate, owing to reducing the errors caused by overlapping of spots and avoiding differential elution from paper, as each fraction represented a simpler mixture of compounds of more or less similar adsorption to cellulose. (+) Catechin, (−) epicatechin and polymeric proanthocyanidins represented the major polyphenols of cashew kernel testa.

Table 3—Chromatographic estimation of polyphenolic spots in cashew kernel testa after solvent fractionation.

	Amount in grams out of 0.2 g of total polyphenols						
Spot No. (Table 2)	I Ether	II Ether-ethyl acetate	III Ethyl acetate	IV Ethyl acetate-alcohol	V Aqueous residue	Total	% Of total
1	0.0030	–	–	–	–	0.0030	1.20
2	0.0010	–	–	–	–	0.0010	0.40
3	0.0067	0.0002	–	–	–	0.0069	2.77
4	0.0145	0.0245	0.0075	–	–	0.0462	18.53
5	0.0171	0.0286	0.0132	–	–	0.0589	23.63
6	0.0027	0.0051	–	–	–	0.0078	3.13
7	–	–	0.0012	–	–	0.0012	0.48
8	–	–	0.0029	0.0024	–	0.0053	2.13
9	–	–	0.0036	0.0040	–	0.0076	3.15
10	–	–	0.0110	0.0040	–	0.0150	6.02
11	–	–	0.0066	–	–	0.0066	2.65
12	–	–	0.0062	0.0100	–	0.0162	6.50
13	–	–	–	0.0090	0.0082	0.0172	6.90
14	–	–	–	0.0066	0.0171	0.0237	9.51
15	–	–	–	0.0041	0.0287	0.0328	13.12

Discoloration of polyphenols

Preliminary examinations showed that testa polyphenols can form a dark-colored complex with iron. Thus, soaking good kernels in polyphenolic extract followed by dipping in ferric chloride solution resulted in discoloration similar to normal darkening. In normal darkening, the discoloration is more against the veins on the periphery of the kernel, where there is close contact of the nut with the testa. Thus, the nature of the surface of the nut also is important. More deformed and undeveloped nuts are more liable to get darkened by the above mechanism than are normal, healthy nuts. Dipping the kernels in iron solution alone did not give discoloration, since kernels themselves do not contain polyphenols.

Washing with dilute acid solutions removed the bluish-black patches in normally darkened nuts; treatment with alkali resulted in a darker brown color. The ether extract of the acid wash showed polyphenolic characteristics on treatment with different spray reagents. Among the different acids tried for removing the discoloration, hydrochloric and citric acids were found to be the most convenient, though each left behind a taste of its own. Estimation of iron content showed that batches of darkened nuts have an iron content of approximately 4.5 mg % compared to 3 mg % of good nuts (Farrer, 1935).

DISCUSSION

IN CASHEWNUT testa, as in most other plant products, leucocyanidin was found to be the predominant leucoanthocyanidin (Bate-Smith, 1954). However, leucopelargonidin was found to occur in larger amounts than leucodelphinidin. This observation, also the presence of leucopelargonidin in other plant materials tested, will prove it is a much more commonly occurring plant constituent than has been supposed (Swain, 1962).

Chromatographic studies show that the main constituents are (+) catechin and (−) epicatechin, which account for about 6 and 7.5%, respectively, of the dried cashew kernel testa. Together they represent more than 40% of the total polyphenols. The polymeric proanthocyanidins form a little less than 40%. The monomeric proanthocyanidins are represented by 2 leucocyanidins and 2 leucopelargonidins, but no leucodelphinidin. Kantamoni (1965) had recently reported the presence of gallic, caffeic and quinic acids apart from a catechin and a leucocyanidin. Gallic acid if present would have occupied the position of spot 4; however, this spot showed full reactivity with vanillin-HCl, suggesting that it is entirely composed of (+) catechin. Furthermore, no gallic acid was detected by the bicarbonate extraction of the ether fraction. Tests for caffeic acid (fluorescence) and quinic acid also were negative.

From the studies it is clear that the black discoloration is due to the complex formation between polyphenol and iron. While the polyphenol is derived from the testa, the source of iron cannot be so clearly established. It may either be from the nut itself, since nuts as a class are found to be fairly rich in iron, or from external sources during processing. The discolored nuts show a higher iron content, which may indicate either absorption of iron from external sources or a higher iron content in nuts susceptible to darkening. Discoloration involving a heavy metal-like iron and polyphenols has been noticed in other cases also (Bate-Smith, et al., 1958; Donath, 1962; Chandler, 1964). That the formed pigment is easily washed away by dilute acid further indicates that the darkening in cashew nuts is probably due to polyphenol-iron complex.

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POST-MORTEM ISOMETRIC TENSION CHANGES AND SHORTENING IN TURKEY MUSCLE STRIPS HELD AT VARIOUS TEMPERATURES

SUMMARY—A study of the physical changes associated with rigor mortis in breast muscle was undertaken to assess the factors that may influence ultimate tenderness. Isometric tension changes and shortening were measured at temperatures 2–37°C. These changes were measured while holding the muscle strips in a phosphate buffer, pH 7.2. Isometric tension was measured by transducers and recorded on a physiograph. A pattern of tension development and gradual relaxation has been demonstrated to occur post-mortem in strips of turkey breast muscle held isometrically. The time to maximum tension development occurs in 3.85 ± 0.19 hr and is not linearly related ($P < .05$) to temperature. The amount of maximum tension developed averaged 25 g/cm^2 and was significantly ($P < .05$) related to temperature. Relaxation to about 50% of maximum occurs in 18 hr. The amount of shortening that occurs post-mortem is linearly related ($P < .01$) to temperature. No "cold shortening" of turkey breast muscle was evident.

INTRODUCTION

RAPID processing with emphasis on continuous chilling is the trend in the poultry industry. After evisceration the birds are placed in a series of mechanical chillers that reduce carcass temperature to 40°F if desired. The processing step of overnight aging in slush ice is rapidly being omitted; the live bird is transformed into frozen meat in a few hours. There are several advantages in handling the product quickly; namely, the maintenance of quality from a microbiological standpoint and the economy afforded in time, labor and space requirements. However, there has been some concern expressed about the effect of rapid chilling and freezing on meat-quality factors, specifically tenderness. Traditionally, it was thought that adequate aging of the carcass must precede freezing to insure tenderness. Although additional muscle relaxation has been demonstrated to occur upon thawing (Klose et al., 1959; 1961a; 1961b), the tenderness of continuously chilled birds has been questioned. The problem is most apparent with young turkeys of the fryer-roaster classification. The turkey fryer exhibits more variability in tenderness and requires longer aging time (Scholtyssek et al., 1967). The rapid process of continuous chilling and immediate freezing without an intermediate aging period is not sufficient to ensure adequate tenderness (Marion et al., 1967).

The biophysical or structural state of muscle is quite relevant to meat tenderness. Lowe (1948) observed that poultry muscles severed soon after death had higher shear values than those aged intact on the carcass. Lowe also suggested an association between shortened or contracted muscles and toughness of meat. More recent work using the bovine species has firmly established this relationship (Marsh, 1964; Herring et al., 1965; Marsh et al., 1966; Howard et al., 1968). Conversely, muscles aged in a restrained or stretched condition are more tender (Partmann, 1963; Herring et al., 1967; Buck et al., 1967). Goll et al. (1964) demonstrated distinctive tenderness patterns for muscles excised and those attached to the carcass. The primary differences are those attributable to the shortening that takes place post-mortem in the excised muscle. Muscles left attached to the carcass are restrained from shortening and tenderize more rapidly when held at the same temperature.

Recent work on bovine and porcine species has used the isometric tension pattern to follow the sequence of physical changes in rigor (Jungk et al., 1967; Busch et al., 1967; Schmidt et al., 1968). The pattern has been shown to closely parallel the tenderness cycle and illustrates the tendency of a muscle to shorten. This study was undertaken to elucidate the physical changes occurring post-mortem in turkey muscle and to assess the influence of temperature on these changes.

EXPERIMENTAL

Source and sample preparation

The birds used in this study were from the University research farm. They included 15 mature Bronze hens from a breeder flock and 25 Large White males. Within each group, the birds had similar genetic backgrounds, had been raised under similar conditions and did not vary appreciably in weight. Exsanguination was accomplished by severing the jugular vein and the carotid artery, the birds being inverted in a metal cone during this process to minimize struggling. No prior stunning or anesthetic was used. Immediately after exsanguination, the skin was incised and a large portion of the pectoralis major removed. Then strips of muscle were dissected parallel to the direction of the muscle fibers. These strips were used in the tension and shortening studies.

Shortening measurements

Muscle strips were measured and placed in a phosphate buffer, pH 7.2, previously adjusted to the desired temperature. The time required to dissect and measure the strips limited the number of temperatures to be studied when an

individual bird was used. Usually 6 temperatures with 5 strips per temperature were used for each. The maximum time from start of exsanguination to placement of the muscle strips in the environmental temperature was 15 min. The tabulated data are expressed as a percentage of shortening from resting length.

Tension measurements

The initial isometric tension measurements on 7 birds were made using a device called an isometer, previously described by Jungk et al. (1967). This instrument was designed to measure post-mortem tension development in a muscle strip held at constant length, thereby simulating the conditions of muscles aging on the carcass. The sensitivity and capacity of this method of measuring rigor has been increased through the use of isometric myographs in conjunction with an E & M Physiograph. Isometric tension patterns were run sumultaneously on 5 excised strips of pectoralis major from each bird; 30 turkeys were measured using the isometric myographs. The muscle strips were immersed in a phosphate buffer, pH 7.2, to prevent spurious tension pattern results due to drying of the strips in air.

RESULTS & DISCUSSION

Isometric tension pattern

A pattern, shown in Figure 1, of tension development and relaxation can be demonstrated while holding muscle strips isometrically. There are 3 characteristics of this pattern that warrant elaboration. They are a) the time lapse between death and maximum tension, b) the amount of tension at maximum and c) the relaxation phase.

The time lapse before maximum tension is important, because during this period the muscle is attempting to shorten. In the animal, since the muscles are attached and prevented from shortening, the antagonistic forces produce stiffness in the carcass. This stiffness is a common attribute of post-mortem muscle. Soon after death, if the muscles are severed or completely excised, they will shorten. In light of the known association between shortening and tenderness, it would seem that conditions conducive to minimum shortening would allow the most tender meat. Also, the long aging might be reduced if it were possible to know the earliest time at which the muscles could be excised without adverse effects on tenderness.

In observing 121 strips from 30 turkeys, we found the average time to maximum tension to be 3.85 ± 0.19 hr. The experiments were conducted at various temperatures 2–37°C. From the regression analysis as shown in Figure 2, no significant ($P < .05$) linear relationship between temperature and time to maximum was observed. This is unusual in that many of the other parameters of rigor (e.g., extent of shortening, loss of extensibility, degradation of ATP and decline in pH) are closely associated with environmental temperature.

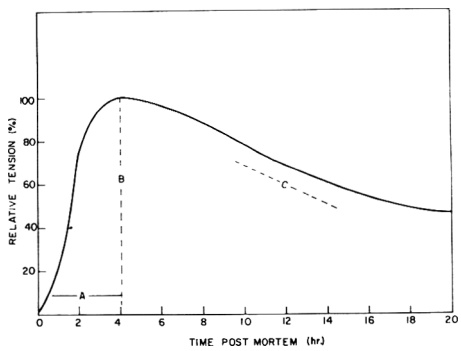

Fig. 1—A typical isometric tension pattern showing: A, the time to maximum; B, maximum tension and C, the relaxation phase.

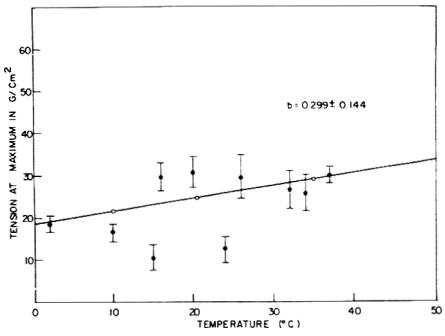

Fig. 3—Effect of environmental temperature on the amount of tension developed in strips of turkey breast muscle held isometrically.

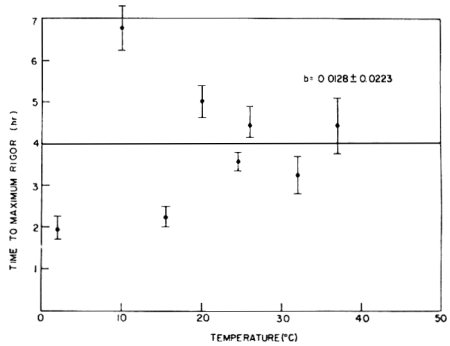

Fig. 2—The lack of effect of environmental temperature on time to maximum rigor.

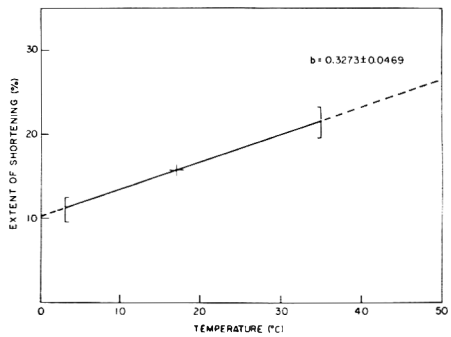

Fig. 4—Effect of environmental temperature on shortening of excised strips of pectoralis major.

The amount of tension that develops post-mortem is small when compared with normal contraction. The maximum strength of tetanic contraction of a muscle operating at normal muscle length is about 3 kg/cm^2 (Huxley, 1958). The tension developed post-mortem was only 24.78 ± 1.45 g/cm^2, or about 1% of the maximum strength for normal tetanic contraction. Temperature of aging environment does influence the amount of tension that develops. This significant ($P < .05$) relationship is shown in Figure 3. It is further substantiated by a similar temperature effect on shortening of excised strips, which will be discussed further. Tension development reflects the attempted shortening, similar to that which would occur on an intact carcass.

The relaxation phase is a characteristic of the post-mortem isometric tension pattern demonstrable at all temperatures. The relaxation phase was evaluated by selecting a common time post-mortem for comparison of the isometric tension curves. The tension values at 18 hr were used for this purpose and expressed as a percentage of the maximum. The mean value was 51.94 ± 2.60% of maximum. No consistent relationship with temperature was found. This phase of the isometric tension pattern bears further scrutiny since it is during this relaxation phase that meat becomes more tender. This conclusion is supported by the parallel pattern of tenderness changes and isometric tension decline (Busch et al., 1967).

Shortening experiments

10 turkeys were used in this facet of the experiment. Muscle strips were excised and placed in environmental temperatures ranging 3–37°C. The percentage shortening at each temperature was noted. From the 263 individual observations, a linear regression analysis was prepared. Figure 4 presents results of this analysis. Special emphasis was made to ensure adequate sampling in the temperature range 10–20°C. It is in this range that a cold-shortening phenomenon has been demonstrated in both the mammalian (Locker et al., 1963) and the avian species (Smith et al., 1969). Our data do not show a cold-shortening effect, but rather a significant ($P < .01$) linear relation between extent of shortening and temperature. This is in conflict with results of Smith et al. (1969). Our experiments were conducted on excised strips of muscle over the temperature range 4–35°C. The previously reported work was done at 0 and 16°C on intact sections of pectoralis major, measured and then excised. Excision of muscles soon

after death stimulates some shortening. This mechanically induced shortening was not included in our data. Such shortening would not normally be imposed on an aging carcass, whereas the thermal stimulus would be.

Final shortening measurements were taken at 5 hr. Most of the shortening was completed within 3–4 hr. This is consistent with results from the isometric tension pattern, showing time to maximum tension to be 3.85 hr.

In summary, the concept of postmortem aging of the carcass to allow tenderization to occur is being changed. The interest is now in controlling the state of contraction of the muscle rather than just allowing sufficient time for it to relax. New processing methods are being devised that will minimize muscle contraction and maximize relaxation. However, they must be amalgamated with conditions maintaining the other quality factors of meat.

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BENZO(A)PYRENE IN SMOKED MEAT PRODUCTS

SUMMARY—A procedure was established for analyzing benzo(a)pyrene (BaP) in smoked meat products, which involves extraction, solvent partition, column chromatography and spectrophotofluorometric measurement. Studies were then made as to the effect of artificial casings on the content of BaP, penetration of BaP through a meat product in smoking, possible presence of the hydrocarbon in unsmoked meats and distribution of the compound after cooking smoked meat. Bologna was used in most experiments except for the one on cooking effect, in which bacon was used. Artificial cellulose casings caused a noticeable reduction in the concentration of BaP. Regardless of whether the smoke was generated in a small-scale or in a larger-scale generator, bologna smoked in casings had much less BaP than that smoked without casings. BaP was concentrated in the outer part of bologna. In smoking with or without casings, the hydrocarbon did not penetrate farther, in most cases, than 1.4–1.6 mm from the surface of the bologna. Several unsmoked meats of beef and pork were analyzed and none seemed to have endogenous BaP, since the detected amount was similar in magnitude to that of the solvent control. More than half the amount of BaP in smoked bacon was found in the fat drippings following cooking.

INTRODUCTION

PRESERVATIVE action, flavor and color have been considered as the major effects of smoking food (Draudt, 1963). With the development of other methods of preservation, foods now are smoked mainly for their sensory qualities.

The presence of carcinogenic polycyclic hydrocarbons in wood smoke has been recognized (Berankova et al., 1953; Hollenbeck et al., 1963; Tilgner, 1958; Gorelova et al., 1961). During the last decade, interest has been increasing in the analysis of smoke and smoked food products for carcinogenic hydrocarbons. Different levels of carcinogens have been found in smoked foods, the amounts being related to many factors, including method of smoke generation, temperature of combustion and oxidation, air supply, length of smoke ducts and density and temperature of the smoking operation (Tilgner, 1968). Because of the historical origin of the problem—the discovery of the first carcinogenic constituent of coal tar, benzo(a)pyrene (BaP) or 3, 4-benzopyrene—much attention has been focused on this polycyclic hydrocarbon.

It has been an open question whether any carcinogenic hydrocarbon can be found in the smoke generated under production conditions in the United States. The most common method currently used in the United States for producing smoke for food processing is burning dampened sawdust in a batch operation (Draudt, 1963). Genest et al. (1964) analyzed smoked fish, frankfurters and cheese for the presence of BaP by a detection method they developed with a reported sensitivity of 10–50 ppb; BaP was not detected. Lijinsky et al. (1965) reported the presence of trace amounts of BaP in smoked fish and of other polycyclic compounds in several liquid-smoke preparations and bacon. Although using an experimental smoke generator and dry sawdust, the authors have found BaP in smoke of hardwood (predominantly maple) sawdust (Rhee et al., 1968).

The present study was undertaken to explore some aspects of smoked meat products in relation to the content of BaP. BaP was chosen as an "arbitrary indicator" of the presence of carcinogenic polycyclic hydrocarbons based on the observation with cigarette smoke that there is a correlation between the concentration of BaP and "tar" tumorigenicity (Wynder et al., 1968). In addition most, if not all, other polycyclic hydrocarbons are pyrosynthesized in wood-smoke production at a favorable generation temperature. Bologna was used as a typical smoked-meat product in this study, unless otherwise indicated, due to its suitability for our study and its homogeneity for sampling.

EXPERIMENTAL

Solvents

All solvents except cyclohexane and nitromethane (practical grade) were reagent grade. Each solvent was purified by treating with activated charcoal followed by distillation (Crosby et al., 1966).

Preparation of smoked bologna

A bologna emulsion was prepared with the following ingredients, in pounds: regular pork trimmings, 9.2. cutter beef trimmings, 9.2. nonfat dried milk, 0.9. and ice; 5.9. And in grams: sodium nitrate, 1.4. sodium nitrite, 1.4. sodium ascorbate, 4.0. sugar, 32.6. and salt (NaCl), 152.0.

The emulsion was stuffed into No. 5 Tee-Pak Clear Zip cellulose casings (Tee-Pak, Inc.). When precooking was necessary to smoke the bologna emulsion without casings, the emulsion was first stuffed into a casing and cooked in water in a steam-jacketed kettle until the internal temperature of bologna reached 145°F (usual cooking time was 2.5 hr). The cooked bologna was showered with cold water to an internal temperature of 90°F, and processed in a smokehouse after removing the casing from the experimental sticks.

The bologna, with or without casings, was processed in an air-conditioned smokehouse having about 5 air changes per minute. Smoke was generated from smoldering dampened hardwood (predominantly maple) sawdust by the smokehouse generator or from dry sawdust by an experimental generator (Porter et al., 1965) connected to the smokehouse. A temperature of 140°F and 23% relative humidity were maintained for the first 2 hr of processing. The temperature was then raised to 160°F with a relative humidity of 31% until the bologna reached an internal temperature of 128°F. The smokehouse temperature was then maintained at 160°F by injecting high-pressure steam, which resulted in 69% relative humidity until an internal bologna temperature of 152°F was attained. The bologna was showered with cold water until the internal temperature was reduced to 90°F, then stored in a 36°F cooler for a few days until sampled.

An extreme outside layer of 1.4–1.6 mm was removed from the sticks of bologna for BaP analysis and this layer designated as the "A" layer. For the experiment in which penetration of the hydrocarbon was studied, an inner layer (1.4–1.6 mm) next to the "A" layer was also removed and called the "B" layer. Bologna samples were ground several times through a 1.5-mm plate of a meat grinder.

BaP analysis

Extraction. A 150-g sample of the ground bologna was thoroughly mixed with 300 g of anhydrous Na_2SO_4 and extracted three times with benzene (300 ml each) and twice with methanol (300 ml each) by refluxing for 30 min after each extraction. The aqueous phase of the benzene extract, if any, was separated. The methanol extract was filtered over a sufficient amount of anhydrous Na_2SO_4. Benzene and methanol were removed from the extracts under reduced pressure using a flash evaporator connected to an aspirator, at a water bath temperature of about 50°C. Concentration of a solution or evaporation of any solvent was as just described, unless otherwise indicated.

Solvent partition. Polynuclear compounds were separated from the bulk of aliphatic material by solvent partition (Lijinsky et al., 1964). The fatty extract residue was dissolved in 100 ml of hexane and shaken with 2, 100-ml portions of nitromethane. The nitromethane layer was separated and concentrated to dryness (I). The extraction of polynuclear aromatic material from the hexane solution was completed with 3, 40-ml portions of dimethylsulfoxide.

To this extract were added 150 ml of hexane and 280 ml of water; the hexane layer was separated, combined with the nitromethane extract residue (I) and concentrated to dryness (II). For samples of very high fat content such as bacon, the amount of solvent or aqueous medium was doubled.

Further removal of impurities by washing. The extract (II) was dissolved in 100 ml of cyclohexane and washed with 110 ml of 90% methanol. The methanol phase was again shaken with 100 ml of fresh cyclohexane and the 2 cyclohexane extracts combined (Grimmer et al., 1965). The cyclohexane solution was washed with 2 N H_2SO_4 (3 times), water, 2 N NaOH (3 times) and water (Cooper et al., 1955) and dried over anhydrous Na_2SO_4. The washed cyclohexane solution was concentrated to dryness and made to a volume of 2 ml with cyclohexane for column chromatography.

Column chromatography. Silicic acid (Mallinckrodt) was washed with acid and activated as described by Grimmer et al. (1965), then partially deactivated by adding 8% (v/w) distilled water. The extract (ca. 2 ml) was applied to a silicic acid column (12 g, 15 mm i.d.) packed with cyclohexane. The column was eluted with cyclohexane. The first 300 ml effluent was collected for BaP determination. During elution the column was protected from light.

Fluorometric determination of BaP

The cyclohexane effluent was concentrated to near dryness and quantitatively transferred to a graduated test tube using cyclohexane and the final volume reduced to 1–3 ml under a stream of nitrogen. The emission spectrum was determined in cyclohexane at the excitation wavelength 381 mμ. An Aminco-Bowman spectrophotofluorometer was used with the following settings: sensitivity 50, slit arrangement No. 2, meter multiplier (MM) 0.1–0.03 and phototube IP 21. The concentration of BaP was calculated from the 403 mμ peak by the "base line" method (Cooper, 1954).

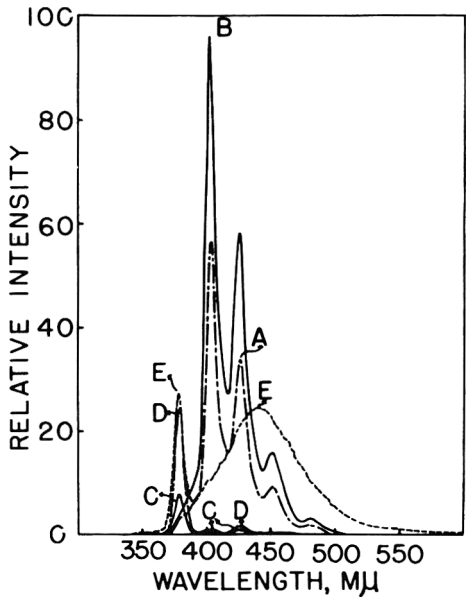

Fig. 1—Emission spectra of cyclohexane column effluent fractions of 12 μg BaP at excitation wavelength 381 mμ. A: 1st 50-ml fraction, MM = 0.1; B: 2nd 100-ml fraction, MM = 0.1; C: 3rd 100-ml fraction, MM = 0.03; D: 4th 100-ml fraction, MM = 0.01; E: 5th 70-ml fraction, MM = 0.01. The final volume of each fraction for spectrum determination was 8 ml.

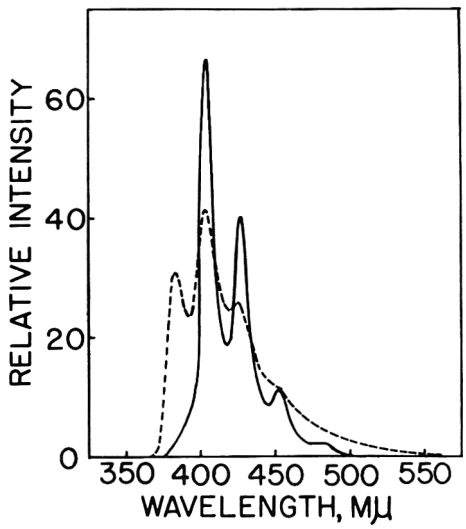

Fig. 2—Emission spectra in cyclohexane at excitation wavelength 381 mμ. Authentic BaP (—): 2 μg/ml, MM = 0.3; the cyclohexane effluent (300 ml) of a bacon sample (100 g) (----): 1 ml final volume, MM = 0.1.

RESULTS & DISCUSSION

Analysis of BaP in meat products

The procedure for analyzing BaP has been modified from that used in the previous experiments of wood smoke (Rhee et al., 1968). Fats were removed from the meat extract by solvent partition, but the TLC step for removing the oily substance(s) in wood smoke of unknown identity and the final alumina column chromatography were omitted. Since the amount of smoke deposited on the smoked product was relatively small as compared with the smoke concentrate we had previously, the oily material associated with smoke did not cause any significant problem, making elimination of the TLC step possible. The polycyclic hydrocarbons present in greater quantities found in the hardwood sawdust smoke are noticeably volatile as shown by evaporation to dryness and much can be lost during the many steps of evaporation or concentration to dryness, at a bath temperature of 50°C or higher (Grimmer et al., 1965; Rhee et al., 1968). In addition, of the hydrocarbons found in wood smoke, only anthracene has an excitation wavelength beyond 370 mμ, but this compound is one of the relatively volatile hydrocarbons and can be mostly volatilized, with the quantity present in smoked foods, during the steps of evaporation to dryness. There is no drying loss of BaP (Grimmer et al., 1965). Therefore, determination of a BaP emission spectrum at excitation wavelength 381 mμ is unlikely to be interfered with by the other polycyclic hydrocarbons of wood smoke. Taking these facts into consideration, the fractionation step of alumina column chromatography could also be eliminated. Thus, the over-all procedure for determining BaP in a meat product includes extraction with benzene and methanol; fat removal by solvent partition using hexane, nitromethane and dimethylsulfoxide; washing the resulting extract with methanol, acid and alkali; silicic acid column chromatography and finally fluorometric determination of the column effluent. With 12 μg of authentic BaP, collection of 250 ml of cyclohexane from the time of sample introduction to the column has proved sufficient for complete elution of the BaP as shown in Figure 1, since there was no 403 mμ peak from the 4th or "D" fraction. The illustration also shows that further elution can contaminate the effluent with unknown impurities, presumably originating from silicic acid. Thus, about 300 ml of cyclohexane effluent were collected from the column for BaP elution in all experiments. Figure 2 presents an emission spectrum of a smoked bacon sample which passed through the above over-all procedure of BaP analysis. Recovery of added BaP through this procedure was satisfactory, resulting in about 80% when 1.0–4.5 μg of the hydrocarbon were added to 150 g of bologna emulsion.

BaP in unsmoked meats

Attempts were made to determine whether the unsmoked bologna emulsion or meat mixture itself contained BaP. A meat mixture was prepared with half pork and half beef trimmings as used for bologna formulation. In analyzing several samples, neither meat mixture nor bologna emulsion appeared to have any endogenous BaP, because the indicated amount of 0.013–0.027 μg/150 g sample or 0.09–0.18 ppb (on wet weight basis) was similar in magnitude to that of the solvent control (0.014 μg).

Effect of casings on BaP permeation

The effect of use of casings on the content of BaP in smoked bologna has been explored. For this study, bologna emulsion was precooked in casings, in 2 or 4 sticks at one trial, and the casings of 1 or 2 precooked bologna sticks were removed before smoking. The other half of cooked bologna was smoked with casings. Using artificial cellulose casings (No. 5 Tee-Pak Clear Zip), it was found that the casing

impeded the transfer of BaP in smoking bologna to a great extent (Tables 1 and 2).

Since much heavier smoke was produced by the smokehouse generator than by the experimental generator, the amount of hydrocarbon absorbed by the "A" layers of bologna sticks differed accordingly. In all cases, regardless of whether the smoke was generated in a small-scale or in a larger-scale generator, bologna smoked in casings had much less BaP. The results expressed are based on dry weight of bologna to balance the differences in moisture content of the samples. The cellulose casing itself did not contain BaP.

It may be noted that whereas α-cellulose is fundamentally crystalline, regenerated cellulose (used for artificial cellulose casings) is fundamentally a gel which shares the properties of other gelatinous materials which are hydrophilic (Tuwinner, 1962). As the permeability of membranes appears to be related directly to swelling and imbibition, it is reasonable to find that BaP, which is hydrophobic and has a high molecular weight, still penetrates in small quantities through the hydrophilic cellulose casing. This is especially true when the BaP concentration outside the casing, i.e., in the smokehouse, is high. Elevated temperatures and humidities would also help the penetration by causing the cellulose casing to swell. Unlike BaP, Simon et al. (1966) reported that cellulose casings did not act as a barrier to smoke components such as acids, carbonyls and phenolic compounds which contribute to smoked flavor and color.

Depth of BaP penetration

Since a preliminary study with a bacon sample showed that whole bacon had much less BaP than outer bacon layers (2 layers taken from flesh side), further exploration was made with smoked bologna to determine the extent of BaP penetration through the product in smoking.

The BaP content of the "A" layer (the outer layer) of bologna was compared with that of the "B" layer (next to the "A" layer). After smoking with or without casings and holding for a few days, there was practically no penetration of the hydrocarbon beyond the "A" layer (Table 2). This is not surprising for a compound of this molecular weight. Phenolic compounds in wood smoke have been found to penetrate a considerable depth into bologna (Bratzler et al.; 1969).

Although Tilgner (1968) indicated that some 38% of BaP of smoked products diffused into the inner parts, detailed information was not given.

BaP in commercial bologna

It was thought that the bologna prepared in our laboratory might differ in BaP content from commercial bologna, since the processing conditions may vary. The determination of BaP content of commercial bologna should give some practical information and should allow for comparisons with our products.

Two well-known brands of commercial bologna were chosen. The outside appearance (casing) of the two brands indicated very little smoking and the sliced bologna did not have an easily detectable smoke aroma. BaP content of the "A" layer and of a whole slice was determined.

BaP values of the two brands were nearly similar for both the "A" layer and a whole slice as shown in Table 3. As compared with our products in casings (Experiments 1, 2 and 3 of Table 1), these "A" layer values are much smaller. Although the processing conditions of the 2 commercial bands were not known, it is reasonable to assume from the results of BaP analysis that these commercial products were processed with little, if any, smoking. BaP values of whole slices were actually similar to those of the solvent

Table 1—Benzo(a)pyrene content of "A" layers of bologna smoked with and without casings.

Experiments	BaP in "A" layer (ppb, on dry wt basis)	
	With casing	Without casing
Experiment 1 (smokehouse generator)	4.6	11.0
Experiment 2 (smokehouse generator)	4.5	12.1
Experiment 3 (smokehouse generator)[1]	1.2	6.6
	1.7	8.4
Experiment 4 (experimental generator)	0.5	4.8
	0.9	5.2

[1] Results of Experiment 3 are of lower values because the thickness of each layer was accidentally 2.2–2.4 mm instead of 1.4–1.6 mm, the routine thickness.

Table 2—Penetration of benzo(a)pyrene through bologna layers.

Experiments	Use of casing in smoking	BaP (ppb, on dry wt basis)	
		"A" layer[1]	"B" layer
Experiment 1 (experimental generator)	With	0.5	0.5
		0.9	0.4
	Without	4.8	0.5
		5.2	0.5
Experiment 2 (smokehouse generator)	With	1.2	0.4
		1.7	0.5
	Without	6.6	0.9
		8.4	0.9

[1] The data on "A" layer of this table and those of Experiments 3 and 4 of Table 1 are identical.

Table 3—Benzo(a)pyrene in commercial bologna.

Bologna Samples	Ingredients	BaP (ppb, on dry wt basis)[1]	
		"A" layer	Whole slice
Brand A	Beef and pork, water, nonfat dry milk, dextrose, salt, corn syrup, flavorings, sodium erythorbate, sodium nitrate, sodium nitrite	0.65	0.26
Brand B	Beef and pork, water, beef hearts, salt, dried corn syrup, dextrose, flavoring, sodium erythorbate, sodium nitrate, sodium nitrite	0.73	0.30

[1] Average of two sticks.

control if expressed on wet weight basis (0.017–0.021 μg/150 g sample); these figures were also very close to those of unsmoked meats mentioned previously.

Distribution of BaP after cooking bacon

Since polycyclic hydrocarbons are soluble in most fat solvents, and smoked products having a fatty flesh had a higher content of BaP than did lean products (Tilgner, 1968), tests were made to determine the loss of BaP from bacon during cooking. Cooking bacon without adding any water, either in a fry pan or in an oven, is actually a process of fat rendering, and much BaP in smoked, uncooked bacon might be in the fat drippings.

In the first experiment, a bacon slab was smoked in our laboratory for about 8 hr and resmoked for an additional 3 hr maintained under conditions similar to those for bologna processing, and the skin removed. Thin slices (about 2-mm thickness) of the anterior and of the posterior half of the smoked bacon slab were analyzed. If the second experiment, a commercial skinless smoked bacon slab was obtained and resmoked in our laboratory for 7.5 hr. Taking the posterior half, odd-numbered slices were used for cooking and even-numbered slices for analyzing uncooked bacon. Cooking was done in a broiler pan at the broiling temperature of an electric oven until the bacon became crisp. After cooking, the fat drippings were drained and the broiler pan rinsed with hexane. The hexane washing was combined with fat drippings and the solvent evaporated by a flash evaporator. Both the cooked bacon and fat drippings were weighed. The crisp cooked bacon was thoroughly crushed and ground in a mortar, since the meat grinder was ineffective in grinding cooked bacon.

Results are shown in Table 4. BaP in smoked bacon was found in both cooked bacon and fat drippings. Starting with 500 g of uncooked bacon, the fat drippings had more BaP than cooked bacon. This may have some practical implication. In many homes, bacon fat is reused for cooking other foods. By discarding the fat, the amount of BaP ingested from smoked bacon can be reduced at least half.

Table 4—Benzo(a)pyrene in the cooked bacon vs. fat drip.

Samples[1]		Weight originating from 500 g uncooked bacon (g)	μg BaP in given weight in the left column
Bacon-I anterior	Cooked bacon	237.2	0.59
	Fat drippings	179.4	06.3
Bacon-I posterior	Cooked bacon	253.8	0.42
	Fat drippings	163.2	0.47
Bacon-II posterior	Uncooked bacon	500.0	1.85
	Cooked bacon	151.6	0.70
	Fat drippings	244.6	0.94

[1]Bacon–I: Bacon slab smoked in the laboratory for about 8 hr in the first smoking and resmoked for 3 hr; skin removed afterward for slicing. Bacon-II: Commercial bacon slab resmoked in laboratory for 7.5 hr.

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SEROLOGIC IDENTIFICATION OF SPECIES OF ORIGIN OF SAUSAGE MEATS

SUMMARY—A partially purified immunoglobulin G (IgG) solution prepared from the serum of a species to be tested was heated to the specifications for sausages. The resulting supernatant fluid was decanted and the precipitate washed with saline and used to immunize rabbits. The supernatant fluid was used to sensitize tanned sheep red blood cells. The immune serum was rendered monospecific by absorptions with heterologous, heated IgG precipitates. A sample of monospecific immune serum was absorbed with a washed homogenate of sausage. Aliquots of the monospecific immune serum, both untreated and sausage absorbed, were tested with cells sensitized with the homologous heated IgG supernatant fluid. A significant reduction of titer by sausage absorption indicated that the sausages contained the meat homologous to the immune serum.

INTRODUCTION

IDENTIFICATION OF the species of origin of heated ground meat samples is an obvious necessity for effective control of such products as frankfurters. While adulteration of unheated ground meats can be easily detected by serologic methods using species-specific immune sera, application of similar methods to heated meats has been only partially successful.

Serologic testing for adulteration of heated meats has been limited almost exclusively to the precipitin technique. Yet meat proteins heated in sausage preparation coagulate and become insoluble, so that they cannot be used in the precipitin test. Instead, a saline extract of the heated meats has been used, usually in combination with an anti-heated whole serum. The homology of such a system is questionable because the immunogen is insoluble heated proteins and the test antigen is the soluble portion of heated meat. Further, the whole serum immunogen contains many protein types with varying degrees of species specificity. These factors led to the failure of earlier specificity tests.

Nuttall (1904) used the precipitin test on blood samples representing 500 animal species and found specificity to be relative rather than absolute. However, workers continued to apply the precipitin techniques to the problem of species specificity until Boyden (1953) showed more conclusively that such techniques were inadequate for measuring serological correspondence.

Early studies on identification of heated meat, heated meat extracts or heated serum proteins involving anti-native whole serum were unsuccessful (Furth, 1925; Pinto, 1961; Ginsberg, 1948). Tests using heated immunogens found greater success. Furth (1925) reported that a serum sample heated to 100°C elicited an antibody specific for heated serum. Rothen et al. (cited by Landsteiner, 1945) found that an immune serum for heat-denatured horse globulin gave no exceptional cross-reactions. Species-specific anti-heated human gamma globulin has been produced by Henney et al. (1968) and Hirose et al. (1967). Both groups agree with Grabar (1958) who found that denatured molecules achieve a new and characteristic specificity which is well-defined rather than random as proposed earlier.

We made this investigation to demonstrate that a simple, purified, heat-denatured serum protein used in conjunction with the sensitive tanned cell hemagglutination (HA) test could be used to identify the species of origin of heated meats. Immunoglobulin G (IgG), the second most abundant serum protein, was found at a concentration of 0.16 mg per gram of wet rabbit muscle tissue. Heating precipitates approximately 70% of the IgG, trapping it within the heated meat sample. Approximately this amount (0.10 mg/g) of residue was presumed to be present in all heated meat samples and was considered sufficient to make heated meat sample absorptions effective in reducing the titer of an homologous anti-heated-IgG-precipitate. The antiserum was tested for titer reduction by micro-HA, using tanned red blood cells sensitized with the homologous heated IgG supernatant fluid. A significant reduction of titer by meat absorption indicated that the meat sample was homologous to the antiserum.

MATERIALS & METHODS

Materials

Whole serum samples were obtained from the following species: chicken (avian, designated A), cow (bovine, B), horse (equine, E), sheep (ovine, O) and swine (porcine, P). Whole sera and serum fractions were stored at −20°C until used.

Rabbits of various breeds, ages and weights and of both sexes were obtained from commercial sources for production of antisera.

Sausages of the following meat types: all chicken, all beef, all pork, chicken and pork, beef and horse, and a beef, sheep and pork mixture were obtained from the U.S. Department of Agriculture. All-sheep meat and all-horse meat sausages were prepared in our laboratory and stored at −20°C until needed.

Antigen preparation

Crude immunoglobulin G (IgG) was prepared from the sera of the 5 species. A 40-ml sample of each serum was precipitated 3 times in a 50% saturated ammonium sulfate solution. The final precipitate was redissolved to 0.5 its original volume in 0.85% NaCl and dialyzed at 4°C against 2 changes of pH 7.2 borate-buffered saline, followed by one change of the appropriate buffer for chromatography. After dialysis, the crude globulin was divided into 2 equal aliquots and stored at −20°C until further fractionated.

IgG was separated from the crude globulin by column chromatography on DEAE cellulose (Williams et al., 1968). The conditions of chromatography yielding the best balance of purity and yield for each globulin species were determined. Phosphate buffers used were:

For bovine and ovine IgG	0.0175 M	pH 6.8
For avian IgG	0.15 M	pH 6.8
For equine IgG	0.0175 M	pH 7.8
For porcine IgG	0.0175M	pH 7.5

The column effluent was monitored with a continuous flow recording ultraviolet (λ = 280 nm) spectrophotometer (Gilson Medical Electronics Co., Middleton, Wisconsin). Only the first material to cause deflection of the recorder needle was collected and concentrated by pressure ultrafiltration (Amicon Corp., Cambridge, Massachusetts) to 10 to 20 mg/ml. The concentration of the IgG solutions diluted as required in 0.1N NaOH was determined by spectrophotometry at λ = 280 nm using the extinction coefficient of 1.5 density units/mg/ml. The extinction coefficient for the five species of IgG was assumed to be the same as that for rabbit IgG found by Steiner et al. (1966).

Tests on antigen

Purity of the IgG preparations was tested by immunoelectrophoresis on glass microscope slides (Scheidegger, 1955). The precipitin lines were developed with homologous rabbit anti-whole serum.

For preliminary tests of IgG interspecies cross-reactivity we employed immunodiffusion in agar on glass microscope slides (Hartmann et al., 1957). A central well and 6 peripheral wells were cut in the agar in a regular (hexagonal) pattern. The central well was filled either with rabbit origin anti-native IgG or with rabbit origin anti-heated IgG precipitate. Circumferential wells contained either native IgG or heated IgG supernatant fluid of the 5 species investigated.

Heat denaturation of IgG

Immunoglobulins from the 5 species were heat-denatured according to the specifications for the maximum time and temperature used in sausage preparation. Globulin aliquots in screw-cap tubes were placed in a water bath at

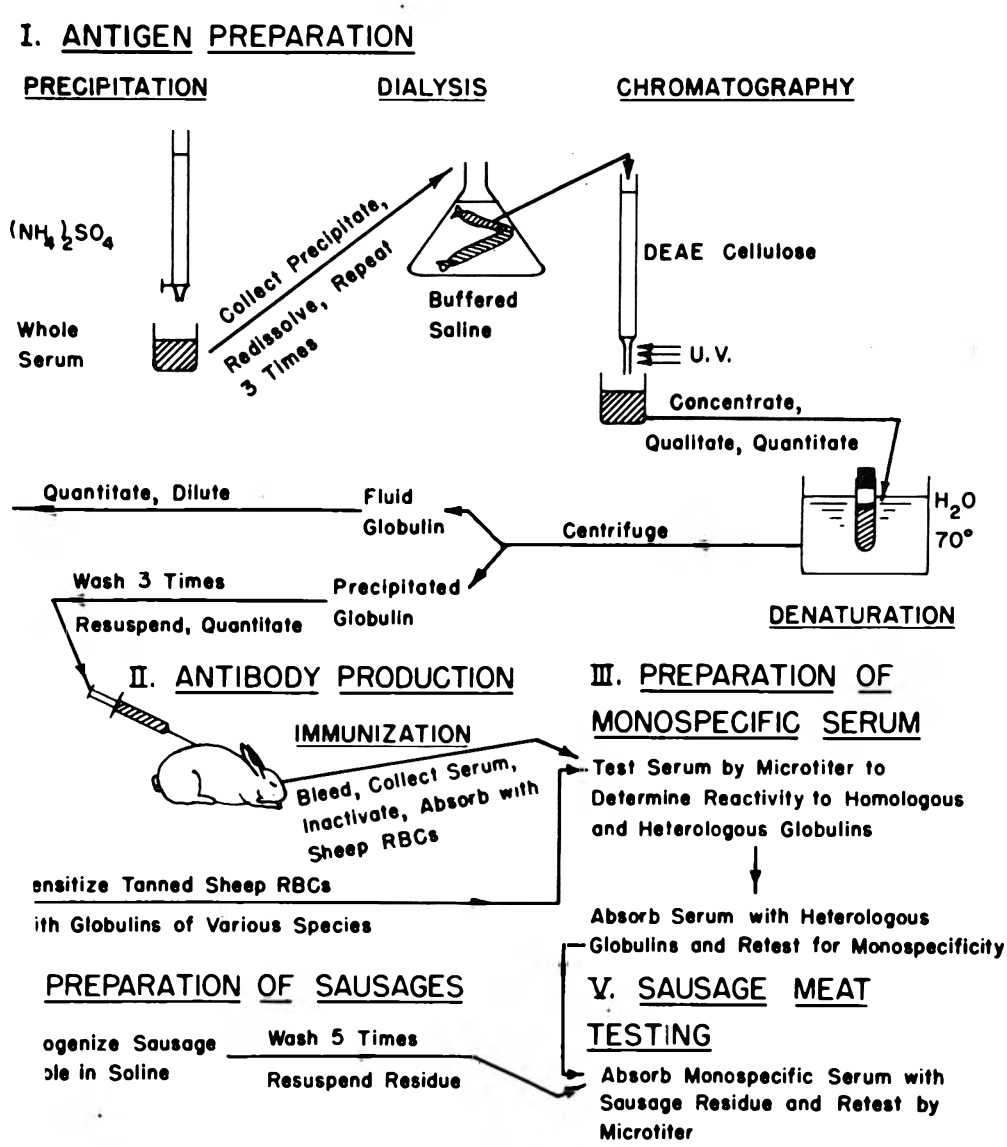

Fig. 1–Flow sheet for preparation of monospecific sera and sausage meat testing.

70°C for 150 min, then removed and centrifuged at 1,500 × g for 5 min and the supernatant fluids drawn off and saved. The precipitates were washed 3 times in 0.85% NaCl solution and finally suspended in a convenient volume of 0.85% NaCl solution. Protein concentrations of supernatant fluids and precipitates were determined by U.V. spectrophotometry as just described, except that 1.0N NaOH was used to dissolve and dilute the precipitates. The heated globulins were designated Ah, Bh, Eh, Oh or Ph supernatant fluid or precipitate, respectively. These materials were stored at −20°C until used. Figure 1, I summarizes the procedure for antigen preparation.

Immunization schedules

Rabbits were immunized as follows. A volume of 0.85% NaCl solution containing 10 mg of purified native IgG was mixed with an equal volume of complete Freund's adjuvant (Difco Laboratories) and injected intradermally into several sites on their backs and repeated 2 wk later. 20 to 30 ml of blood were collected by cardiac puncture 1 wk after the last injection. Thereafter, either intradermal booster injections of 10 mg of IgG without adjuvant were given, or 20 to 30 ml of blood were collected on alternate weeks. Two different schedules were used to produce antisera to heated IgG precipitates. By the first method, a volume of IgG precipitate suspension containing 3.3 mg of IgG was injected subcutaneously 3 times a week on alternate weeks for 2 such series. A week after the last injection, 20 to 30 ml of blood were collected by cardiac puncture, or booster injections were given subcutaneously with 10 mg of precipitate suspensions, on alternate weeks for up to 7 times. By the second method, a volume of IgG precipitate suspension containing 10 mg of IgG was mixed with an equal volume of Freund's complete adjuvant. The emulsion was injected subcutaneously into rabbits at several sites, and the injection was repeated 2 wk later. 1 wk after the last injection, 20 to 30 ml of blood were collected by cardiac puncture or the animal was given a booster injection intramuscularly with thrice-weekly injections, each of 5 mg on IgG precipitate on alternate weeks. The serum was separated from whole blood samples and kept frozen until used.

Antibody testing procedures

To determine the antibody titers and species specificities of the various antisera we used the micro-technique modification (Sever, 1962) of the hemagglutination (HA) test of Boyden (1951) as modified by Stavitsky (1954). Sheep red blood cells were obtained from whole sheep blood collected in sterile modified Alsever's solution (Buhantz et al., 1946) and stored at 4°C for no longer than 21 days. 12.5 μg of heated IgG supernatant fluid was used to sensitize each milliliter of 2.5% tanned cells. After sensitization, the cells were diluted 1:5 to obtain a 0.5% suspension for use in the micro-technique. When specificity tests were performed, aliquots of cells were sensitized simultaneously with heated IgG supernatant fluid of each species to insure identical handling. Titers were expressed as the number of wells showing agglutination in a series of twofold serum dilution. The first well contained a 1:2 dilution of serum.

Preparation of formalinized red blood cells

Formalinized sheep red blood cells were prepared by the method of Csizmas (1960) from sheep whole blood stored in Alsever's solutions

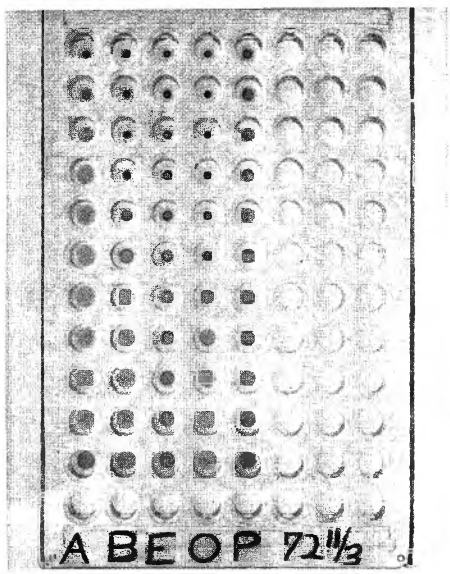

Fig. 2—Microtiter patterns of anti-Ph-IgG serum No. 72—11—3 prior to absorption to monospecificity. Tanned sheep RBC's sensitized with fluid-heated globulins: A(vian), B(ovine), E(quine), O(vine), P(orcine). Titers: A=10, B= 7, E = 6, O = 6, P = 8. Well No. 1 of each row does not contain sensitized RBC's.

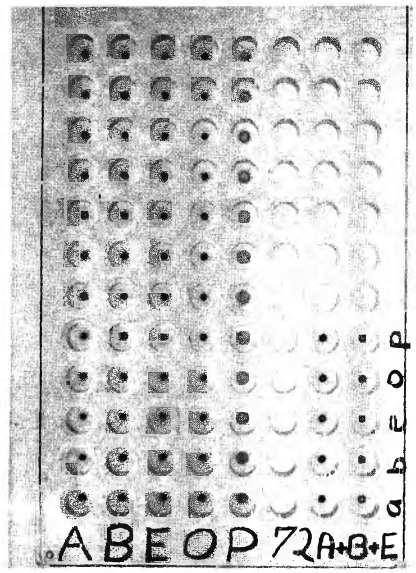

Fig. 3—Microtiter patterns of anti-Ph-IgG serum No. 72—11—3 following absorption to monospecificity with a mixture of avian, bovine, and equine heated globulin precipitates. A, B, E, O, P: See legend, Fig. 2. a, b, e, o, p: negative controls for A, B, E, O, P. Titers: A, B, E, O = negative; P = 8.

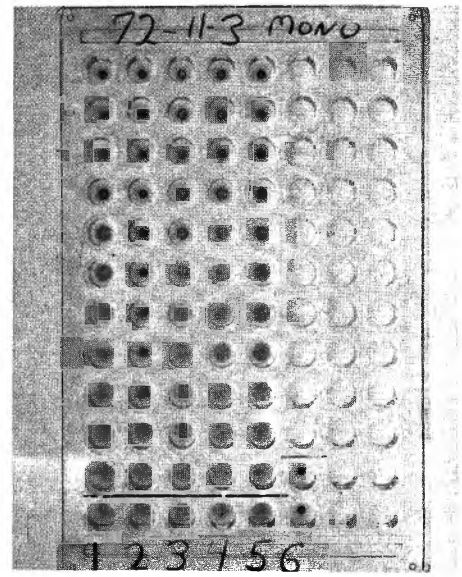

Fig. 4—Microtiter patterns of monospecific anti-Ph-IgG serum No. 72—11—3 before and after absorption with various sausage homogenate residues. Monospecific serum absorbed with: 1, no absorption; 2, pork sausage; 3, beef-horse sausage; 4, beef sausage; 5, horse sausage. 6 = negative controls. Titers: 1 = 8; 2 = 3; 3 = 7; 4 = 7; 5 = 7.

no longer than 5 days. Formalinized cells were stored at 25% concentration in phosphate buffered saline, pH 7.2, at 4 C for up to 6 months. Use of formalinized red blood cells in HA was similar to that of fresh red blood cells except that the diluent was 1% bovine serum albumin (BSA), rather than 1% normal rabbit serum, in 0.85% NaCl solution, and the red blood cell concentration for the micro-HA test was 1%.

Preparation of monospecific sera

Species cross-reactive antisera were made monospecific by absorption with purified heterologous IgG precipitates. Antisera were first tested by micro-HA with cells sensitized with homologous and heterologous heated IgG supernatant fluids. If the homologous titer was high enough, the serum was first absorbed with the IgG precipitate of the species giving the greatest heterologous titer. If monospecificity was not obtained, the absorption was repeated with the major cross-reacting IgG, usually up to 3 times. If necessary, the serum was then absorbed with the second and the third most cross-reactive IgG's. An antiserum was considered monospecific only if it had a homologous titer of 5 wells or more, and a heterologous titer not exceeding 1 well. Absorptions were usually performed on 1 ml of antiserum heated at 56°C for 30 min and absorbed with red blood cells. An aliquot of IgG precipitate suspension containing 2 mg of protein was placed in a 13- by 100-mm round-bottomed test tube and centrifuged at 900 × g for 5 min and the supernatant fluid discarded. 1 ml of the prepared serum was added to the residue and the tube rotated at an angle at 190 rpm for 30 min on a rotary shaker. The tube was centrifuged at 900 × g and the supernatant fluid recovered and tested by micro-HA. Figures 2 and 3 illustrate microtiter patterns of an antiserum before and after absorption to monospecificity.

Preparation of sausages

All-sheep and all-horse sausages were prepared from raw materials. Horse and sheep meat and fat were obtained from animals given postmortem examinations at the University of Illinois College of Veterinary Medicine. The following recipe was scaled down to suit the meat sample sizes available (horse meat—174 g; sheep meat—301 g): meat—12 lb (including 20% fat); ice—4 lb; salt—100 g; dextrose—28.4 g; white pepper—7.0 g; mace—3.0 g; coriander—3.0 g; sodium nitrate—1.0 g; sodium nitrite—1.0 g; ascorbate—4.0 g; nonfat dry milk solids—20.0 g; water—20.0 g. These materials were mixed in a blender and stuffed into frankfurter skins. The sausages were heated in a water bath at 70°C to an internal temperature of 50°C (approximately 40 min) and kept frozen until used.

Preparation of sausage homogenates and supernatant fluids

10-g aliquots of each sausage sample were added to 20 ml of 0.85% NaCl solution and homogenized (VirTis Company, Inc., Gardiner, New York) at approximately 22,500 rpm for 3 min. The homogenate was centrifuged at 1,500 × g and the supernatant fluid recovered. The residue was washed 3 to 5 times with 30-ml aliquots of 0.85% NaCl solution. The washings were repeated until no oil or fat layer formed at the meniscus. The washed residue was resuspended in 20 ml of 0.85% NaCl solution and kept frozen until used. The fat layer was lifted off the first supernatant fluid with an applicator stick and the aqueous portion kept frozen until used.

Testing of sausage

1 ml of sausage homogenate suspension was placed in a 13- by 100-mm round-bottomed test tube and centrifuged at 900 × g. The supernatant fluid was drawn off, leaving a residue volume of 0.2 to 0.3 ml. 0.1 ml of monospecific antiserum was added to the residue. The tube was shaken and the supernatant fluid recovered as described for absorption with heated IgG

Table 1.—Numbers of homologous and heterologous lines obtained in testing by double diffusion each of 5 anti-native IgG and 5 anti-heated IgG sera against 5 native and 5 heated IgG preparations.

	Number of precipitin lines			Cross-reactivity index
	Total	Heterologous	Homologous	Heterologous / Homologous
Anti-Native IgG Sera				
vs. Native IgG	21	9	12	0.75
vs. Heated IgG	18	11	7	1.57
Anti-heated IgG Sera				
vs. Native IgG	7	2	5	0.4
vs. Heated IgG	6	1	5	0.2

Table 2.—Numbers and types of heated IgG precipitate absorptions required to render several antisera monospecific.

Antisera	Number of absorptions and IgG type
Anti-Eh[a]	1 × Ah
Anti-Ah	1 × Bh
Anti-Oh	3 × Bh + 1 × Eh
Anti-Ph	2 × Eh + 2 × Bh + 2 × Ah

[a] Ah: heated avian IgG: Bh: heated bovine IgG; Eh: heated equine IgG; Oh: heated ovine IgG; Ph: heated porcine IgG.

precipitate. The sausage-absorbed monospecific serum thus prepared was tested by micro-HA with cells sensitized with homologous heated IgG supernatant fluid. The HA titer was compared with that of untreated monospecific serum. Figure 4 illustrates the microtiter patterns of sausage-absorbed monospecific serum and Figure 1 summarizes the procedure for testing sausages.

Micro-HAI test

To test for the presence of avian meat in sausages, we used the micro-hemagglutination inhibition test (HAI) of Boyden (1951) as modified by Stavitsky (1954) and Sever (1962). Sausage supernatant fluids served as the inhibiting agent. The monospecific anti-avian-heated-IgG serum was prepared as for the HA test just described. The HA titer of the serum was 12 wells, corresponding to a dilution of 1:4096. One drop of serum diluted 1:500, corresponding to 8 HA units of antibody, was added to each of a series of twofold dilutions of sausage supernatant fluid. The first well of the series contained undiluted material. Red blood cells sensitized with heated Ah-IgG supernatant fluid were added and the test read as usual. Appropriate controls indicated that the sausage supernatant fluid caused red blood cells to agglutinate nonspecifically. The nonspecific agglutinin was removed by shaking the sausage supernatant fluid with an equal volume of chloroform and recovering the aqueous phase. Treatment with chloroform was repeated 2 or 3 times, until testing of the aqueous phase showed absence of nonspecific agglutination.

RESULTS

DOUBLE DIFFUSION studies were conducted on the interspecies cross-reactivity of gamma globulins using anti-native IgG and anti-heated IgG sera. Each serum was tested against all 5 native IgG's and all 5 heated IgG's. The anti-native IgG sera were generally more potent than the anti-heated IgG sera. Despite the bias created by the stronger antisera's ability to develop a greater number of precipitin lines, some general trends were discernible. Results are summarized in Table 1.

Anti-native IgG sera revealed twice the cross-reactivity among heated IgG's of that among native IgG's, while anti-heated IgG sera completely reversed this trend by revealing twice as much cross-reactivity among native IgG's as among heated IgG's.

The HA testing system chosen was anti-heated IgG serum and cells sensitized with heated IgG supernatant fluid. Because of the low titers displayed by this system, serial antiserum dilutions were started with undiluted material. To conserve antisera, we used the micro-HA test. Initially, we used formalinized red blood cells for HA testing because they can be stored for up to 6 months without deterioration and can be kept frozen or freeze-dried for even longer periods. This would have allowed testing of various materials with a single preparation of cells over a long period. However, after 4 months of successful use, the formalinized cell system suddenly became inoperative; cause of this failure could not be determined. Since a second batch of formalinized red blood cells was also found to be unusable, testing with formalinized cells was abandoned.

Absorption of anti-native IgG sera with a great excess of homologous heated IgG precipitate failed to remove all antibody detectable by red blood cells sensitized with native IgG. Similarly, absorption of anti-heated IgG sera with either homologous heated IgG precipitate or native IgG failed to remove all antibody detectable by micro-HA using red blood cells sensitized with native IgG. However, antibody detected in anti-heated IgG sera by testing with red blood cells sensitized with heated IgG supernatant fluid was generally removed by absorption with homologous heated IgG precipitate. For this reason the latter system was selected as the most specific.

The number and types of absorptions with heated IgG precipitates required to render an antiserum monospecific varied and had to be determined for each individual antiserum (Table 2). Examples of an antiserum monospecific without pretreatment, an antiserum rendered monospecific and an antiserum which could not be rendered monospecific are given (Table 3).

Of 12 sera tested, 3 could be made monospecific, 3 could not be and 6 were monospecific, or almost so, without pretreatment. Factors which influenced the effectiveness of absorption included the initial titer of the serum, the use of homologous or heterologous antigens and their sequence, and the quantity and quality of heat-precipitated IgG used for each absorption.

Monospecific antisera were used to test for the presence of a specific IgG in a sausage sample homogenate. Examples of tests on sausage samples with 4 monospecific sera are shown (Table 4).

The monospecific anti-Bh-IgG serum behaved as expected, showing complete removal of homologous titer by absorptions with sausage samples containing beef and no significant reduction of titer

Table 3—Homologous and heterologous HA titers of several antisera before and after absorptions with various numbers and types of heated IgG precipitates.

Antiserum type and number		Number of absorptions and IgG type	Titer to cells sensitized with IgG type				
			Ah	Bh	Eh	Oh	Ph
Anti-Bh[a]	102-8-28	None	0	12	0	0	0
Anti-Ph	115-8-28	None	10	9	10	8	10
		1 × Ah	6	6	5	4	5
		1 × Ah + 1 × Bh	2	0	0	0	0
Anti-Ph	59-6-22	None	7	8	8	4	9
		2 × Eh	7	3	3	2	8
		2 × Eh + 2 × Bh	7	0	0	0	8
		2 × Eh + 2 × Bh + 2 × Ah	0	0	0	0	8

[a] See footnote, Table 2.

Table 4—Homologous HA titers of four monospecific antisera after absorption with homogenates of various sausage types.

Monospecific antiserum to IgG species[a]	Micro-HA titer						
	Prior to absorption	After absorption with sausage meat type					
		C	B + E	B + O + P	P	P + A	E[a]
Bh[b]	9	0	ND[d]	0	ND	8	ND
Eh	7	ND	0	5	ND	5	0
Oh	8	ND	6	2	ND	ND	ND
Ph	8	7	?	0	3	0	7

[a] See footnote, Table 2.
[b] Anti-Bh-IgG serum was monospecific without pretreatment.
[c] B, bovine; E, equine; O, ovine; P, porcine; A, avian.
[d] Not done.

Table 5—Hemagglutination inhibition titers of Anti-Ah-IgG serum No. PII 68-5-24 with sausage supernatant fluids and Ah-IgG supernatant fluid.

Inhibitor	Micro-HAI titer[a]
Ah-IgG[b]-S.[c]	16
Saline	0
A[d]-S.[e]	2
AP-S.	2
BE-S.	0
BOP-S.	0

[a] 8 HA units of antiserum were used.
[b] 6.9 mg/ml.
[c] Supernatant fluid.
[d] Abbreviations used here are cited in Table 4.
[e] Supernatant fluids were chloroform extracted to remove nonspecific agglutination.

by absorption with non-beef sausage. The monospecific anti-Ph-IgG serum showed almost complete removal of homologous titer with absorptions by a sausage sample containing only pork, complete removal of titer by 2 sausage samples containing pork and meat from other species and no significant reduction of titer by absorptions with non-pork sausages. The monospecific anti-Eh-IgG serum behaved as expected, showing complete removal of homologous titer by sausages containing horse meat and only slight reduction of titer by sausages not containing horse meat. The monospecific anti-Oh-IgG serum is an example of a poor but usable antiserum. A sausage sample containing sheep meat reduced the homologous titer but did not abolish it, and a sausage sample containing no sheep meat caused a slight reduction in titer.

In the course of micro-HA testing, a large pool of anti-heated avian-IgG antisera was found that showed no significant reduction of titer upon absorption with homologous sausage solids. This finding was verified by serial absorptions of the antiserum pool with heated avian IgG precipitate. Because the pool was both large and easily made monospecific, an attempt was made to use it in a hemagglutination inhibition test. The resulting inhibition titers are summarized in Table 5.

DISCUSSION

CONDITIONS CHOSEN for chromatography of each of the 5 globulins represented a compromise between purity and yield. Though it was demonstrated that immunogens of partial purity can elicit both monospecific antisera and antisera which can be rendered monospecific, a more highly purified immunogen might be more advantageous. Conceivably, some of the impurities in the immunogens may represent antigens that are more species cross-reactive than IgG. Elimination of such impurities from the immunogens may result in the production of antisera that can be rendered monospecific more easily.

Results of the immunodiffusion analysis (Table 1) suggest that heating of IgG causes an increase of species cross-reactivity as well as the acquisition of new, species-specific antigens (Furth, 1925; Landsteiner, 1945; Maurer, 1954; Zinsser, 1924; Henney et al., 1968; Grabar, 1958). Results support the tentative conclusion that anti-heated IgG sera are more apt to reveal species specificity of heated IgG's than are the anti-native IgG sera. This conclusion was confirmed in preliminary trials in which the 2 types of sera were absorbed with heterologous heated IgG's and tested by hemagglutination with red blood cells sensitized with heated IgG supernatant fluid.

The testing system chosen was the most homologous among those tried, in that it employed only heated IgG components. The testing system used anti-heated IgG precipitate, red blood cells sensitized with heated IgG supernatant fluid and heated IgG precipitate absorptions to make the antisera monospecific. The success in obtaining monospecific sera was credited to the homology between immunogens, antiserum and test antigen since, as discussed above, heating of IgG seems to cause the appearance of new species-specific antigenic determinants.

Sera of rabbits immunized with heated IgG precipitate may contain species-specific antibody, interspecies cross-reacting antibody or a mixture of the two. When an antiserum containing mostly cross-reacting antibodies is absorbed with a mixture of all heterologous heated IgG precipitates, its reactivity for both heterologous and homologous antigens is removed. Such antisera could not be rendered monospecific and, therefore, could not be used in this investigation.

The greatest problem encountered in obtaining monospecific sera was the marked cross-reactivity between the closely related bovine and ovine IgG's. Cross-immunization between these species might be a far superior method of developing monospecific sera when compared to the absorption method used here. Absorptions cannot in all cases render a serum monospecific. In sera successfully made monospecific, the remaining titer was usually low, and sometimes the absorbed serum could not be used. Sera from cross-immunization, that is, those elicited by injection of Oh-IgG into a cow and of Bh-IgG into a sheep, would differentiate between these species quite clearly and would be expected to show high titers.

The purification procedures employed in this work yielded immunogens in which IgG was the major component but not always the only one as judged by immunoelectrophoresis. In spite of the presence of impurities, it was possible to produce sera which were monospecific or could be made so by absorption with heterologous IgG's. Since there was great variation in the quality of the antisera produced by individual rabbits, it was found expedient to immunize several rabbits with IgG of each species. It was necessary to test the rabbit sera at frequent intervals during the course of immunization. Sera obtained during the early phase of immunization were often more specific than those obtained later from the same rabbit. The broadening of specificity during the course of immunization is a well-recognized phenomenon (Nuttall, 1904; Proom, 1943; Landsteiner, 1945). It was also found that antisera produced by immunization with IgG emulsified in complete Freund's adjuvant were often more cross-reactive than those elicited by IgG alone. Two causes may have contributed to this result: 1) the property of Freund's adjuvant to enhance the antigenicity of minor antigenic components (Kabat et al., 1961), such as the impurities present in the immunogen; 2) the possibility that immunization with Freund's adjuvant may elicit antibodies of greater cross-reactivity than those obtained by immunization with the antigen alone (Dawe et al., 1965).

Several factors require individual attention to ensure a successful test. Before bleeding, rabbits should be fasted for 12 hr. If the serum is cloudy, fats should be removed by chloroform extraction. All antisera should be screened to determine the extent of homologous and heterologous reactions. Only those antisera which can be rendered monospecific and shown to react as desired to absorption with homologous heated globulin precipitate should be used to test sausage samples. Sausage homogenates must be freshly prepared for testing, as frozen homogenates tend to partially reaggregate. For each antiserum found to perform as required, the optimum antigen dose for sensitization of RBC's should be determined as that giving the highest titer with a positive serum. In addition, the optimum amount of sausage homogenate residue used for absorption and the number of absorptions to be performed should be predetermined with homologous heated globulin precipitate.

The yield of avian-IgG obtained by chromatography of avian serum was low, and much of this protein remained in solution upon heating. For these reasons, rabbits were injected with both the Ah-IgG precipitate and the supernatant fluid to reach an immunizing dose level. The resulting antiserum pool showed reactivities to both the soluble and insoluble protein portions. For this reason antibodies detected by cells sensitized with Ah-IgG supernatant fluid could not be absorbed out with Ah-IgG precipitate, either pure

or as found in sausage. Therefore, it was necessary to use the hemagglutination inhibition test for avian meat-containing sausages. The low inhibitory titers obtained reflect the low concentration of soluble protein remaining in the sausage.

Although results obtained with avian sausage indicate that HAI can be used for meat identification, tests based on serum absorptions with sausage solids should be generally preferred, for two reasons. First, as stated above, most of the globulin in sausage should be insoluble. Secondly, there is no theoretical limit to the amount of insoluble antigen that can be used to absorb a serum sample, since absorptions can be done repeatedly. In contrast, the HAI procedure is dependent on the concentration of inhibitor present in the sausage supernatant fluid, which cannot be increased without additional manipulations. Therefore, HAI is not recommended as a general procedure for identification of heated meat.

Some of the sera used in this pilot project had undesirable characteristics such as low titers, prozones, and weak agglutination patterns. There seems to be little doubt, however, that screening of large numbers of rabbit antisera should permit the selection of satisfactory reference sera.

Serologic identification of the species of meats used in the preparation of sausages was accomplished. Undoubtedly, additional problems will arise in the course of the application of the methods to routine identification of sausage meats. However, the success experienced in solving the problems encountered in this work suggests that such application should be undertaken with confidence.

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COMPARATIVE GROWTH OF CLOSTRIDIUM PERFRINGENS IN CARBON DIOXIDE AND NITROGEN ATMOSPHERES

SUMMARY—Relative growth rates of 8 food poisoning strains of Clostridium perfringens *in a 100% nitrogen atmosphere and in a 100% carbon dioxide atmosphere at 760-mm pressure were determined. Growth was measured by the pour plate method. Substrate was fluid thioglycollate medium flushed with 100% nitrogen gas or 100% carbon dioxide gas at atmospheric pressure and incubation was at 43°C. Headspace gas in the culture flask was analyzed during the growth period. The nitrogen was reduced at the end of 5 hr but the carbon dioxide remained at approximately 100% during the 3.5–4-hr exponential growth phase. Generation time of the* Clostridium perfringens *strains grown in 100% nitrogen atmosphere varied from 12.9–16.9 min and in the 100% carbon dioxide atmosphere from 12.9–17.2 min. When comparing individual strains in carbon dioxide versus nitrogen, no significant difference was noticed. When each of the 8 strains was compared with one another in a nitrogen atmosphere, as well as in a carbon dioxide atmosphere, significant differences were noticed in growth rates.*

INTRODUCTION

DURING 1968, 56 outbreaks were reported and 5,966 persons were affected by *Clostridium perfringens* food poisoning in the United States (Anon. 1969). The major vehicles associated with *Clostridium perfringens* food poisoning were turkey, beef, chicken and pork. *Clostridium perfringens* food poisoning was second only to staphylococcus as the cause of food-borne outbreaks, but led all others in number of persons affected. The wide distribution of this organism results in the contamination of many foods and food products consumed by humans.

The survival of both vegetative and spore forms of *Clostridium perfringens* has been reported under conditions varying from below-freezing temperatures to boiling-point temperature (Hall et al., 1965; Hobbs, 1957).

Hall et al. (1965) reported that extremely rapid growth of these organisms would occur in food at temperatures between 43.3 and 46.6°C. Smith (1963) indicated that 35°C was optimal for growth of *Clostridium perfringens*. Hobbs (1957) found that temperatures in the range 39–49°C allowed rapid growth in meat slices and gravy. Hall et al. (1965) reported that the excellent growth obtained at 35° was preceded by a lag of 2–4 hr, whereas no lag phase was noted at 46°C. Temperatures at 46°C and above have been used for isolation purposes (Chapman, 1928). Barnes et al. (1963) found an interesting relationship existing between pH, composition of medium and growth temperature. Using Robertson's cooked meat broth, they found growth of *Clostridium perfringens* at 20°C variable at pH 5.8 but rapid at pH 7.2. In a study of the relationship between pH and generation time by Smith (1963) no growth was evident at pH 5.0 or 8.5. Fuchs et al. (1957) and Boyd et al. (1948) studied the nutritional requirements of *Clostridium perfringens*. Angelotti et al. (1962) reported that 90% nitrogen and 10% carbon dioxide created anaerobiosis for growth of *Clostridium perfringens* in anaerobic jars. There is no information available concerning the growth of *Clostridium perfringens* in higher nitrogen and high carbon dioxide atmospheres. This paper presents a comparative study of growth for several food poisoning strains of *Clostridium perfringens* in a 100% nitrogen atmosphere and in a 100% carbon dioxide atmosphere in fluid thioglycollate medium at 43°C.

EXPERIMENTAL

EIGHT strains of *Clostridium perfringens* were used. Strains NCTC 8235, 8237 and 8247 were obtained from Dr. Betty Hobbs, Central Public Health Laboratory, London, England; strains 1362, S–80, S–88 and 6867 from Dr. H. E. Hall, Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio.

Stock cultures were carried in Angelotti's modification of Noyes Veal Broth (Angelotti et al., 1962). Every 3 months cultures were transferred to freshly prepared modified Noyes Veal Broth, incubated in a constant-temperature water bath at 43°C for 24 hr and stored at refrigerated temperature.

Preparation of inoculum

To prepare inocula, 1 ml from a stock culture tube was inoculated into 10 ml of freshly prepared fluid thioglycollate medium (BBL). The inoculated tubes were incubated at 43°C for 24 hr. 1 ml of a 24-hr culture was diluted in 9 ml of fluid thioglycollate medium and 0.1 ml of this dilution inoculated into 100 ml of fluid thioglycollate medium in a Nephloflask (Bellco Glass, Vineland, New Jersey) to initiate each growth study.

To prepare a 100% nitrogen atmosphere, sterile fluid thioglycollate medium was flushed with nitrogen gas (Fig. 1). A manometer was used during the flushing of nitrogen gas to maintain 760 mm Hg pressure in the flasks. The 100% nitrogen atmospheric condition in the Nephloflask was checked by injecting a 1-ml headspace sample at room temperature into a gas chromatograph (A–90 P3, Aerograph) fitted with a molecular sieve column ¼ in. in diameter and 5 ft long. Analysis for both nitrogen and oxygen was carried out. When no oxygen peak was detectable, it was assumed the nitrogen atmosphere was complete and flushing was terminated. Time required to reach the end point varied from 1.5–2 hr.

The 100% carbon dioxide atmosphere was obtained in Nephloflasks by following the flushing procedure previously described for nitrogen. To maintain the pH of the medium at 6.8, 5 ml of 1.0 N NaOH were added to the fluid thioglycollate medium before flushing with 100% carbon dioxide.

Headspace analysis for both air and carbon dioxide was accomplished using a Porpak Q column. When the air peak detected became

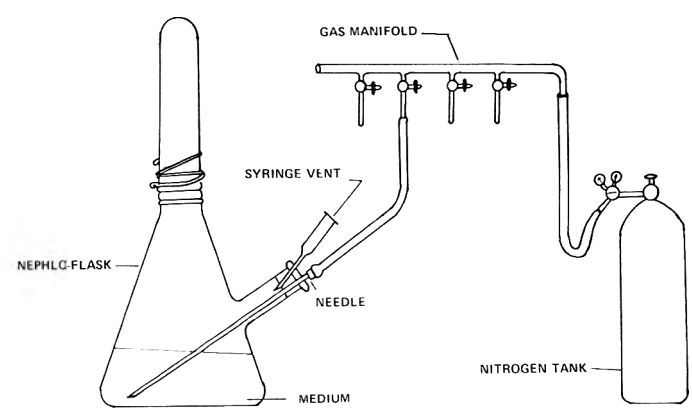

Fig. 1—Gas flushing system.

very small it was assumed that the carbon dioxide atmosphere was complete and flushing was terminated. The time required to reach the end point varied from 1.5–2 hr.

Flushed Nephloflasks were inoculated with 0.1 ml of a *Clostridium perfringens* culture through the flask septum using a sterile tuberculin syringe (B + D disposable, 23–G needle, 2.5 cc). The flasks were incubated in a constant-temperature water bath at 43°C. An uninoculated flushed control was carried in every experiment.

The growth rate of *Clostridium perfringens* was evaluated by the pour plate method. Every 30 min the culture flasks were shaken and 1 ml of the growing culture withdrawn from the Nephloflask with a sterile disposable syringe (B & D tuberculin, 23–G needle, 2.5 cc). Serial dilutions were made in pH 7.2 phosphate buffer (Standard Method for the Examination of Dairy Products, 1967) and plated in Sulfite Polymyxin Sulfadiazine (SPS) agar (Difco, BBL) (Angelotti et al., 1962). After the initial pour plate had solidified, a few milliliters of SPS agar were poured into each petri dish to cover the entire previously poured surface. After solidification the plates were inverted and placed into Case anaerobic jars (Case Laboratories, Inc., Chicago, Ill.), under an atmosphere of 100% nitrogen gas. Nitrogen was used for all pour plate incubations regardless of the original fluid thioglycollate growth atmosphere, to permit valid comparisons without complications due to changes in colony formation potential and morphology. Anaerobiosis was accomplished by connecting the jars to a vacuum line to obtain a reading of 20–25 in. on the attached gauge, then filling the jar to atmospheric pressure with 100% nitrogen. This procedure was repeated 4 times before incubation of the petri dishes. A duplicate series of plates was incubated in a carbon dioxide atmosphere during one complete experiment involving cultures under both atmospheres. There was no significant difference in cell counts between the two sets of plates. Therefore, all subsequent plates were incubated under nitrogen atmosphere throughout the experiments. The jars were then placed into a 37°C incubator for 24–48 hr. Black colonies of *Clostridium perfringens* were counted with a Quebec colony counter (Darkfield Quebec Colony Counter, American Optical Co., N. Y.).

The atmosphere within the flasks was monitored during the course of microbial growth. 1-ml headspace samples were removed from the Nephloflasks at 1-hr intervals. The flasks were kept at atmospheric pressure by periodic release of gases generated by the microorganisms. Either an A90–P3 or a G.C.5 (Beckman Instrument Company) gas chromatograph equipped with a thermal conductivity detector was used for the analysis. For the analysis of oxygen, nitrogen and carbon dioxide a G.C.5 gas chromatograph with dual columns was used. The conditions of operations were Column 1. Molecular sieve (80–100 mesh). Column size–¼ in. by 6 ft. Injector temperature–45°C. Detector temperature–250°C. Column temperature–65°C. Carrier gas–Helium 40 ml/min. Column 2–Porpak Q (150–200 mesh).

Operating conditions of the A90–P3 gas chromatograph for the analysis of carbon dioxide and air as used in the 100% carbon dioxide atmospheric growth studies were as follows: Column–Porpak Q (20–40 mesh). Column size–¼ in. by 6 ft. Injector temperature–90°C. Detector temperature–110°C. Column temperature–50°C. Carrier gas–Helium 50 ml/min.

Table 1–Duration of adjustment phase for Clostridium perfringens in 100% nitrogen and 100% carbon dioxide atmospheres.

	Duration of adjustment phase			
	Nitrogen		Carbon dioxide	
Strain	Initial level (org/ml)	Time (min)	Initial level (org/ml)	Time (min)
6867	6.4×10^3	60	1.4×10^3	90
	4.5×10^3	60	1.9×10^3	60
8235	4.0×10^3	90	8.0×10^3	60
	1.4×10^3	60	5.8×10^4	60
8247	1.0×10^3	60	1.3×10^3	30
	1.1×10^3	60	1.8×10^3	60
1362	1.1×10^3	90	2.8×10^5	120
	9.2×10^3	60	2.7×10^5	90
8797	3.4×10^2	60	1.4×10^3	60
	2.0×10^3	60	2.4×10^3	60
S–88	1.2×10^4	120	8.2×10^3	150
	1.1×10^3	150	2.2×10^4	90
8237	1.0×10^3	30	1.1×10^3	60
	1.3×10^3	30	1.5×10^3	60
S–80	4.2×10^3	120	1.0×10^5	90
	3.6×10^3	90	9.8×10^4	90

The data accumulated during the exponential growth phase of *Clostridium perfringens* for all strains in each environmental condition were subjected to regression analysis. The numerical value for the slope of the best straightline was determined. Slopes from replicated experiments were subjected to a "t" test at the 95% confidence level. Average slope values were converted into generation times for each strain in each of the two growth conditions and were expressed in minutes per generation.

Growth of all strains of *Clostridium perfringens* in 100% nitrogen and 100% carbon dioxide were compared with one another by analysis of variance of the average slopes.

Tukey's multiple comparison test at the 95% confidence level was used to compare growth rates of the various strains in each of two atmospheres.

RESULTS & DISCUSSION

DURATION of the adjustment phase for strains of *Clostridium perfringens* in both nitrogen and carbon dioxide atmospheres is presented in Table 1. Strains 6867, 8235 and 8797 showed approximately 60-min adjustment phase durations in both nitrogen and carbon dioxide atmospheres. Extended adjustment phase durations were observed for strains S–88 and S–80. They ranged from 90 to 150 min, with S–88 averaging approximately 120 min and S–80 approximately 105 min. There was no difference between the response in nitrogen or carbon dioxide. Strain 1362 tended towards an extended adjustment phase duration in carbon dioxide but not in the nitrogen atmosphere. This is accentuated further by the higher initial level of cells in the carbon dioxide atmosphere, which would be expected to reduce the adjustment phase time. A similarly reduced adjustment time was observed for strain 8237 in 100% nitrogen atmosphere compared to the same strain in carbon dioxide. The time required to enter the exponential growth phase for strain 8237 in nitrogen was only 30 min compared to 60 min in a carbon dioxide atmosphere.

There appeared to be some adjustment phase duration differences between atmospheres and definite differences between some strains.

Gladstone et al. (1935) stated that carbon dioxide is an essential factor for certain bacteria including *Clostridium perfringens* and that carbon dioxide must be present in all media before multiplication begins. If this were true, some inhibition should have been evident in the nitrogen atmosphere systems. The data indicated some possibility that growth was initiated even earlier for some strains in the absence of carbon dioxide and that there was some delay in growth initiation in a carbon dioxide atmosphere.

Table 2 represents the generation time of 8 strains of *Clostridium perfringens* in the two atmospheres. There was no significant difference in the growth rate at the 95% confidence interval within any strain whether grown in a carbon dioxide or nitrogen atmosphere. Generation time of *Clostridium perfringens* in the 100% nitrogen atmosphere ranged from 12.9–16.9 min. In the 100% carbon dioxide atmosphere the generation time ranged from 12.9–17.2 min. Analysis of variance of average slopes for all of the strains, shown in Table 3, indicated there were significant growth rate differences between

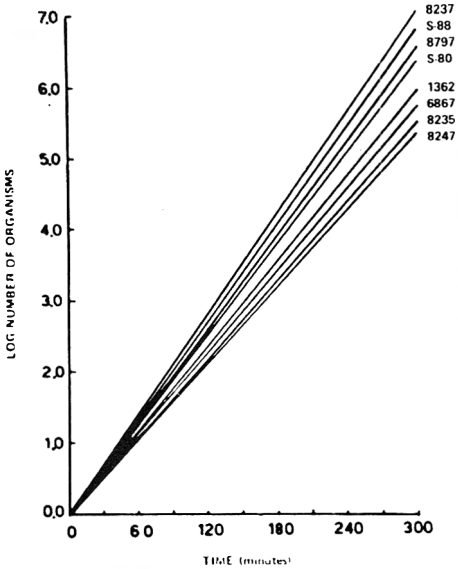

Fig. 2—Growth curve of Clostridium perfringens in the exponential growth phase at 43°C in a 100% nitrogen atmosphere.

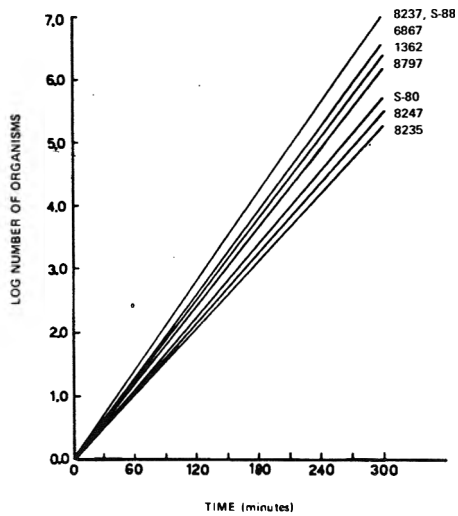

Fig. 3—Growth curve of Clostridium perfringens during the exponential growth phase at 43°C in a 100% carbon dioxide atmosphere.

Table 2—Growth rates and generation times for Clostridium perfringens in 100% nitrogen and 100% carbon dioxide atmospheres.

	100% Nitrogen atmosphere		100% Carbon dioxide atmosphere	
Strains	Average slopes ± C.I.	Generation time (min) ± C.I.	Average slopes ± C.I.	Generation time (min) ± C.I.
6867	0.0193 ± 0.0019	15.6 ± 1.4	0.0222 ± 0.0020	13.6 ± 1.3
8235	0.0183 ± 0.0025	16.4 ± 2.6	0.0175 ± 0.0011	17.2 ± 1.1
8247	0.0177 ± 0.0008	16.9 ± 0.8	0.0180 ± 0.0012	16.7 ± 1.2
1362	0.0197 ± 0.0005	15.3 ± 0.4	0.0208 ± 0.0029	14.4 ± 2.2
S-80	0.0212 ± 0.0031	14.2 ± 2.4	0.0188 ± 0.0015	16.0 ± 1.2
8797	0.0217 ± 0.0037	13.8 ± 2.9	0.0217 ± 0.0021	13.8 ± 1.5
S-88	0.0230 ± 0.0025	13.1 ± 1.6	0.0232 ± 0.0039	12.9 ± 2.6
8237	0.0233 ± 0.0011	12.9 ± 1.1	0.0232 ± 0.0012	12.9 ± 1.3

$$\text{Generation time} = \frac{\text{Log } 2}{\text{Slope}} \cdot \text{C.I. at 95\% level.}$$

strains in either atmospheres. This may be seen in Figure 2, which represents the idealized exponential growth phase for the 8 strains of *Clostridium perfringens* in nitrogen and Figure 3, which represents the same information for carbon dioxide.

Figure 4 compares average slopes for 8 strains using Tukey's multiple comparison test with 95% confidence interval in nitrogen and carbon dioxide atmospheres. It appears there are 3 types of *Clostridium perfringens* strains: One which grows faster than the other two, i.e., strains 8237 and S–88; a second which is slower growing, i.e., strains 8235 and 8247 and a third type which grows at a rate between the two others.

The generation time for *Clostridium perfringens* in bacteriological medium at 45°C reported by Smith (1963) was about 18.0 min. Neither strain nor type of medium used was reported.

Preliminary studies in which Fluid Thioglycollate Medium was used without gas flushing yielded growth curves with extended adjustment phases and increased generation times. These effects were probably due to the regular disturbance of the anaerobic system by the mixing of the culture at each sampling time.

The large surface volume and the large headspace within the culture flasks added to the oxygen incorporation. This was observed through the indicator material present in the medium.

Fuchs et al. (1957); Collee et al. (1961) and Hauschild et al. (1967) reported that maximum growth of *Clostridium perfringens* occurred in 4.5–5 hr. This is in agreement with the data obtained in this study for those growth curves followed to the stationary phase.

During growth of *Clostridium perfringens*, gas production was evident. *Clostridium perfringens* obtains energy needed for growth from carbohydrates. Friedemann et al. (1941) studied the breakdown products formed from glucose after growth of a number of pathogens in complex media. For *Clostridium perfringens*, lactic acid, acetic acid, ethyl alcohol, carbon dioxide and hydrogen were the principal fermentation products and some butyric acid was found. Carbon dioxide production was noticed during the growth of *Clostridium perfringens* in nitrogen. The levels of hitrogen and carbon dioxide in the inoculated flasks during the growth studies were determined by analyzing headspace samples in a gas chromatograph. Carbon dioxide production lowered the nitrogen level, as shown in Figure 5. The initial nitrogen level began to fall after the 3rd hr of growth, in the middle of the exponential phase. The carbon dioxide level remained constant throughout the growth study. Some hydrogen sulfide, hydrogen and other gases were separated by gas chromatography. Fuchs et al. (1957) documented the utilization of several inorganic and organic sulfur compounds and the production of hydrogen sulfide from these compounds by 3 strains of *Clostridium perfringens*. Hydrogen sulfide was produced from thiosulfate and cystine. Other gases produced during the growth were not identified. There was no noticeable change in initial level of carbon dioxide, as seen in Figure 5. This may be due to the sensitivity of the measuring technique.

CONCLUSIONS

MODEL system evidence presented indi-

Table 3—Analysis of variance table.

	Degree of freedom	Sum of square	Mean of square	F
Total	31	0.0001725	–	–
Strain (S)	7	0.0001186	0.00001694	7.1597*
Condition (C)	1	0.0000021	0.0000021	0.8875
Interaction (S × C)	7	0.00001591	0.000002273	0.9607
Error	16	0.00003786	0.000002366	–

*Significantly different at 95% confidence level.

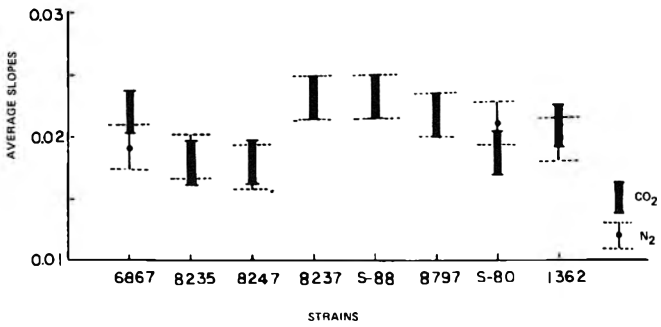

Fig. 4—Average slopes representing the exponential growth phase for 8 strains of Clostridium perfringens in fluid thyoglycollate broth at 43°C in atmospheres of 100% nitrogen and 100% carbon dioxide. The limits exhibited were determined by application of Tukey's test at the 95% confidence level.

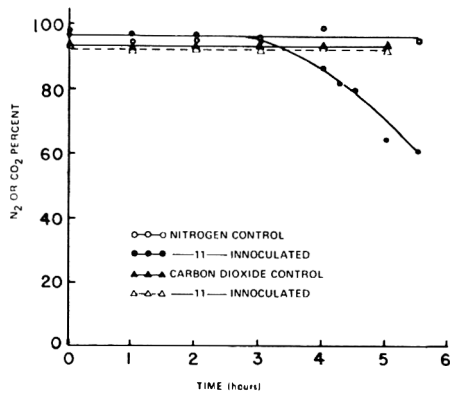

Fig. 5—Culture flask headspace analysis during growth of Clostridium perfringens after prior replacement of air with carbon dioxide or nitrogen.

cates that gas-flushed systems, whether carbon dioxide or nitrogen, will permit growth of *Clostridium perfringens*, all other conditions being favorable. Generation time for *Clostridium perfringens* in a 100% nitrogen atmosphere was 12.9–16.9 min and in a 100% carbon dioxide atmosphere 12.9–17.2 min.

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BIOCHEMISTRY OF TEA FERMENTATION: CONVERSION OF AMINO ACIDS TO BLACK TEA AROMA CONSTITUENTS

SUMMARY—^{14}C-amino acids were added to fresh tea-leaf homogenate undergoing conversion to black tea. After conversion (30 min, 25°C), the volatile compounds present in the headspace over the reaction mixture were collected and analyzed by gas chromatography. Results showed that leucine, isoleucine, valine and phenylalanine were partially converted to the aldehydes expected from a Strecker degradation. These aldehydes are constituents of black tea aroma. Further, drying of the fermented mixture caused an additional amount of the aldehydes to be formed. In contrast, no detectable volatile compounds were formed from aspartic acid, glutamic acid, glutamine, arginine, threonine, serine or theanine under the same conditions. Production of aldehydes from amino acids was shown to be dependent on the enzymic conversion process: Tea leaf which had been inactivated by steam treatment was not effective in causing formation of volatile aldehydes from the amino acids. Identical results were obtained in a model tea fermentation system composed of a crude soluble enzymes extract from tea leaves, purified epigallocatechin gallate and ^{14}C-amino acids. Ascorbic acid was found to inhibit formation of aldehydes from amino acids in this model tea fermentation system; dehydroascorbic acid by itself was found to be effective in causing formation of volatile aldehydes from amino acids.

INTRODUCTION

IT HAS been shown that the concentrations of free amino acids undergo appreciable changes during the various stages of conversion of fresh tea-shoot tips to the black tea of commerce: Namely, during the first (withering) stage of black tea manufacture there is an over-all increase in the concentration of free amino acids (Roberts et al., 1951; Bhatia et al., 1965); during the second (fermentation) stage the concentrations of leucine, isoleucine, serine, glutamic acid, glutamine, threonine, phenylalanine and theanine (theanine = 5-N-ethyl glutamine) are appreciably reduced whereas other amino acids undergo little change (Roberts et al., 1966); during the last (drying) stage there is a small general decrease in free amino acid concentration (Roberts et al., 1966). Wickremasinghe et al. (1965) also reported a decrease of amino acids during fermentation.

The over-all decrease which occurs in the level of free amino acids during fermentation suggests they are being converted to other substances. Several researchers (Bokuchava et al., 1954; Nakabayashi, 1958; Wickremasinghe et al., 1964; 1965) have suggested that the amino acids are being converted, at least in part, to volatile compounds likely to be important constituents of tea aroma. Bokuchava et al. (1954) reported that when certain amino acids are incubated with tea flavanols in hot aqueous solutions, pleasant aromas are formed which are different for each amino acid, and a mechanism to explain these results was proposed (Popov, 1956). The purpose of our investigation was to verify the conversion of amino acids to volatile aldehydes by the tea fermentation system using radiotracer techniques and to obtain additional information about the mechanism of this conversion. This is part of an ongoing investigation of the mechanism by which black tea aroma is formed during the conversion of fresh tea-shoot tips to black tea.

EXPERIMENTAL

Materials

Fresh tea leaf was picked at Lipton's Experimental Tea Garden near Charleston, South Carolina, and air freighted to us so as to arrive in our laboratory the same day. The leaf was placed in a freezer at -40°C on arrival in the laboratory, for use when required.

Radioactive (^{14}C) amino acids, generally labelled, were purchased from Schwarz BioResearch, Inc.; epigallocatechin gallate (EGCG) was prepared from fresh green tea leaves by procedures described below. Dehydroascorbic acid was purchased from Pierce Chemical Company and theanine was synthesized in our laboratory according to the procedure of Sakato et al. (1950).

Preparation of enzymes solution

Fresh frozen green-tea leaf (100 g) in 500 ml of phosphate buffer (pH 7.4, 0.1 M) containing 50 g of Polyclar AT was macerated in a Waring Blendor for 5 min. The macerate was filtered through 2 layers of cheese cloth and the leaf residue discarded. The filtrate was treated with ammonium sulfate and the 30–90% saturation precipitate collected. This precipitate was dissolved in a minimum amount of phosphate buffer (pH 7.4, 0.1 M). The solution was dialyzed against a large volume of phosphate buffer (pH 7.4, 0.1 M), with 2 changes of buffer daily, until the outside solution was colorless. This required about 3 days of dialysis.

All of the above-mentioned steps were carried out at 4°C using cold reagents and equipment. Finally, the soluble tea enzymes preparation, a yellowish, cloudy solution, was stored frozen at −40°C until required for use.

Preparation of flavanols

The method used was a modification of that reported by Vuataz et al. (1959).

a. Preparation of crude tea polyphenol extract from fresh tea leaves. Four batches of 500 g fresh-frozen tea leaves were macerated in a Waring Blendor for 2 min with 1,000 ml of cold (0°C) acetone. The suspension was filtered on a Büchner funnel. The residue was macerated 2 more times with 1 liter of 80% acetone and finally with 1 liter of absolute acetone.

The acetone extracts were combined and mixed with one-half their combined volume of chloroform in a separatory funnel. Distilled water was added until 2 layers formed and the chloroform layer was extracted 3 times with 1/4 its volume of distilled water. The combined aqueous layers containing most of the polyphenols free of fat-soluble materials were saturated with NaCl and extracted 4 times with 1/3 their volume of ethyl acetate. The ethyl acetate extracts were combined, dried with anhydrous Na_2SO_4 and taken to dryness under reduced pressure. The solids obtained were extracted twice with dry ethyl acetate to extract the unoxidized tea flavanols from oxidized (colored) material which is left behind.

The ethyl acetate extract was evaporated to dryness and the polyphenols were dissolved in distilled water and freeze dried. The final product was a light-tan powder.

b. Preparation of the flavanol-cellulose charge. The freeze-dried crude tea polyphenols were mixed thoroughly with an equal weight of cellulose under an atmosphere of nitrogen. To this mixture, distilled water (1/2 the dry weight of cellulose used) was added in small increments. Solvent "A" [ethyl propionate: ligroine 66–75°C. (3:1) mixture saturated with water] was added to thoroughly wet the flavanol-cellulose charge.

c. Operation of the column. The charge was packed on a prepared cellulose column (80- by 10-cm i.d.) and the column eluted overnight with solvent "A" at a flow rate of 6.5–6.7 ml/min. The eluate was collected in 340-ml fractions maintained at 4°C. Following elution of epicatechin gallate (ECG) from the column, gradient elution was started by diluting solvent "A" (2,000 ml) with solvent "B" [ethyl propionate: ligroine (9:1) mixture saturated with water] to maintain a constant volume in the mixing reservoir. When epigallocatechin gallate (EGCG) eluted from the column, the elution gradient was changed so that the mixing reservoir contained 2,000 ml of solvent "B" which was continuously diluted with solvent "C" (ethyl acetate saturated with water) to maintain a constant volume in the mixing reservoir. The elution was continued until epigallocatechin (EGC) eluted from the column. The eluant was monitored spectrophotometrically at 275 nm.

All fractions from the cellulose column containing a single flavanol, as determined from the UV absorption curve and paper and thin-layer chromatography, were combined and taken to dryness under reduced pressure at 30°C. The solids obtained were dissolved in distilled water and extracted with methylene chloride to remove any residual ligroine. The methylene chloride-washed aqueous solutions were freeze dried. dried.

d. **Crystallization of the flavanols.** The freeze-dried flavanols were twice crystallized from water, dried in an evacuated desiccator over P_2O_5 and stored at −40°C in sealed evacuated vials until required for use.

Model tea fermentation system

Oxidations were carried out in jacketed reaction vessels (Fig. 1) at 25°C. The reaction mixture was made up as follows: 10.0 ml substrate/buffer solution containing 5.0 mM epigallocatechin gallate in citrate-phosphate buffer (0.1 M, pH 5.4), 10 μliters (1 μc) ^{14}C-amino acid solution and 1.0 ml of the soluble tea enzymes preparation. The enzymic oxidation was continued for 30 min, during which time the reaction mixture was aerated by stirring vigorously with a magnetic stirrer. Since ^{14}C-theanine was not available, a higher concentration of theanine (30 mM) was used in the model tea fermentation system to improve the chance of detecting any volatile compound formed therefrom.

Enzymic oxidation of fresh green-tea homogenate

Fresh-frozen tea leaves (16 g) were mixed with 64 ml of cold citrate-phosphate buffer (0.1 M, pH 5.4) and macerated in a cold base (0°C) Waring Blendor for 3 min. The homogenates were transferred to the reaction vessel (Fig. 1) and brought to 25°C. Then 10 μliters (1 μc) of the ^{14}C-amino acid solution was added to the homogenate and oxidation (called fermentation) allowed to continue for 30 min, during which time the solution was aerated continuously by stirring vigorously with a magnetic stirrer.

Headspace volatile analysis by gas-liquid chromatography

At the end of the oxidation period, a headspace volatiles trap was attached to one of the side arms of the vessel and the vessel was otherwise closed. Hot water (95°C) was circulated through the jacket. The sample was stirred continuously with a magnetic stirrer. 3 min after the hot water was turned on, the headspace volatiles in the vessel were flushed into the trap with N_2 gas flowing at a rate of 100 ml/min for 5 min. The headspace volatiles trap consisted of stainless steel tubing, 1/4 in. o.d. by 7 in. long, packed with 0.9 g of 5% Apiezon L on Gaschrom CL, 100–120 mesh. The trap was maintained at −80°C with dry ice.

Gas chromatography

A Barber-Colman Series 5000 Selectra System with dual hydrogen flame detectors was used.

The trap containing the headspace volatiles was attached to the inlet of the GLC unit and heated to 220°C with a heating tape.

Development of the chromatogram was started when the temperature of the trap reached 100°C (carrier gas = He; flow rate = 65 ml/min). The column temperature was programmed from 70 to 200°C at 2°C/min with hold at 200°C for at least 0.5 hr. The columns used were of stainless steel, 3/16 in. o.d. by 50 ft long, packed with 10% Carbowax 20 M on Gas-chrom Q, 60–80 mesh.

Part of the column effluent was diverted to a radioactivity monitoring unit (Barber-Colman Model 5190 Radioactivity Monitoring System).

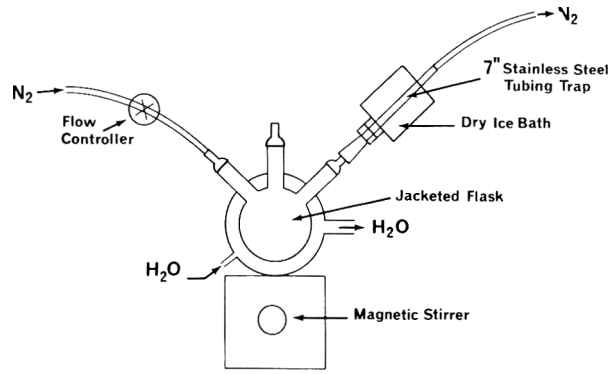

Fig. 1—Schematic diagram of headspace volatiles collection apparatus.

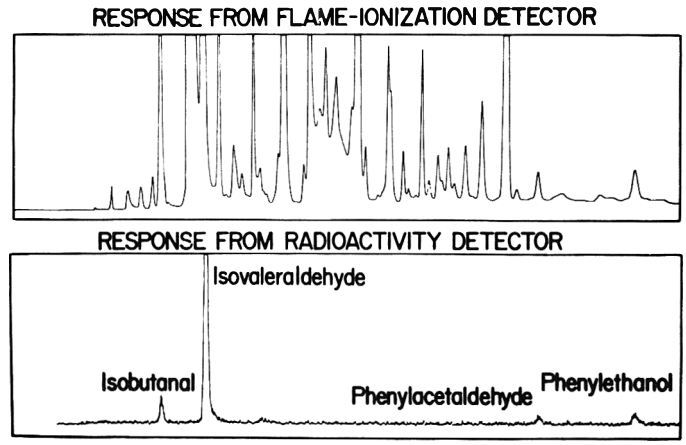

Figure 2—Gas-liquid chromatogram of the headspace volatiles obtained from a green-tea-leaf homogenate fermented in the presence of ^{14}C-valine, ^{14}C-leucine and ^{14}C-phenylalanine.

RESULTS

FRESH green-tea-leaf homogenate was fermented in the presence of ^{14}C-amino acids at 25°C for 30 min. The headspace volatiles over the fermented (oxidized) mixture were collected and analyzed by gas-liquid chromatography as described in the Experimental section. A typical set of gas-liquid chromatograms produced simultaneously by the flame ionization detector and the radioactivity detector are shown in Figure 2. In this case ^{14}C-valine, ^{14}C-leucine and ^{14}C-phenylalanine were added to the fermenting tea-leaf homogenate. The chromatogram produced by the flame ionization detector shows the typical complex aroma pattern of fermented tea (Bondarovich et al., 1967), whereas the chromatogram produced by the radioactivity detector shows only 4 radioactive peaks, identified by comparison of retention times to be isobutanal, isovaleraldehyde, phenylacetaldehyde and phenylethanol. These 4 compounds are constituents of black tea aroma (Bondarovich et al., 1967, and references contained therein) and they correspond to the Strecker degradation products of the ^{14}C-amino acids (except for phenylethanol) added to the fermenting tea-leaf homogenate.

The effect of the fermentation system composition on formation of volatile compounds from amino acids was studied both in whole tea-leaf homogenates and in model tea fermentation systems.

Table 1—Effect of reaction mixture composition on formation of ^{14}C-isovaleraldehyde from ^{14}C-leucine.

Reaction mixture composition	Color of reaction mixture at end of incubation	^{14}C-isovaleraldehyde recovered (dpm)
Experiment I		
^{14}C-Leucine + green-tea homogenate	Brown	5,100
^{14}C-Leucine + green-tea homogenate (enzyme inactivated)	Green	950
^{14}C-Leucine + green-tea homogenate, dried[1]	Dark-brown	9,100
Experiment II		
^{14}C-Leucine	Colorless	200
^{14}C-Leucine + enzymes	Light yellow[2]	200
^{14}C-Leucine + EGCG	Colorless	880
^{14}C-Leucine + EGCG + enzymes	Yellow-brown	4,640
^{14}C-Leucine + EGCG + enzymes, inactivated	Light yellow[2]	930

(All reaction mixtures were allowed to oxidize for 30 min at 25°C prior to collection of the headspace volatiles for analysis by GLC. The procedures used are described in the Experimental Section.)

[1]Taken to dryness on a steam bath after 30 min oxidation.

[2]Due to color of enzyme solution.

The first experiment (i.e., Experiment I in Table 1) clearly demonstrated that ^{14}C-leucine is actively converted to ^{14}C-isovaleraldehyde in a fermenting tealeaf system. These results also show that prevention of the tea fermentation process by inactivation of the enzymes present in the tea leaf markedly reduced the amount (rate) of isovaleraldehyde formed from leucine. Further, drying of the fermented mixture caused an additional amount of ^{14}C-isovaleraldehyde to be formed.

The model systems studies (Experiment II in Table 1) showed that the conversion of ^{14}C-leucine to ^{14}C-isovaleraldehyde takes place in the presence of a tea enzyme extract when it is oxidizing tea flavanols (EGCG was used in this set of experiments). Incubation of leucine with the tea enzymes preparation alone produced only a very small amount of isovaleraldehyde, no more than obtained by incubating leucine by itself. Incubation of leucine with EGCG by itself or with inactivated tea enzymes preparation produced a small amount of isovaleraldehyde: This system appears to be analogous to that when leucine is incubated with an inactivated green-tea homogenate (Experiment I, Table 1).

The several amino acids reported to decrease most during the fermentation stage of black-tea manufacture (Roberts et al., 1966) were incubated with the tea flavanol EGCG and a soluble tea enzymes preparation to determine their ability to form volatile aldehydes. Table 2 shows a list of the ^{14}C-amino acids tried, both in the model tea fermentation system and in an oxidizing green-tea homogenate and the volatile aldehydes produced. Valine, isoleucine, leucine and phenylalanine were converted into their corresponding Strecker degradation aldehydes; namely, isobutanal, 2-methylbutanal, isovaleraldehyde and phenylacetaldehyde, respectively, in both of the tea fermentation systems tried. It is noteworthy that all of these aldehydes have been reported to be constituents of black-tea aroma (Bondarovich et al., 1967). In contrast, aspartic acid, glutamic acid, glutamine, arginine, threonine, serine and theanine did not give rise to any detectable volatile compounds under these conditions.

The effect of ascorbic acid and dehydroascorbic acid on conversion of ^{14}C-leucine to ^{14}C-isovaleraldehyde was investigated. Results are shown in Table 3. When ascorbic acid was added to the reaction mixture consisting of EGCG, tea enzymes preparation and ^{14}C-leucine, only a very small amount of ^{14}C-isovaleraldehyde was produced. When no ascorbic acid was present, the amount of ^{14}C-isovaleraldehyde formed was more than 10 times greater. Dehydroascorbic acid was able to cause conversion of ^{14}C-leucine to ^{14}C-isovaleraldehyde in the absence of EGCG and tea enzymes preparation.

DISCUSSION

ISOBUTANAL, isovaleraldehyde, 2-methylbutanal and phenylacetaldehyde were formed from valine, leucine, isoleucine and phenylalanine, respectively, in the presence of fermenting (oxidizing) fresh tea-leaf homogenate. Since these aldehydes are present in black-tea aroma (Bondarovich et al.,1967), the results of our investigation clearly show that a portion of the black-tea aroma components come from amino acids. Further, at least part of the decrease of free amino acids during the conversion of green tea to black tea (Wickremasinghe et al., 1965; Roberts et al., 1966) must be accounted for by this transformation.

Results of our experiments also show that the production of aldehydes from amino acids is largely dependent on the tea fermentation process (Roberts, 1962; Sanderson, 1965): Tea leaf which has been activated by steam treatment produced only a small amount of volatile aldehydes from amino acids. Further, our model tea fermentation system studies clearly showed that oxidation of tea flavanols by tea enzymes markedly increases the amount of aldehydes formed from amino acids.

These results are interpreted to be a reflection of a rate effect; that is, they reflect the rate of production of oxidized

Table 2—List of amino acids tested in model tea fermentation system and in fermenting green-tea-leaf homogenates and the volatile products formed.

^{14}C-Amino Acid	Volatile ^{14}C-products
Valine	Isobutanal
Isoleucine	2-Methylbutanal
Leucine	Isovaleraldehyde
Phenylalanine	Phenylacetaldehyde
Aspartic Acid	None
Glutamic Acid	None
Glutamine	None
Arginine	None
Threonine	None
Serine	None
Theanine[1,2]	None

(The experimental methods are described in the Experimental Section.)

[1]5-N-Ethyl glutamine.

[2]This experiment was carried out with nonradioactive theanine only.

tea flavanols which are the oxidants causing the Strecker degradation of amino acids in the tea fermentation system. Only when the tea leaf enzyme system is operating is there an appreciable amount of volatile aldehydes produced from amino acids in the period of time normally allowed for tea fermentation, i.e., 0.5 to 3 hr (Eden, 1965; Harler, 1963; Keegel, 1958). The relatively slow nonenzymatic conversion of amino acids to volatile aldehydes in the presence of tea flavanols is probably analogous to the situation existing in the development of chocolate aroma (Biehl, 1967; Rohan et al., 1967).

It was mentioned above that the ability of oxidized flavanols to catalyze the oxidation of amino acids has been shown by several investigators (Bokuchava et al., 1954; Popov, 1956; Nakabayashi, 1958; Wickremasinghe et al., 1964) and that Popov (1956) has proposed a mechanism for the oxidative deamination of amino acids during tea fermentation. Popov's (1956) scheme proposes that orthoquinones produced by the action of catechol oxidase on tea flavanols during the tea fermentation process are required for the production of aldehydes from amino acids.

Popov (1956) did show that carbon dioxide, ammonia and aldehydes were formed in model tea fermentation systems consisting of amino acids, tea tannin and acetone dried powder of tea leaf as source of tea-leaf enzyme system. However, the results of these experiments did not exlude the possibility that specific enzymes were causing the observed changes. Results of our investigation have extended these earlier findings by demonstrating that a) specific enzymes do not appear to be operating during tea fermentation to bring about the conversion of amino acids to their corresponding Strecker degradation aldehydes, i.e., the crude tea enzymes preparation alone was not capable of bringing about this change, and b) the oxidation of tea flavanols is sufficient in itself to cause the conversion of amino acids to aldehydes.

It is noteworthy that enzyme systems capable of converting amino acids to aldehydes have been extracted from tomato fruit (Yu et al., 1968a; Yu et al., 1968b) and microorganisms (MacLeod et al., 1956; Sasaki, 1962), but that the likely presence of phenolic compounds in the test systems used raises the possibility that the mechanism in these systems is the same as that operating in fermenting tea leaf.

A study of the ability of different amino acids to be converted into their corresponding aldehydes by the tea fermentation system showed that valine, leucine, isoleucine and phenylalanine were converted into volatile aldehydes corresponding to their respective expected Strecker degradation products. On the other hand, aspartic acid, glutamic acid, glutamine, arginine, threonine, serine and theanine did not give rise to any detectable volatile aldehydes. The results obtained with this latter group of amino acids could well be expected if one considers the low volatility or the reactivity, or both, of their expected Strecker degradation products. Similar negative results were reported by Yu et al. (1968b) for alanine and aspartic acid and by Rooney et al. (1967) for lysine, arginine, histidine, tryptophan, glutamic acid and proline in their investigation of similar systems.

Table 3—Effect of ascorbic acid and dehydroascorbic acid on formation of ^{14}C-isovaleraldehyde from ^{14}C-leucine.

Reaction mixture composition	Color of reaction mixture at end of incubation	Amount of ^{14}C-isovaleraldehyde recovered (dpm)
^{14}C-Leucine + EGCG + enzymes + ascorbic acid	Light yellow[1]	440
^{14}C-Leucine + EGCG + enzymes	Yellow-brown	4,630
^{14}C-Leucine + dehydroascorbic acid	Light brown	1,540

[The experimental methods are described in the Experimental Section. Ascorbic (5.0 mM) and dehydroascorbic acid (5.0 mM) were added to the substrate/buffer solution.]
[1] Due to color of enzyme solution.

Popov (1966) suggested that dehydroascorbic acid formed by nonenzymatic oxidation of ascorbic acid can cause formation of aldehydes from amino acids by oxidative deamination. Our experiments confirm and extend these results by showing that dehydroascorbic acid can indeed cause the formation of aldehydes from amino acids and that the aldehydes formed are those expected from a Strecker degradation reaction.

The presence of ascorbic acid itself in the tea fermentation system prevented both color formation and appreciable conversion of amino acids to volatile aldehydes. Ascorbic acid is known to prevent enzymic browning caused by oxidation of phenolic compounds by reducing the quinones formed back to their original oxidation level, thus preventing polymerization. In the system investigated here, it is postulated that the quinone formed from EGCG is preferentially converted by ascorbic acid back into EGCG, with the result that no quinone is available to convert amino acids to aldehydes.

The results of our investigation fit well in a scheme for tea fermentation proposed previously (Sanderson, 1965). The part of this scheme concerned with the present investigation has been amplified to incorporate the results discussed above and is shown in Figure 3. Essential features of this scheme may be summarized as follows: During the conversion of fresh green-tea leaf to black tea, flavanols are oxidized by catechol oxidase to quinones (or possibly to semiquinones), which polymerize to form colored products such as theaflavins and thearubigins (Roberts, 1962; Brown et al., 1969). If ascorbic acid is present, no colored oxidation products are formed until such time as essentially all of the ascorbic acid present is oxidized to dehydroascorbic acid: The oxidation of ascorbic acid by oxidized tea flavanols is favored over other reactions possible in the tea fermentation system. The quinones formed from flavanols are strong oxidizing agents and are capable of converting amino acids to their corresponding Strecker degradation products (i.e. aldehydes). Further, dehydroascorbic acid formed from ascorbic acid may also act as an oxidizing agent capable of converting amino acids into their corresponding aldehydes.

The interrelationship which exists among the several tea leaf constituents studied in this investigation, and shown in Figure 3, points out again (Sanderson, 1965) the importance of the chemical composition of the tea leaf in determining the organoleptic properties of the finished black tea. Unfortunately, it must be stated that as yet all too little is known about what the composition of a tea leaf should be to enable the best black tea to

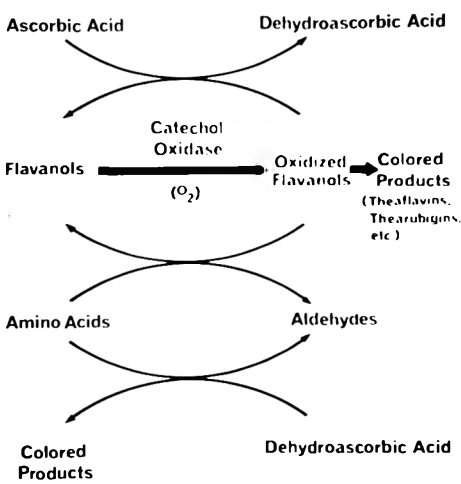

Fig. 3—Scheme showing relationship between tea flavanol oxidation and formation of aldehydes from amino acids during tea fermentation.

be produced. And, of course, much remains to be learned about how the black-tea manufacturing conditions (Eden, 1965; Harler, 1963; Keegel, 1958) affect the organoleptic properties of the final product. It has also been shown (Finot et al., 1967) that amino acids can give rise to volatile aldehydes through interaction with black-tea solids under conditions existing during the normal brewing of a cup of tea. Perhaps the method of brewing tea is another black-tea aroma determinant.

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VARIATIONS OF RELATIVE RETENTION TIMES IN OPEN–TUBULAR GAS–CHROMATOGRAPHIC COLUMNS: RELEVANCY TO FRUIT VOLATILES

SUMMARY—Open-tubular gas chromatographic columns made from stainless steel tubing, coated with methyl silicone oil, give variable retention times for individual alcohols, dependent on the size of sample. This difficulty is largely overcome by using a small proportion of adsorption-reducing material with the methyl silicone. There are cases of reversal of peak order, caused by differences in column temperature or minor differences in the coating. However, with care, relative retention times are nearly constant and are helpful as an aid in identification of compounds. A procedure for precise comparison of the retention times of unknown fruit volatiles with known compounds is described. Relative retention times of representative compounds are given for twelve different stationary liquids.

INTRODUCTION

ONE DESIRABLE feature of an analytical gas-chromatographic column is constancy of relative retention time of each encountered solute, referred to a standard compound or set of standards. Although identification of compounds by relative retention times alone is dubious, good retention data are helpful along with other methods. In research on fruit volatiles an individual compound may occur at widely different concentrations in different preparations. Therefore, possible variation of retention time with amount of solute must be considered along with other variables. A number of factors which affect constancy of relative retention times have been described.

Porter et al. (1956) discussed the effects of sample size, nonlinearity of vapor-pressure variation with concentration of the solute in the stationary phase, and charging technique on peak size and shape. Although sample size of a given solute affects the retention time to the peak maximum, the time to the front of the peak would appear to change very little in most cases if other independent variables are unchanged (and if adsorption is not a factor).

The effect of adsorption of the solute by the solid support on relative retention times with packed columns was discussed in the original paper on gas chromatography by James et al. (1952) and more recently by others (Eggertsen et al., 1958; Averill, 1962; Mon et al., 1966). Scholz et al. (1962) showed with a packed column which exhibited adsorption that the relative retention times (to both the maximum and peak front) of low-molecular-weight alcohols may be changed considerably by variation of the sample size. Elimination or minimizing of adsorption effects in both packed and open-tubular columns has been described. This was accomplished by including small proportions of surface active agents in the stationary phase (James et al., 1952; Averill, 1962, 1965; Mon et al., 1967), by treatment of the solid support (Scholz et al., 1962; Ottenstein, 1968), or by saturating the carrier gas with water vapor (Knight, 1958; Teranishi et al., 1962; Mon et al., 1966).

An example of reversal of peak order at different column temperatures has been presented by Littlewood (1962a), with a discussion of the thermodynamic principles involved. More recently a discussion of the temperature effect and a compilation of considerable pertinent retention data on hydrocarbons have been presented by Ettre et al. (1967).

This paper deals only with open-tubular columns. It shows cases of peak-order reversal with variation in sample size, as a result of adsorption, and the degree of improvement observed when a surface active agent is included in the stationary phase.

The second part of this paper is concerned with desired changes in relative retention times obtained with different stationary liquids. Considerable data on this topic have been published for packed columns (Tenney, 1958; Wehrli et al., 1959; Littlewood, 1962b; Institute of Petroleum, 1966) and open-tubular columns (Mon et al., 1966; Institute of Petroleum, 1966).

EXPERIMENTAL

THE COLUMNS were made in this laboratory from three sizes of stainless steel tubing, 0.01, 0.02 and 0.03 in. ID. Except where noted otherwise, the tubing was rigorously cleaned with a series of liquids including nitric acid, ammonia and solvents (Mon et al., 1967). The columns were coated by the procedure described by Teranishi et al. (1964). With the exception of the earliest experiments (Figs. 1 and 2), the columns were operated in altered Thermotrac ovens (Beckman Instruments, Inc., Fullerton, Calif.), with on-column inlets (Carle Instruments, Inc., Anaheim, Calif.) in which the sample is vaporized in a 2-in. × 0.07-in. ID tube.

The inlet temperature was maintained sufficiently high (e.g., 225°C for sesquiterpenes) to give essentially instantaneous vaporization and minimize adsorption, and thus avoid peak broadening. Samples were injected with a 1-µl syringe (excepting the experiments for Figs. 1 and 2). Flame-ionization detectors made in this laboratory were used (Teranishi et al., 1962). The carrier gas was helium.

RESULTS & DISCUSSION

Undesired peak-order reversals

Figure 1 shows chromatograms of a solution of 1-propanol and 3-methylpentane in ethanol. The column used was coated with methyl silicone oil without additive. Although alcohols tailed badly, this column was once regarded as one of the best for the study of orange essence. The three chromatograms were all run under the same conditions of temperature and linear velocity of helium. The only difference was in the size of sample injected.

The hydrocarbon (peak 2) shows the same retention time in all three runs, regardless of sample size, but the alcohol, 1-propanol, (peak 1) appears before peak 2 when the sample is large but after peak 2 when the sample is smaller.

For the experiment shown in Figure 2, benzene was substituted for the ethanol and other conditions remained the same. As before, the aliphatic hydrocarbon shows the same retention time in all runs, but 1-propanol appears before peak 2, between peaks 2 and 3, or after peak 3, depending on the sample size. A similar movement of the 1-propanol peak was observed when heptane, which appears at 16 min, was substituted for the benzene. The conclusion from these runs and others is that hydrocarbons show the same retention time regardless of peak size while low molecular weight alcohols, at least up to C_6, show widely different retention times depending on the amount present, on columns of this type. This effect is believed to be due to adsorption of alcohols on the walls of the tubing. However, above a certain limit, further increases in sample size have only a slight effect on retention time. Only when the amount of alcohol is relatively high does it exceed the adsorptive capacity of the column inner wall, and then the retention time of the alcohol is controlled mainly by partitioning between the silicone oil and the carrier gas.

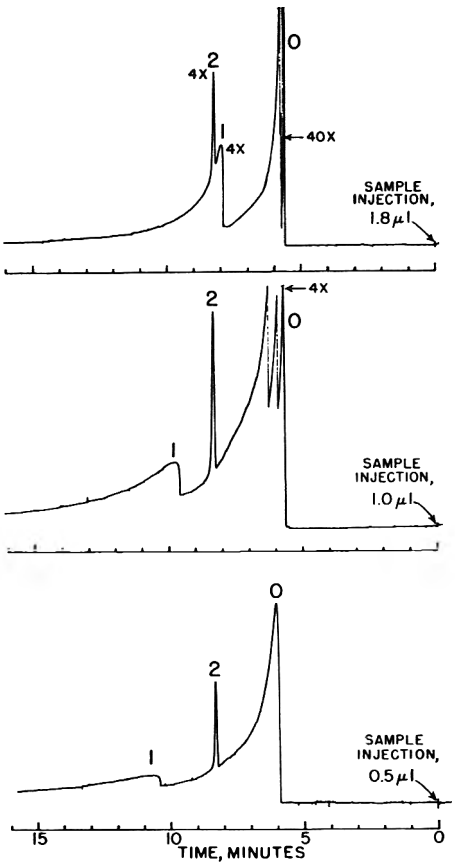

Fig. 1—Chromatograms of a solution consisting of 65% ethanol, 34% 1-propanol, and 1% 3-methylpentane. Column: 200-ft, 0.01-in. ID, coated with methyl silicone SF-96(50), no tailing reducer, new tubing, cleaned with organic solvents only before coating. Stream splitter at column inlet: 1/200. Column temperature: 40°C. Peaks: 0, ethanol; 1, 1-propanol; 2, 3-methylpentane.

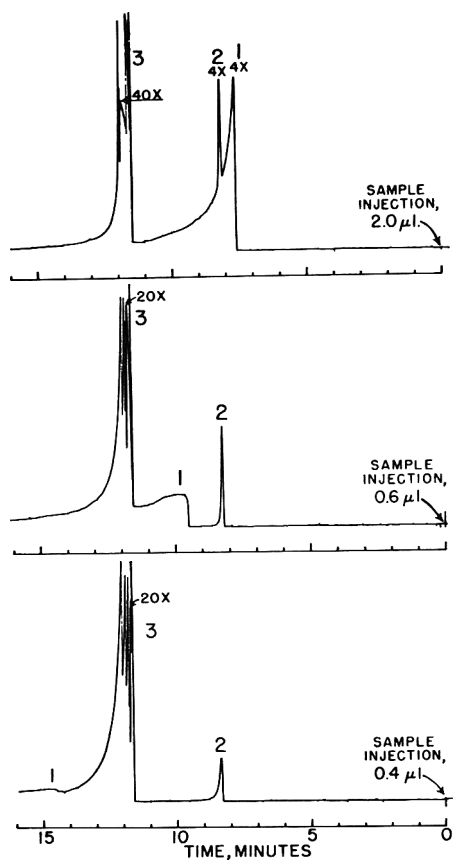

Fig. 2—Chromatograms of a solution consisting of 65% benzene, 34% 1-propanol, and 1% 3-methylpentane. Column and splitter: Same as in Fig. 1. Column temperature: 40°C. Peaks: 1, 1-propanol; 2, 3-methylpentane; 3, benzene.

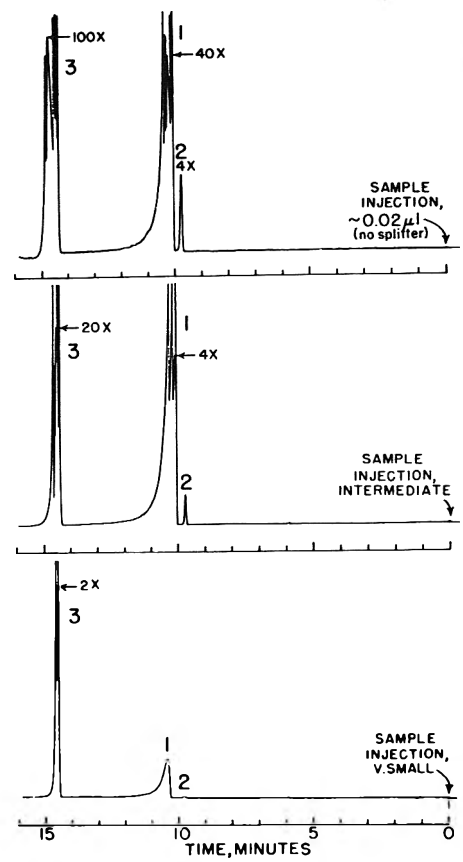

Fig. 3—Chromatograms of a solution consisting of 65% benzene, 34% 1-propanol, and 1% 2-methylpentane. Column: 500-ft, 0.02-in. ID, coated with methyl silicone SF-96(50) plus 5% of Igepal CO-880. No stream splitter. Column temperature: 40°C. Peaks: 1, 1-propanol; 2, 2-methylpentane; 3, benzene.

Use of adsorption-reducing material

We included a small proportion of polar material such as Carbowax or Igepal with silicones and hydrocarbons used as stationary liquids (Averill, 1965; Mon et al., 1967). The original purpose of the polar material was to eliminate or reduce tailing of alcohols and other oxygenated compounds and to give peaks that have areas more nearly proportional to the amount of compound injected. This is accomplished by preferential adsorption of the polar coating material on the column walls.

Figure 3 shows the behavior of 1-propanol in a column coated with methyl silicone SF-96(50) plus 5% of Igepal CO-880. The test compounds were mixed in the same proportions as before. (Instead of 3-methylpentane the 2-methyl isomer was used because it is more clearly separated from 1-propanol on this column.) In this column the retention time to the peak front, of 1-propanol, is constant with sample size variation from large to intermediate and is only 0.2 min later when the sample size is less than one tenth the smallest amount used in the earlier column. The samples for the middle and bottom chromatograms were too small to measure but can be estimated from the size of the benzene peaks. With a still smaller amount, so small that the 1-propanol peak could barely be seen, with the detector set at five times higher sensitivity, the response came 0.7 min late (chromatogram not shown). This is in contrast with a shift of 7 min with the earlier column. Thus, the small proportion of Igepal applied with the stationary liquid acts not only to reduce tailing but also to give nearly constant retention times for alcohols, except at very low levels.

In a similar experiment with a 0.02-in. column coated with Carbowax 20 M only, operated at 75°C, and with a solution containing only 1% of 1-propanol and the usual 1% of 2-methylpentane in benzene, the retention time of the 1-propanol was constant within 0.1 min, for peaks from large size down to the smallest that could be observed.

With the newer silicone columns and peaks of moderate size, one must still be careful to avoid reversal of peak order. Figure 4 shows portions of chromatograms of two similar samples of apple essence (Flath et al., 1967). The columns were nominally the same: 0.02-in. ID, 500 ft long, coated with methyl silicone SF-96(50) containing 5% of Igepal CO-880. The only known difference is that column A had been fully cleaned with nitric acid, ammonia, and solvents before coating, while column B was simply washed out with solvent and recoated. It had previously been coated with the same material. Apparently all the Igepal was not removed by the solvent and this resulted in a higher concentration of Igepal after recoating. The effect was that the relative retention times of alcohols and unsaturated aldehydes were increased with respect to esters. In Figure 4 the

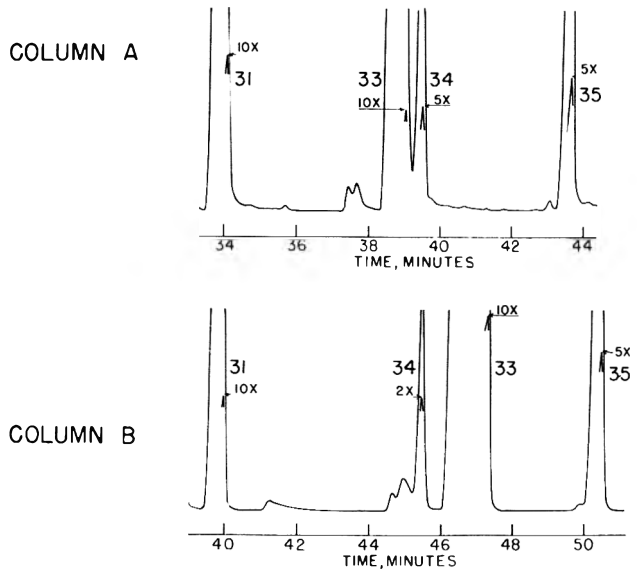

Fig. 4—Portions of chromatograms of samples of apple essence (Flath et al., 1967). Column A: 500-ft, 0.02-in. ID, coated with methyl silicone SF-96(50) plus 5% Igepal CO-880 after complete rigorous cleaning. Column B: Same specifications as column A, but coated once, then washed out with organic solvents only, and recoated. Column temperature: 50° during first 10 min, then programmed at 1° per min. Peaks: 31, butyl acetate; 33, 2-hexenal, trans; 34, ethyl 2-methylbutyrate; 35, 2-methylbutyl acetate.

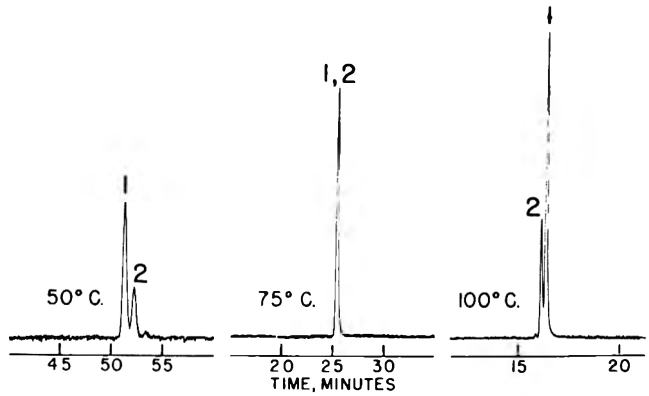

Fig. 5—Chromatograms of a mixture of 75% 2-hexenal, trans and 25% ethyl 2-methylbutyrate. Column: 650-ft, 0.03-in. ID, coated with methyl silicone SF-96(50). Carrier gas saturated with water vapor at room temperature. Peaks: 1, 2-hexenal, trans; 2, ethyl 2-methylbutyrate.

three esters, peaks 31, 34 and 35 are in about the same relative positions on columns A and B, but 2-hexenal, peak 33, is later on column B than on A, and its order with respect to ethyl 2-methylbutyrate is reversed. Differences of this kind, but of lesser degree, are known to occur with columns of this type after long use, compared to performance when the column was new.

Figure 5 shows peak-order reversal as a result of variation of column temperature, with the same two compounds, 2-hexenal and ethyl 2-methylbutyrate. All three runs were made on the same column. At 50°C, the aldehyde appears first; at 75° there is no separation; at 100° the ester appears first.

Precise comparison of retention times

In actual practice with a methyl silicone-5% Igepal, 0.02-in. ID × 500-ft column, operated repeatedly with the same temperature program and helium pressure, relative times to the peak front for fruit essence constituents vary only slightly, or not at all, with size of sample. Relative retention times to the peak maximum show considerably more variation in many cases. These latter variations appear to be caused by nonlinearity of vapor pressure with concentration of solute in the stationary phase, as discussed by Porter et al. (1956). The small elongated inlets (injectors) and the injection technique used should preclude any appreciable effect on retention times from this part of the process.

Because of the situation noted above, peak fronts (the lower front face) are frequently more useful than peak maxima for helping to identify compounds. In a recent study on orange essence (Schultz et al., 1967) by combined gas chromatography and mass spectrometry, supporting retention time data were obtained by the peak enhancement (enrichment) procedure. The column was the same as noted in the paragraph above. A 0.30-μl sample of the nonaqueous essence was co-injected with 0.01–0.02 μl of a mixture of 5 known compounds of widely different retention time.

The chromatogram was superimposed on a plain chromatogram of the essence, with successive lining up on different sharp peaks near the peaks representing the added known compounds, respectively. Where there was peak enhancement, i.e., where the added compound and one of the essence constituents were the same, the enhanced peak was usually considerably larger, and its lower front face a little earlier than that of the simple peak. With hydrocarbons, esters and aldehydes, the lower front face of the enhanced peak was 0–0.05 min early (excepting benzaldehyde, 0.15 min early) but with alcohols it was 0.02–0.25 min early (0.08 min with 1-propanol; 0.25 min with 1-octanol). When there is any doubt, a more precise matching may be attempted by diluting the known compound further with noninterfering hydrocarbons so that the degree of enhancement will be small.

Relative retention times with various stationary liquids

Table 1 presents relative retention times of four compounds chosen from constituents found in fruit essences, representing alcohols, esters, aldehydes and terpene hydrocarbons. (The individual times were corrected by subtracting the time for carrier gas to pass through the column.) The retention times are given relative to that of decane. The stationary liquids are listed in order of increasing interaction with 1-hexanol. Data were taken from an earlier publication (Mon et al., 1966).

Methyl silicone has been the principal stationary phase for studying fruit volatiles at this laboratory, because of its good stability, low background when used with mass spectrometry and generally good separation ability for the compounds present. Table 1 will be of assistance when a different stationary liquid is used to separate compounds of different chemical classes, which are close together on methyl silicone SF-96(50).

Carbowax 20 M is usually a good choice because of its large difference from methyl silicone. However, from this table it may be predicted that any of the Apiezons, Oppanol B 10, polyphenol ether, silicone OV-225 or Tween 20 would do better than Carbowax 20 M in resolving an ester and an aldehyde that are not separated by methyl silicone. Of

Table 1—Relative retention times of representative compounds. Open-tubular columns at 125°C.

Stationary liquid	1-Hexanol	Amyl acetate	Octanal	Limonene	Decane
Apiezon L[3, 1, 2]	0.28	0.35	0.75	1.4	1.0
Apiezon C[3, 1, 2]	0.29	0.33	0.75	1.3	1.0
Apiezon J[3, 1, 2]	0.30	0.37	0.78	1.4	1.0
Oppanol B 10[4, 1, 2]	0.35	0.44	0.88	1.4	1.0
Silicone SF-96(50)[5, 2]	0.44	0.53	0.94	1.2	1.0
Silicone OV-17[6, 2]	0.9	1.1	2.0	2.0	1.0
Silicone QF-1[7, 2]	1.2	1.7	3.3	1.6	1.0
Polyphenyl ether[8]	1.3	1.4	2.8	2.6	1.0
Silicone OV-225[9, 2]	3.3	2.2	4.9	2.6	1.0
Igepal CO-880[10]	4.1	2.0	3.8	2.8	1.0
Carbowax 20 M[11]	4.5	2.2	4.2	3.0	1.0
Tween 20[12]	4.9	2.0	4.3	2.9	1.0

[1]Purified with alumina (Mon et al., 1966).
[2]With small proportion of Igepal CO-880.
[3]Hydrocarbon, refined by molecular distillation. Associated Electrical Industries Ltd., Manchester, England.
[4]Polyisobutylene. Badische Anilin & Soda Fabrik AG, Ludwigshafen Am Rhein, Germany.
[5]Methyl silicone. General Electric, Waterford, N.Y.
[6]Phenyl methyl silicone. Ohio Valley Specialty Chemical Co., Marietta, Ohio..
[7]Trifluoropropyl methyl silicone. Dow Corning Corp., Midland, Michigan.
[8]"Six-ring." Supplied by Applied Science Laboratories, Inc., State College, Pa.
[9]Cyanopropyl methyl-phenyl methyl silicone. Ohio Valley Specialty Chemical Co., Marietta, Ohio.
[10]Nonylphenoxy polyoxyethylene ethanol. General Aniline and Film Corp., New York, N.Y.
[11]Polyoxyethylene glycol (Polyethylene glycol). Union Carbide Corp., New York, N.Y.
[12]Polyoxyethylene sorbitan monolaurate. Atlas Chemical Industries, Inc., Wilmington, Del.

this group of liquids, silicone OV-225 or Tween 20 would be expected to make a good separation of ester, aldehyde and alcohol where the three compounds are not separated by methyl silicone. These predictions are based on the assumption that the percent change in relative retention time given by two different stationary liquids is the same for all compounds within a chemical class. This assumption may be useful as an approximation.

Trifluoropropyl methyl silicone, QF-1, compared with methyl silicone, is the only stationary phase considered here that substantially increases the relative retention times of amyl acetate and octanal more than it does for hexanol. The silicone with cyano groups, OV-225, gives a high relative retention time for octanal.

Relative retention times are similar with the three purified Apiezons L, C and J (Table 1) which differ in average molecular weight. For work with fruit volatiles J or L is preferable because C bleeds from the column at a faster rate at the temperatures required. The melting temperature of L is near 50°C, so J should be used for columns which are at times operated near or below that temperature.

The last three lines of Table 1 show similarity among stationary liquids which are mainly polyoxyethylene. The other chemical groups present in Igepal CO-880 and Tween-20 appear to have minor effects on the relative retention times.

When two similar compounds of the same chemical class are difficult to separate with a given stationary liquid, it cannot be predicted which other stationary liquid would give the best separation. This is illustrated by the following examples. Table 2 (Teranishi et al., 1967) presents data on the separation of two sesquiterpene hydrocarbons which are quite similar. Ylangene is a geometrical isomer of α-copaene. These compounds are separated by SF-96(50) but only slightly, as the retention times differ by only 2%. In preparative work a 2% difference is usually insufficient to enable ready separation and collection of the two components in a reasonable state of purity. From Table 2 it is evident that equal improvement is given by either the nonpolar Apiezon or the polar Igepal or Carbowax. There is no reversal of peak order. In high-resolution preparative work, a few mg of each pure sesquiterpene were collected by use of a 1000-ft, 0.03-in. ID, open tubular, Igepal column, with repeated 0.5-μl injections of the binary mixture (Teranishi et al., 1967).

Table 3 shows another example of the separation of two sesquiterpene hydrocarbons. This time there is a greater difference in structure, but again the retention times with methyl silicone differ by only 2 or 3% at 175° or 150°C, the temperatures we have used for preparative work on sesquiterpenes. In contrast to data in Table 2, there is considerable improvement in separation with Apiezon but scarcely any separation with Carbowax, and the latter reverses the peak order except at 175° where there is no separation. Apiezon and the silicones show considerably better separation when the column temperature is lower. Separation with Carbowax improves somewhat at 125°, but is poorer again at 100°, although the relative retention times are about the same at these temperatures.

Data from the same runs presented in a different manner are shown in Table 4, which presents the resolution (ratio of horizontal distance between peak maxima to mean peak width at base). Of course, the resolution depends not only on the relative retention times, but also on column efficiency and sample size. These tables illustrate the degree of improvement of separation we have obtained by employing different stationary phases for closely similar fruit volatiles.

In both examples of pairs of sesquiterpenes, Apiezon gave fairly good separations. However, there are other cases of pairs of both mono- and sequiterpene hydrocarbons which are separated poorly, if at all, on both methyl silicone and Apiezon, but are resolved fairly well by silicones OV–17 or OV–225 or by Carbowax 20 M. This behavior illustrates again the necessity of employing several different stationary phases for a thorough study of complex mixtures.

Table 2—Relative retention times of α-copaene and ylangene (Teranishi et al., 1967). Open-tubular columns at 150°C.

Stationary liquid	Ylangene	α-Copaene
Apiezon C[1, 2]	1.00	1.05
Oppanol B 10[1, 2]	1.00	1.04
SF-96(50)[2]	1.00	1.02
OV-17[2]	1.00	1.03
QF-1[2]	1.00	1.03
Igepal CO-880	1.00	1.05
Carbowax 20 M	1.00	1.05

[1]Purified with alumina (Mon et al., 1966).
[2]With small proportion of Igepal CO-880.

Table 3—Relative retention times of β-copaene. Caryophyllene = 1.00.

Stationary liquid	Column temperature, °C			
	100	125	150	175
Apiezon J[1, 2]	1.12	1.10	1.08	1.06
Silicone SF-96(50)[2]	1.06	1.05	1.03	1.02
Silicone OV-17[2]	1.05	1.03	1.02	1.01
Carbowax 20 M	0.98	0.98	0.99	1.00

[1]Purified with alumina (Mon et al., 1966).
[2]With 5% of Igepal CO-880.

Table 4—Resolution of β-copaene and caryophyllene.

Stationary liquid	Column temperature, °C			
	100	125	150	175
Apiezon J[1, 2]	8.4	6.6	4.4	4.0
Silicone SF-96(50)[2]	3.0	1.9	2.1	1.1
Silicone OV-17[2]	2.0	1.8	1.1	~0.3
Carbowax 20 M	~0.6	~0.7	~0.4	0

[1]Purified with alumina (Mon et al., 1966).
[2]With 5% of Igepal CO-880.

Columns: 0.02 in. ID; efficiency in the order of 100,000 theoretical plates at 150°C.

Sample size: 0.02 μl of a 25% β-copaene-75% caryophyllene mixture.

Linear velocity of helium: 31 cm per sec at 150°C.

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EFFECT IN RATS OF PARTIAL REPLACEMENT OF COW'S MILK PROTEIN BY SUPPLEMENTARY NITROGEN

SUMMARY—A number of experiments studied growth of weanling rats and urinary nitrogen excretion in young adult rats when cow's milk protein was partially replaced by varying levels of supplementary nitrogen. A mixture of nonessential L-amino acids (NEAA) or a mixture of diammonium citrate and glycine (DAC-Gly) was used as the source of nitrogen. Substitution of the 15% milk protein diet to the extent of 10% slightly reduced growth; significant growth reduction occurred with substitutions of 20% and greater. Fortification of the diets containing 10.5% milk protein and the supplementary nitrogen sources with sulfur amino acids did not restore growth to the maximum rate obtained with the 15% milk protein diet. Additional supplementation with tryptophan further improved growth slightly but not to the maximum rate. Several other essential amino acids, alone or in combination, had no apparent effect. Based on urinary nitrogen excretion, comparable results were obtained with young adult rats by substituting nitrogen for milk protein in the diets. The reduced performance following substitution of milk protein with supplementary nitrogen may be due partly to decreased utilization of sulfur amino acids and possibly to decreased utilization of all essential amino acids.

INTRODUCTION

WHEN ESSENTIAL amino acids form the sole dietary source of protein for rats, a lower growth rate is observed than when the same level of dietary N is supplied by a well-balanced amino acid mixture containing approximately equal amounts of essential and nonessential amino acids (Rose et al., 1948; Stucki et al., 1962; Young et al., 1968).

Animal proteins of high biological value may contain some or all of the essential amino acids, expressed per unit of total protein nitrogen, in excess of required amounts for the rat. Bender (1965) suggested that egg protein contains a surplus of 10–20% of all essential amino acids. In this case, essential amino acids are catabolized to meet calorie needs or are used for synthesis of nonessential amino acids and other N-containing compounds. Therefore, isonitrogenous replacement of the excess essential amino acids with supplementary nitrogen (from nonessential amino acids or other utilizable N sources) should not decrease dietary N utilization and animal performance.

For man, studies in this laboratory (Scrimshaw et al., 1966; Huang et al., 1966) and in others (Snyderman et al., 1962, Kofranyi et al., 1964) suggested that high quality protein N can be partially replaced with supplementary nitrogen without reducing nitrogen retention.

In the present series of experiments, weanling rats were fed dried skim milk as the source of dietary protein. A mixture of nonessential L-amino acids (NEAA), patterned as in cow's milk, or a mixture of diammonium citrate and glycine (DAC-Gly) was used as supplementary nitrogen to replace part of the protein nitrogen. The influence of the protein nitrogen replacement on growth rate was studied. Some experiments evaluated the effects of adding several essential amino acids to the diets containing the supplementary N sources.

The influence of age was also evaluated because available data indicate that for both man (Rose, 1957; Holt et al., 1965) and rats (Rama Rao et al., 1964; Smith et al., 1967) the requirements for essential amino acids per g total nitrogen differ in young and adults.

EXPERIMENTAL & RESULTS

Methods

A preliminary experiment (Table 1) confirmed general experience that maximum growth rates are achieved with a diet containing 15% protein from unsupplemented skim milk. This concentration of dietary protein was used, therefore, as a control level in the growth studies. The net protein utilization (NPU) of skim milk protein by rat assay (Bender et al., 1957) was 71 ± 2 compared with 63 ± 2 for unsupplemented casein as a standard.

Male weanling rats (Charles River Laboratories, North Wilmington, Mass., CD strain) were fed the experimental diets ad libitum for 14 days. The basal diets contained: dried skim milk (Dried Nonfat Skim Milk, Carnation Company, Van Nuys, California), 28.1 to 50.5% (10–18% protein); corn oil, 10%; salt mixture, 5% (Rogers et al., 1965); vitamin mixture, 0.5% (Rogers et al., 1965); choline chloride, 0.2%; and dextrin:sucrose (2:1 weight ratio) to make a total of 100%. In all diets lactose was partially substituted for the dextrin:sucrose mixture to achieve the lactose level present in the control diet (15% skim milk protein). Weekly weight gain and daily food intake were recorded.

The essential amino acid content of the basal diet, calculated from the data of Orr et al. (1957), and the essential amino acid requirements for rat growth as reported by Rama Rao et al. (1964) are given in Table 2. The diet appears to meet all requirements although it is borderline for the sulfur amino acids, methionine and cystine. In some experiments methionine and cystine (all L-amino acids purchased from General Biochemicals, Inc., Chagrin Falls, Ohio) were added in a ratio by weight of 3 to 1 in order to reach a calculated sulfur amino acid content of 0.65% in the diet.

Experiments 1 and 2

Experiment 1 was designed to determine the extent to which a mixture of NEAA could isonitrogenously replace a 15% milk protein diet without affecting growth in weanling rats. Body weight gains are summarized in Table 3. A highly significant ($P < 0.01$) decrease in growth, PER, and food efficiency was observed only for

Table 1—Effect of dietary level of skim milk protein on the 14-day growth rate of weanling rats.

Group No.	Dietary skim milk protein %	Food intake g/day	Weight gain[1] g/14 days	FE[2] %	PER[3]
1	10	8.8	45.5 ± 3.3	36.9	3.69
2	12	8.7	56.4 ± 1.5	46.2	3.86
3	15	9.9	76.4 ± 4.0	54.8	3.67
4	18	9.6	77.9 ± 2.1	57.8	3.22

[1] Beginning weight of rats was 45 ± 2 g. Weight gain is mean ± standard error for 8 rats per group.
[2] Food efficiency (FE) is (g gain per g food intake) × 100.
[3] Protein efficiency ratio (PER) is g gain per g protein consumed.

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Table 2—Calculated % of essential amino acids in experimental diets compared with requirements for young growing rats.

Amino acid	Experimental diets					Amino acids required[2]
	Basal[1]	% of milk protein N replaced				
		10	15	20	30	
	(% of Diet)					
Isoleucine	0.96	0.86	0.82	0.77	0.67	0.55
Leucine	1.47	1.32	1.25	1.18	1.03	0.70
Lysine	1.17	1.05	0.99	0.94	0.82	0.90
S-amino acids (Methionine + cystine)	0.50	0.45	0.43	0.40	0.35	0.50
Aromatic amino acids (Phenylalanine + tyrosine)	1.49	1.34	1.27	1.19	1.04	0.72
Threonine	0.69	0.62	0.59	0.55	0.48	0.50
Tryptophan	0.20	0.19	0.18	0.17	0.15	0.11
Valine	1.03	0.93	0.88	0.83	0.72	0.55
Arginine	0.55	0.50	0.47	0.44	0.39	—
Histidine	0.39	0.35	0.33	0.31	0.27	0.25

[1] Calculated values from data of Orr et al. (1957).
[2] From Rama Rao et al. (1964).

the 30% substitution in this experiment.

Therefore, a second experiment was conducted with larger groups of rats to evaluate the effect of 20% and 30% replacements of milk protein N by two sources of supplementary nitrogen. The results are summarized in the lower half of Table 3. Significant ($P < 0.01$) decreases in growth rate, FE, and PER occurred for both sources of nitrogen at the two substitution levels.

Experiment 3

Because the sulfur amino acids, methionine and cystine, are limiting in cow's milk protein for weanling rats, a third experiment was conducted to determine the influence of DAC-Gly substitution of milk protein N in diets supplemented with sulfur amino acids. Table 4 summarizes these results. Comparison of groups 5 and 9 reveals that inclusion of DAC-Gly in the diet resulted in a lower weight gain ($P < 0.05$) than when the diet contained 10.5% milk protein alone. This effect appeared to be related to the reduced food intake, particularly because efficiency of food conversion was similar for both groups.

Growth rates were similar for diets containing 10.5% milk protein, with or without added DAC-Gly, supplemented with 0.15–0.28% sulfur amino acids (compare group 6 vs. 10, 7 vs. 11). The highest level of S-amino acid supplementation reduced body weight gain significantly when the diet contained DAC-Gly (group 8) but not when the only other dietary N was milk protein (groups 4 and 12). These findings suggest that the DAC-Gly mixture affected utilization of sulfur amino acids at the high and possibly at the low dietary sulfur amino acid levels.

Although addition of 0.15–0.28% S-amino acids to 10.5% milk protein diets containing DAC-Gly allowed weight gains which were similar to the 10.5% milk protein diets without the added nitrogen, growth rate remained lower than that on the 15% milk protein diet which supplied the same level of total N and was supplemented with sulfur amino acids.

Experiments 4 and 5

A study was conducted with addition of various essential amino acids to diets containing 30% of total dietary N from the NEAA mixture. The added amino acids achieved the concentrations calculated to be present in the 15% milk protein diet. Arginine, lysine, or threonine addition to the diets (Expt. 4, Table 5) failed to improve weight gain significantly.

The effects of adding tryptophan, tryptophan and threonine, or a combination of several essential amino acids, were also studied (Expt. 5, Table 5). Tryptophan significantly ($P < 0.05$) improved the growth rate, mainly as a consequence of higher food intake (group 4). Addition of tryptophan and threonine together or in combination with arginine and lysine did not result in a higher growth rate than that achieved with tryptophan alone.

Experiment 6

DAC-Gly replaced from 10–50% of skim milk protein N while concentrations of 0.65% sulfur amino acids and 0.21% tryptophan were maintained as in the control diet. The results, summarized in Table 6, show that a 10% replacement of milk protein produced a small but nonsignificant ($P > 0.05$) decrease in growth rate of weanling rats. A significant reduction ($P < 0.01$) in growth rate did occur when 20% of the protein was replaced by DAC-Gly. Proportionately greater reductions were observed with still higher rates of replacement by DAC-Gly despite the unchanged amounts of sulfur amino acids and tryptophan in the diet.

Experiment 7

In young adult rats isonitrogenous replacement of milk protein with the DAC-Gly mixture was studied by monitoring changes in urinary nitrogen excretion. The basal diet contained 4% milk protein, while the remaining

Table 3—Fourteen-day growth of weanling rats during isonitrogenous replacement of skim milk by nonessential amino acids or glycine and diammonium citrate.

	Level of milk protein in diet %	Nitrogen source	% total dietary N from added N source	Food intake g/day	Weight gain g/14 days	FE %	PER
Expt. 1[1]	15.00	—	0	9.4	73.8 ± 4.0	56.1	3.74
	13.50	NEAA[2]	10	9.3	69.1 ± 2.2	53.1	3.54
	12.75	NEAA	15	9.4	66.8 ± 1.9	50.8	3.38
	12.00	NEAA	20	10.0	67.0 ± 2.3	47.9	3.19
	10.50	NEAA	30	9.3	56.0 ± 2.4	43.0	2.87
Expt. 2[3]	15.0	—	0	9.8	68.0 ± 2.4	49.6	3.30
	12.0	NEAA	20	9.3	53.4 ± 2.2	41.0	2.73
	12.0	DAC-Gly[4]	20	9.9	56.8 ± 2.1	41.0	2.73
	10.5	NEAA	30	8.9	48.3 ± 2.0	37.8	2.58
	10.5	DAC-Gly	30	9.5	49.5 ± 2.5	37.2	2.48

[1] Beginning weight of rats was 36 ± 2 g. Weight gain is mean ± standard error for 8 rats per group.
[2] Amino acid (g) per 100 g mixture: L-alanine, 6.5; L-aspartic, 13.72; L-glutamic, 44.03; glycine, 3.72; L-proline, 20.90; L-serine, 11.09.
[3] Beginning weight of rats was 48 ± 3 g. Weight gain is mean ± standard error for 14 rats per group.
[4] Diammonium citrate and glycine mixture. Each compound supplied equal amounts of N per unit weight of mixture.

Table 4—Fourteen-day growth of weanling rats on diets containing skim milk protein supplemented by S-amino acids, with and without added glycine and diammonium citrate (Experiment 3).

Group No.	Level of milk protein in diet %	% total dietary N from added DAC-Glycine	Methionine & cystine added[1]	% dietary methionine & cystine[2]	Food intake g/day	Weight gain[3] g/14 days	FE %	PER
1	15.0	–	–	0.50	10.3	73.3 ± 4.5	50.8	3.49
2	15.0	–	0.15	0.65	10.6	79.8 ± 3.2	53.8	3.55
3	15.0	–	0.28	0.77	10.6	81.1 ± 4.1	54.6	3.58
4	15.0	–	0.43	0.92	10.7	81.3 ± 3.1	54.3	3.52
5	10.5	30	–	0.35	8.9	49.0 ± 4.1	39.3	2.62
6	10.5	30	0.15	0.50	9.8	62.7 ± 5.1	45.7	3.02
7	10.5	30	0.28	0.65	11.1	73.3 ± 3.2	47.2	3.09
8	10.5	30	0.43	0.77	10.7	64.6 ± 2.1	43.1	2.79
9	10.5	–	–	0.35	11.2	61.0 ± 4.2	38.9	3.71
10	10.5	–	0.15	0.50	10.9	67.1 ± 3.5	44.0	4.13
11	10.5	–	0.28	0.65	11.3	73.7 ± 4.8	46.6	4.32
12	10.5	–	0.43	0.77	12.2	78.9 ± 5.1	46.2	4.23

[1]Methionine and cystine were added in a weight ratio of 3:1.
[2]Calculated values.
[3]Beginning weight of rats was 45 ± 3 g. Weight gain is mean ± standard error for 7 rats per group.

dietary components were similar to those in previous experiments. Lactose was maintained at the basal (4% milk protein) level in all diets.

Except for sulfur amino acids, the basal diet contained concentrations of essential amino acids in excess of those suggested by Smith et al. (1967) for maintenance in young adult rats. These authors suggested 0.29% for the total sulfur amino acids, whereas the present 4% milk protein diet supplied 0.13%. However, with a 30% DAC-Gly substitution, isoleucine and threonine also were probably deficient.

The rats, housed individually in metabolic cages, were given 10 g of the basal diet daily for an initial 10-day adaptation period followed by a 10-day control interval (Period 1). For 10 days (Period 2) after the control phase, group 1 was maintained on the basal diet, while groups 2, 3 and 4 were given diets in which the DAC-Gly mixture replaced 10, 15 and 20% of the milk protein, respectively. During the final 10 days (days 31–40, Period 3) all groups were returned to the basal 4% milk protein diet.

On day 11 of the experiment, daily urine collections began. Two 5-day pooled samples of urine were prepared for each period and analyzed for nitrogen by the Kjeldahl method.

Table 5—Fourteen-day growth of weanling rats on diets containing skim milk protein supplemented with sulfur amino acids, NEAA, and various essential amino acids.

	Group No.	Level of milk protein in diet[1] %	% total dietary N from added NEAA mixture[2]	Essential amino acid added %		Food intake g/day	Weight gain[3] g/14 days	FE %	PER
Experiment 4									
	1	15.0	–	–	–	12.1	85.6 ± 3.0	50.5	3.34
	2	10.5	30	–	–	12.1	68.4 ± 2.3	40.4	2.64
	3	10.5	30	L-Arg.	0.12	12.3	71.0 ± 3.1	41.2	2.67
	4	10.5	30	L-Lys.	0.13	11.9	69.0 ± 2.2	41.1	2.68
	5	10.5	30	L-Threo.	0.10	12.3	75.4 ± 2.7	43.8	2.84
	6	10.5	–	–	–	12.2	70.4 ± 3.1	41.2	3.81
	7	10.5	–	L-Arg.	0.12	13.4	74.9 ± 3.3	39.9	3.66
	8	10.5	–	L-Lys.	0.13	13.6	70.2 ± 2.5	36.9	3.37
	9	10.5	–	L-Threo.	0.10	12.2	71.8 ± 2.3	42.0	3.86
Experiment 5									
	1	15.0	–	–	–	11.7	80.7 ± 2.7	49.2	3.25
	2	10.5	30	–	–	11.3	65.4 ± 2.9	41.3	2.70
	3	10.5	30	L-Threo.	0.10	11.1	63.0 ± 3.3	40.5	2.63
	4	10.5	30	L-Try.	0.06	12.5	74.0 ± 4.8	42.3	2.75
	5	10.5	30	L-Threo.	0.10	11.5	71.0 ± 4.2	44.1	2.85
				+ L-Try.	0.06				
	6	10.5	30	L-Threo.	0.10	11.1	68.3 ± 4.2	44.0	2.80
				+ L-Try.	0.06				
				+ L-Arg.	0.12				
				+ L-Lys.	0.13				

[1]15% milk protein diets were supplemented with 0.15% methionine and cystine (3:1) and the 10.5% milk protein diets with 0.3%.
[2]Composition of the NEAA mixture is given in Table 3.
[3]Beginning weight of rats was 45 ± 3 g. Weight gain is mean ± standard error for 8 rats per group in Experiment 4 and 7 rats per group in Experiment 5.

Table 6—Fourteen-day growth of weanling rats fed diets with different levels of DAC-Glycine replacement of skim milk protein supplemented with sulfur amino acids and tryptophan (Experiment 6).

Group No.	Level of milk protein in diet %	Amino acid added %	% total dietary N from added DAC-Glycine	Food intake g/day	Weight gain[1] g/14 days	FE %	PER
1	15.0	Met-Cys 0.15	0	11.2	84.8 ± 2.4	54.1	3.57
2	13.5	Met-Cys 0.20 + Try 0.02	10	11.1	81.2 ± 2.5	52.3	3.43
3	12.0	Met-Cys 0.25 + Try 0.04	20	10.3	68.4 ± 4.4	47.4	3.10
4	10.5	Met-Cys 0.30 + Try 0.06	30	9.7	61.0 ± 2.3	44.9	2.92
5	7.5	Met-Cys 0.40 + Try 0.10	50	8.3	35.0 ± 2.8	30.1	1.94

[1] Beginning weight of rats was 55 ± 3 g. Weight gain is mean ± standard error for 7 rats per group.

Rats were weighed at the beginning of the study (mean weight 200 g) and at the end of each dietary period. They showed little change in weight during the entire 40-day span.

Table 7 shows that 15 or 20% substitution of milk protein with DAC-Gly resulted in a significant increase ($P < 0.01$) in levels of urinary nitrogen excretion during the first five days of Period 2 (days 21–25). During the last five days of this period, urinary nitrogen excretion decreased for groups 2 and 3 (10 and 15% substitution) but was still high for group 4 (20% substitution).

When the rats were returned to the basal diet (Period 3) a sharp reduction in urinary N output occurred in groups 2–4, indicating improved retention of dietary nitrogen on the basal diet as compared with partial replacement of skim milk protein N by supplementary nitrogen.

Urinary nitrogen excretion during the last five days of Period 2 was significantly greater ($P < 0.01$) for rats fed the diet containing 20% of N from DAC-Gly compared with other groups. However, the level of urinary nitrogen excretion was not significantly higher ($P > 0.05$) for rats fed diets with the 10 and 15% substitution than for rats given the basal diet throughout (group 1).

In contrast to these results, the reduced urinary N excretion following return to the basal diet was highly significant ($P < 0.01$) for all groups given DAC-Gly. The control group (group 1), maintained throughout the experiment on the basal diet, did not show a significant change in urinary N excretion ($P > 0.1$) between Periods 2 and 3.

Experiment 8

Because the maintenance requirement of young adult rats for S-amino acids (methionine and cystine) may be higher than the amount supplied by the basal diet (Smith et al., 1967), the effect of partial replacement of milk protein nitrogen by DAC-Gly was studied while a constant dietary level of S-amino acids and tryptophan was maintained. In this study, the basal diet and 20% and 30% levels of substitution were utilized. The experimental design was similar to that of Experiment 7, except that six rats were used per dietary group.

Table 8 shows values of urinary nitrogen excretion for the last five days (15–20) of Period 1 during feeding of the basal 4% protein diet, for days 25–30 of Period 2, and for the first five days (days 31–35) of Period 3. The results show that even with the concentration of S-amino acids and tryptophan maintained at the level present in the basal diet, urinary nitrogen excretion increased for both levels of substitution with DAC-Gly. However, maintenance of the dietary S-amino acid and tryptophan levels appeared to result in a smaller urinary nitrogen increase following 20% replacement than was observed in the earlier experiment without supplemental dietary S-amino acids (Table 7).

DISCUSSION

GROWTH depression resulted from isonitrogenous substitution of cow's milk protein by supplementary nitrogen, either a mixture of nonessential L-amino acids, patterned as in cow's milk, or DAC-Gly.

Various investigators have examined the interrelationship between glycine and methionine. Glycine is known to alleviate the growth depressing effects of high levels of dietary methionine (Hardin et al., 1951; Roth et al., 1950; Benevenga et al., 1967; Wertz et al., 1968) and methionine protects against excess glycine (Wertz et al., 1968).

In the present studies, DAC-Gly was utilized as a source of supplementary nitrogen. The additional dietary glycine may have limited the utilization of the sulfur amino acids and resulted in the lower growth rates achieved when DAC-Gly was added to the 10.5% milk protein diet. Growth rate was reduced to the same extent following the 20% or 30% substitutions of milk protein nitrogen by both DAC-Gly and NEAA mixture.

Also, in rats given the methionine-supplemented diets containing 10.5% milk protein plus nitrogen, growth rates remained lower than the rate of rats on the 15% milk protein diets with supplementary methionine and cystine. Therefore, in addition to possible alterations in sulfur amino acid metabolism, decreased utilization of all essential amino acids may have been responsible for the re-

Table 7—Effect of 40-day replacement of milk protein by DAC-Glycine on urinary nitrogen excretion in young adult rats (Experiment 7).

Days of expt.[2]	% of Milk protein N replaced[1] 0 (1)	10 (2)	15 (3)	20 (4)
	(Mg urine N per day)[3]			
16–20	40.3 ± 1.2	42.8 ± 2.0	42.4 ± 1.6	40.4 ± 1.5
21–25	40.0 ± 1.0	43.7 ± 2.0	46.7 ± 1.6	45.9 ± 1.3
26–30	38.0 ± 1.4	39.7 ± 1.9	41.1 ± 1.2	44.5 ± 1.1
31–35	37.0 ± 0.9	35.6 ± 1.0	36.0 ± 1.1	36.0 ± 1.3
36–40	38.7 ± 0.8	35.6 ± 1.8	36.6 ± 1.3	37.3 ± 1.0

[1] Group number is in parentheses.

[2] During days 16–20 and 31–40 all rats were given the basal 4% milk protein diet. During days 21–30 groups of rats were fed the basal diet or a diet containing 10, 15 or 20% of milk protein N replaced with DAC-Glycine.

[3] Mean ± standard error for 10 rats per group.

Table 8—Urinary nitrogen excretion in young adult rats fed milk protein diets, supplemented with sulfur amino acids and tryptophan, following partial replacement with diammonium citrate and glycine for 40 days (Experiment 8).

Days of expt.[1]	% of Milk protein N replaced 0	20	30
	(Mg urine N per day)[2]		
16–20	44.0 ± 1.1	42.3 ± 1.6	42.5 ± 1.7
25–30	40.9 ± 2.1	45.5 ± 2.3	47.8 ± 1.2
31–35	39.5 ± 1.6	38.9 ± 2.7	35.5 ± 1.8

[1] During days 16–20 and 31–35 all rats were given the basal diet and during days 25–30, rats were fed the basal diet or diets containing 20 and 30% of total dietary N from DAC-Glycine. All diets contained 0.13% methionine and cystine and 0.06% tryptophan.

[2] Mean ± standard error for 6 rats per group.

duced growth rates following replacement of the milk protein by the nitrogen.

The essential amino acid pattern of the test protein probably is an important factor modifying response to substitution of that protein by supplementary nitrogen. Braham et al. (1968) observed that addition of nonessential amino acids or DAC to egg protein did not influence weight gain of rats. Addition of either or both of these nitrogen sources to casein, soybean, or cottonseed protein decreased weight gain. These authors suggest that the response to nonessential amino acids or DAC depends largely on the essential amino acid pattern of the protein tested.

Moran et al. (1967) observed a depression in egg production and weight gain of hens when DAC was added to a 10% protein diet of which half the protein was supplied by corn meal and half by soybean meal. Recently Daniel et al. (1968) have reported that addition of L-glutamic acid to diets containing 5.0 to 8.5% milk protein adversely affected growth rates of rats. On the other hand, Venkat Rao et al. (1964) showed that, although weight gain was proportional to the quantity of egg protein in the diet, addition of L-glutamic acid to the egg protein diets did not adversely affect growth rates in this case.

The present findings showed that tryptophan supplementation of the 10.5% milk protein diet containing added DAC-Gly and sulfur amino acids resulted in a small improvement of growth rate. Addition of several other essential amino acids suspected as being limiting in the methionine-supplemented 10.5% milk protein diets, with or without added nitrogen, failed to improve growth rates. However, Swendseid et al. (1962), using an 8% casein diet supplemented with methionine, found that the growth depression resulting from addition of several sources of nonspecific nitrogen disappeared after addition of tryptophan and threonine, except when glycine was the source of supplementary nitrogen.

In the present study the higher growth rates of control rats fed the 15% milk protein diet, compared with the controls used by Swendseid et al. (1962), may explain the failure of tryptophan and threonine supplementation of diets containing the nitrogen to restore growth to the maximum rate achieved with the 15% milk protein diet.

A 10% substitution of milk protein N, with or without supplementary sulfur amino acids, only slightly and nonsignificantly reduced the growth rate in the present study. Proportionately greater, significant reductions were observed at higher levels of substitution. These results differ from those in children (Snyderman et al., 1962) and adult men (Kofranyi et al., 1964; Scrimshaw et al., 1969), who apparently are able to tolerate at least a 10–20% replacement of milk protein with supplementary nitrogen.

When urinary nitrogen excretion was the major parameter used for determining the effects of milk protein substitution by nitrogen in young adult rats, the results also indicated lessened N utilization (Tables 7 and 8). The increase in urinary nitrogen excretion was greater with higher levels of substitution. This adverse effect on nitrogen balance was not prevented by maintenance of the S-amino acid and tryptophan content of the diets at the level present in the control 4% milk protein diet.

Substitution of the milk protein nitrogen by supplementary nitrogen sources at the 10 or 15% levels, without additional sulfur amino acids, resulted in increased urinary nitrogen excretion. However, a partial adaptation to protein replacement with DAC-Gly appeared to occur, as indicated by the decreased rate of urinary nitrogen excretion during the latter part of the replacement period. These results with young adult rats are, therefore, generally similar to results obtained with weanling rats.

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LOW–TEMPERATURE, LONG–TIME HEATING OF BOVINE MUSCLE
1. Changes in Tenderness, Water-Binding Capacity, pH and Amount of Water-Soluble Components

SUMMARY—Relationships between the tenderness of very slowly cooked meat and its water-holding capacity, pH and the amount of water-soluble components were studied. Beef muscle portions from the longissimus, semitendinosus and rectus femoris muscles were heated under fixed temperature programs with samples from each analyzed at 1-hr intervals between the 3rd and the 10th hr of heating. Weight losses after holding at the final temperature to the 24th hr were determined. During the first 4 hr of heating there were only minor changes in tenderness. The major decrease in shear values occurred between the 4th and 6th hr, when the meat was warming from 50–60°C. The weight losses increased rather linearly to the 7th hr and remained constant for the longissimus and the semitendinosus muscle. The pH values gradually increased during heating. During the first 3 hr of heating, up to 45°C, there was only a slight decrease in the amount of the water-soluble fraction. During the following 3 hr, from 45 to 58°C, the water-soluble fraction decreased more rapidly and the decrease was only slight during a following 4-hr holding period. After 6 hr of heating to 60°C there were still uncoagulated water-soluble proteins. These studies indicate that the final temperature of meat has great influence on tenderness and weight loss. The significance of the shrinkage of collagen in long-time, low-temperature cooking is considered.

INTRODUCTION

THE TENDERIZING effect of long-time, low-temperature cooking has been widely studied since Cover (1937) found that well-done roasts were more tender when cooked at 124 than at 225°C. Bramblett et al. (1959; 1964) found that muscles cooked at 68°C were more tender and had slightly better appearance and flavor than those cooked at 93°C, although they were less juicy. These workers concluded that the length of holding between 57 and 60°C was closely related to increased tenderness. Marshall et al. (1960) found that during low-temperature roasting, evaporation losses increased with the internal temperature of the meat, drip losses increased with increasing oven temperature and with increasing internal temperature and that the total losses were greater in the lowest-temperature oven. Schoman (1960) found that juice losses and power consumption could be reduced by roasting at 121 instead of 149°C in a moisture-tight forced-convection oven. These results indicate that low-temperature cooking affects the water-binding capacity of the meat.

Dymit (1961) introduced the "delayed service" method of meat cookery. He browned beef ribs for 1.5 hr at 178°C, then held them for 3–48 hr at 60°C. Flavor, tenderness and juiciness improved during the first 24 hr and remained constant during the next 24 hr at 60°C. Only 15% shrinkage was observed. Gaines et al. (1966) used a much higher browning temperature, 218°C, and the internal temperature of the roasts never reached the collagen melting point and they were more raw and less tender than the control samples. Funk et al. (1966) also used a higher browning temperature than Dymit, and also reported a decreased quality using the delayed service method. It would appear that the more severe browning used by Gaines et al. and Funk et al. impeded subsequent heat penetration at the lower temperatures, probably due to a severely coagulated surface layer. Korschgen et al. (1963) roasted large cuts of beef to an internal temperature of 43°C in a 149°C oven. The meat was then cooled and sliced. They then broiled slices for 3 min on each side on a 204°C grill. This procedure resulted in good tenderness even of the visible connective tissue.

It is evident from these studies that the rate of heating can affect various properties of the meat associated with tenderness. Yet the extent and nature of the changes are not clear, due primarily to the use of different heating rates by different workers. In the present study, well-defined conditions of low-temperature cooking were followed. At regular intervals, the water-holding capacity, pH, amount of water-soluble components and tenderness were measured. The objective was to detect relationships between tenderness and these parameters.

EXPERIMENTAL

Raw materials

Longissimus, rectus femoris and semitendinosus muscles from both sides of 3 Hereford steer carcasses were used. The carcasses graded either Prime or Choice and had Moderately Abundant or Slightly Abundant marbling. After aging 5-7 days at 0 ± 1°C, the muscles were separated and trimmed of fat and epimysium. They were cut into 2.5-cm-thick slices weighing 100–130 g and sealed under vacuum in Cryovac bags. They were then returned to 0°C until each muscle had been aged for 2 wk from slaughter.

Heating rates

A preliminary experiment showed that the center temperature of a 15.7-kg steamship round roast increased about 0.1°C/min when cooked at 121°C in an institutional gas-heated oven. Therefore, the experimental samples were cooked in the sealed plastic bags by submerging in a 30°C water bath and increasing the bath temperature 0.1°C/min until the bath reached 60°C. This temperature was maintained until the total cooking time was 10 hr. From the 3rd to the 10th hr samples were removed from the water bath every hour. In addition, samples were removed from the water bath at 36 and 44°C and heated in 2 other baths at 37 and 45°C until total cooking periods of 6 and 10 hr were reached. One control sample from each muscle was at first tempered 1 hr at 30°C in a water bath, then heated up to 80°C in 1 hr, and kept at this temperature for 1 hr. This is similar to the schedule used by Marsh et al. (1966). The approximate meat temperature-time curves and the sampling points are shown in Figure 1.

Meat evaluation

Tenderness was measured as shear value in pounds using a Warner-Bratzler apparatus. Test cores were 2.5 cm in diameter, and an attempt was made to keep the axis of the core parallel

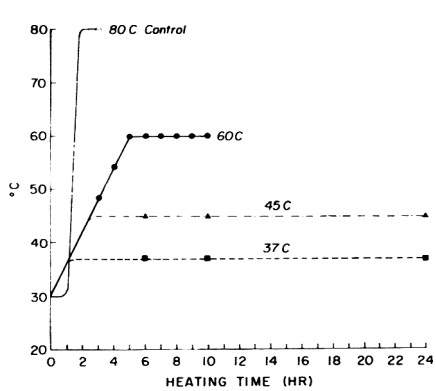

Fig. 1.—Approximate heating curves for muscles cooked at different rates. The dots represent times and temperatures when samples were withdrawn for analysis.

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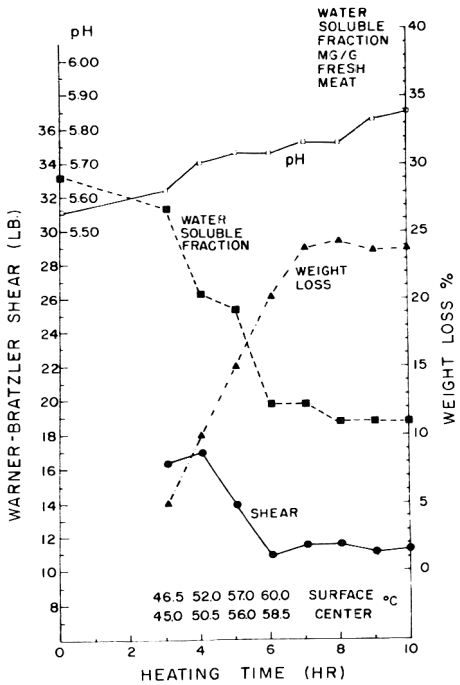

Fig. 2.—Shear value readings, weight loss, pH and amount of freeze-dried water-soluble fraction in longissimus muscle heated at 0.1°C/min to 60°C and held for 10 hr total heating time.

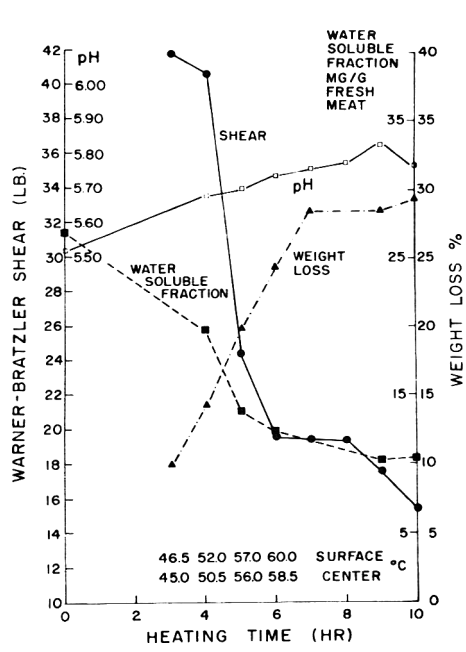

Fig. 3.—Shear value readings, weight loss, pH and amount of freeze-dried water-soluble fraction in semintendinosus muscle heated at 0.1°C/min to 60°C and held for 10 hr total heating time.

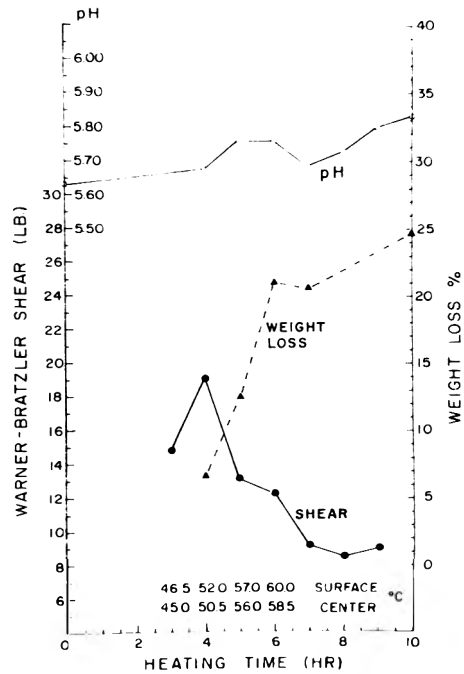

Fig. 4.—Shear value readings, weight loss and pH in rectus femoris muscle heated at 0.1°C/min to 60°C and held for 10 hr total heating time.

to the muscle fiber. 3 such cores were taken at each sampling point and each core was first sheared into halves and these were sheared again, thus giving 9 shear values for each muscle sample.

Water accounts for the major portion of the weight loss during meat cooking, especially with low-fat cuts. Therefore, the weight loss reflects the water-binding ability of the meat proteins. The samples were weighed before and after heating and the weight loss reported as percent of original weight.

The water-soluble material, largely protein, was extracted according to the scheme suggested by Maier et al. (1966). 15 g of meat were cut into strips with scissors and 60 ml distilled water added. The mixture was homogenized for 30 sec in a Waring Blendor. The pH of the homogenate was determined. The homogenate was then centrifuged 30 min at 2,000 X g at 0 ± 1°C. The supernatant was filtered and freeze dried. The dry matter was weighed and is reported herein as milligrams per gram uncooked meat.

RESULTS & DISCUSSION

Relationships between factors studied

Values for tenderness, weight loss, pH and water-soluble fraction observed throughout cooking the longissimus are shown in Figure 2. Similar values for the semitendinosus muscle and rectus femoris are shown in Figures 3 and 4, respectively. Values shown are means of measurements made on 3 different samples of each muscle, except for the amount of water-soluble material, which is the mean of 2 determinations. These values appear in Figure 4. The water-soluble material was not determined for rectus femoris.

During the first 4 hr of heating there were only minor changes in the tenderness. Both the longissimus and rectus femoris appeared to increase slightly in shear value between the 3rd and 4th hr, although the differences were within the standard deviations of the observations. Both muscles were significantly more tender (lower shear values) than the semitendinosus. The major decrease in shear values occurred between the 4th and 6th hr, when the meat was warming from 50 to 60°C. The rectus femoris continued to show decreasing shear values to the 8th hr, but the longissimus did not show further changes after the 6th hr. Shear values of the semitendinosus muscle remained constant between the 6th and 8th hr, then decreased further during the remainder of the heating schedule. The final shear values of the semitendinosus muscle were close to values obtained for the 2 other muscles after 3 hr of heating.

Weight loss upon cooking is a usable, if imprecise, measure of the water-holding capacity of meat (Hamm, 1960). Each muscle showed a slightly different pattern in weight loss during cooking. The weight losses increased rather linearly to the 7th hr, then remained constant for the longissimus and the semitendinosus muscles. After 6 hr, when the temperature of the meat had just reached 60°C, the weight loss of the rectus femoris held constant for 1 hr, then increased more gradually to the end of cooking. Between the 4th and 6th hr, rates of weight loss were 5.3, 7.2 and 4.4%/hr for the longissimus, semitendinosus and rectus femoris muscles, respectively.

The pH values gradually increased during heating, although there were fluctuations in the rate of rise. These fluctuations may have been due to pH variations between different parts of the same muscle (Lawrie, 1966) as well as to the use of samples from different animals. The largest fluctuations and slightly higher average pH of the unheated muscle were found in the rectus femoris. Since there were also fluctuations in tenderness and weight loss of this muscle during heating, all these factors may reflect some special properties of its proteins.

During the first 3 hr of heating, up to 45°C internal temperature, there was only a slight decrease in the amount of the water-soluble fraction. During the following 3 hr, 45.0–58.5°C internal temperature, the water-soluble fraction decreased more rapidly. The decrease was only slight during the following 4-hr holding period. The final values were higher than for the control samples, showing that there were still uncoagulated water-soluble proteins in the meat. The water-soluble fraction includes both proteins and salts. It is estimated from data presented by Lawrie (1966) that about 2/3 of the water-soluble material are sarcoplasmic proteins. As these proteins coagulate, they can no longer be extracted. It follows that changes due to heating represent a measure of protein

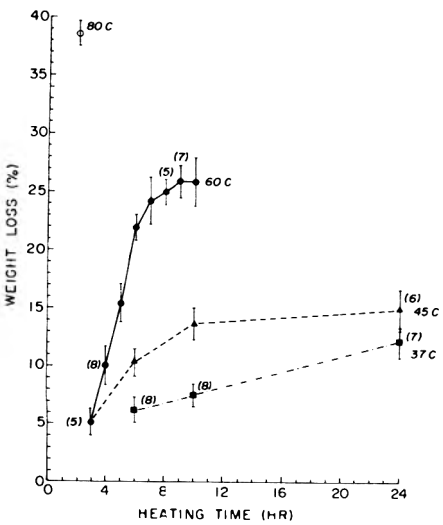

Fig. 5.—Effect of heating temperature on the average weight loss of 3 muscles (longissimus, semitendinosus and rectus femoris) from 3 Hereford steers. The data represent means and standard deviations of 9 measurements, except when the number of measurements is indicated in parentheses.

coagulation. The total water-soluble fraction of the unheated longissimus was 2.91%, considerably lower than the value obtained from Lawrie (1966). One reason for this may be differences in extraction methods, but another reason is probably the influence of aging and freeze-drying of the samples (Fujimaki et al., 1964). Kronman et al. (1960) reported 2.51 ± 0.17% water-soluble protein in aged muscle.

After 6 hr of heating, the samples had reached approximately 60°C, and represented the conditions in rare meat. At this temperature the largest decrease in shear value had just been completed. Authorities agree that the degree of solubility of collagen increases with temperature and that at about 60°C collagen A shortens and is converted into collagen B (Lawrie, 1966). There were still uncoagulated water-soluble proteins, since 12.22 mg/g water-soluble material was obtained from the experimental samples of longissimus and only 6.45 mg/g was obtained from the well-done controls. The weight loss for this muscle was only 21.9% as compared with 38.7% for the well-done controls. It is reported that the combination of low pH and high temperature precipitates sarcoplasmic proteins on to the myofibril, lowering the water-holding capacity (Bendall et al., 1962). Accordingly, reduced coagulation in this study resulted in juicier meat. During the subsequent holding period, further coagulation must have occurred, yet the amount of water-soluble material did not decrease nor did the meat express more juice. This does not preclude a shift in the location of the juice within the microstructure of the meat. The retention of the meat juice, together with the shrinkage of the collagen, may explain the increased tenderness of meat cooked for a long time at low temperatures.

Figure 5 shows the effect of heating temperature on the total weight losses for all muscles included in the study. All samples were heated at 0.1°C/min to the holding temperatures of 37, 45 and 60°C. The average of the control values is also shown. As the temperature increased, weight loss increased. Both the 37 and 45°C samples continued to lose weight after the holding period began. The 37°C samples lost weight at a constant rate throughout the entire holding period. The 45°C samples lost weight more rapidly during heating and the early part of the holding period, but little weight was lost after a total cooking time of 10 hr. The 60°C samples lost weight even more rapidly during the first 9 hr, but lost little during the last hour of cooking.

Marsh (1952a; 1952b) and Hamm (1956) concluded that the post-mortem increase in expressible water is accompanied by a tightening of fibrous structure and shrinkage of the tissue. Marsh (1962) has reported that shortening of muscle fibers during rigor mortis is directly proportional to temperature, up to 43°C. The effect of temperature on weight loss during cooking may be related to similar changes in meat structure.

From these studies, it would seem that the final temperature of the meat is extremely critical in affecting tenderness and weight loss. If the temperature is below the temperature at which collagen shrinks, the major decrease in tenderness does not occur. If the temperature is higher than the shrinkage temperature of collagen, the more severe coagulation will cause a higher weight loss and more tightly packed, less tender tissue will be formed. If the meat is heated to the collagen shrinkage temperature, there will be less weight loss, yet the major increase in tenderness will have occurred. If the meat is to be well done, it should be held at that temperature rather than heated further. Although not apparent from these data, the literature cited and the experiences of the authors indicate that slow heating may be essential if one is to obtain the benefits of holding meat no hotter than 60°C.

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LOW–TEMPERATURE, LONG–TIME HEATING OF BOVINE MUSCLE
2. Changes in Electrophoretic Patterns

SUMMARY–Polyacrylamide gel electrophoresis was used to follow changes in the nature of the water-soluble proteins and juices of bovine muscle during low-temperature heating. The slowest-moving anodic proteins were coagulated first. The myoglobins and myoalbumins were altered significantly only by holding the meat at 60°C. The largest changes in tenderness and amount of water-soluble material in the meat occurred at the same temperatures under which the slow-moving proteins were denatured. The most heat-sensitive proteins detected were denatured before there were significant changes in tenderness or water-soluble substance content.

INTRODUCTION

RANDALL et al. (1967) found 3 cathodic bands and 13 anodic bands using starch gel electrophoresis of the water-soluble fraction of bovine skeletal muscle. Scopes (1964) has shown that myoalbumin is the fastest-moving anodic band in sarcoplasmic extracts. Quinn et al. (1964a; 1964b) found at least 3 myoglobin bands, the slowest being the most predominant. Randall et al. (1967) observed 2 pigmented bands, the 6th and 7th from the anodic end, the faster being the more intensely colored. Rowland et al. (1968) found 1 major myoglobin band and several more rapid forms using polyacrylamide gel electrophoresis.

Most of the sarcoplasmic proteins coagulate when bovine muscle reaches 40–60°C (Hamm, 1966). Grau et al. (1963) showed that the proteins migrating in an electric field with the greatest velocity were denatured the most easily, although the cathodic proteins were more stable (Lee et al., 1966). The juice from cooked meat shows different electrophoretic patterns from the cooked juice of raw meat, which Lee et al. (1966) suggested was due to an influence of the myofibrillar proteins. Quinn et al. (1964a; 1964b) found that the 3 myoglobins evident in their electrophoretic patterns were not affected by heating to 55°C for 5 min.

The objective of this study was to relate changes in the electrophoretic patterns of water-soluble proteins and juices obtained from bovine muscle during low-temperature cooking to changes observed in the tenderness, water-holding capacity, pH and amount of water-soluble protein.

EXPERIMENTAL

LONGISSIMUS, rectus femoris and semitendinosus muscles from Hereford steers were heated at 0.1°C/min to holding temperatures of 37, 45 and 60°C. Samples were withdrawn periodically during the heating program for the various measurements. The freeze-dried water extracts were used for the electrophoresis. Details on muscle preparation, heating program and extraction of the water-soluble material were presented in the preceding paper (Laakkonen et al., 1969).

The method for vertical polyacrylamide gel electrophoresis presented by Thompson et al. (1964) was modified for this study. The gels were 6% in polyacrylamide and 4.5 M in urea. The electrophoresis cell was buffered to pH 8.2. Gel formation was catalyzed by addition of 0.2% ammonium persulfate. 40 mg of the freeze-dried water-soluble fraction was dissolved in 0.5 ml distilled water, the solution saturated with sucrose and 2 drops of bromphenol blue added. Electrophoresis was done at 6°C for 7 hr. The initial current was set to 50 mA and increased 5 mA every 15 min until the voltage reached 250 v. The voltage was then kept constant to the end of the run. The gels were stained with Amido Black 10B and destained with 7% acetic acid. They were stored in the destaining solution until photographed. Histograms were constructed such that the width of the bar indicates the width of the band and the height indicates the estimated intensity. The distance migrated relative to the front is indicated by the position of the bar on the abscissa.

RESULTS

AS EXPECTED, the electrophoretic patterns of the water-soluble material from raw meat shown at the top of Figure 1 are similar to those obtained by Randall

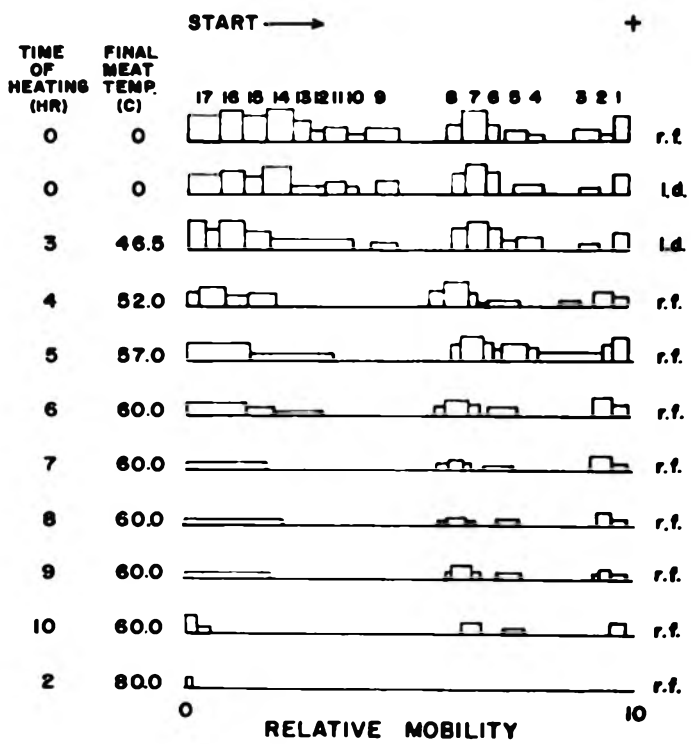

Fig. 1–Electrophoretograms of freeze-dried water-soluble proteins from samples heated to 60°C at 0.1°C/min and held for 10 hr total heating time. Muscles: l.d. = longissimus, r.f. = rectus femoris. Control: rectus femoris heated to 80°C.

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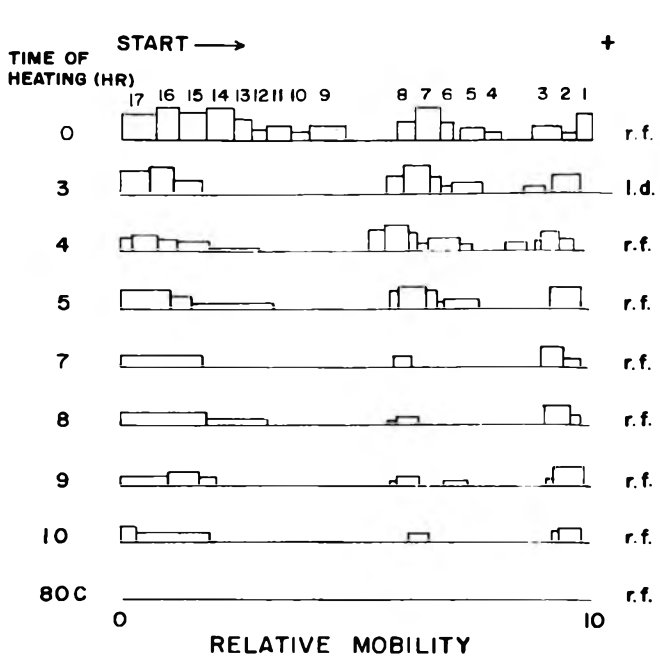

Fig. 2.—Electrophoretograms of freeze-dried meat juices (drip) exuded from samples heated to 60°C at 0.1°C/min and held for 10 hr total heating time. Muscles: l.d. = longissimus, r.f. = rectus femoris. Control: rectus femoris heated to 80°C. The pattern for water-soluble proteins from unheated rectus femoris is included for reference.

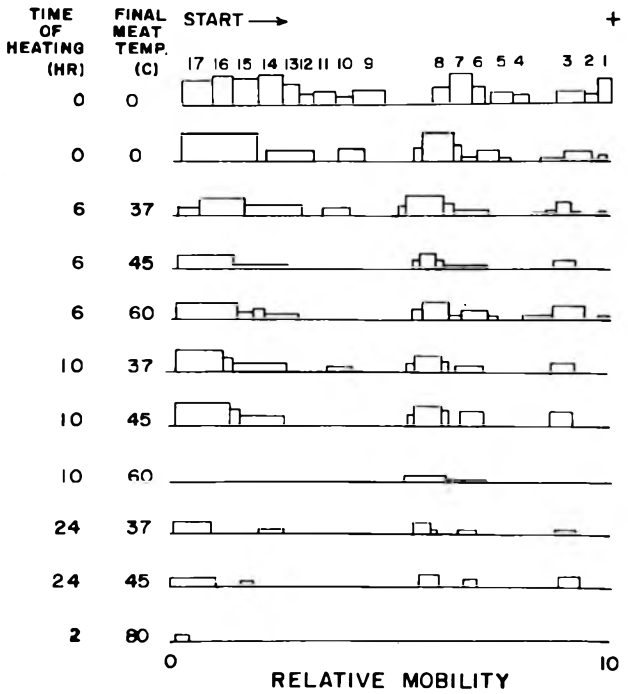

Fig. 3.—Electrophoretograms of freeze-dried water-soluble proteins from samples heated to 37, 45 and 60°C at 0.1°C/min and 80°C at 0.8°C/min. Muscle: longissimus (r.f. = rectus femoris shown for reference).

et al. (1967). Although 17 anodic bands are present, the higher pH used in the present study may have changed some proteins from a net positive to a net negative charge. The 5 slowest-moving bands were difficult to differentiate, but they included 2 intensively stained zones, bands 14 and 16. Band 7 had the most intense meat color prior to staining, but band 8 was also colored. Bands 5 and 6 were more weakly colored. This agrees with the results of Quinn et al. (1964a; 1964b) and Rowland et al. (1968). In fact, it confirms Quinn's postulation of 4 myoglobin bands. Sharp bands were not always seen, but stained areas are depicted in the figures since they disappeared with continued heating.

Figure 1 shows the changes in the electrophoretic patterns during heating. Bands 9–11 were the most heat sensitive and could not be extracted from muscle heated for 4 hr (50.5°C in the center and 52.0°C on the surface). Bands 14–17, the slowest-moving, started to blur and disappear from extracts of meat cooked for 5 hr. The myoglobin bands, 5–8, appeared to remain unchanged until the holding temperature of 60°C had been reached. Following this, the amounts of myoglobins decreased gradually for the rest of the holding time. The myoalbumins, bands 1–3, were not affected until the meat reached 52°C. Band 3 was diffuse in extracts of meat heated to 57°C, then disappeared. In raw and partially rare meat, band 1 was more intense than band 2. At 60°C, this was reversed. Extracts from the control sample, heated to 80°C in 1 hr and held 1 hr, had only a trace of protein-like material and that did not leave the origin.

Figure 2 shows the changes in the proteins of the meat juice (drip) exuded from the samples. Below 45°C, the amount of juice was very small, as reported in the preceding paper. The most heat-sensitive bands, 9–11, did not appear in these electrophoretograms. The slowest group of proteins was more clearly defined and unchanged in the juice

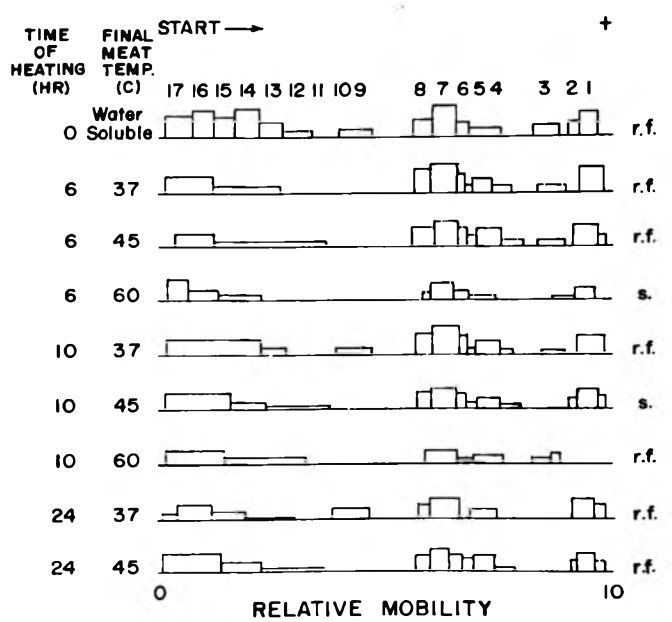

Fig. 4.—Electrophoretograms of the freeze-dried juices (drip) exuded from samples heated to 37, 45 and 60°C at 0.1°C/min and 80°C at 0.8°C/min. Muscles: r.f. = rectus femoris, s = semitendinosus. The pattern for water-soluble proteins from unheated rectus femoris is included for reference.

than in the extracts. After 7 hr of cooking, the myoglobin bands were less intense and less clearly resolved in the juice than in the water extracts. The juice appeared to have a slightly higher concentration of the myoalbumin group. No mobile proteins were found in the juice from the control sample. The difference between the electrophoretograms of meat juice and of water-soluble material from cooked meat is clear but small, as stated by Lee et al. (1966).

Figures 3 and 4 show the electrophoretograms of water-soluble proteins and juices, respectively, of meat samples held at different temperatures. Band 11 could not be seen in any of the heated samples, but band 9 was detectable in the meat held at 37°C. The myoglobins and myoalbumins were little affected by holding the meat at temperatures below 60°C.

Loss of solubility of the slowest-moving proteins occurs at the same points in the heating schedule as do the largest decreases in shear values and total amount of water-soluble material reported in our first paper. During the 60°C holding period, shear values and content of water-soluble material remained essentially constant in spite of the apparent decrease in extractable myoglobins. The weight loss of the meat does not seem to be related to the electrophoretically observable changes. There did seem to be fewer extractable proteins in samples having the higher weight loss.

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LOW–TEMPERATURE, LONG–TIME HEATING OF BOVINE MUSCLE
3. Collagenolytic Activity

SUMMARY—Naturally occurring collagenolytic activity was found in the water-soluble fraction of bovine muscle. General proteolytic activity determined with Azocoll indicated that this total activity was much greater than the collagenase activity specifically determined according to the method of Wünsch and Heidrich. The collagenase fraction was concentrated by polyacrylamide gel electrophoresis and the activity of the enzyme was studied under various pH and temperature conditions. This collagenase could remain active in the meat at cooking temperatures experienced in long-time, low-temperature cooking, $<60°C$. With faster heating and higher internal temperatures, $>70-80°C$, the collagenase observed in this study is inactivated.

INTRODUCTION

BY DEFINITION collagenase is an enzyme attacking native collagen at or near physiological pH (Mandl, 1961). However, collagen is easily denatured and then is susceptible to almost any unspecific proteolytic enzyme.

Proteolytic enzymes are present in muscle (Whitaker, 1964) and the pH optima of some of them are within the range of meat. Koszalka et al. (1960) reported a proteolytic enzyme in rat skeletal muscle having optimal activity at pH 8.5–9.0. Nogochi et al. (1966) found that this enzyme is located mainly in the myofibrils.

Davey et al. (1968) describe the aging of meat as a loss of the tensile strength of the myofibrillar components of the muscle cell brought about by disintegration of the Z bands. This dissolution could lead to a change in the extractability of proteins.

Solovyov et al. (1967) isolated muscular cathepsins at pH 5.6. This complex of enzymes possessed a relatively low but clearly pronounced elastase activity. When studying catheptic action on the pure elastin obtained from fresh warm meat, they found more than a threefold increase in the N-terminal residues of the 6 amino acids investigated. Simultaneously, soluble products of elastin breakage appeared. There was a 68% increase of N-terminal amino acids. The amount of elastolysis-soluble products had a nearly twofold increase during meat aging.

Gross et al. (1965) reported a collagenolytic enzyme in anuran tadpole tissue cultures. This enzyme has a pH optimum of 6–8, loses activity upon heating for 10 min at 50–60°C and is inhibited by EDTA and cysteine. Lazarus et al. (1968) extracted a collagenase operative at neutral and alkaline pH from the granule fraction of human granulocytic leukocytes. Woods et al. (1965) demonstrated far larger collagenolytic activity in the bone of rat than in any other of the tissues studied; namely, kidney, leukocytes, brain and liver.

The objective of this study is to determine whether collagenolytic activity could be measured in meat and to relate any such activity to changes occurring in bovine muscle during low-temperature cooking.

EXPERIMENTAL

LONGISSIMUS, rectus femoris and semitendinosus muscles from Hereford steers were heated at 0.1°C/min to holding temperatures of 37, 45 and 60°C. Samples were withdrawn periodically for analysis. Both the exuded meat juice and the water-soluble fractions were freeze dried and held frozen until used in the present study. Details on muscle preparation, heating program and extraction of the water-soluble material were presented in a preceding paper (Laakkonen et al., 1969).

Collagenolytic activity

Collagenolytic activity of both the water-soluble fraction and the drip from muscles given various heat treatments was determined quantitatively according to a modification of the method by Wünsch et al. (1963), using 4-phenyl-azo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (Mann Research Laboratories, Inc., 136 Liberty St., N.Y. 10006) as substrate.

40 mg of the freeze-dried sample were dissolved in 0.5 ml of 0.01 M calcium acetate. 10 mg substrate was dissolved in 0.1 ml methanol and diluted to 10 ml with acetate-veronal buffer, pH 7.7. 3 identical tubes were preincubated at 37°C for 15 min, and 3 additional tubes kept at 0°C. The substrate solution was similarly preincubated, then 2 ml was mixed with each of the protein solutions. After holding for 15 min, 0.5 ml of each reaction mixture was pipetted into 1 ml of 0.5% citric acid then 5 ml ethylacetate added. The mixture was shaken for 15 sec. The phases were allowed to separate and 4 ml of the ethylacetate phase were removed and dried over 0.3 g of anhydrous sodium sulfate. The difference in absorbance at 320 mμ between each sample incubated at 37° C and an average sample incubated at 0°C was used to calculate collagenase activity in γ collagenase/mg dried sample from a standard curve. This standard curve was established using 4-phenyl-azo-benzyloxycarbonyl-prolyl-leucine in ethylacetate. The samples incubated at 0°C were used as the reference because preliminary experiments showed that the ethylacetate also extracted various amounts and types of meat colors, depending upon the sample and heat treatment.

Proteolytic activity

Azocoll (Calbiochem, Box 54282, Terminal Annex, Los Angeles, California) is an insoluble but hydrophilic complex of collagen and an azo dye (Schubert et al., 1968). Proteolytic enzymes release the dye from the complex, the rate of release reflecting the proteolytic activity of the sample.

40 mg of the freeze-dried sample were dissolved in 5 ml of 0.1 M sodium phosphate buffer, pH 7.5, at 0°C. Then 25 mg Azocoll were added and the mixture incubated for 15 min. Triplicate preparations were incubated at both 37 and 0°C. After incubation, each sample was filtered and the absorbance of the filtrate at 580 mμ was determined. The difference between the absorbance of each solution incubated at 37°C and the absorbance of an average solution incubated at 0°C, indicative of the dye released by the enzymic activity of the sample, was converted to collagenase activity using a standard curve supplied by the substrate manufacturer. The samples incubated at 0°C were used as references since myoglobin, λ max = 555 mμ, and oxymyoglobin, λ max = 575–585 mμ, are both soluble under these conditions.

Isolation and characterization of collagenolytic activity

The freeze-dried water-soluble fraction obtained from rectus femoris heated at 0.1°C/min for 4 hr to 52°C was chosen for the attempts to characterize the collagenolytic activity. 40 mg of the dried material were dissolved in 250 μl of 0.1 M calcium acetate and stored at 0°C for 2 hr. The solution was then evenly distributed among 20 slots across the width of a polyacrylamide gel and subjected to electrophoresis. Details of the electrophoresis have already been described. The unstained gel was cut transversely into 10-mm-wide segments, 11 altogether. The strong myoglobin band reported previously (Laakkonen et al., 1969) appeared in the 6th segment from the origin. Each segment was soaked in 10 ml of 0.1 M calcium acetate at 0°C. After a 5-day soaking period, 0.5-ml portions of the solution were subjected to the Wünsch et al. (1963) collagenase assay.

Triplicate determinations of the collagenolytic activity of material dissolved from the 7th and 8th strips were made at pH 4, 6, 7, 8, 9 and 10. The acetate-veronal buffer used was

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adjusted to the desired pH values with 0.1 N HCl or NaOH.

3 to 5 determinations at pH 7.7 of the collagenolytic activity dissolved from the same gel strips were made at each of 3 incubation temperatures: 37, 45 and 60°C.

RESULTS & DISCUSSION

COLLAGENOLYTIC activity, as shown in Figure 1, was detected in all 3 muscles examined and was not completely eliminated by heating the meat to 58–60 °C. The activity detected in the water-soluble fraction decreased as heating progressed. The longissimus lost collagenolytic activity more rapidly during the early stages of heating, whereas the semitendinosus lost activity in the later stages of heating. The exuded meat juices (drip) had higher collagenolytic activities than the corresponding water-soluble fraction except for the longissimus heated to 58–60 °C.

One reason for the higher activities in the drip may be that in the cut surface of the muscle there may be a mechanism similar to that which releases collagenase activity in healing wounds (Ross, 1968, and Grillo, 1967). Another reason may be a greater increase in calcium ion in the drip than in the meat, as calcium ion is an activator of collagenase. Van den Berg et al. (1964) found increasing amounts of calcium in meat juices exuded during cooking of poultry meat.

Houck et al. (1968) reported that when whole normal skin excised from rats had been incubated in sterile-organ culture at 56°C for 16 hr, this in vitro burned tissue demonstrated a translation of 30% of the insoluble collagen to a form extractable in 0.15 M NaCl. The normally bound, inactive collagenase is converted to a free, active form. This conversion is associated with the release of proteolytic activities. A similar release of collagenase may occur in muscle tissue during aging and during low-temperature heating. The calcium ion may play a role, since Webb et al. (1967) reported a 3× increase in extractable calcium after 9 days of aging at 3.3°C.

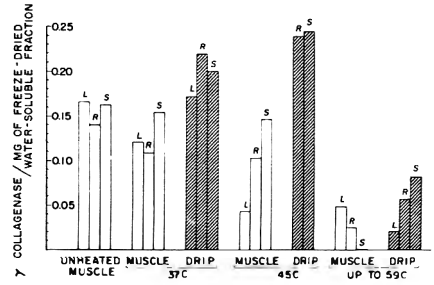

Fig. 1—Amounts of collagenase in the freeze-dried water-soluble fraction and in the freeze-dried exuded meat juice (drip) from unheated muscles, and after 6 hr of heating at a rate of 0.1°C/min to 37, 45 and 59°C. L = longissimus; R = rectus femoris; S = semitendinosus.

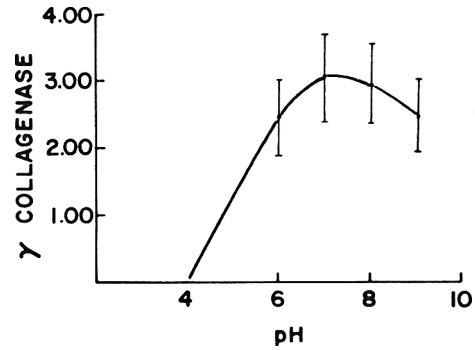

Fig. 2—Collagenase activity at 37°C at different pH values of the 0.1 M calcium acetate solution obtained by soaking collagenase-active polyacrylamide gel strips. Activity is expressed as γ collagenase in 0.5 ml of 0.1 M calcium acetate solution. Small vertical lines indicate the standard deviations of 3 to 5 determinations.

The relatively high amount of collagenolytic activity after heating for 6 hr at 37 and 45°C, and the rapid decrease upon heating to 60°C may explain Cover's (1941) early finding that heating for 23 hr in a 90°C oven gave shear values of 1/2 to 1/3 those obtained by heating for 3 hr in 90°C water. The slower penetration of heat causes less loss of meat juice; therefore, more collagenolytic activity will be retained in the meat.

Table 1 shows the results of the determination of proteolytic activities, reported as γ collagenase/mg dried sample. These values are about 10 times those obtained using the specific collagenase substrate of Wünsch et al. (1963). This wide difference confirms the expectation that proteolytic enzymes other than collagenase also are present. The total proteolytic activity did not change greatly upon heating to 37°C for 6 or 10 hr. Heating for 4 hr to 50.5°C internal temperature seemed to increase the proteolytic activity, although not in all samples. Heating for 6 hr to a 58.5°C internal temperature caused a clear decrease in activity.

The pH activity curve obtained for the collagenolytic material concentrated by electrophoresis is shown in Figure 2. The pH optimum seemed to be about pH 7–8 and there was no activity at pH 4. These results are consistent with those reported by Lazarus et al. (1968). The temperature activity curve is shown in Figure 3. Of the 3 temperatures actually studied, the enzyme was most active at 37°C, but was significantly active at 45°.

There is probably little collagenolytic activity in meat during aging when the pH is around 5.5 and the temperature is close to 0°C. This may be the reason collagenase has not been reported in meat.

Results of the present study seem to show that in aged meat there is a collage-

Table 1—Proteolytic activity in freeze-dried water-soluble extracts of heated muscle samples as measured with Azocoll and expressed as gamma quantities of collagenase.

Muscle sample	Heating		Collagenase activity present per mg of freeze-dried water-soluble fraction (gamma)
	Temperature (C)	Time (hr)	
Rectus femoris, right[1]	—	0	1.36
Rectus femoris, left[1]	—	0	1.38
Rectus femoris[2]	37	6	1.24
Rectus femoris[1]	37	10	1.54
Rectus femoris[1]	52	4	2.46
Longissimus dorsi[1]	52	4	1.50
Semitendinosus[1]	52	4	1.60
Rectus femoris, drip[1]	60	6	.75

[1] Steer A.
[2] Steer B.

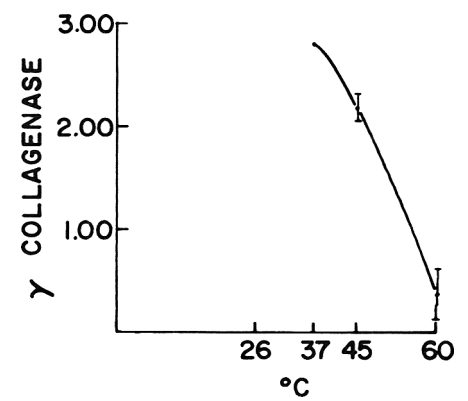

Fig. 3—Collagenase activity at pH 7.7 at different temoeratures of the 0.1 M calcium acetate solution obtained by soaking collagenase-active polyacrylamide gel strips. Activity is expressed as γ collagenase in 0.5 ml of 0.1 M calcium acetate solution. Small vertical lines indicate the standard deviations of 3 to 5 determinations.

nase-like enzyme capable of attacking the peptide chain L-prolyl-L-leucyl-glycyl-L-prolyl between leucine and glycine. Neither trypsin, chymotrypsin, carboxypeptidase A & B nor amidase has this potential (Wünsch et al., 1963). In most meat-cooking methods, however, the rate of rise in temperature is fast and when the final internal temperature of 70–80 °C is reached this enzyme is obviously inactivated. If meat is heated slowly to an internal temperature of 60°C, this collagenase-like enzyme is probably capable of producing tender meat.

Once the triple helix of collagen is unfolded, several other proteolytic enzymes besides collagenase may attack it. Results appearing in Table 1 indicate that proteolytic activity in addition to collagenase was much greater than the collagenase activity specifically determined according to the method of Wünsch et al.

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POULTRY PRODUCT QUALITY. 1. Compositional Changes During Cooking of Turkey Roasts

SUMMARY—White and dark turkey roasts, averaging 7.1 and 3.1 lb respectively, were made with meat taken from selected samples of the various entries in the Pennsylvania Turkey Random Sample Meat Test. Additional roasts were made from sample birds from the University research flock. Roasts were wrapped in aluminum foil, then cooked in a Telkes oven. All roasts were cooked to an internal temperature of 170°F. There were no sex differences in cooking losses except when skin was examined separately. Differences in cooking losses were observed in the breast meat but not in the thigh meat of roasts prepared from Bronze and White turkeys. Losses were higher for breast meat than for thigh meat. There was an indication that size of bird was not a significant factor in determining percentage cooking losses for breast and thigh roasts. When fat drippings from cooked skin were analyzed for carbonyl content high skin yielding males were characterized by the high concentration of the 2-enals in relation to the methyl ketones. Low skin groups consisted mainly of methyl ketones.

INTRODUCTION

MacNEIL et al. (1968) reported that in addition to differences in meat yield between different strains of turkeys, thickness of the epidermal skin may also influence the amount of usable raw material. In their material, the portion of eviscerated carcass represented by skin varied from 5.8 to 12.4 percent in different strains. Similarly the spongy mass of neck skin covering the crop area varied from 2.4 to 6.3 percent. Hoke et al. (1967) pointed out that differences in cooking losses exist between white and dark meat of turkeys. Earlier Marquess et al. (1963) observed differences in the response of white and dark meat to different cooking systems.

Martinsen et al. (1968) stated that total precooking losses of turkey roast were not significantly affected by the use of foil. However, the relative amount of drip and evaporation were changed because, as expected, the foil decreased the amount of volatile material that was vaporized. Further documentation of differences in cooking responses between white and dark meat was presented by Wilkinson et al. (1967). They reported that the shear force of dark meat decreased constantly and significantly as the temperature of the cooked meat increased. Values for white meat declined about one-third as the temperature increased from 60 to 66°C.

Goertz et al. (1962) reported that cooking times and cooking losses were similar for Bronze and White turkeys but that there were sex differences in cooking losses in breast and thigh meat. In addition, breast meat had a shorter cooking time and resulted in smaller cooking losses than thigh meat.

The significance of carbonyl compounds in lipid systems has been recognized as making a major contribution to flavor. Removal of these compounds from the volatile fraction of chicken meat slurries resulted in a loss of the chickeny flavor (Minor et al., 1965). As a result, this class of compounds was studied extensively (Gadbois et al., 1967). Pippen et al. (1965) reported flavor panel differences between leg and breast muscles of broilers.

The purpose of this investigation was to observe and report cooking losses of turkey roasts prepared from different strains of turkeys and to initiate preliminary studies to examine those chemical components involved in off flavor development in cooked turkey products.

EXPERIMENTAL

TURKEY ROASTS were prepared using white and dark meat from Bronze and White turkeys in the Pennsylvania Turkey Random Sample Meat Test as well as samples selected from the University flock. Breast and thighs were removed from the turkey carcass and each component (white and dark meat) was made into a roast using the skin which normally covers, in one case the two thighs, and the other the plate. As cooking progressed, the temperatures were monitored and when any roast reached 170°F it was quickly removed from the oven, placed on a drip rack for 1 min, then weighed. The meat and skin were separated to measure losses occurring in each component.

breast area but not including the neck skin. Temperatures were monitored by using a wooden plug containing three thermocouples which were inserted into the thickest portion of each roast. The roasts with the attached thermocouples were wrapped in aluminum foil and held at 37 to 38°F for 24 hr. White meat roasts averaged 7.1 lb while dark meat roasts averaged 3.1 lb.

Cooking trials were conducted using a Telkes cooking oven maintained at 325°F. After removal from the storage refrigerator and having the thermocouple lead wires connected to a 24 point automatic recording potentiometer, the roasts were placed on the lower oven plate. As cooking progressed, the temperatures were monitored and when any roast reached 170°F it was quickly removed from the oven, placed on a drip rack for 1 min, then weighed. The meat and skin were separated to measure losses occurring in each component.

During the cooking tests there appeared to be some differences in odor among the samples being evaluated. Since taste and flavor observations had not been included in the experimental design, an additional test was designed to explore areas of possible flavor difference between cooked turkeys. The logical place to begin such tests would be to examine high- and low-skin yield groups. Two such groups of comparable size and age were selected from the University flock. Immediately after slaughtering and picking, the skin was removed and placed in a freezer for several minutes then ground in a refrigerated grinder. Samples of the ground skin were cooked in a beaker at 325°F for 15 min.

RESULTS & DISCUSSION

DATA TREATED to analysis of variance (Snedecor, 1956) showed no significant sex differences in percent cooking losses

Table 1—Sex and variety comparisons of cooking losses in turkey roasts.

	N	Cooking loss breast meat %	Cooking loss thigh meat %	Cooking loss total skin %
Sex comparisons				
Males (mean)	75	27.4	33.6	17.4
Females (mean)	75	27.7	34.5	20.2
Indication of significance		—	—	*
Variety comparisons				
Bronze (mean)	70	27.1	34.1	17.1
White (mean)	80	28.0	33.9	20.3
Indication of significance		*	—	**

* Significant at the 5% level (Analysis of variance).
** Significant at the 1% level (Analysis of variance).

of either breast or thigh meat (Table 1). Significant differences ($P < .05$) were found in skin cooking losses between sexes. When cooking losses from roasts prepared from Bronze and White turkeys were examined significant differences ($P < .05$) were found to exist in breast meat losses but not in the thigh meat. Skin cooking losses were significantly different among roasts prepared from the different strains of turkeys. However, since an interaction was found between sex and variety ($P < .10$) for this variable, its significance as a true indicator of cooked yield differences is questionable.

Cooking losses from samples observed during the 1966 tests (Table 2) supports the data previously reported (Marquess et al., 1963) on the difference in cooking losses between light and dark meat. Values ranging from 24.6 to 31.7 percent were observed in cooking losses for breast meat; whereas, the range for thighs was from 34.7 to 37.6 percent. As expected these differences were also evident when calculated on a roast basis. Both breast and thigh skin cooking losses were similar in most cases. Data collected the previous year (MacNeil, unpublished data) showed breast and thigh cooking losses similar to those reported above. Cooking losses of the skin varied greatly possibly due to problems in determining end point temperatures.

In another test, meat and skin were taken from non-commercial strains maintained at The Pennsylvania State University. Three strains and weight classification groups were represented. Analysis of Variance, Snedecor (1956) and Multiple Range Test, Duncan (1955) showed significant differences between groups in all of the yield variables shown (Table 3). The main point noted in this study was that bird size did not play a role in determining cooking losses when breast and thigh meat were fabricated into a natural roast type product. Cooking losses of roasts containing breast and thigh meat were not significantly different for the highest and lowest weight groups.

The fat drippings from the skin were analyzed for carbonyl content according to the methods of Schwartz et al. (1963). The results (Table 4) indicate that there were differences in the relative concentration of the individual monocarbonyl classes. High-skin yield males were characterized by the high concentration of the 2-enals in relation to the methyl ketones and alkanals; whereas, the drippings from the low-skin yield groups consisted mainly of methyl ketones.

To evaluate the possible role of the individual monocarbonyl classes with regard to odor, the carbonyls were regenerated (Bassette et al., 1960) with the following odor responses (Table 5). The methyl ketones were described as being oily or minty; the alkanals meaty or turkey-like; the 2-enals had a strong oxidized broth-like odor; the 2,4-dienal had a strong, painty, nutmeg or spicy odor. The variability in amounts and concentrations of these classes were sufficiently high to make them detectable by taste (Day et al., 1963) and as a result strongly implicates the differentiation in flavor response between the high and low skin-yielding turkeys.

Studies are currently underway to fully characterize the monocarbonyl compounds in cooked turkey and chicken fat under various processing and storage conditions in order to improve the utilization of this turkey component in processed poultry products.

Table 2—Mean cooking losses for turkey meat and skin from 1966 random sample test males.

	Group* Mean Eviscerated Weight (lb)	Breast meat loss %	Breast skin loss %	Thigh meat loss %	Thigh skin loss %	Raw roast weight (lb)	Breast roast loss %	Raw roast weight (lb)	Thigh roast loss %
	21.2	31.7	34.1	37.0	34.4	7.22	31.9	3.28	36.9
	20.5	30.4	27.8	37.0	33.7	6.83	30.2	3.09	36.6
	22.1	29.8	31.0	37.6	29.4	7.35	29.9	3.18	36.8
	19.9	30.0	23.4	36.3	23.0	7.34	29.8	2.94	35.3
	21.9	27.2	28.0	35.5	32.8	7.31	27.3	3.33	35.2
	20.2	26.0	24.9	34.9	20.5	6.82	26.0	3.06	33.7
	21.1	29.5	24.0	35.1	20.6	6.89	29.3	3.13	34.0
	21.9	29.9	36.1	35.6	25.4	7.75	30.1	3.22	34.9
	21.1	29.8	24.6	35.6	20.8	7.80	29.6	3.02	34.4
	20.0	28.4	31.7	34.7	18.6	7.38	28.5	2.87	33.6
	20.8	28.7	27.7	36.9	22.8	6.99	28.6	3.11	35.7
	18.7	27.0	26.2	36.5	26.4	6.02	26.9	2.82	35.5
	19.4	27.8	25.5	36.5	21.2	6.72	27.7	2.91	35.2
	20.2	26.5	26.5	37.3	29.5	7.00	26.5	3.02	36.7
	21.7	28.1	28.0	35.3	27.0	7.54	28.1	3.35	34.7
	19.4	24.6	25.9	36.2	21.6	6.48	24.7	2.89	35.0
	21.2	28.2	30.2	36.6	29.8	6.89	28.2	3.32	36.1
Average	20.7	28.44	27.99	36.15	25.74	7.08	28.44	3.09	35.32

* Each sample group contains five birds.

Table 3—Cooking losses[1] of turkey roasts made from turkeys taken from the University flock.

N	Eviscerated weight (lb)	Total skin %	Breast meat loss %	Thigh meat loss %	Breast skin loss %	Thigh skin loss %	Raw breast roast (lb)	Breast roast loss %	Raw thigh roast %	Thigh roast loss %
65	20.5[a]	6.4[a]	31.0[a]	37.3[a]	10.7[a]	14.7[a]	6.58	30.0[a]	3.22	35.8[a]
48	15.3[b]	8.0[b]	28.9[b]	34.7[b]	14.2[b]	22.3[b]	4.56	28.1[b]	2.38	33.6[b]
65	12.8[c]	10.0[c]	30.5[a]	37.5[a]	17.1[c]	29.0[c]	3.95	29.6[a]	2.04	36.7[a]
Ave.	16.2	8.1	30.1	36.5	14.0	22.0	5.03	29.2	2.55	35.4

[1] Means having the same letter are not significantly different from each other $P < .05$.

Table 4—Concentration of different carbonyl compounds in drippings from high and low skin-yield turkeys.

Sample	N	% Fat	Micromoles per 10 g fat: Total carbonyl	Mono-carbonyl	Methyl ketone	Alkanal	2-Enal	2, 4-Dienal
♂ –heavy	3	57.9	84.2	7.2	0.9	1.7	2.2	2.6
♂ –light	4	38.7	73.2	9.2	6.2	–[a]	1.3	1.7
♀ –heavy	3	67.2	57.0	5.8	4.0	–	0.8	0.3
♀ –light	3	66.1	44.4	6.9	4.3	0.9	0.5	0.2

[a] No detectable level.

Table 5—Odors detected in regenerated carbonyl compounds isolated in turkey skin drippings from cooked roasts.

Monocarbonyl	Odor response
Methyl ketone	Oily, minty
Alkanal	Meaty, turkey-like, C_8 aldehyde
2-Enal	Strong, oxidized, broth-like, C_8 and C_9 enal
2,4-Dienal	Strong, painty, nutmeg, spicy

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POULTRY PRODUCT QUALITY. 2. Storage Time-Temperature Effects on Carbonyl Composition of Cooked Turkey and Chicken Skin Fractions

SUMMARY—The carbonyl compounds in cooked turkey and chicken skin fractions after storage were isolated as their 2,4-dinitrophenylhdrazones. The monocarbonyl class was separated into methyl ketones, 2-enals and 2,4-dienals and measured spectrophotometrically. The turkey skin residue fraction contained higher concentrations of carbonyls than did the chicken samples. The oil extract from the skin of both groups was similar in carbonyl concentration. Lower storage temperature dramatically lowered the development of carbonyls. Phospholipid phosphorus determinations indicated the residue contained high levels of polar lipid; whereas, negligible amounts were in the oil. Thin-layer chromatography of the carbonyl classes from the skin residue indicated mainly C_7-C_9 2-enals and C_8, C_9 2, 4-dienals in the unsaturated aldehyde fractions. Changes in fatty acid composition of the residue polar lipids during storage suggested linoleic and arachidonic acids as the probable substrates in autoxidative deterioration.

INTRODUCTION

THE PRODUCTION of further processed poultry products is steadily increasing. The economic importance of proper utilization of turkey skin for the expanding poultry product area requires an assessment of its quality. MacNeil et al. (1968) reported that skin yields varied from 6 to 12% by weight of the eviscerated carcass in different strains of turkeys. This percentage of raw material is too large to be considered as waste by-product and may be a potential source of raw material for further processed products.

The actions of heat and oxygen on lipids during cooking are known to accelerate autoxidation which may result in carbonyl formation and affect product quality. Lineweaver et al. (1961) identified numerous carbonyl compounds in cooked chicken broth and adipose tissue. Minor et al. (1965) attributed the "chickeny" aroma of cooked chicken muscle to the volatile carbonyls; whereas, the "meaty" aroma was thought due to the sulfur compounds. Mecchi et al. (1956) demonstrated the inferior oxidative stability of turkey fat as compared to chicken fat. This stability difference was attributed to the more efficient deposition of the antioxidant, tocopherol, in the carcass fat of the chicken.

Preliminary studies (MacNeil et al., 1969) indicated that the differentiation in flavor response between heavy and light skin yield turkeys may be due to the levels of individual monocarbonyl classes isolated from cooked skin of the respective strains.

Our objectives were to compare the composition and concentration of the highly flavorful carbonyls produced during storage in cooked turkey and chicken skin fractions.

EXPERIMENTAL

Sample description

24 bronze-colored male turkeys representing progeny produced from a three-way cross were selected. All turkeys were April-hatched, reared under natural day-length conditions, fed a pelleted commercial ration and slaughtered from 29 to 31 wk of age over a 2-wk period. 100 ready-to-cook, 9-week-old broilers including both males and females, were obtained from a commercial processing plant.

The skin was removed following slaughter, evisceration, placed on stainless steel trays and frozen in an air-blast freezer at -30°F. While still frozen, the skin was ground twice in a Hobart meat grinder equipped with a 1/8-in. plate. 1-lb portions of ground skin were then spread in a 1/4-in. layer on 9- by 13-in. trays and cooked in a Telkes oven at 325°F for 20 min. The cooked skin and oil were scraped into a double thickness of cheese cloth and manually pressed to extract as much oil as possible. The extracted oil from each portion (approximately 200 ml) was stored in Erlenmeyer flasks covered with aluminum foil. The corresponding skin portions, denoted as residues, were reground, placed in Cryovac bags and air-evacuated for storage. These portions were held at temperatures of 0, 40 or 60°F and samples for analyses taken periodically during 3, 7, 13 or 23 wk of storage.

Chemical analyses

A measured amount of extracted oil (approximately 10 g) was dissolved in 200 ml of purified hexane. The skin residue (50 g) was slurried in purified hexane in a Waring Blendor and the resulting solution filtered for further analysis. Fisher Certified hexane was purified by refluxing with sulfuric acid, distilling in glass over KOH pellets and rendering carbonyl-free according to the method of Schwartz et al. (1961). The hexane-lipid solutions were then

Table 1—Amounts and types of carbonyl compounds isolated from cooked turkey skin residue (R) and extracted oil (O) stored at 40°F.

Storage (wk)	μMoles/10 g fat									
	Total carbonyls		Monocarbonyls		Methyl ketones		2-Enals		2,4-Dienals	
	R	O	R	O	R	O	R	O	R	O
0	111.2	60.2	39.6	3.9	24.3	2.0	2.0	0.3	1.4	[a]
1	[b]	52.6		3.7		2.4		0.2		0.1
2		40.0		6.5		4.7		0.2		0.2
3		54.6		6.7		2.9		0.6		0.2
5	127.6	54.8	24.4	4.0	13.4	2.8	3.2	0.3	1.2	0.2
7	292.0	56.7	34.6	4.6	21.2	3.3	10.4	0.2	2.6	0.3

[a] Not detectable.
[b] Not determined.

passed over a Celite column impregnated with 2,4-dinitrophenylhydrazine, H_3PO_4 and H_2O as described by Schwartz et al. (1963) to convert the carbonyls to DNP-hydrazones. The DNP-hydrazones were eluted from the column with 200 ml of purified hexane. The total concentration of the carbonyl derivatives was determined by reading the absorbency of this hexane solution at 340 mμ and converting to μmoles using E = 22,500. All readings were conducted on a Beckman Recording Spectrophotometer, Model DBG.

The lipids were removed from the DNP-hydrazones by passing the mixture over a Celite 545-Sea Sorb 43 (Fisher Scientific Co.) column (1:1, w/w) using 20 g of packing material. 200 ml of hexane rendered the DNP-hydrazones free of lipids. The absorbed monocarbonyl derivatives were eluted from this column with 140 ml (3:1, v/v) of chloroform-nitromethane. Ketoglyceride derivatives were separated from the monocarbonyl DNP-hydrazones on an alumina column. 40 g of neutral alumina (80-100 mesh), activated by heating 24 hr at 150°C, partially deactivated by addition of 6% (w/w) distilled H_2O and allowed to equilibrate 16 to 20 hr, was found necessary for good separation. The resulting monocarbonyl DNP-hydrazones were evaporated to dryness, dissolved in a known volume of chloroform and the concentration determined spectrophotometrically at 365 mμ using E = 22,500.

The DNP-hydrazone classes were isolated from the monocarbonyl fraction by the procedure of Schwartz et al. (1962). A 20-g column was prepared using Magnesia 2665 (Fisher Scientific Co.) and Celite 545 (1:1, w/w) and separation of the DNP-hydrazone classes accomplished with the following solvents: 150 ml of 15% chloroform in hexane, 100 ml of 30% chloroform in hexane, 100 ml of 60% chloroform in hexane and 150 ml of 100% chloroform. A fraction collector equipped with a UV source to monitor the eluant from the column aided in determining the separation. The DNP-hydrazone derivatives were evaporated to dryness, pooled according to class, dissolved in known volume of chloroform and read spectrophotometrically to determine concentration. Classes were established on the basis of the following absorption maxima: methyl ketones, 365; saturated aldehydes, 355; 2-enals, 373; 2,4-dienals, 390.

Separation of the individual compounds within a class was carried out on Kieselguhr G (Brinkmann Instruments, Inc.) thin-layer plates impregnated with Carbowax-400 with methyl cyclohexane as a solvent (Badings et al., 1963).

Phospholipid phosphorus was determined according to the procedure of Rouser et al. (1966) on the total lipids extracted from the oil and the skin residue (Folch et al., 1957). Polar lipids were separated on silicic acid absorption columns (Hirsch et al., 1958) and methyl ester analysis of their corresponding fatty acids was determined by GLC (Metcalfe et al., 1966).

RESULTS & DISCUSSION

CONCENTRATIONS of the carbonyl classes for the turkey skin oil and residue samples stored at 40°F are presented (Table 1). Lack of data for the residue samples in this study was because the extracted oil was originally thought to be the most unstable fraction and most susceptible to autoxidation. However, as this initial study progressed, the analytical data demonstrated that the residue, which contained 10% fat, yielded higher concentrations of carbonyls expressed in μmoles/10 g of fat than the oil extract from the same sample. The concentration of total carbonyls in the turkey residue increased to 181 μmoles/10 g fat during the 7 wk of storage; whereas, the concentration in the oil remained relatively constant at below 60 μmoles/10 g fat.

Interestingly, throughout the entire study no measurable amounts of alkanals were found either in the cooked turkey or in the chicken samples. The 2-enal and 2,4-dienal classes increased strikingly in the residue as compared to the extracted oil. Observations of the odors regenerated (Bassette et al., 1960) from these fractions are presented in Table 2. Data reported by Day et al. (1963) and Langler et al. (1964) demonstrated that the flavor threshold values of the unsaturated aldehydes may be 10 times lower than the saturated aldehydes and methyl ketones, implicating their influence on flavor response of autoxidized lipids.

The carbonyl concentrations resulting from the chicken skin fractions stored at the same temperature (Table 3) reflect similar differences in the stability between the residue and oil. However, much lower concentrations of the unsaturated aldehydes were evident in the residue even after 13 wk of storage. In contrast, the chicken oil contained levels of carbonyls comparable with the turkey oil with the exception of the 2,4-dienal class. Only 2 of the 7 test samples indicated measurable amounts of these unsaturated aldehydes. It is evident from the 40°F studies that the chicken residue samples are more stable than the turkey with reference to formation of total carbonyls and unsaturated aldehydes.

Figure 1 is a histogram representing the concentrations of the monocarbonyl classes isolated from chicken and turkey skin fractions held at 60°F storage temperatures. These data readily demonstrate the formation of higher concentrations of the unsaturated aldehydes in the residue as compared to the oil for both groups. Dramatic increases in the total carbonyl concentration were evident in the residue during 3 wk of storage at this temperature. The turkey residue increased from 91.6 to 348.9 μmoles/10 g fat; whereas,

Table 2—Odor descriptions for regenerated carbonyl compounds isolated from cooked chicken and turkey skin residue samples.

Carbonyl	Odor response
Methyl ketones	Minty, fruity, sweet, oily
2-Enals	Oxidized, poultry-like, painty
2, 4-Dienals	Poultry-like, oxidized, putrid, spicy

Table 3—Amounts of carbonyl compounds isolated in cooked chicken skin residue and extracted oil stored at 40°F.

Storage (wk)	μMoles/10 g fat									
	Total carbonyls		Monocarbonyls		Methyl ketones		2-Enals		2,4-Dienals	
	R	O	R	O	R	O	R	O	R	O
0	145.4	55.3	46.0	4.9	20.1	2.9	[a]	0.2	–	–
1	90.5	74.6	30.4	21.3	32.2	17.8	1.9	0.3	–	0.1
3	101.1	58.8	29.3	2.9	16.4	1.3	1.9	0.3	1.5	–
5	99.2	56.7	33.7	5.8	21.2	3.5	1.0	0.5	–	–
7	97.3	51.9	26.8	5.6	14.8	3.6	1.7	0.5	–	–
11	131.0	58.8	44.9	11.0	27.1	8.4	2.1	0.6	0.6	–
13	190.1	66.4	36.2	7.5	21.0	5.9	2.5	0.4	0.6	0.2

[a] Not detectable.

the chicken increased from 91.1 to 240.2 μmoles/10 g fat. Similarly, the concentration of the 2-enals in the turkey residue was three times greater than in the chicken residue, being 22.3 and 6.7 μmoles/10 g, respectively. The increase in the 2-enals during storage throughout these studies was indicative of an autoxidative breakdown and the accumulation of unsaturated end products.

Variability in the levels of methyl ketones in both the oil and the residue cannot be explained in these studies; however, the ability of microorganisms to selectively attack and utilize carbonyl compounds (Smith et al., 1968) may be the causative factor for the differences in concentration.

The influence of storage temperature on carbonyl development can be seen by comparing these data with the carbonyl data obtained for turkey skin fractions stored at 0°F (Table 4). During this extended storage period, concentrations of the respective carbonyl classes in the oil increased rather consistently; however, they remained considerably lower than those for the residue samples. Figure 2 demonstrates the influence of temperature on the 2-enal formation for the turkey residue samples. Tentative identification of the individual compounds within this 2-enal class is presented in Figure 3. An increase in the level of 2-heptenal, 2-octenal and 2-nonenal was evident during the 60°F storage study, even though considerable detail was lost in the thin-layer chromatographic plates during reproduction. Similarly, the 2,4-dienal class during storage was composed largely of 2,4-nordienal and 2,4-decadienals (data not shown). In addition to the tentative identification of these compounds on Kieselguhr absorbent, the R_f of these carbonyl derivatives also correspond to knowns when chromatographed using Micro Cel T-38 as the stationary phase and pure hexane as the solvent (Schwartz et al., 1968).

The proposed origin of these highly unsaturated aldehydes is the unsaturated fatty acids, namely linoleic and arachidonic acids (Badings, 1960; Patton et al., 1959). The polar lipids, consisting primar-

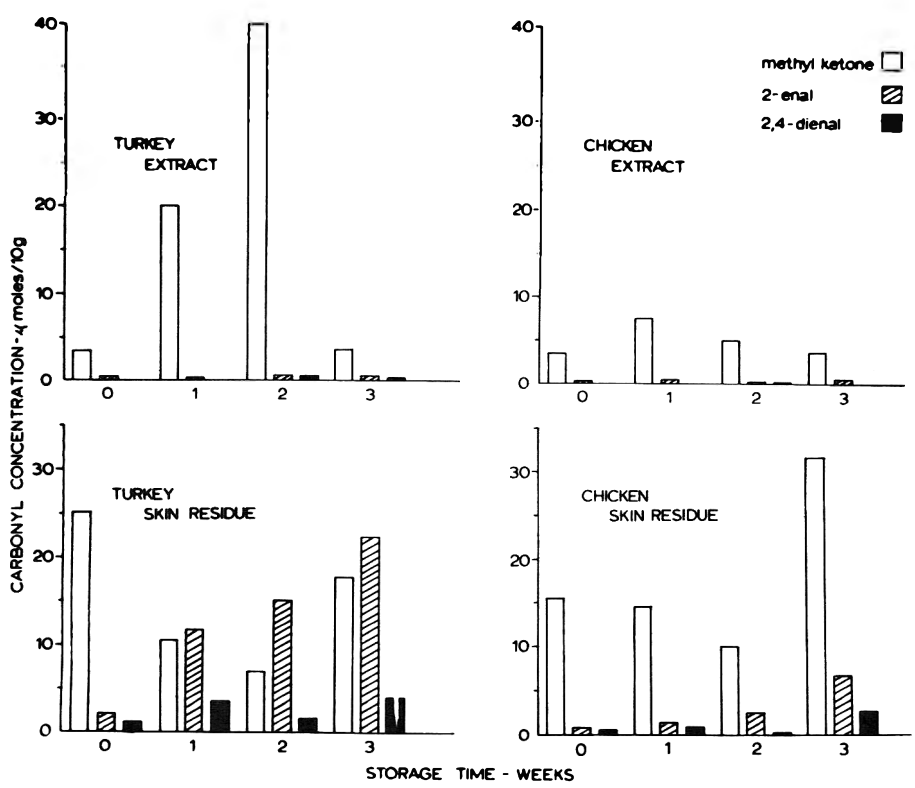

Fig. 1—Concentration of carbonyl classes in cooked turkey and chicken skin fractions stored at 60°F.

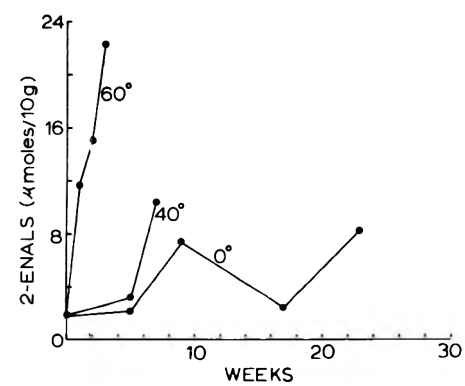

Fig. 2—Concentration of the 2-enals isolated from cooked turkey skin residue following storage at 0, 40 and 60°F.

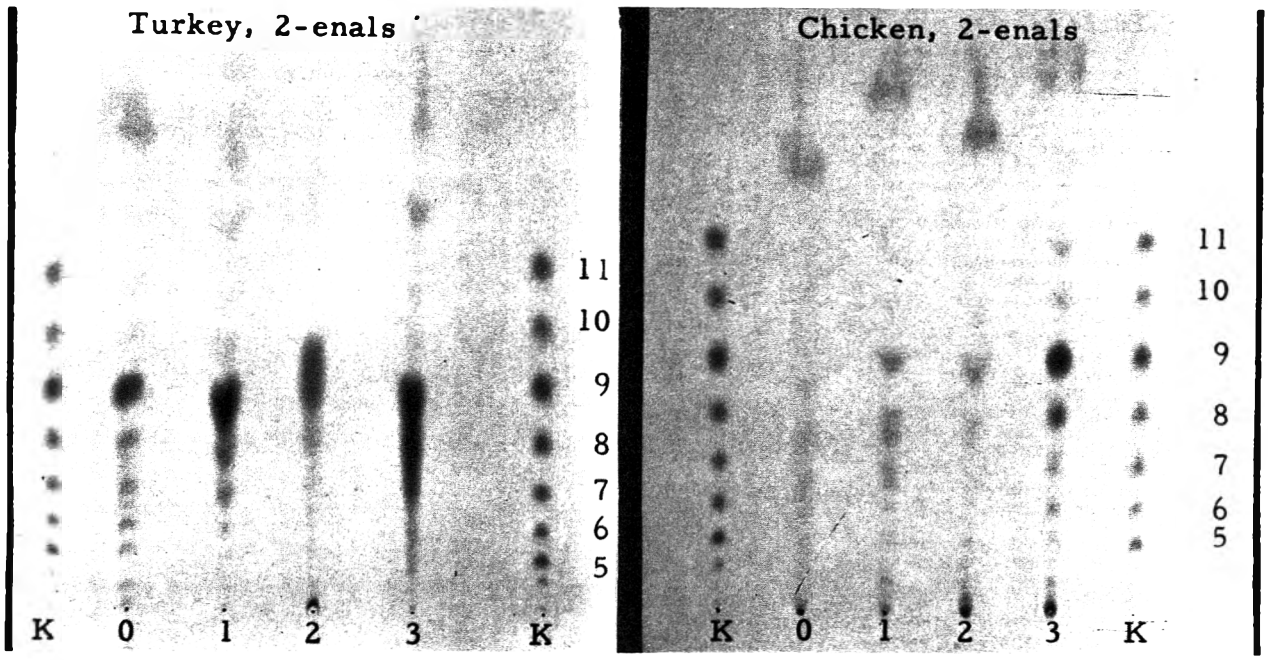

Fig. 3—Thin-layer plates of 2-enals as their 2,4-dinitrophenylhydrazones isolated from cooked turkey and chicken skin residue stored at 60°F. K—Reference alk-2-enals C_5 through C_{11}; 0, 1, 2 and 3 are weeks of storage.

Table 4—Comparison of amounts of carbonyl compounds isolated in cooked turkey skin residue and extracted oil stored at 0°F.

Storage (wk)	μMoles/10 g fat									
	Total carbonyls		Monocarbonyls		Methyl ketones		2-Enals		2,4-Dienals	
	R	O	R	O	R	O	R	O	R	O
0	130.8	47.6	38.8	2.4	23.4	0.6	1.8	–[a]	1.6	–
5	124.8	53.0	42.0	2.4	27.0	0.7	2.2	–	0.6	–
9	227.9	76.6	55.1	5.1	29.8	3.8	7.4	0.3	2.0	–
17	132.1	57.0	43.2	4.4	22.9	2.4	2.5	0.4	0.4	0.2
23	355.2	94.0	60.5	8.9	34.2	6.5	8.3	1.3	3.7	–

[a] Not detectable.

ily of phospholipids and a highly potential source of these labile fatty acids, were determined on the turkey and chicken residue samples (Table 5).

No significant change in the levels of phospholipids was evident during the storage period; however, the data demonstrated the lack of phospholipids in the extracted oil of both groups as compared to the skin residue. This large difference could account for the variations in carbonyl development between the two fractions. The similarity in phospholipid content between species indicates that the difference in stability is not due to the amounts of substrate available. This supports the carlier findings of Nutter et al. (1943) and Mecchi et al. (1956). These similarities are also reflected in the fatty acid compositional data for the polar lipids (Table 6).

Using stearic acid as a stable component of the lipid, the ratios of weight percentage data for 18:2/18:0 and 20:4/18:0 indicate a greater loss of linoleic and arachidonic acids in the turkey residue polar lipids as compared to the chicken residue. The apparent instability of the turkey samples could be due to the low levels of the antioxidant, tocopherol, present (Mecchi et al., 1956). However, the inferior stability of the residue samples for both groups may be attributed to the possible extraction of this fat-soluble antioxidant into the oil before storage, thereby increasing carbonyl development in the residue. The data illustrated that the degree of keeping quality of turkey skin oil as separated and stored in this study is comparable to chicken skin oil.

Table 5—Phospholipid content of cooked turkey and chicken skin fractions.

	μg Phosphorus/g fat					
	Residue			Oil		
	Mean	S.D	n	Mean	S.D.	n
Turkey	2,005	523	7	2.8	0.8	13
Chicken	2,017	195	11	0.9	0.5	10

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Table 6—Fatty acid composition of polar lipids of cooked turkey (T) and chicken (C) skin residues.

Fatty acid	Weeks of storage at 60°F							
	0		1		2		3	
	T	C	T	C	T	C	T	C
14:0	2.2	0.3	0.7	0.4	0.3	0.6	0.6	0.7
14:1	3.2	1.7	2.7	1.8	1.6	1.3	1.4	2.1
16:0	22.1	32.5	30.6	27.7	25.9	31.2	24.0	21.3
16:1	4.7	0.4	1.0	0.3	0.3	0.4	0.6	0.6
16:2	5.0	1.9	3.0	1.2	0.6	0.4	0.7	0.3
18:0	17.9	25.0	19.3	22.2	24.4	22.9	25.0	23.1
18:1	13.7	23.1	16.9	20.6	19.1	31.2	20.3	23.3
18:2	17.1	11.9	14.4	12.6	18.4	10.7	18.3	13.5
20:4	14.1	13.1	11.3	13.3	9.4	11.4	9.1	15.3
18:2/18:0	.96	.48	.75	.57	.75	.50	.73	.58
20:4/18:0	.79	.52	.59	.60	.39	.50	.36	.66

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POULTRY PRODUCT QUALITY. 3. Organoleptic Evaluation of Cooked Chicken and Turkey Skin Fractions as Affected by Storage Time and Temperature

SUMMARY—Cooked turkey and chicken skin residue and separated drippings or oil were stored at various temperatures then presented to a trained taste panel for flavor evaluation. Panel members were able to discriminate between a control (unstored) and a sample of residue after 3 wk of storage at 40°F. They were not able to differentiate between control and treatment (stored) oil even after 7 wk of storage. When chicken skin residue and oil were evaluated after storage at 40°F the panel members could detect differences between the residue samples at 3 wk, but unlike the turkey oil stored at the same temperature they indicated discriminatory ability after 1 wk of storage. When both cooked chicken and turkey skin fractions were presented to the panel at the same time without a reference control (unstored) they were able to identify differences but could not indicate a clear preference for either one.

INTRODUCTION

THE STEADILY INCREASING number and volume of further processed poultry products offered to consumers emphasize the expanded use of these products. Many of these newer products contain poultry lipids. The carbonyl compounds in lipid materials, as suggested by Minor et al. (1965), contribute to the flavor of foods. The economic importance of using large amounts of skin from turkeys was cited by MacNeil et al. (1968) when they noted that the skin can amount to as much as 12% of the dressed carcass weight.

While initial flavor quality is important, its stability (or shelf life) must be maintained at an adequate level during storage and while in the consumer's hands. Martinsen et al. (1968) could not detect any increase in oxidative rancidity, as measured by TBA values, for cooked turkey meat during storage. A trained taste panel of 6 indicated a significant decrease in flavor for these samples over a 7-month period. Cash et al. (1968) found that rancidity was significantly affected by precooking. They reported that TBA values for fresh frozen controls were always lower than for precooked stored samples. Hoke et al. (1968) showed a significant continuous decrease in the flavor of cooked dark turkey meat. Pickett et al. (1968) presented evidence that oxidative rancidity is inhibited by implantation of turkey with vitamin E. Turkeys treated with vitamin E and stored 2 months showed less change in TBA values than did stored turkeys receiving no vitamin E. Cook et al. (1949) detected rancidity in flesh from birds fed a tocopherol-free diet, tocopherol being the precursor of vitamin E. The apparent instability of turkey fat, as suggested by Mecchi et al. (1956), possibly is due to its low level of the antioxidant tocopherol.

Bradley (1964) suggested the use of a modified triangle test in which the taste panel member selected the odd sample from three samples, one of which is a variant or odd, and two are standard or alike. In addition the panel members scored the degree of difference between the paired samples and the odd sample. This procedure enabled Bradley to utilize information on the number of correct selections in the triangle tests as well as the information contained in the degree of difference scores. Brant et al. (1967), using a dual-standard panel comparison, reported that panel members detected individual bird differences within either control or treatment samples. Stier et al. (1967) pointed out that the same concentration of flavor enhancers will not necessarily result in the same flavor response when tested in different food products.

Our purpose was to study the effects of storage times and temperatures on the organoleptic responses of a trained taste panel testing cooked turkey and chicken oil and residue.

EXPERIMENTAL

TURKEY SKINS were obtained from 24 bronze-colored male progeny produced from a three-way cross and processed in the manner described by Dimick et al. (1970). 100 broilers of both sexes, originating from one flock, were obtained from a commercial dressing plant and brought to the University poultry products laboratory, where the skin was removed in the manner previously cited (Dimick et al., 1970). For taste panel evaluation the extracted oil (turkey or chicken) and the corresponding residue were removed from the appropriate storage cooler (0, 40 or 60°F) and allowed to reach room temperature. At the same time a pound of frozen raw control skin was removed from the freezer, thawed completely, cooked at 325°F for 20 min, then the oil and residue collected in the same manner as the treated skins. In the long storage experiment lasting for 35 wk the skin from a freshly slaughtered turkey was used instead of the raw frozen control. Both control and treated samples were heated to 105°F in a water bath before being evaluated by panel members.

TBA values were determined according to the method of Tarladgis et al. (1960) as a measure of the degree of oxidative rancidity.

To examine the long-term effects of storage on turkey residue and oil a study was designed to observe flavor changes in these materials over 35 wk at 0°F storage.

Panel selection

The panel consisted of 12 women, most of whom had previously participated in organoleptic determinations with beef products. Training sessions were conducted using various cooked skin residue and oil samples that might be similar to some of those anticipated in the various experiments. All of the panel members were faculty wives from various departments within the College of Agriculture, selected because of their previous experience with organoleptic tests or because of their interest and willingness to participate. These women were from 24 to 59 years of age, with 2 identified as smokers.

Evaluations were made on both cooked skin residue and the resulting drippings or oil using a modified triangle and hedonic test. Members were seated in separate cubicles numbered 1 through 12. 6, 25-w red lights provided overhead illumination. Panel members were encouraged to sit at a different position each week. Samples were prepared in an adjoining room and carried to them.

The triangle tests consisted of three parts: 1) Is there a difference? 2) If there is a difference, complete the triangle test in the usual manner and 3) indicate a preference among the samples tested. This modified triangle test was similar to that outlined by Bradley (1964) except that a preference determination was required rather than a degree of difference. In subsequent statistical analyses of preference data only the preferences associated with correct triangle judgments were used. The hypothesis used in the Chi-square analysis of the triangle test was that if there were no differences between control and treatment or stored samples the expected probabilities would be these: 1/3 would indicate no difference; 1/3 would make a correct judgment and 1/3 would select the incorrect sample pair. The expected probabilities used with the preference data were 1/2 for a preference of treatment and 1/2 for preference of control, assuming no difference between samples. Hedonic values were analyzed by the analysis of variance.

Consistency scores were computed for all panel members. They were considered able to measure the panel members' ability to be consistent in their judgment in the triangle test and the hedonic test, since identical samples were used.

RESULTS & DISCUSSION

RESULTS OF PANEL evaluation of cooked turkey residue and oil stored at 40°F for 7 wk are presented in Table 1. The Chi-square analysis of the triangle tests showed that the panel members were not able to discriminate between control and treated oil extract samples after storage for 7 wk. After 3 wk they could discriminate between control and treated residue. When asked to indicate a preference for either the control or treated samples, after 1 wk of storage, the panel members had no difficulty in picking out the control residue sample as the one they preferred. This same discriminatory ability was not evident when oil samples were tested. Hedonic values for all of the samples in this test show a distinct separation of scores between the control and treated residue samples but only very slight differences between control and treated oil samples. That there are similar differences between control and treated hedonic scores for each of the storage test periods, and panel members did not indicate this difference until the 3rd wk, cannot be explained fully at this time. We must mention that this was the first of 4 experiments and although the panel had been subjected to training sessions some of them could still have been in the learning process for these particular products.

The analysis of variance (Table 2) of the hedonic scores indicates significant differences between treated and control (stored and unstored) samples, storage times, panel members, judgments, as well as an interaction between treatment and panel members in both residue and oil samples. A significant interaction between storage and panel member existed in the oil samples. If the significant interactions were used as the error term to test significance, the treatment effects would still be significant, but it would invalidate the significance of storage differences for both residue and oil. The significant effect of panel member differences with regard to residue samples also would be cancelled.

Cooked chicken residue and oil were stored at 40°F for 13 wk with taste panel evaluations at various intervals. The triangle test analysis by Chi-square (Table 3) showed that after 3 wk the panel could discriminate between control and treated residue. When oil samples were evaluated the panel indicated an ability to detect differences between control and treatment for the first 2 storage periods of 1 and 3 wk. If a significant level of 10% were selected, the only storage periods in which the panel members could not discriminate between control and treated oil were 7 and 11 wk. It would appear that the treated chicken oil contained compounds which enabled the panel members to distinguish it from the control sample, which was not true for the turkey oil samples. Panel members were presented the chicken samples without any previous exposure to tasting chicken skin and oil and only after several sessions of evaluating turkey residue and oil.

From the two preceding experiments it would appear that 1) there are differences in flavor response between turkey residue and the corresponding oil fraction after several weeks of storage, the skin having a decidedly poorer taste response, and 2) while similar conditions existed with chickens the degree of discrimination in chicken oil samples was greater than in the turkey oil samples. The marked difference between control and treatment for both chicken and turkey residue is portrayed graphically in Figure 1. The oil samples did not follow this same pattern (Figure 2), with control and treated samples overlapping in some instances. This further supports results reported by Dimick et al. (1969) showing the high levels of 2-enals and 2,4-dienal

Table 1—Taste panel evaluation of cooked turkey residue and oil stored at 40°F for 7 wk.

Storage time	Triangle test		Preference for control (unstored)		Mean hedonic scores[a]			
					Residue		Oil	
(wk)	Residue	Oil	Residue	Oil	Control	Stored	Control	Stored
1	NS	NS	NS	NS	3.2	5.1	3.1	3.6
2	NS	NS	+	NS	2.0	4.9	2.1	3.2
3	*	NS	+	NS	2.7	4.8	2.5	3.6
5	*	NS	+	NS	2.8	4.1	2.4	3.6
7	**	NS	+	NS	2.6	5.5	3.1	3.9

* Significant discrimination between control and treatment 5% level.
** Significant discrimination between treatment and control 1% level.
NS—No significant discrimination between treatment and control.
+—Preference for control samples significant at 5% level.
[a]—Hedonic scores from 1, Extremely Good, to 7, Extremely Poor.

Table 2—Analysis of variance of hedonic scores of cooked turkey residue and oil stored at 40°F for 7 wk.

Source of variation	df	Residue Mean square	Oil Mean square
Treatment (stored vs. unstored)	1	126.58**	25.54**
Storage (wk)	4	3.19*	2.49*
Treatment x storage	4	2.15	0.45
Panel members	10	4.39**	7.22**
Treatment x panel members	10	5.60**	1.62*
Storage x panel members	40	1.51	1.62**
Error		1.17	0.68

* F value significant at 5% level.
** F value significant at 1% level.

Table 3—Taste panel evaluation of cooked chicken residue and oil stored at 40°F for 13 wk.

Storage time	Triangle test		Preference for control		Mean hedonic scores[a]			
					Residue		Oil	
(wk)	Residue	Oil	Residue	Oil	Control	Stored	Control	Stored
1	NS	**	++	+	2.8	5.3	2.9	5.4
3	**	*	++	NS	2.0	5.0	2.7	3.7
5	**	NS	++	NS	2.0	3.9	4.2	4.8
7	*	NS	+	NS	2.7	4.4	2.9	3.6
10	**	NS	++	NS	2.1	6.1	2.9	3.7
11	**	NS	++	NS	3.7	6.0	3.7	3.7
13	**	**	++	NS	2.6	5.8	4.5	3.7

* Significant discrimination between treatment and control 5% level.
** Significant discrimination between treatment and control 1% level.
\+ Preference for control samples significant at 5% level.
++ Preference for control samples significant at 1% level.
NS No significant discrimination between treatment and control.
[a] Hedonic scores from 1, Extremely Good to 7, Extremely Poor.

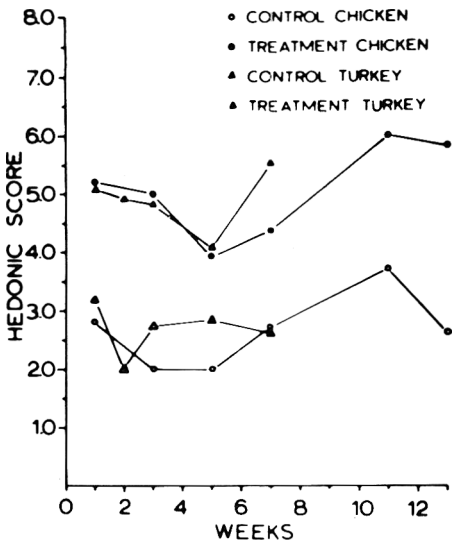

Fig. 1—Hedonic scores for control and treatment chicken and turkey residue samples. (1, Extremely Good; 7, Extremely Poor.)

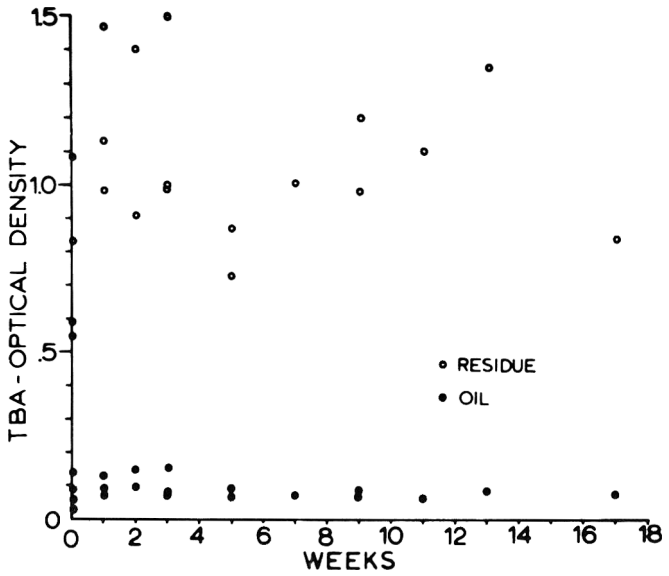

Fig. 3—TBA values reported as optical density for chicken and turkey samples of skin residue and oil.

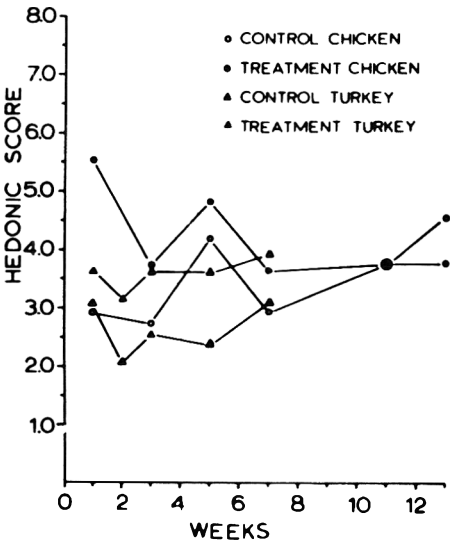

Fig. 2—Hedonic scores for control and treatment chicken and turkey oil samples. (1, Extremely Good; 7, Extremely Poor.)

Table 4—Taste panel evaluation of cooked chicken and turkey residue and oil after storage at 60°F for 3 wk.

Storage time	Triangle test		Preference for chicken over turkey		Mean hedonic scores[a]			
					Residue		Oil	
(wk)	Residue	Oil	Residue	Oil	Chicken	Turkey	Chicken	Turkey
0	**	*	NS	NS	4.6	4.5	3.6	3.4
1	**	NS	NS	NS	4.8	4.2	5.3	5.3
2	*	**	NS	+	4.7	4.4	2.6	5.4
3	**	NS	NS	NS	4.3	4.4	3.1	3.3

* Significant discrimination between chicken and turkey 5% level.
** Significant discrimination between chicken and turkey 1% level.
\+ Preference for chicken over turkey significant at the 5% level.
NS No significant discrimination between turkey and chicken.
[a] Hedonic scores from 1, Extremely Good to 7, Extremely Poor.

carbonyl classes in residue samples as compared to the much lower levels observed in oil. The striking taste panel differences are believed due to the demonstrated association of these compounds with disagreeable flavor.

TBA values are presented in Figure 3. The instability of the residue samples is apparent and it should be pointed out that the oxidative deterioration took place immediately upon heating as indicated by the high TBA values at 0 time. With this in mind a third experiment was conducted comparing chicken and turkey residue and oil. To speed up development of undesirable flavor compounds the residues and oils were stored at 60°F.

The triangle tests analyses (Table 4) showed that the panel members made a significant discrimination between chicken and turkey skin at 0 storage time and were able to distinguish between these samples throughout the remainder of the study. The panel did not significantly prefer chicken skin over turkey skin even though the former was slightly favored. The hedonic scores indicated similarities between chicken and turkey skin residue, but it was surprising that both 0 storage time hedonic values were higher than those observed for either turkey or chicken control residue in the two preceding experiments. In every storage period except 2 the hedonic value in these 2 experiments for the control residue of turkey and chicken was between 2 and 3. It was possible that when samples of similar taste response were given to the panel they had difficulty in establishing a point of reference.

Taste panel scores presented in Table 5 show that panel members distinguished between residue samples but not between oil samples in samples stored at 0°F for 35 wk. The preference data given in the table showed no significant preference for control either in the residue or in the oil, the highest significance level being 10%. Perhaps a higher significance level would be justified, since only those preferences associated with correct judgments were used. For example, at the storage period of 9 wk, 6 panel members preferred control and 2 preferred the treated, but 2 recorded no differences between the samples, which made the preference differ-

ence not significant. The hedonic scores must be taken into consideration to get a realistic appraisal of the flavor response.

The consistency scores for the panel members are presented in Table 6. It is of interest that in most cases the panel members making the most incorrect judgments were consistent through all experiments. The test in which chicken and turkey were compared—in which there was no control sample—gave panel members the most difficulty.

Table 5—Taste panel evaluation of cooked turkey residue and oil stored at 0°F for 35 wk.

Storage time	Triangle test		Preference for control		Mean hedonic scores[a]			
					Residue		Oil	
(wk)	Residue	Oil	Residue	Oil	Control	Stored	Control	Stored
5	*	NS	NS	NS	3.8	5.2	2.8	3.6
9	**	*	≠	NS	2.4	4.4	2.2	2.8
17	**	**	NS	+	3.3	3.9	2.3	4.1
23	**	NS	NS	NS	4.0	5.0	2.9	4.0
35	**	NS	NS	NS	1.9	4.8	3.0	4.0

* Significant discrimination between treatment and control 5% level.
** Significant discrimination between treatment and control 1% level.
\+ Preference for control samples significant at 5% level.
≠ Preference for control samples significant at 1% level.
NS No significant discrimination between treatment and control.
[a] Hedonic scores from 1, Extremely Good to 7, Extremely Poor.

Table 6—Consistency scores of panel members.

Panel member identification number	Percent consistency				
	Test 1	Test 2	Test 3	Test 4	Avg
6	86.0	81.7	88.6	95.0	87.8
5	92.0	76.7	85.7	90.0	86.1
12	100.0	80.0	91.4	70.0	85.4
9	86.0	78.3	87.1	85.0	84.1
1	78.0	85.0	87.1	77.5	81.9
3	78.0	63.3	91.4	87.5	80.0
2	82.0	80.0	91.4	65.0	79.6
11	92.0	90.0	76.7	55.0	78.4
10	80.0	63.3	74.3	80.0	74.4
8	54.0	75.0	95.0	57.5	70.4
4	52.0	66.0	78.3	75.0	67.8
7	62.0	61.7	76.7	65.0	66.4

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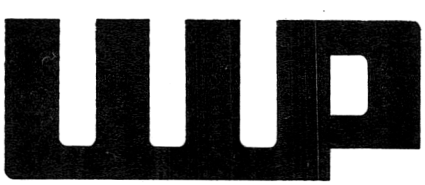

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