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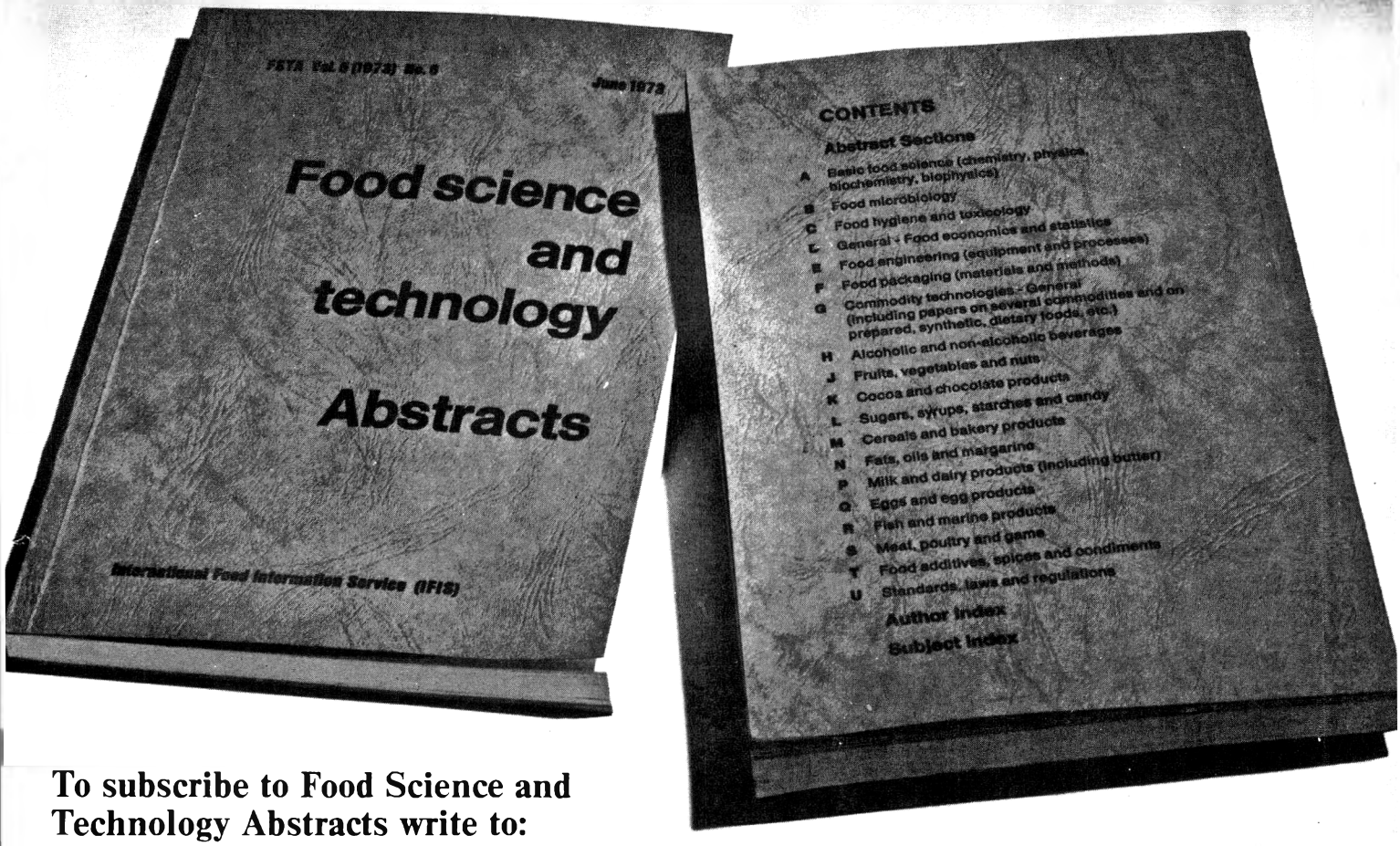

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ABSTRACTS:
IN THIS ISSUE

Clostridium perfringens **INHIBITION BY SODIUM NITRITE AS A FUNCTION OF pH, INOCULUM SIZE AND HEAT.** W.E. RIHA JR. & M. SOLBERG. *J. Food Sci.* **40,** 439–442 (1975)–The effect of heat, pH inoculum size and nitrite concentration on the inhibition of various *C. perfringens* strains was characterized. The inhibition of six strains in filter sterilized medium was correlated to the amount of undissociated nitrous acid. The same was not true in autoclaved medium where a more potent inhibitor appeared to exist. A 4-log cycle increase in inoculum cell concentration increased nitrite tolerance five to tenfold in filter sterilized medium and 10 to 100 fold in autoclaved medium. The relationship between log inoculum size and log nitrite concentration necessary for inhibition was linear for strain 8797. For every 6.8 min exposure of a nitrite containing culture medium to heat at 121°C, the inhibitory concentration of nitrite for strain 8797 was diminished by 90%.

Clostridium perfringens **GROWTH IN A NITRITE CONTAINING DEFINED MEDIUM STERILIZED BY HEAT OR FILTRATION.** W.E. RIHA JR. & M. SOLBERG. *J. Food Sci.* **40,** 443–445 (1975)–The growth of *Clostridium perfringens* 8797 in a nitrite containing chemically defined medium at pH 6.3 was characterized with respect to the method of medium sterilization and nitrite concentration. All cultures which grew in either autoclave or filter sterilized medium demonstrated equivalent generation times and reached equivalent maximum cell concentrations regardless of the nitrite concentration. A significant difference in adjustment phase duration existed between autoclaved and filter sterilized medium, although within each type of medium the duration of the adjustment phase was homogeneous regardless of the nitrite concentration. The effect of nitrite appeared to be cellular and was permanent.

PURIFICATION AND PROPERTIES OF ANTIMICROBIAL SUBSTANCES PRODUCED BY Streptococcus diacetilactis AND Leuconostoc citrovorum. A.L. BRANEN, H.C. GO & R.P. GENSKE. *J. Food Sci.* **40,** 446–450 (1975)–This study was initiated to further elucidate the properties of the antimicrobial substances produced by *Leuconostoc citrovorum* and *Streptococcus diacetilactis* and to determine the feasibility of isolating these substances. A medium consisting of 5% spray-dried whey plus 0.5% yeast extract was found to be excellent for the production and subsequent purification of the antimicrobial substances. The antimicrobial activity of *S. diacetilactis* and *L. citrovorum* was similar in many respects; however, cation exchange and gel chromatography indicated that distinctly different substances accounted for the activity of the two organisms. A low molecular weight antimicrobial preparation was obtained from a *S. diacetilactis* fermentation liquor using methanol extraction followed by cation exchange and gel chromatography. The purified material showed antimicrobial activity towards several *Pseudomonas* species and was peptidyl in nature.

SOME ADDITIONAL STUDIES ON THE THERMAL DENATURATION OF LIGHT MEROMYOSIN FRACTION 1. K. SAMEJIMA & T. YASUI. *J. Food Sci.* 40, 451–455 (1975)–We have studied the thermal denaturation of LMM Fr 1 in further detail under various conditions. The results obtained show: (1) Changes in solubility at 0.1μ of thermally treated LMM Fr 1 in 0.6M KCl as a function of pH are substantially in accordance with the pH-dependence of tryptic activity; (2) When LMM Fr 1 in 0.6M KCl is heated at 45°, 50° or 60°C while maintaining pH at 5.4 (the value at which the protein isoelectrically precipitates), a drastic decrease in solubility occurs at 60°C and the majority of the protein remains insoluble even after shifting the pH to the neutral region; (3) When LMM Fr 1 was heated at 40°C and between pH values of 5.6–6.0, aggregation of the protein took place. The reaction was found to proceed according to second order kinetics; (4) Thermodynamic data calculated from the melting curves of LMM Fr 1 (which were drawn by measuring viscosity, helical contents and difference spectrum in the UV region), indicate that ΔH is 41.5 kCal/mole and ΔS is 125 e.u. at the transition temperature; and (5) The denatured product obtained at 60°C is different from that at 70°C.

FREE RADICALS IN LYSOZYME REACTED WITH PEROXIDIZING METHYL LINOLEATE. K.M. SCHAICH & M. KAREL. *J. Food Sci.* **40,** 456–459 (1975)–Production of free radicals in lysozyme due to reactions with peroxidizing methyl linoleate was studied as a function of water activity in freeze-dried emulsions. Electron spin resonance (ESR) was used to measure the free radicals, and the effects of reaction with linoleate peroxides were compared with effects of γ-irradiation of lysozyme. There were similarities as well as some differences between these two mechanisms for producing free radicals in lysozyme. Free radical concentrations in lysozyme decreased with increasing water activity probably due to radical recombination and crosslinking.

ANTIOXIDANT ACTIVITY OF ACETONE EXTRACTS OBTAINED FROM A CARAMELIZATION-TYPE BROWNING REACTION. C. RHEE & D.H. KIM. *J. Food Sci.* **40,** 460–462 (1975)–Antioxidant activity of the acetone extracts obtained from successive stages of a caramelization-type browning reaction mixture, a 2.0M glucose solution heated at 100°C for 96 hr, was determined, using an edible soybean oil as substrate. Relationships between the color intensity (absorbance at 470 nm) of the browning reaction mixture and the length of reaction time, and the antioxidant activity and the length of reaction time were studied. All the extracts exerted considerable antioxidant activity on the autoxidation of the substrate which was kept at 45 ± 0.7°C for 18 days. While the color intensity appeared to increase in proportion to the length of reaction time, the antioxidant activity did not follow the same pattern. The antioxidant activity of the extracts obtained from the reaction mixture which had been heated for 96 hr, was not much greater than that of the extracts obtained from the reaction mixture heated for 9 or even 3 hr. These results seem to suggest that although the brown-colored pigments produced in the later stages of the browning reaction might have some antioxidant activity, the major antioxidant compounds were probably colorless or almost colorless intermediates such as reductones formed in the fairly earlier stages of the browning reaction.

FLUOROMETRIC ASSAY FOR TOTAL VITAMIN C USING CONTINUOUS FLOW ANALYSIS. J.R. KIRK & N. TING. *J. Food Sci.* **40,** 463–466 (1975)–A continuous flow fluorometric procedure is described for the quantitative determination of dehydroascorbic and total ascorbic acid, which results in a significant reduction in analysis time. Dehydroascorbic acid in sample extracts were determined by the AOAC o-phenylenediamine microfluorometric procedure adapted to continuous flow analysis. Total ascorbic acid levels were determined as dehydroascorbic acid following the oxidation of reduced ascorbic acid with 2,6-dichlorophenolindophenol. A comparison of the automated o-phenylenediamine procedure with the AOAC manual procedure for total ascorbic acid in orange juice, milk, dry breakfast cereal and canned tomatoes indicated good agreement between the two methods. Further studies with orange juice indicated less than 1% difference between the automated procedure and the manual 2,6-dichlorophenolindophenol titration for reduced ascorbic acid.

EFFECT OF CALCIUM, MAGNESIUM AND WHEY PROTEINS ON THE ACTIVITY OF β-GALACTOSIDASE (*A. niger*) IMMOBILIZED ON COLLAGEN. J. JAKUBOWSKI, J.R. GIACIN, D.H. KLEYN, S.G. GILBERT & J.G. LEEDER. *J. Food Sci.* **40,** 467–469 (1975)–The specific activity for collagen-bound β-galactosidase (*Aspergillus niger*) was determined in 5% lactose, acid whey and ultrafiltered acid whey as substrates.

The bound enzyme showed inhibited activity in the whey substrates, as compared to aqueous lactose. While the reactor constructed from a glutaraldehyde cross-linked film exhibited a decrease in activity upon contacting with acid whey, the inhibition was reversible in nature. Magnesium ion was found to inhibit enzymatic activity of an immobilized enzyme reactor constructed from an untanned membrane. Neither magnesium or calcium ion inhibition was observed for reactors constructed from tanned films. Whey proteins were shown to have an adverse effect on the activity of the bound lactase, irrespective of the immobilization procedure.

FUNGAL FERMENTATION OF PEANUT FLOUR: EFFECTS ON CHEMICAL COMPOSITION AND NUTRITIVE VALUE. M.R. QUINN, L.R. BEUCHAT, J. MILLER, C.T. YOUNG & R.E. WORTHINGTON. *J. Food Sci.* **40**, 470–474 (1975)—Solvent defatted peanut flour was fermented with *Rhizopus oligosporus, Aspergillus oryzae, Mucor hiemalis, Neurospora sitophila* and *Actinomucor elegans.* Increased percentages of crude protein and ash in ferments were attributed to disproportionately higher losses of nonnitrogenous volatiles during fermentation. Increases were greatest for *N. sitophila* ferments. Amino acid analyses substantiated the increases in percent protein in fermented as compared to nonfermented samples. Amino acid compositions of ferments did not differ greatly from each other or from control samples. Fatty acids reflected changes from normal peanut oil fatty acid profiles toward profiles more representative of those expected in fungal mycelia. Linolenic acid was detected in *A. oryzae* and *N. sitophila* ferments. All of the fungi used in this study significantly increased the levels of riboflavin and thiamin but not pantothenate over autoclaved nonfermented peanut flour. Significant increases in niacin were noted in *N. sitophila* ferments. Fermentation did not significantly change the protein efficiency ratio of peanut flour for rats and had no significant effect on liver lipid content.

FUNCTIONAL PROPERTY CHANGES RESULTING FROM FUNGAL FERMENTATION OF PEANUT FLOUR. M.R. QUINN & L.R. BEUCHAT. *J. Food Sci.* **40**, 475–478 (1975)—Solvent defatted peanut flour (SDPF) was supplemented with 0.5% tapioca, 0.8% NaCl and 2.0% citric acid, combined with tap water, and sterilized. The substrate was fermented for 4 days with *Rhizopus oligosporus, Aspergillus oryzae, Mucor hiemalis, Neurospora sitophila,* and *Actinomucor elegans,* lyophilized and pulverized. Functional property changes were characterized by nitrogen solubility, viscosity, emulsifying capacity, water and oil retention and equilibrium moisture adsorption isotherms. SDPF nitrogen solubility was increased markedly in the isoelectric pH range of 4.0–5.0 as a result of fungal fermentation. The greatest change was noted for *M. hiemalis* which increased the 5% nitrogen solubility of nonfermented SDPF to 34%. Heating SDPF adversely affected emulsion capacity and protein solubility but these properties were somewhat restored after fermentation. Water adsorbing capacity of test materials increased with increasing atmospheric relative humidities (RH) and with elevated temperature at specific RH. Moisture adsorption at the same temperature-RH conditions was greater in ferments than in controls.

COMPOSITIONAL DIFFERENCES IN WHEY SYSTEMS. R.V. JOSEPHSON, S.S.H. RIZVI & W.J. HARPER. *J. Food Sci.* **40**, 479–483 (1975)—Fresh cheese whey systems and commercial UF-processed whey powders were evaluated for differences in physical/chemical composition and properties which may affect the ultimate stability and nutritional value of whey. Cheddar, cottage and control pH 4.6 acidified wheys were prepared from the same whole milk or skim milk by standard procedures. Five lots of UF-processed spray-dried sweet whey protein concentrates were supplied by a commercial source. Total solids, pH, ash, lactose and total nitrogen data from three trials were within normal ranges for all fresh wheys. Both Cheddar and cottage wheys were higher in 12% TCA-soluble nitrogen than their comparable pH 4.6 wheys. Cheddar whey was highest in pH and solids, but lowest in calcium, phosphorus and ash. Cottage whey was lowest in total nitrogen, citrate and lactose. Dialysis against water revealed higher nondialyzable calcium for Cheddar and cottage wheys and different ratios of nondialyzable components among whey systems. UF whey powders varied little in composition except for lactic acid content and lactose which showed wide variations in analyses by polarimetry and Dubois methods. UF whey powders were considerably higher in protein but lower in most low molecular weight components except calcium than Cheddar whey on an equivalent solids basis. Alkaline gel electrophoretic and Sephadex G-100 patterns were normal for fresh wheys but showed significant whey protein denaturation/aggregation in UF powders. These observations revealed new evidence of changes in whey composition resulting from cheese making and UF and/or spray drying processes.

FORMATION OF N-NITROSOPYRROLIDINE FROM PROLINE AND COLLAGEN. J.I. GRAY & L.R. DUGAN JR. *J. Food Sci.* **40**, 484–487 (1975)—Proline and collagen as potential N-nitrosopyrrolidine (N-Pyr) precursors were investigated in model system studies. Proline, in the presence of sodium nitrite was decarboxylated in low moisture carboxymethylcellulose (CMC) systems at elevated temperatures to produce N-Pyr, maximum formation occurring at 180°C. Collagen when heated with nitrite in a CMC system at 200°C or in an oil-water system simulating the frying of bacon produced N-Pyr. Confirmation was achieved by gas chromatography-mass spectrometry. Connective tissue of a cured, smoked ham was also shown to be a precursor of N-Pyr, with and without additional nitrite.

EFFECT OF SODIUM NITRITE AND NITRATE ON Clostridium botulinum GROWTH IN A SUMMER STYLE SAUSAGE. L.N. CHRISTIANSEN, R.B. TOMPKIN, A.B. SHAPARIS, R.W. JOHNSTON & D.A. KAUTTER. *J. Food Sci.* **40**, 488–490 (1975)—Two experiments were conducted to determine the growth potential of *Clostridium botulinum* in fermented sausage. The first test demonstrated that growth and toxin production did not occur during fermentation. Product stored at 27°C did not become toxic. This may be explained by acid production which occurred with sufficient rapidity to negate any nitrate or nitrite effect. The relative effects of nitrite, dextrose and starter culture on *C. botulinum* growth in thuringer stored at 27°C were then evaluated. Growth of *C. botulinum* was prevented in sausage formulated with dextrose and 50 μg or more of nitrite per g of meat. The average pH of these samples decreased from an initial 5.63 to 4.68 within 1 wk at 27°C. Omitting dextrose from the sausage formulation resulted in samples in which the pH remained at the initial level throughout storage. In these samples, increased nitrite levels up to 150 μg per g of meat retarded but did not completely prevent toxin production.

EFFECT OF ADDED SODIUM NITRITE AND SODIUM NITRATE ON SENSORY QUALITY AND NITROSAMINE FORMATION IN THURINGER SAUSAGE. A.E. DETHMERS, H. ROCK, T. FAZIO & R.W. JOHNSTON. *J. Food Sci.* **40**, 491–495 (1975)—The effects of added sodium nitrite and sodium nitrate on sensory qualities and nitrosamine formation in fermented thuringer sausage were investigated. Thuringer was stored under select temperature and time conditions and tested fresh, fried and baked. Trained sensory panels indicated that flavor and appearance qualities were improved with 50 ppm added nitrite and that desirable flavor and appearance characteristics were obtained with 100 ppm or more added nitrite. Added nitrate produced detectable improvements only in the absence of nitrite. No nitrosamines were detected in thuringer regardless of added nitrite, added nitrate, storage condition, or kitchen preparation method.

PERSISTENCE OF STAPHYLOCOCCUS. Bruised Tissue Microenvironment Affecting Persistence of Staphylococcus aureus. C.T. ROSKEY & M.K. HAMDY. *J. Food Sci.* **40**, 496–499 (1975)—Tissue extracts of the pectoralis major muscle from contused chickens (8–10 wk old) were prepared and inoculated with *S. aureus.* Extracts of normal (nonbruised) chicken muscle were treated in similar manner and served as controls. Bacterial growth, cell respiration and virulence factors were determined. Traumatized tissue extracts (TTE) supported active microbial growth but coagulase, β-hemolysin, hyaluronidase and enterotoxin B were inhibited, particularly during the early stages of healing (1–4 days). These aforementioned factors increased after 6–8 days to reach the level noted in normal tissue extract. Increased respiration of bacterial cells was noted in TTE whereas hemoglobin (0.0035 μmoles/ml) inhibited both respiration and enterotoxin B formation. The accelerated growth of staphylococcal cells in the bruised tissue creates a greater opportunity for selection of those viable cells that are resistant to natural tissue bactericidal substances and may therefore account for their prolonged persistence in bruised tissue.

COMPARISON OF SARCOMERE LENGTH MEASUREMENT OF COOKED CHICKEN PECTORALIS MUSCLE BY LASER DIFFRACTION AND OIL IMMERSION MICROSCOPY. J.E. RUDDICK & J.F. RICHARDS. *J. Food Sci.* **40,** 500–501 (1975)—Sarcomere length measurements were made on samples of cooked chicken Pectoralis muscle aged for 10 min, 3 hr and 24 hr postmortem using laser diffraction and oil-immersion microscopy. A strong and significant correlation was shown to exist between the two methods of measurement. Laser diffraction was found to be a more rapid and accurate method for obtaining sarcomere length measurements on a large number of samples although it provides only limited information on the morphology of the muscle fiber.

QUALITY CHARACTERISTICS OF SOY-SUBSTITUTED GROUND BEEF, PORK AND TURKEY MEAT LOAVES. C.W. WILLIAMS & M.E. ZABIK. *J. Food Sci.* **40,** 502–505 (1975)—Ground beef, pork (50:50 mixture of ham and pork) and turkey meat loaves containing 0 or 30% soy-substitution were evaluated for sensory characteristics of flavor, juiciness, mouthfeel and overall acceptability. In addition, cooking losses, moisture content, total lipid and TBA values during short-term storage at 5 or −11°C were determined. 30% soy-substitution did not adversely affect the quality characteristics of the ground beef and turkey systems. However, the soy-substitution did lower the flavor, juiciness and overall acceptability scores of the pork meat loaves. The use of 30% soy-substitution decreased the total and drip loss, whereas it did not affect the volatile losses. Although 30% soy-substituted meat systems appeared to have slightly lower TBA values during refrigerated and frozen storage, soy does not appear to appreciably reduce the amount of TBA reactive compounds developing in the meat systems.

A COMPARISON OF HYDROGEN SULFIDE EVOLUTION FROM COOKED LAMB AND OTHER MEATS. J.E. KUNSMAN & M.L. RILEY. *J. Food Sci.* **40,** 506–508 (1975)—Ground lamb, beef, pork and game loaves were cooked to well done and the hydrogen sulfide (H_2S) evolution measured. Under the cooking conditions used lamb gave off significantly more ($P < 0.05$) H_2S than beef. This condition also prevailed when lamb and beef adipose tissue and lean tissue were compared. The adipose tissue contributed more H_2S gas than the lean. No significant differences were noted between the amounts of H_2S evolution from lamb and those from elk and deer. The amount of H_2S evolved from lamb was significantly higher ($P < 0.05$) than moose and significantly lower ($P < 0.05$) than antelope.

ULTRASTRUCTURE OF THAW RIGOR BOVINE MUSCLE. A.O. OKUBANJO, J.R. STOUFFER, A. BENSADOUN & W.O. SACK. *J. Food Sci.* **40,** 509–515 (1975)—Studies based on light and electron microscopy showed that both stretched and contracted bovine muscle strips exhibited similar extensive and drastic structural damage especially in thin fibers after thaw rigor. Specifically noted were the alternate banding pattern of zones of superstretched and supercontracted sarcomeres, wavy and kinky fibers, lateral clumping of actin filaments, extreme disorganization of the myofibrillar structure, degradation of the sarcoplasmic reticulum and the triad system, presence of dark granular precipitates of insoluble materials and conspicuous absence of glycogen granules. Myosin filaments of supercontracted sarcomeres were observed to either penetrate the Z discs or crumple on impingement on the Z lines.

EFFECT OF STORAGE TIME AND TEMPERATURE ON HISTAMINE CONTENT AND HISTIDINE DECARBOXYLASE ACTIVITY OF AQUATIC SPECIES. W.J. EDMUNDS & R.R. EITENMILLER. *J. Food Sci.* **40,** 516–519 (1975)—Since it has been reported that formation of histamine and histamine like substance in fish muscle is responsible for scombroid poisoning, an investigation was undertaken to determine the effect of storage time and temperature on histamine content and histidine decarboxylase activity in Spanish mackerel (*Scomberomorus maculatus*), common mullet (*Mugil cephalus*), speckled trout (*Cynoscion nebulosus*), white shrimp (*Penaeus setiferus*) and channel catfish (*Ictalurus punctatus*). Fillets of the four fish species and deheaded white shrimp were held at 4°C for 0, 7 and 14 days and at ambient temperatures (24 ± 2°C) for 0, 1 and 2 days. Muscle histamine was determined fluorometrically after perchloric acid extraction and ortho-phthaldialdehyde reaction. Histidine decarboxylase activities were determined by radioisotopic assay. At 4°C, speckled trout, mullet, white shrimp and channel catfish showed slight but significant ($P < 0.05$) increases in histamine content with time. There was no significant change with time in enzyme activities in any of the species. Comparison of the species within a given storage temperature showed there were slight but significant ($P < 0.05$) differences in both histamine content and enzyme activities. The highest histamine content observed was 3.40 μg/g muscle in channel catfish, and the highest enzyme activity was 19.37 nmoles/min/g muscle also in channel catfish. At ambient temperature Spanish mackerel had significantly ($P < 0.05$) higher histamine content than all species except mullet and significantly ($P < 0.05$) higher enzyme activity than all other species. All species showed significant ($P < 0.05$) increases in histamine content with time. Channel catfish, speckled trout and mackerel showed significant ($P < 0.05$) increases in enzyme activity with time while common mullet and white shrimp showed no significant ($P < 0.05$) changes. The highest level of histamine observed was 333 μg/g muscle in Spanish mackerel and the highest enzyme activity, also in the Spanish mackerel was 135.6 nmoles/min/g muscle. The study shows that even though measurable enzyme activity occurs in speckled trout, common mullet, white shrimp and channel catfish, there is little chance that these species would develop sufficient histamine levels to lead to histamine intoxication. Mackerel could be capable of developing sufficient histamine to produce symptoms of intoxication although advanced spoilage would probably be necessary to reach this stage.

LIPID-PROTEIN INTERACTION DURING AQUEOUS EXTRACTION OF FISH PROTEIN: FISH ACTIN PREPARATION AND PURIFICATION. S.Y.K. SHENOUDA & G.M. PIGOTT. *J. Food Sci.* **40,** 520–522 (1975)—A method was developed to produce a pure actin from fish. The procedure consists of four steps: preparation of acetone powder; extraction of G-actin from the acetone powder; purification of G-actin by repeated polymerization (to F-actin) and depolymerization to G-actin; and final purification by using Sephadex-G200 column and lyophilization. The purified G-actin showed a single band on sodium dodecylsulphate polyacrylamide gel electrophoresis. The amino acid analysis of fish actin was reported and showed the absence of 3-methylhistidine.

LIPID-PROTEIN INTERACTION DURING AQUEOUS EXTRACTION OF FISH PROTEIN: ACTIN-LIPID INTERACTION. S.Y.K. SHENOUDA & G.M. PIGOTT. *J. Food Sci.* **40,** 523–532 (1975)—Interactions between fish actin and C-14 labeled polar and neutral fish lipid were investigated in aqueous media. The results showed that actin interacts with polar lipid (PL) or neutral lipid (NL) at room temperature or cold temperature. Actin interacts in monomer form (G-actin) or polymer form (F-actin). F-actin interacts more strongly than G-actin (2–3 times). Any treatment which induces the transformation of G-actin into F-actin (i.e., Mg^{++}, Ca^{++}, temperature, etc.) increases the lipid-actin complex formation. Agitation and/or heating increased the hydrophobic interaction between actin and NL. The effect of pH and ionic strength indicates the participation of hydrophobic and electrostatic interaction between actin and lipid. The SDS and urea treatments suggest either the existence of covalent bonding or a strong electrostatic and/or hydrophobic bonding between actin and lipid in the actin-lipid complexes.

REPRESSION OF *Vibrio parahaemolyticus* BY *Pseudomonas* SPECIES ISOLATED FROM PROCESSED OYSTERS. L.J. GOATCHER & D.C. WESTHOFF. *J. Food Sci.* **40,** 533–536 (1975)—45 cultures isolated from processed Maryland oysters stored at 5°C were examined for inhibitory activities against various strains of *Vibrio parahaemolyticus* by a spot-plate method. Nine oyster isolates which demonstrated inhibitory activity were identified as *Pseudomonas* species. Inhibition was more pronounced at 25°C than at 35°C and increased with decreasing levels of *V. parahaemolyticus*. Type and composition of plating medium, including pH, salt and peptone content were important factors. Inhibition was maximal at 0.5% NaCl and decreased with increasing salt concentrations until little or no inhibition was observed at 2.5% NaCl. Effect of pH was variable according to strain of *V. parahaemolyticus* used. Addition of trypticase or phytone to tryptone-glucose-yeast extract agar decreased inhibition to different degrees. Pigmentation of *Pseudomonas* cultures was strong when production of inhibition was maximal. In general, pathogenic strains of *V. parahaemolyticus* tested were inhibited to a lesser degree than were nonpathogenic strains.

FOOD USE OF SOYBEAN 7S AND 11S PROTEINS. Heat Denaturation of Soybean Proteins at High Temperature. K. SAIO, M. TERASHIMA & T. WATANABE. *J. Food Sci.* **40,** 537–540 (1975)—Qualitative changes in 7S and 11S proteins during heat treatment at 100–170°C were studied. Protein paste of 25% concentration was autoclaved and the resultant heat-induced gel was submitted to measurement of its solubility, ultracentrifugal characteristics and disc polyacrylamide gel electrophoresis after dissolving with sodium dodecyl sulfate and 2-mercaptoethanol. The results showed: (1) heating over 100°C resulted in the formation of an insoluble gel; (2) during heating up to 140°C the gel gradually became soluble but the gross-structure of subunits remained unchanged, 11S-gel being more soluble than 7S-gel even at lower temperature; and (3) during heating at above 150°C, the gel became highly soluble, showing degradation of the gross-structure of subunits.

FOOD USE OF SOYBEAN 7S AND 11S PROTEINS. Changes in Basic Groups of Soybean Proteins by High Temperature Heating. K. SAIO, M. TERASHIMA & T. WATANABE. *J. Food Sci.* **40,** 541–544 (1975)—Protein paste of 25% concentration from cold insoluble fraction (CIF) or crude 7S was autoclaved at 100–170°C to prepare heat-induced gel. After solubilization of gel, quantitative changes in Amido Black 10B bound to protein, basic amino acids and amide groups during heating, were investigated. In these experimental conditions, no significant change in basic amino acids were recognized but decreases of amide groups and the amount of Amido Black 10B bound were significant as temperature of heating increased. The decrease began from 105°C in CIF-gel and from 140°C in crude 7S-gel. From the results on Amido Black 10B bound to protein and on SDS-disc polyacrylamide gel electrophoresis, the gross-structure of subunits derived from gel was degraded into lower molecular substances by heating at above 150°C. The relationship of these results with those of our previous reports on gel properties, solubility and others are also discussed.

EFFECTS OF FEEDING OXIDIZED OR HEATED SOYBEAN OIL ON TISSUE COMPOSITION AND HEMATOLOGICAL STATUS OF RATS. J. MILLER & D.R. LANDES. *J. Food Sci.* **40,** 545–548 (1975)—Rats were fed diets containing 40% soybean oil that was either aerated at room temperature, heated in the presence of carbon dioxide or air, or supplied as the fresh product. When the protein in the diet was comprised of 20% casein, both growth and feed efficiency were depressed by the damaged oils. Feeding the oil that was aerated during heating reduced the number of hepatic cells and increased the quantity of protein per cell. Liver lipid, especially triglyceride, was decreased by the damaged oils. The number of red blood cells formed and their hemoglobin content and absorption of iron were also curtailed by the heated oil. Studies indicated that the effects were not due solely to reduced food intake. Increasing the protein in the diet containing heated oil reduced but did not eliminate the extrahepatic effects while further increasing the protein content of the liver cells. There is evidence that damaged oil could aggravate the nutritional quality of diets having marginal protein sufficiency.

ULTRASONIC EXTRACTION OF PROTEINS FROM AUTOCLAVED SOYBEAN FLAKES. L.C. WANG. *J. Food Sci.* **40,** 549–551 (1975)—Amounts of proteins extracted from soybean flakes by applying ultrasonic waves and by conventional stirring were compared. Respective yields of the total proteins from unautoclaved and autoclaved flakes were 60% and 16% by conventional stirring and 88% and 58% by sonication in a single 1:10 meal-to-water extraction. From autoclaved flakes sonication in a single extraction dispersed up to 78% total proteins in water with 1:40 meal-to-water ratio. Sonication recovered a portion of proteins from autoclaved flakes ordinarily unattainable by conventional stirring extraction. Proteins obtained by either method revealed no differences in their ultracentrifuge patterns.

SPROUTING OF SEEDS AND NUTRIENT COMPOSITION OF SEEDS AND SPROUTS. J.R. FORDHAM, C.E. WELLS & L.H. CHEN. *J. Food Sci.* **40,** 552–556 (1975)—A method of using cellulose sponges as a medium for sprouting of seeds is reviewed in this article. Six varieties of peas and 12 varieties of beans were used for sprouting, and graded as to their ability to produce sprouts. Proximate analysis, determination of vitamins (vitamin C, thiamin, niacin, riboflavin and tocopherols), carotenes and minerals (iron, calcium, magnesium, manganese, potassium and phosphorus) were carried out with each variety of seed before and after sprouting. Ash, protein and lipid showed a 1.5- to threefold variability between varieties in both seeds and sprouts. The dry seeds were relatively low in ascorbic acid, but this was very varietal dependent as was the ratio of reduced to oxidized ascorbic acid. Total ascorbic acid ranged from 13–50 mg/100g of sprouts. Similar variability between varieties was obtained with tocopherol (0.024–2.3 mg/100g sprouts) and carotene (0.2–4 mcg/100g sprouts). Thiamin, riboflavin, niacin and minerals varied to a much smaller extent between varieties.

SEVERAL FACTORS AFFECTING COLOR, TEXTURE AND DRAINED WEIGHT OF CANNED DRY LIMA BEANS. B.S. LUH, C. WANG & H.N. DAOUD. *J. Food Sci.* **40,** 557–561 (1975)—Two varieties of dry large lima beans (*Phaseolus lunatus* L.) B-51-114 and White Ventura 65 were used for investigation of their canning quality. Addition of 0.25% citric acid to the soaking solution improved the color of the canned product. It is postulated that the beans contain small amounts of iron and copper ions which can combine with the sulfides derived from the sulfur-containing amino acids during the heat sterilization process. The metal sulfides thus formed may be related to the discoloration. The reaction of polyphenols with iron and copper in the beans may also contribute to the discoloration problem. Addition of 0.25% citric acid or 300 ppm EDTA · Na_2 (ethylenediaminetetraacetic acid disodium salt) to the brine improves the color of the canned product; and addition of small amounts of calcium ions to the brine improves the firmness of the canned product. The drained weight of the canned beans was decreased by acidification of the brine.

FOLIC ACID CONTENT OF CANNED GARBANZO BEANS. K.C. LIN, B.S. LUH & B.S. SCHWEIGERT. *J. Food Sci.* **40,** 562–565 (1975)—Total and free folic acid in dry and canned garbanzo beans was determined with *Streptococcus faecalis* (ATCC 8043) as test organism. A modified assay medium was developed which can support excellent growth of *S. faecalis* by incubation at 37°C for 24 hr. There was a correlation between concentration of folic acid and growth of the organism (absorbance at 650nm). The coefficient of correlation was 0.980. The recovery of added folic acid to the test medium was 96%. The loss of free folic acid in the beans during soaking was 4.6%. The corresponding loss in total folic acid on a dry basis was 6.1%. Total folic acid retention in garbanzo beans was 78.1% when steam blanched for 10 min and 74.9% when water blanched for 10 min. Folic acid in garbanzo beans was quite stable to heat processing. There was no significant decrease in either free or total folic acid activity even though the heat processing time at 118.3°C was increased from 29.8 min (F_o = 8) to 53.4 min (F_o = 20). The retention of total and free folic acid in the canned beans was 70.0% and 73.5% respectively, of that present in the original dry bean.

RED LIGHT INTENSITY AND CAROTENOID BIOSYNTHESIS IN RIPENING TOMATOES. R.L. THOMAS & J.J. JEN. *J. Food Sci.* **40,** 566–568 (1975)—Carotenoid biosynthesis was higher in mature, detached tomatoes illuminated with Gro-Lux fluorescent lamps than with cool white fluorescent lamps, even though the light intensity of the Gro-Lux lamps was much lower. Gro-Lux fluorescent lamps emit light predominantly in the red region, whereas cool white lamps emit very little red light. Tomatoes illuminated with varying intensities of red light, showed increased carotenoid production as light intensity increased. Total carotenoid biosynthesis was found to be proportional to the logarithm of the radiant energy received up to a saturation point. Red light increased and far-red light suppressed carotenoid biosynthesis when compared to a dark control. These data suggest the involvement of phytochrome in carotenoid biosynthesis in ripening tomatoes.

FACTORS INFLUENCING BLOATER FORMATION IN BRINED CUCUMBERS DURING CONTROLLED FERMENTATION. J.L. ETCHELLS, H.P. FLEMING, L.H. HONTZ, T.A. BELL & R.J. MONROE. *J. Food Sci.* **40,** 569–575 (1975)—Conditions were provided whereby the fermentation of brined cucumbers was directed to one of predominant microbial growth by *Lactobacillus plantarum,* the added starter culture and a microbe that produces relatively little CO_2. Comparisons were made between microbial activities using this procedure and natural fermentations as practiced commercially. Significant differences in the microbial flora were noted between the two fermentation types. Also, directed (controlled) fermentations resulted in less CO_2 and a more rapid

and complete conversion of fermentable sugars to acid; these fermentations consistently were completed within about 10 days at 27 or 32°C. Natural fermentations were more variable in rates of acid production; and, often fermentable sugars remained after the primary fermentation by lactic acid bacteria. Although bloater damage was much less in the directed than in natural fermentations, 24–48% of the cucumbers had some damage due to the CO_2 produced by *L. plantarum* and the cucumber tissue. Bloater damage was directly related to the depth at which cucumbers were brined in 55-gal drums, probably because more dissolved CO_2 was retained at greater brine depths. Bloater damage was greater at 32°C than at 27°C, and the depth effect on damage was enhanced at the higher temperature. Damage also was directly related to pack-out ratios of cucumbers:brine (65:35 > 55:45 > 45:55). These studies served as a basis for the "controlled fermentation" process which recently was outlined for commercial briners.

FRACTIONATION AND CHARACTERIZATION OF PEROXIDASE FROM RIPE BANANA FRUIT. N.E. NAGLE & N.F. HAARD. *J. Food Sci.* **40,** 576–579 (1975)–Isoperoxidases from banana fruit were fractionated into anionic and cationic forms. The pH optimum for the peroxidatic reaction was found to be 4.5–6.0 for both fractions. The pH optima for the oxidation of IAA was 4.5–5.0 for the anionic and 5.5–6.0 for the cationic form. Gel electrophoresis has shown six anionic forms and one cationic form. Isoelectric focusing in gels revealed eight anionic and two cationic forms. Column isoelectric focusing elucidated eight zones of peroxidase activity and nine of IAAoxidase activity. All zones had isoelectric points between pH 3.5–5.0. All isoenzyme species exhibited low IAAoxidase activity as compared to the crude extract.

BREAD BAKING PROPERTIES OF AQUEOUS PROCESSED PEANUT PROTEIN CONCENTRATES. M.N. KHAN, K.C. RHEE, L.W. ROONEY & C.M. CATER. *J. Food Sci.* **40,** 580–583 (1975)–Bread baking properties of three experimental peanut protein concentrates (PPC) produced by an aqueous extraction process were compared with a commercial defatted peanut and a commercial full fat soy flour. An experimental defatted peanut flour was also included for comparative purposes. Protein (N × 6.25) and fat contents of PPC were 56 and 17%, respectively. Brabender Farinograph absorption increased with the addition of protein source compared to 100% wheat flour. Farinograph peak time was not affected due to the addition of the protein source. Bread loaf volume containing PPC and baked with short-time dough system was on an average 10, 11 and 13% less than the commercial defatted peanut, experimental defatted peanut, and full fat soy flours, respectively. Crumb color of the bread containing commercial defatted peanut flour was darker than the one containing PPC. Commercial full fat soy flour imparted an unacceptable yellow color to the bread crumb. Bread baked with full fat soy and commercial defatted peanut flours had a softer crumb than that baked with PPC. On an average, bread baked with PPC, commercial defatted peanut, experimental defatted peanut, and full fat soy flours increased the bread protein by 32, 38, 36 and 21%, respectively. Taste panel studies indicated that bread with PPC had better organoleptic qualities than that baked with the commercial defatted peanut and full fat soy flours. All three forms of PPC did not show any significant difference in their bread baking properties.

PROTEIN CONTENT AND AMINO ACID COMPOSITION OF DEVELOPING PEAS. E.T. GRITTON, Y. POMERANZ & G.S. ROBBINS. *J. Food Sci.* 40, 584–586 (1975)–Ovuies for three pea cultivars were harvested at 12–48 days after pollination. Physiological maturity was attained about 25 days after pollination, when moisture content averaged 62.7%. During the period studied, ovule dry weight increased 6.2–11.6 times. Accompanying changes in protein concentration were small. The major changes in concentrations of amino acids in pea proteins were increases in lysine, histidine, aspartic acid, serine, proline, glycine, isoleucine, leucine, tyrosine and phenylalanine; and decreases in threonine, glutamic acid, alanine, cystine and methionine. Concentration of arginine increased at early stages of development and then decreased. Cultivar differences in patterns of protein concentration and amino acid composition were small. Changes in concentrations of amino acids in pea proteins are compared with the diametrically different changes in concentrations of these amino acids in proteins of cereals.

INTERRELATIONSHIPS BETWEEN STORAGE, SOAKING TIME, COOKING TIME, NUTRITIVE VALUE AND OTHER CHARACTERISTICS OF THE BLACK BEAN (Phaseolus vulgaris). M.R. MOLINA, G. DE LA FUENTE & R. BRESSANI. *J. Food Sci.* 40, 587–591 (1975)–Black beans (*Phaseolus vulgaris*) were stored under ambient conditions for a period of time up to 6 months. Although storage increased methionine and available lysine contents of the raw and processed beans, it had, in general, a detrimental effect on protein quality. Cooking time significantly ($P < 0.05$) decreased protein quality of both the recently harvested and the beans stored for 3 months. The same was true for the beans stored for 6 months when subjected to a soaking treatment of 16 or 24 hr prior to cooking. Soaking decreased significantly ($P < 0.05$) protein quality in the case of the beans stored for 6 months. Storage had an opposite effect on the protein digestibility and on the nitrogen solubility in 1N NaCl, 0.05N NaOH and H_2O of the processed beans. Preliminary results indicate that the nitrogen fraction soluble in 1N NaCl is capable of lowering bean protein digestibility in vitro when using pepsin as the digestive enzyme.

FROZEN STORAGE KEEPING QUALITY OF MINCED BLACK ROCKFISH (*Sebastes* spp.) IMPROVED BY COLD-WATER WASHING AND USE OF FISH BINDER. D. MIYAUCHI, M. PATASHNIK & G. KUDO. *J. Food Sci.* **40,** 592–594 (1975)–Black rockfish (*Sebastes* spp.) is not suitable for production of conventional fillet blocks owing to a shelf life at −18°C of less than 4 months. Use of minced fish muscle offers greater flexibility of processing to improve flavor, texture and storage stability. Modified fish blocks prepared by mixing the minced muscle with a fish binder had a storage life of 8–12 months. Modified blocks made with cold water-washed minced muscle were significantly better in color and flavor than those made with unwashed minced muscle. Use of 0.001% BHA and 0.001% BHT did not significantly improve the cold-storage life of the modified minced fish blocks.

FUNCTIONAL PROPERTIES OF ADDED PROTEINS CORRELATED WITH PROPERTIES OF MEAT SYSTEMS. Effect of Concentration and Temperature on Water-Binding Properties of Model Meat Systems. A.-M. HERMANSSON & C. ÅKESSON. *J. Food Sci.* 40, 595–602 (1975)–The effects of some functional properties (e.g., solubility, viscosity, swelling and gel strength) of added proteins on moisture loss properties of model meat systems were studied. Besides moisture loss, some additional studies were made on penetration depth. The protein preparations soy protein isolate (Promine-D), caseinate and whey protein concentrate were added to pork shoulder and beef brisket systems, and changes were observed with respect to temperature and percent exchanged protein. Observed changes in moisture loss properties were correlated with the corresponding changes in functional properties by certain regression procedures. The best statistical solution from changes on raw meat systems had a correlation coefficient of 0.99, with solubility explaining 79%, swelling 10% and viscosity 10% of the variance. The best statistical solution for heat-treated systems had a correlation coefficient of 0.98, with gel strength alone explaining 94% of the variance.

FUNCTIONAL PROPERTIES OF ADDED PROTEINS CORRELATED WITH PROPERTIES OF MEAT SYSTEMS. Effect of Salt on Water-Binding Properties of Model Meat Systems. A.-M. HERMANSSON & C. ÅKESSON. *J. Food Sci.* **40,** 603–610 (1975)–The effects of solubility, swelling, viscosity and gel strength properties of added proteins on moisture loss properties of raw and heat-treated model meat systems with varying salt content were studied. Proteins added were soy protein isolate (Promine-D), caseinate and whey protein concentrate. Quantitative interrelationships of functional properties were calculated by a general metric hierarchical clustering technique, and correlations between functional properties and moisture loss properties by multiple regression analysis. In addition, penetration studies were made. The addition of salt decreased the moisture loss of all the meat systems tested. The functional properties of the added protein were, however, very differently affected by the addition of salt. Although complex behavior occurred due to salt addition, good statistical correlations were obtained between differences in functional properties and differences in moisture loss properties. The best statistical solutions on raw and heat-treated meat systems had correlation coefficients of 0.82 and 0.98, respectively.

FUNCTIONAL PROPERTIES OF ADDED PROTEINS CORRELATED WITH PROPERTIES OF MEAT SYSTEMS. Effect on Texture of a Meat Product. A.-M. HERMANSSON. *J. Food Sci.* **40,** 611–614 (1975)—Texture changes observed when 4% proteins were added to a commercial meatball recipe were correlated with changes in the functional properties of the added proteins. Proteins added were untreated soy protein isolate, caseinate and whey protein concentrate, preheat-treated soy protein isolate and whey protein concentrate. Good statistical correlations were found between texture changes of meatballs and moisture loss changes of model meat systems. When regression equations calculated from moisture loss studies were used on the texture changes of meatballs, correlation coefficients as high as 0.88 were obtained. Of the functional properties, swelling and gel strength were shown to be of great importance for the texture changes of meatballs.

VITAMIN B_6 CONTENT OF TURKEY COOKED FROM FROZEN, PARTIALLY FROZEN AND THAWED STATES. P.P. ENGLER & J.A. BOWERS. *J. Food Sci.* **40,** 615–617 (1975)—Vitamin B_6 content of muscles and drip and other selected objective measurements from raw turkey and turkey roasted from the frozen, partially frozen and thawed states were determined. Vitamin B_6 contents of breast and thigh muscles did not differ calculated on a wet-weight basis. When vitamin B_6 content was calculated on a moisture- and fat-free basis, uncooked breast muscle and muscle cooked from the partially frozen state had significantly ($P < 0.05$) more vitamin B_6 than muscle cooked from the thawed state. Muscle cooked from the frozen state was intermediate in vitamin B_6 content. Uncooked thigh muscles had significantly ($P < 0.05$) more vitamin B_6 than did muscles subjected to heat treatments. Meat cooked from frozen and partially frozen states had significantly ($P < 0.01$) longer cooking times than meat roasted from the thawed state. Uncooked breast and thigh muscles had significantly ($P < 0.01$) higher moisture contents and uncooked thigh muscles had lower ($P < 0.05$) ether extract than did muscles roasted from frozen, partially frozen or thawed states.

SURVIVAL OF SELECTED PATHOGENS DURING PROCESSING OF A FERMENTED TURKEY SAUSAGE. W.L. BARAN & K.E. STEVENSON. *J. Food Sci.* **40,** 618–620 (1975)—The purpose of this study was to determine the potential survival of selected pathogens during processing of dry fermented turkey sausage. Bacterial cultures were inoculated into the sausage mixture and microbiological analyses were conducted before and after processing. The sausages were heated to 46°C and dried at 10°C and 72% relative humidity for 8 days. The numbers of salmonellae, *C. perfringens* and enteropathogenic *E. coli* decreased as a result of processing. The degree of destruction varied according to the strain and amount of inoculum used. When cells of *S. aureus* were inoculated into the sausage mixture, growth occurred. The results indicated salmonellae, *C. perfringens, S. aureus* and enteropathogenic *E. coli* may survive in fermented dry turkey sausages which receive a "low-heat" process.

GRILLED FREEZE-DRIED STEAKS. Effects of Mechanical Tenderization Plus Phosphate and Salt. L.C. HINNERGARDT, S.R. DRAKE & R.A. KLUTER. *J. Food Sci.* **40,** 621–623 (1975)—The excised semimembranosus muscles from USDA choice top rounds were randomly allotted to the following treatments: (a) mechanical tenderization; (b) injection with 3% Na tripolyphosphate and 7.5% NaCl solution to 10% of the raw meat weight; (c) mechanical tenderization followed by injection with 3% Na tripolyphosphate and 7.5% NaCl solution to 10% of the raw roast weight; and (d) no mechanical tenderization, nor phosphate and NaCl addition, prior to slicing (1.27 cm thick) and freeze-drying. Both objective and subjective evaluations revealed the most desirable steak tenderness resulted from roasts which were mechanically tenderized and injected with a phosphate and salt solution. Mechanical tenderization without the phosphate injection produced the next most acceptable tenderness. Texture was enhanced by injection of the phosphate-salt solution, but not enough to be of practical consequence without mechanical tenderization.

FRESHLY COOKED AND COOKED, FROZEN, REHEATED BEEF AND BEEF-SOY PATTIES. J.A. BOWERS & P.P. ENGLER. *J. Food Sci.* **40,** 624–625 (1975)—Ground beef and beef-soy (15 and 30% soy) patties were prepared and frozen raw or cooked and then, after cooking or reheating were evaluated by a taste panel. Percentages of moisture and fat and TBA values were determined. Adding soy decreased cooking losses, and the reheating process increased cooking losses. Beef patties contained less moisture but more ether extract and had higher TBA values than beef-soy blends. Beef patties were less firm and their meaty flavor and aroma were more intense than those of beef-soy patties but their cereal-like flavor and aroma were less intense. Reheated beef-soy patties had less stale flavor and aroma than reheated beef patties.

INFLUENCE OF SODIUM NITRITE ON THE CHEMICAL AND ORGANOLEPTIC PROPERTIES OF COMMINUTED PORK. J.P. HADDEN, H.W. OCKERMAN, V.R. CAHILL, N.A. PARRETT & R.J. BORTON. *J. Food Sci.* **40,** 626–630 (1975)—Objectives of a study undertaken to further develop present knowledge of the action of sodium nitrite in meat involved an examination of organoleptic and chemical differences occurring between cooked, canned pork emulsions processed with or without added sodium nitrite in samples with or without added salt (NaCl). It appears that sodium nitrite added to cooked, canned comminuted pork plays a vital role in developing and maintaining cured pork flavor. Nitrite was found to retard the rate of oxidative rancidity (TBA value) in this model system. However, it is important to note that in the model system studied, spices, sweeteners and extenders were excluded from the formulation and that the average fat level of the finished product was approx 17% which is leaner than that used in most commercial formulations.

EFFECTS OF FROZEN STORAGE, COOKING METHOD AND MUSCLE QUALITY ON ATTRIBUTES OF PORK LOINS. A.W. FLYNN & V.D. BRAMBLETT. *J. Food Sci.* **40,** 631–633 (1975)—Pork loins classified as normal; pale, soft, exudative (PSE); and dark, firm, dry (DFD) were frozen for 9 months. Chops cut after frozen storage from each type were deep-fat fried, oven-broiled, or left raw, and evaluated for extent of rancidity, quantity of lipids, relative quantities of major fatty acids, and palatability. Results indicated that PSE muscles were the most rancid and DFD the least. Oven-broiled samples were significantly ($p < 0.01$) more tender, flavorful and acceptable than the deep-fat fried pork, and DFD pork was more juicy than normal or PSE. No significant difference in tenderness due to muscle type was observed.

EFFECTS OF CURING INGREDIENTS AND HOLDING TIMES AND TEMPERATURES ON ORGANOLEPTIC AND MICROBIOLOGICAL PROPERTIES OF DRY-CURED SLICED HAM. J.D. KEMP, B.E. LANGLOIS, J.D. FOX & W.Y. VARNEY. *J. Food Sci.* **40,** 634–636 (1975)—Hams were dry cured using salt and sugar only, and salt and sugar plus either nitrite, nitrate, nitrite and nitrate or Prague Powder. After aging, the hams were sliced and vacuum packed. Slices were evaluated 3 days after packing and after a month's storage at either 1°C or 24°C. All groups treated with nitrite and/or nitrate developed more desirable color than controls and maintained this color at 1°C but not at 24°C. Organoleptic scores favored the treated groups. All scores were lower after 24°C storage. Nitrite levels decreased with storage. Microbiological counts were affected only slightly by cure treatment but increased greatly when slices were stored at 24°C.

FACTORS AFFECTING SHOWCASE COLOR STABILITY OF FROZEN LAMB IN TRANSPARENT FILM. M.C. HUNT, R.A. SMITH, D.H. KROPF & H.J. TUMA. *J. Food Sci.* **40,** 637–640 (1975)—Display color stability of frozen lamb chops of three marbling levels was studied using all combinations of two freezing temperatures (−40°C and −26°C), two display temperatures (−29°C and −21°C), two packaging films (oxygen permeable L-300 and low oxygen permeable Saran) and two lighting systems (deluxe cool white fluorescent and incandescent). Packaging in oxygen permeable L-300 film resulted in brighter visual scores for fresh packaged chops and frozen chops after display 1 or 42 days, but chops in Saran film had more desirable color after unpackaging and thaw. Reflectance data indicated less metmyoglobin (Mb^+) in chops in Saran when displayed 7 or more days. Freezing at −40°C, compared with −26°C, resulted in more desirable visual color at all time periods post-freezing, confirmed by higher 630 nm reflectance and resulting in no bleach. Display at −29°C, compared with −21°C improved visual color only after 21 days display and after the thaw-bloom period. Reflectance suggested more oxidation of myoglobin occurred at −21°C. Chops displayed and scored under incandescent lighting had more desirable visual color but lighting apparently masked color deterioration as reflectance suggested less Mb^+ in chops displayed under deluxe cool white fluorescent lighting.

Marbling level did not affect weight loss or color stability. Drip losses were less in chops frozen at −40°C than at −26°C. Weight loss, from fresh packaged to frozen displayed for 6 wk, averaged 0.71%.

IMMOBILIZED CATALASE REACTOR FOR USE IN PEROXIDE STERILIZATION OF DAIRY PRODUCTS. H.D. CHU, J.G. LEEDER & S.G. GILBERT. *J. Food Sci.* **40,** 641–643 (1975)–The effect of H_2O_2 treatment on the bacteriological content of cheese milk was studied at various temperatures. Using 0.05% H_2O_2 at room temperature reduced the total bacterial counts by 97%. The performance of the catalase-collagen reactor was investigated. It was found that the mass transfer is the rate-controlling mechanism in the reactor. The reactor may be scaled-up for industrial use by considering both space time and flow rate.

STORAGE STABILITY OF DRIED SWEET CHEESE WHEY. N.L. KEHRBERG & J.M. JOHNSON. *J. Food Sci.* **40,** 644–646 (1975)–Dried whey was stored in sealed plastic bags, in glass jars and open, under room conditions and refrigeration for 30, 60 and 90 days. Samples were analyzed for moisture, pH, color and protein solubility (Kjeldahl nitrogen). Greatest changes occurred in open, room temperature stored whey. After 30 days the samples were visibly browned, and after 90 days moisture content had increased and pH and solubility had decreased significantly. Less rapid and extensive changes in moisture, pH, color and solubility occurred in whey stored under all other conditions. Changes in pH, color and solubility were attributed largely to nonenzymatic browning. Cakes were prepared from all whey stored under room conditions, using 100% substitution for nonfat dry milk (ndm). Initially, a taste panel found no significant difference between ndm cakes and whey cakes. After 30 days ndm cakes were preferred to cakes made from open-stored whey, and at 90 days panel members preferred ndm cakes over all whey cakes. Cake volume decreased in cakes from open-stored whey after 30 days, and in jar- and bag-stored whey after 90 days. Open stored whey cakes were less tender and had darker crumbs than other whey cakes.

A REFLECTANCE METHOD FOR THE ENUMERATION OF SURFACE BACTERIA. G.J. JEDLICKA, W.M. HILL & J.G. HECK. *J. Food Sci.* **40,** 647–648 (1975)–Increased emphasis on sanitation and quality control has caused concern in many food processing operations. Present methodology for making bacterial surface counts on equipment and carcasses is complicated, time consuming and requires considerable skill on the part of the person performing the test. There is need for a simple test which can be performed by relatively unskilled persons. A method has been developed consisting of a contact plate filled with a reflectance medium and a reflectometer. To perform the test, the surface of the plate is pressed against the surface to be tested, transferring bacteria from the surface to the medium. The culture medium is then incubated. Reflectance of the medium is then measured in a reflectometer. Change in reflectance is related to the bacterial count. The advantages of the method are: (1) Does not require visual counting of bacteria colonies; (2) Estimates viable bacteria on a per square inch basis; (3) Reproducible; (4) Does not require skilled operator; (5) Inexpensive; and (6) Compares favorably with standard swab methods.

EFFECT OF PRECOOKING ON COPPER CONTENT, PHENOLIC CONTENT AND BLUEING OF CANNED DUNGENESS CRAB MEAT. J.K. BABBITT, D.K. LAW & D.L. CRAWFORD. *J. Food Sci.* **40,** 649–650 (1975)–Precooking reduced the copper content and slightly reduced the phenolic content of canned crab meat. No significant blueing was observed in the cooked or precook-cooked canned crab meat when the crab were processed immediately after harvesting. The higher pH of canned meat from crab held 2 or 4 days prior to processing may promote the oxidation and polymerization of phenols to colored melanins particularly in the presence of copper. Precooking followed by the addition of citric acid to the canned crab meat will help prevent blueing when the crab are in good condition and handled promptly after harvesting.

A STUDY OF THE RATE-LIMITING FACTORS IN THE RESPIRATORY OXYGEN CONSUMPTION OF INTACT POST-RIGOR BOVINE MUSCLE. D.P. DeVORE & M. SOLBERG. *J. Food Sci.* **40,** 651–652 (1975)–The relationship between the oxygen consumption rate, tissue NADH concentration and cytochrome c reductase activity was determined in intact post-rigor bovine muscle. Oxygen uptake was measured manometrically at 5°C under a constant oxygen headspace pressure of one atmosphere. The decay in oxygen consumption rate was associated with a coincident reduction in respiratory enzyme activity and tissue NADH concentration.

JFS BASIC SCIENCE

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Clostridium perfringens INHIBITION BY SODIUM NITRITE AS A FUNCTION OF pH, INOCULUM SIZE AND HEAT

INTRODUCTION

FOOD POISIONING cases attributed to *C. perfringens* continue to occur in significant numbers. Numerous studies have been conducted to more completely understand *C. perfringens* and its relation to man and his food. Most *C. perfringens* food poisoning outbreaks have been associated with meat, poultry, or meat products as a vehicle. Cured meat products have only occasionally been implicated. The influence of the curing salts, especially sodium nitrite, has been hypothesized as the factor providing a margin of safety.

The inhibitory effect of sodium nitrite upon members of the genus *Clostridium* was reported by several investigators (Perigo and Roberts, 1968; Gough and Alford, 1965; Perigo et al., 1967; Johnston et al., 1969; Pivnick et al., 1967, 1969, 1970; Duncan and Foster, 1968; Wasserman and Huhtanen, 1972). Perigo and Roberts (1968), Gough and Alford (1965) and Riha and Solberg (1973) reported the inhibition of *C. perfringens* by sodium nitrite. Perigo and Roberts (1968) and Riha and Solberg (1973) demonstrated a significant enhancement of the inhibitory effect when $NaNO_2$ was autoclaved in microbiological media. This study was undertaken to characterize the effect of sodium nitrite as an inhibitor on several strains of *C. perfringens* as a function of pH, inoculum size, sterilization technique and nitrite concentration.

MATERIALS & METHODS

Media

All nitrite studies were conducted in a chemically defined medium (R & S Medium) described by Riha and Solberg (1971) as capable of supporting growth of low inocula levels of *C. perfringens*. After preparation, the medium was adjusted to pH 6.3 or 7.2 with NaOH. $NaNO_2$ was then added to the medium from a stock solution. Filter sterilization was accomplished using a 0.22μ pore size micropore filter (Millipore Filter Corp., Bedford, Mass.). Glucose was added to the medium prior to filtration. After filtration, the sterile medium was aseptically dispensed into appropriate sterile containers. Autoclave sterilization was accomplished by heating at 121°C for 15 min after which glucose was aseptically added from a stock autoclave sterilized solution to the autoclave sterilized medium to a concentration of 1.25% glucose.

Cultures of *C. perfringens* were maintained on Cooked Meat Medium (Difco) and grown on Fluid Thioglycollate Medium (FTM, Difco) in preparation of an inoculum. Plate counts were made on SPS Agar (Difco).

Inocula

Seven strains of *C. perfringens* were used throughout most of the study. Six strains (8797, 1362, 8235, 8237, S-80, S-88) have been associated with foods and the seventh strain, BP6K, is a medically significant strain. To prepare an inoculum, the organisms were grown twice for 24 hr in FTM at 37°C. A tube containing 10 ml of FTM was inoculated with 0.1 ml from the second 24 hr culture and subsequently incubated at 43°C for 6 hr, after which serial dilutions were prepared in sterile 0.1% peptone water.

Incubation

Plate counts were made after 18–24 hr incubation at 43°C in an Anaero-Jar (Case Labs Inc., Chicago, Ill.). Anaerobiosis was attained by evacuating and flushing the jar three times with nitrogen. All tubes and culture flasks of R & S Medium were incubated at 43°C for at least 72 hr unless otherwise stated.

Effect of pH, inoculum size, heat and nitrite conc

Seven strains were visually observed for growth during a 72-hr incubation at 43°C. An inoculum consisting of 0.1 ml, containing approximately 10^3 cells, was delivered into 10.0 ml of R & S Medium containing various levels of nitrite. This resulted in a cell concentration of approximately 10^2 cells/ml. The medium was at pH 6.3 or 7.2 and had been sterilized by either filtration or autoclaving. The highest $NaNO_2$ concentration at which growth was observed and the lowest $NaNO_2$ concentration at which no growth occurred after 72 hr was recorded. Some tubes which did not demonstrate growth were incubated for 7–14 days prior to declaring them negative. The experiment was conducted in duplicate.

The effect of inoculum level upon nitrite tolerance for six strains of *C. perfringens* was examined by inoculating 10 ml of both filter sterilized and autoclave sterilized R & S Medium at pH 6.3 with either 0.1 ml of a 6-hr 43°C FTM culture containing approximately 10^7 cells or 0.1 ml of a peptone water dilution of the same culture containing approximately 10^3 cells. Growth was observed during a 72-hr incubation at 43°C after which the highest level of nitrite permitting growth and the lowest level inhibiting growth were recorded.

Further inocula studies were conducted with *C. perfringens* 8797 in pH 6.3 R & S Medium. The medium was filter sterilized, 10 ml were dispensed into sterile test tubes, after which the tubes were heated at 121°C for 5, 10 and 15 min. Sterile glucose was added after heating. From a 6-hr 43°C FTM culture of strain 8797, six serial dilutions were made in 0.1% peptone water and 0.1 ml from each dilution was inoculated into duplicate tubes containing various levels of nitrite. Plate counts of the inocula were made on SPS Agar. The tubes were incubated at 43°C for 72 hr and examined visually for growth. The maximum nitrite concentration at which growth occurred was recorded.

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Table 1—The highest concentrations of $NaNO_2$ which allowed growth and the lowest concentrations of $NaNO_2$ which prevented growth in R & S Medium for seven strains of C. perfringens after incubation at 43°C for 72 hr as determined by visual observation. (The initial cell concentration was approximately 10^2 cell/ml.)

	$NaNO_2$ Concentration (ppm)							
	Filter sterilized				Autoclaved			
	pH 7.2		pH 6.3		pH 7.2		pH 6.3	
Strain	Growth	No Growth	Growth	No Growth	Growth	No Growth	Growth	No Growth
8797	600	800	160	180	25	50	10	20
1362	1000	1500	200	250	25	50	8	10
BP6K	1000	1500	90	100	10	25	*	*
8235	1000	1500	200	250	50	75	10	20
8237	1000	1500	200	250	50	75	8	10
S-80	1000	1500	250	400	50	75	10	20
S-88	600	800	60	80	25	50	0	2

* Not determined

RESULTS & DISCUSSION

TABLE 1 presents the levels of $NaNO_2$ which inhibited the seven strains of *C. perfringens* in R & S Medium as a function of pH and mode of sterilization. Each value in Table 1 is the average of at least two experiments. It is apparent that nitrite inhibition is more effective at a lower pH and in autoclaved medium. It required 3.8–15.0 times more nitrite to inhibit growth in pH 7.2 filter sterilized medium than in pH 6.3 filter sterilized medium.

The mean average ratio ($\bar{R}$) of sodium nitrite concentration required for inhibition at pH 7.2 as compared to pH 6.3 in filter sterilized medium was 6.8.

Comparison of pH effects and sterilization method effects were accomplished using a mean average ratio ($\bar{R}$)concept.

The computing formulae were as follows:

(1) for pH comparison with the sterilization method being constant

$$\bar{R} = \frac{(G_{7.2}/G_{6.3}) + (I_{7.2}/I_{6.3})}{2n}$$

(2) for sterilization method comparison with the pH being constant

$$\bar{R} = \frac{(G^F/G^A) + (I^F/I^A)}{2n}$$

where: n = number of strains; G = highest $NaNO_2$ conc permitting growth; I = lowest $NaNO_2$ conc inhibiting growth; F = filter sterilization; and A = autoclave sterilization. It therefore required an average of 6.8 times as much nitrite to inhibit growth at pH 7.2 as was required at pH 6.3 in filter sterilized medium. The $\bar{R}$ of five strains in autoclaved medium was 4.4. Only five strains were used in this computation since strain BP6K was not evaluated at pH 6.3 and strain S-88 did not grow at any experimental nitrite level above 0.

There appears to be a relationship between nitrite inhibition and the nitrous acid content of the medium. The amount of undissociated nitrous acid formed at the inhibitory sodium nitrite concentration for both pH levels is approximately the same since there is an eightfold decrease in hydrogen ion concentration from pH 6.3 to pH 7.2. This agrees with the report of Castellani and Niven (1955) who demonstrated that the amount of undissociated nitrous acid necessary to inhibit *S. aureus* in unheated medium remained constant over a wide pH range. Based on the assumption that nitrite behaves in equilibrium as follows:

$$[H^+]\,[NO_2^-] \rightleftharpoons [HNO_2] \text{ and } pk_a$$

$$= [H^+]\,[NO_2^-]/[HNO_2];$$

$$\text{therefore, } [HNO_2] = [H^+]\,[NO_2^-]/pk_a$$

where pk_a is constant. As the pH changes from 7.2 to 6.3, an eightfold increase in $[H^+]$ and a 6.8-fold decrease in $[NO_2^-]$ will permit the concentration of undissociated nitrous acid to remain nearly constant. The nitrous acid therefore seems to be an important factor in the inhibition of *C. perfringens* in unheated medium and contributes to a lesser extent by approximately 30% but nevertheless significantly to the pH related effect in the heated medium.

The mean average ratio (R) of $NaNO_2$ required to inhibit *C. perfringens* in pH 7.2 filter sterilized medium was 30.7 times that required in autoclaved medium. At pH 6.3, the $NaNO_3$ concentration required for inhibition was 20.3 times greater in filter sterilized medium than it was in heat sterilized medium. These re-

Table 2—Growth supporting and growth inhibiting concentrations of $NaNO_2$ for six strains of Clostridium perfringens in 10 ml of filter or autoclave sterilized R & S Medium at pH 6.3 as a function of inoculum size

		Sodium nitrite conc (ppm)			
	Approx inoculum[a]	Filter sterilized		Autoclave sterilized	
Strain	(Viable org/ml)	Growth[b]	No growth[b]	Growth	No growth
8797	10^2	160	180	10	20
	10^6	800	1000	100	150
1362	10^2	200	250	8	10
	10^6	1000	1500	75	100
8235	10^2	200	250	10	20
	10^6	1000	1500	300	>300
8237	10^2	200	250	8	10
	10^6	1000	1500	300	>300
S-80	10^2	250	400	10	20
	10^6	1000	1500	300	>300
S-88	10^2	60	80	0	2
	10^6	600	800	25	50

[a] Inoculum was prepared from a 6-hr culture in Fluid Thioglycollate Medium incubated at 43°C.

[b] Growth was evaluated visually after 3 days incubation at 43°C.

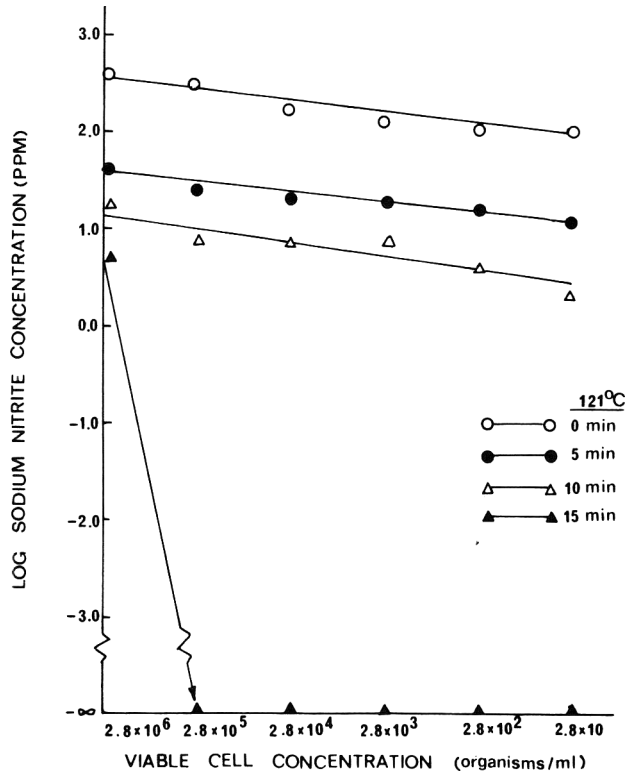

Fig. 1—The effect of heating time at 121°C and sodium nitrite concentration upon the growth of various cell concentrations of Clostridium perfringens 8797 in R & S Medium incubated at 43°C for 48 hr. Each point represents the maximum sodium nitrite level at which growth occurred for the cell concentration indicated.

sults confirm the report of Perigo et al. (1967) who found that autoclaving nitrite in microbiological medium produced a potent inhibitor and demonstrate clearly that the effect is over and above that which is attributable to pH alone.

It is evident that nitrite inhibition of *C. perfringens* in complex microbiological media (Perigo and Roberts, 1968; Gough and Alford, 1965) is comparable to nitrite inhibition in the chemically defined R & S Medium. The inhibition levels of 800–1500 ppm at pH 7.2 and 100–400 ppm at pH 6.3 for filter sterilized media correspond to the levels reported by Perigo and Roberts (1968) at pH 7.0 and pH 6.0. The pH 7.2 levels in this study are slightly lower than the pH 7.1 FTM studies of Gough and Alford (1965). In autoclaved medium, a two–three times greater tolerance occurred in this study at pH 7.2 than that reported by Perigo and Roberts (1968) at pH 7.0. The same differential existed for strains 8797 and 8237 which were common to both studies. At pH 6.3, the inhibitory levels were similar to those of Perigo and Roberts (1968).

The effect of inoculum size on nitrite inhibition is illustrated in Table 2. The medium was sterilized by filtration or by heat prior to inoculation. A 4-log cycle increase of inoculum size in filter sterilized medium increased the nitrite concentration required for inhibition at pH 6.3 to approximately that required for the smaller inoculum at pH 7.2. (Compare Table 2 with Table 1.) The five to tenfold increase of the inhibitory concentration of $NaNO_2$ in filter sterilized medium as a result of the higher inoculum level was not as great as the 10–100 fold increase in nitrite tolerance observed in autoclaved medium when the inoculum level was increased from 10^2 organisms per ml to 10^6 organisms per ml. At the 10^6 cells per ml initial inoculum level, strains 8235 and S-80 tolerated autoclaved nitrite at a level equivalent to an initial inoculum of 10^2 cells per ml in filter sterilized medium. This demonstrated that previous work by Duncan (1970) indicating that the level of spore concentration is an important factor in the spoilage of cured meats is also an important consideration when nitrite inhibition of vegetative cells is being studied. The difference in inocula levels may explain many of the discrepancies in the literature concerning the nitrite tolerance of microorganisms under similar conditions (Gough and Alford, 1965; Perigo and Roberts, 1968). Gough and Alford (1965) used inocula of approximately 10^6-10^7 cells per ml whereas Perigo and Roberts (1968) used 10^4-10^5 cells per ml.

To further illustrate the effect of inoculum size as well as the effect of heat, one strain, 8797, was selected for intensive study. A series of six serial dilutions of a 6-hr, 43°C FTM culture of the organism was inoculated into tubes of filter sterilized pH 6.3 R & S Medium which had received heat treatment at 121°C for 0, 5, 10 or 15 min after which sterile glucose was added aseptically. The mean cell concentrations at each dilution level (determined by SPS Agar pour plate counts) and the mean maximum nitrite levels at which growth occurred after 72 hr at 43°C are plotted in Figure 1.

Regression analysis applied to the three lines showed that the log of sodium nitrite concentration tolerance of *C. perfringens* 8797 dropped 0.13-log cycles for every 90% reduction in inoculum size in the medium when no heat was applied. For medium which was heated at 121°C for 5 and 10 min this decrease was 0.10 and 0.17-log cycles of nitrite respectively for each serial dilution. Analysis of covariance for homogeneity of the regression lines showed that the slopes are representative of the same population. These results further indicate the dependency of culture outgrowth on inoculum size when nitrite is present.

Figure 1 also illustrates that outgrowth in the presence of nitrite depends upon the amount of heat applied to the medium. Since all cell concentrations yield nitrite tolerance curves of equivalent slopes the results can be averaged to obtain mean heating time response information. The log of nitrite tolerance drops from 0.13 to 0.18-log cycles for every heating minute at 121°C. This gives a mean "D_{121}" value of 6.8 min. Therefore, as the heating time of the nitrite containing medium increases 6.8 min, the log of the sodium nitrite concentration required to inhibit a given number of cells decreases by 90%.

Perigo et al. (1967) showed that the addition of heat to a bacteriological medium yielded a first order response with respect to inhibitory nitrite levels for *C. sporogenes* at one inoculum level. The responses observed in these experiments were similar. However, no increases in nitrite tolerance levels were found with prolonged heating as were observed by Perigo et al. (1967). This is probably due to insufficient heating since Perigo et al. (1967) heated the medium up to 120–130°C for 20 min.

It is apparent that heating a microbial growth medium containing nitrite produces a potent inhibitor. This observation may be significant in canned cured meat products that generally receive a very

short, sublethal heat treatment (F_0 = 0.2). This treatment may be enough to reduce the nitrite tolerance of the indigenous clostridia to a level which is less than the federally permitted maximum amount of added sodium nitrite, 200 ppm. Johnston et al. (1969) found that the water-soluble portion of meat extracts interfere with the formation of the potent inhibitor during autoclaving of medium containing nitrite. Johnston et al. (1969) state that the inhibition is due in fact to the effect of residual nitrite and curing salts and not so much to the presence of a Perigo inhibitor. The effect of residual nitrite is unlikely in these studies however since the amount of residual nitrite needed to inhibit *C. perfringens* 8797 is far greater than those levels needed for inhibition in autoclaved medium and far greater than those levels expected to be recovered from R & S Medium after autoclaving (Riha and Solberg, 1973). The effect of heating in R & S Medium must therefore be attributed to the formation of some sort of inhibitor. Wasserman and Huhtanen (1972) have shown similar inhibition of *C. botulinum* due to the formation of a Perigo inhibitor which was not a nitrosamine. The nature of the inhibitor remains unknown.

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Clostridium perfringens GROWTH IN A NITRITE CONTAINING DEFINED MEDIUM STERILIZED BY HEAT OR FILTRATION

INTRODUCTION

C. perfringens continues to be responsible for a considerable number of food poisoning outbreaks annually. Recent studies demonstrated that the illness response is the result of microbial growth in the vector food product, consumption of the product containing vegetative cells of *C. perfringens*, growth and sporulation within the enteric system, followed by lysis of the vegetative cells releasing an enterotoxin (Hauschild, 1970; Duncan et al., 1972). Cured meat products have only occasionally been involved in *C. perfringens* food poisoning outbreaks. The presence of sodium nitrite in these products may be responsible for their safety. Sodium nitrite is an inhibitor of spore outgrowth and cell division for clostridia (Duncan and Foster, 1968). Sodium nitrite, heated in microbiological media has a dramatically increased inhibitory action on clostridia when compared to the unheated form (Perigo and Roberts, 1968; Riha and Solberg, 1973).

These experiments were designed to characterize the growth of *C. perfringens* in a chemically defined nitrite containing microbiological medium at a pH approximating that of cured meat products and to assist in the development of an hypothesis relating to the mode of action of sodium nitrite as a clostridial inhibitor.

MATERIALS & METHODS

CULTURES were maintained in Cooked Meat Medium (Difco). Inocula were prepared in Fluid Thioglycollate Medium (FTM) (Difco). *C. perfringens* 8797 was grown in FTM for two successive transfers at 43°C for 24 hr after which 0.1 ml was inoculated into 10 ml of FTM which was incubated at 43°C for 4 hr. The 4-hr culture was centrifuged and the resultant pellet was resuspended in sterile peptone water (0.1%) to 16% transmission at 550 nm (Spectronic 20, Bausch and Lomb, Rochester, N.Y.). 1.0 ml of a 10^{-4} dilution served as the inoculum for 200 ml of pretempered culture medium in a 300 ml Nephelo flask, modified with a side sampling septum (Buchanan and Solberg, 1972). Pour plate samples were drawn through the septum by syringe. Pour plates for colony counting were prepared using SPS Agar (Difco). Solidified plates were over-laid with SPS Agar. Growth curves were carried out in R & S Medium (Riha and Solberg, 1971) at pH 6.3. Media were filter sterilized using a 0.22μ pore size micropore filter (Millipore Filter Corp., Bedford, Mass.) and heat sterilization was at 121°C for 15 min. Glucose was heat sterilized separately and added aseptically to the heated media. All incubations were at 43°C. Anaero-Jars, (Case Laboratories, Chicago, Ill.) evacuated and flushed with nitrogen three times, were used for pour plate incubation.

RESULTS & DISCUSSION

VARIABILITY of growth response of *C. perfringens* 8797 to nitrite was observed during the growth curve studies. In filter sterilized media, growth occurred at 0, 100, 150, 175 and 200 ppm in some experiments and not at 150 and 200 ppm during other experiments. All flasks containing 300 ppm failed to support growth; four of six flasks containing 200 ppm did not support growth; and two of ten containing 150 ppm did not support growth. In autoclaved media growth was observed at 0, 2, 4, 5, 7.5, 10, 15 and 20 ppm $NaNO_2$ and was not apparent in some experiments at 10 and 20 ppm. All flasks containing 30 ppm $NaNO_2$ were unable to support growth, in two of seven flasks, 20 ppm $NaNO_2$ prevented growth and in two of eight flasks, 10 ppm of $NaNO_2$ prevented growth.

This variability was probably the factor which prompted Perigo et al. (1967) to express nitrite inhibition results in terms of ED_{50} values, which represented levels at which 50% of the inoculated culture failed to grow.

The effect of both filter sterilized and autoclaved nitrite appeared to be permanent. No culture which failed to reproduce during the growth curve studies demonstrated growth during prolonged incubation at 43°C. No viable colonies were ever recovered from the culture flasks in which growth was restricted, even after collection of all cells on a 0.22μ micropore filter after 72 hr incubation, removal of inhibitory medium by washing with 50 ml of 0.1% peptone water and then reculturing on nitrite-free SPS Agar with an SPS Agar overlay and incubating anaerobically for 48 hr at 43°C. This failure to reproduce on nitrite-free medium as well as the lack of evidence of a nitrite reaction with essential amino acids (Riha and Solberg, 1973), tends to rule out the interaction of nitrite with a nutrient which might render the medium nutritionally deficient. The inhibition of *C. perfringens* 8797 is therefore probably due to the reaction of some inhibitory agent formed by nitrite, or the reaction of nitrite itself with the cell to render the cell incapable of growth.

The adjustment phase duration was defined as the time required for the original cell concentration in the culture flask to double. The mean adjustment phase durations for *C. perfringens* 8797 in all filter sterilized and autoclave sterilized media are presented in Table 1. Analysis of variance of the individual adjustment phases showed that all cultures which grew in filter sterilized medium had a mean adjustment phase duration of 9.1 hr and all those cultures which grew in autoclaved medium had an adjustment phase duration of 4.3 hr. The adjustment phase duration for each culture which grew was not significantly different than any other culture sterilized in a similar manner, regardless of nitrite content; therefore, the difference is not nitrite dependent. The absence of a progressive $NaNO_2$ concentration effect upon the duration of the adjustment phase is contrary to the reports of Buchanan and Solberg (1972) and Nurmi and Turunen (1970) for *Staphylococcus aureus*. The difference in results may be attributed to altered metabolic pathways involved in the growth of organisms of different generic classification.

Analysis of variance of the adjustment phase durations for both modes of sterilization demonstrated a significant (99.0%) difference between the two populations. The shorter adjustment phase in autoclaved medium may be the result of less dissolved O_2 in the medium since pre-

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liminary studies conducted in our laboratory have shown that heated or steamed R & S Medium contains lower amounts of dissolved O_2 after cooling as compared to filter sterilized medium. Further investigation is required to substantiate this hypothesis.

The exponential phase of the individual growth curves were subjected to regression analysis and are expressed as mean generation times in Table 2. Tests of homogeneity of each population of regression lines (filter sterilized, autoclaved) showed that the regression coefficients of each sterilization method were homogeneous. The mean generation time for all cultures grown on filter sterilized medium was 23.0 min and for cultures grown on autoclaved medium was 20.2 min. When the regression coefficients of both populations were pooled, the overall mean regression time was 21.6 min and the pooled population was homogeneous. This value is somewhat greater than the 13.8 min generation time reported for *C. perfringens* 8797 in FTM at 43°C (Parekh and Solberg, 1970), but is in agreement with the mean generation times of 20.8 and 21.2 min reported by Riha and Solberg (1971) for growth of *C. perfringens* in R & S Medium.

Although the mean generation time did not compare to generation times of *C. perfringens* 8797 in another medium, the significant fact remained that all regression lines from both modes of sterilization were representative of the same population. Regardless of nitrite concentration or mode of sterilization, if the organism was capable of growth, it reproduced at a constant rate. *C. perfringens* 8797 therefore, behaved much like the micrococci study by Nurmi and Turunen (1970) in that the rate of growth was not suppressed by nitrite. This is in contrast to the concentration dependent response reported by Buchanan and Solberg (1972) for *S. aureus* and Nurmi and Turunen (1970) for *Lactobacillus* sp.

There were no significant differences in the maximum cell concentration reached in all culture flasks which supported growth in either filter sterilized or autoclaved nitrite containing media. The log mean maximum cell concentration for each nitrite level is presented in Table 3. Analysis of variance showed that no differences existed in either sterilization population and that the log mean maximum concentration of cells found in all filter sterilized cultures was 8.11 and the log mean maximum cell concentration found in autoclave sterilized cultures was 8.54. Analysis of variance of the maximum cell concentration found during the stationary phase of each population (filter sterilized and autoclaved) indicated that the populations were not significantly different. The log mean maximum cell concentration for the combined population was 8.31 organisms/ml.

A composite growth curve, made from adjustment phase, exponential phase, and stationary phase data for filter sterilized and autoclaved R & S Medium is shown in Figure 1. The curve illustrates the difference in adjustment times for the two types of sterilization, previously shown to be significant. Those curves showing cultures which did not grow are approximations of the loss of viability of the culture. All autoclaved media which failed to support growth of *C. perfringens* 8797 contained no viable organisms at a mean of 2.3 hr after inoculation. No organisms were found in filter sterilized medium after an average incubation time of 5.6 hr. Differences in the death curves indicate the presence of a more potent inhibitory mechanism in media autoclaved with nitrite as compared to media containing cold sterilized or filtered nitrite.

There were differences in the pH pattern during culture development when heated medium was compared to filtered medium. The differences seemed related to the medium and independent of the nitrite since they were equivalent even in those samples which contained no nitrite. The pattern of events is illustrated in Figure 2 which represents the average curves for all nitrite levels in heated or filtered medium adjusted to a mean zero time representing the time after the pH of the medium dropped to pH 6.1. This required 7.2 hr in heated medium and 11.8 hr in filtered medium. The sodium nitrite disappeared from both autoclaved and filter sterilized medium during the exponential growth phase. The $NaNO_2$ was probably metabolized by the organisms.

Table 1—The mean adjustment phase duration for C. perfringens 8797 in R & S Medium at pH 6.3 containing various concentrations of $NaNO_2$ incubated at 43°C

Mode of sterilization	$NaNO_2$ conc (ppm)	No. of experiments	Adjustment phase duration (hr)
Filter	0	10	7.8
	100	8	0.2
	150	4	9.5[a]
	175	4	9.8
	200	4	∝[b]
	300	4	∝
Autoclave	0	8	4.3
	2	2	3.9
	4	2	4.0
	5	6	5.9
	7.5	2	3.0
	10	6	3.0[a]
	15	2	2.5
	20	4	5.3[c]

[a] Adjustment phase was of infinite duration for two experiments which are not included in value presented.
[b] Adjustment phase was 60 hr for two experiments which are not included in the value presented.
[c] Adjustment phase was of infinite duration for four experiments which are not included in the value presented.

Table 2—The generation times of C. perfringens 8797 grown at 43°C in R & S Medium at pH 6.3 containing various amounts of $NaNO_2$. Generation times were determined by regression analysis of exponential segment of growth curves

Mode of sterilization	Nitrite conc (ppm)	No. of experiments	Generation time (min)
Filter	0	9	23.9
	100	7	25.4
	150	8	23.2
	175	4	30.3
	200	1	13.2
Autoclave	0	7	20.0
	2	2	21.1
	4	2	20.3
	5	4	18.7
	7.5	2	22.4
	10	6	20.2
	15	2	20.0
	20	4	22.6

Table 3—Log mean maximum cell concentrations reached during the stationary phase of growth of C. perfringens 8797 in R & S Medium containing various concentrations of sodium nitrite and incubated at 43°C at pH 6.3

Mode of sterilization	Sodium nitrite (ppm)	No. of samples	Log mean max cell conc (cell/ml)
Filter	0	14	8.23
	100	10	8.35
	150	8	7.78
	175	3	7.50
	200	2	8.20
Autoclave	0	7	8.48
	2	2	8.73
	4	2	8.58
	5	6	8.65
	7.5	2	8.42
	10	6	8.48
	15	2	8.72
	20	4	8.49

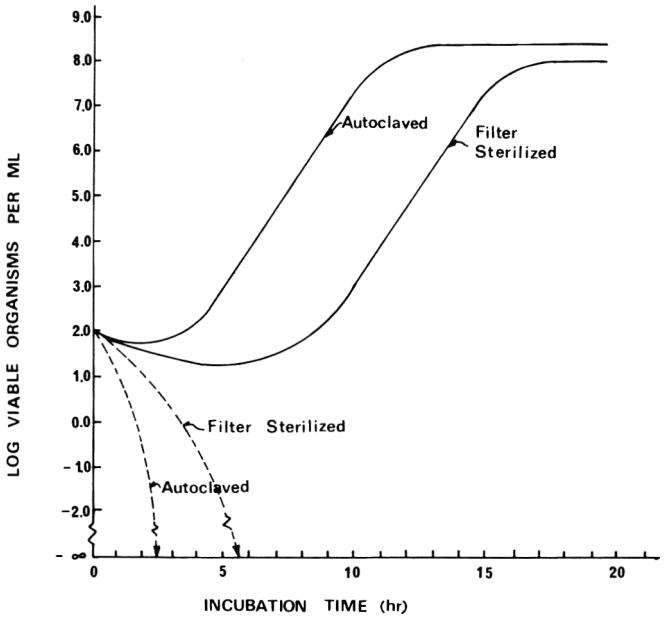

Fig. 1—Composite growth curve made from adjustment phase, exponential phase and stationary phase data of all growth curve analyses of C. perfringens 8797 in various concentrations of filter sterilized and autoclaved sodium nitrite in R & S Medium at pH 6.3 incubated at 43°C. Solid lines (——) represent all flasks which supported growth and broken lines (------) represent all flasks which did not support growth.

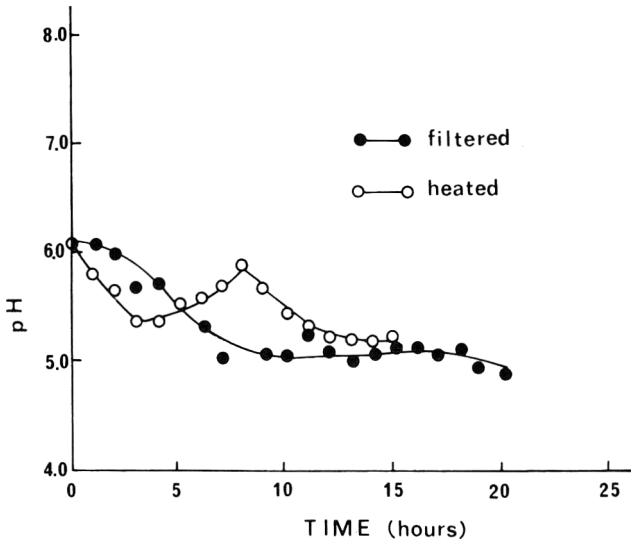

Fig. 2—Average pH changes in heated or filtered R & S Medium prepared at pH 6.3 and containing various levels of sodium nitrite which permitted the growth of C. perfringens 8797 during incubation at 43°C. Zero time is adjusted to the time when the culture medium reached pH 6.1.

The inhibitory action of sodium nitrite on *C. perfringens* was apparently at the cellular level since all cultures which supported growth demonstrated similar responses and there were no nutritional differences between media which were capable or incapable of supporting growth (Riha and Solberg, 1973). Recovery studies of cells exposed to nitrite showed that these organisms were unable to initiate growth, even in nitrite-free medium. Microscopic examination of these organisms indicated no visible difference between inhibited cells and normal cells, thus damage was probably at a sub-microscopic level. Previously published data (Riha and Solberg, 1973) as well as experiments conducted by Saville (1958) indicated that nitrite can react with sulfhydryl containing compounds. It is hypothesized that nitrite, in some form, may be reacting with enzymes containing functional sulfhydryl groups, thus resulting in growth inhibition. Further work will have to be conducted to substantiate this hypothesis.

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PURIFICATION AND PROPERTIES OF ANTIMICROBIAL SUBSTANCES PRODUCED BY Streptococcus diacetilactis AND Leuconostoc citrovorum

INTRODUCTION

NUMEROUS WORKERS have reported on the ability of *Streptococcus diacetilactis* and *Leuconostoc citrovorum* to produce antimicrobial substances which are active against certain pathogenic and spoilage organisms. Both *S. diacetilactis* and *L. citrovorum* inhibit the growth of a wide range of organisms, but are particularly effective against *Pseudomonas* species (Genske and Branen, 1973; Marth and Hussong, 1963). Daly et al. (1972), Lundstedt and Fogg (1962), Mather and Babel (1959), Reddy et al. (1970), Radick et al. (1969) and Vedamuthu et al. (1966) have reported that cultures of *L. citrovorum* or *S. diacetilactis* extend the shelf life of cottage cheese, meat and numerous other food products presumably by preventing the growth of *Pseudomonas* species. In growth association studies, *S. diacetilactis* has significantly reduced the growth of *Pseudomonas* species, *Staphylococcus aureus, Escherichia coli, Clostridium perfringens* and *Alcaligenes* species (Daly et al., 1970, 1972). *Salmonella* species have also been inhibited by both *L. citrovorum* and *S. diacetilactis* (Daly et al., 1972; Park and Marth, 1972; Vedamuthu et al., 1966; Sorrells and Speck, 1970).

The mechanism by which *L. citrovorum* and *S. diacetilactis* inhibit microbial growth is still not clear. Several workers have attributed the inhibitory activity to organic acids (Daly et al., 1972; Pinheiro et al., 1968a, b; Sorrells and Speck, 1970) while others have pointed out the possibility that hydrogen peroxide contributes to the activity (Daly et al., 1972). Other workers have indicated that not all of the inhibitory activity can be accounted for by organic acids and hydrogen peroxide and have suggested that other antimicrobials are present (Collins, 1961; Daly et al., 1971, 1972; Mather and Babel, 1959; Pinheiro et al., 1968a; Reddy et al., 1970). Vedamuthu et al. (1966) was unable to demonstrate inhibition in cell free extracts of *S. diacetilactis* and reported that viable cells were necessary for activity.

It was the purpose of this study to further explore the properties of the antimicrobials produced by *L. citrovorum* and *S. diacetilactis* and to determine the feasibility of isolating these substances free from the cells.

MATERIALS & METHODS

Microorganisms

The single-strain cultures used in this study were obtained from the departmental culture collection. Cultures of *S. diacetilactis* were maintained in sterile, reconstituted 11% solids nonfat milk medium, while all other cultures were maintained in trypticase soy broth or nutrient broth (Difco). All cultures were transferred biweekly and checked for purity monthly by streaking on an appropriate agar medium.

Bioassay

A standard bioassay procedure was used for detecting antimicrobial activity. 15 ml of nutrient agar (Difco) were dispensed into screw cap tubes. These tubes of agar were then autoclaved, equilibrated at 45°C, mixed with 0.2% by volume of a 24-hr broth culture of the test organism, and poured into petri plates. With the exception of the study on antimicrobial spectra, *P. fluorescens* #23 was used as the test organism. After the agar solidified, the plates were immediately used or stored under refrigeration (5°C) and used within 2 days. For bioassay, a standard paper assay disc (Schliecher and Schnell, 12.7 mm diam) was dipped into the sample to be tested, touched to the side of the container to remove excess liquid, and placed on the assay plate. The plates were then incubated at the optimum growth temperature for 20 hr. The diameter of the clear zones around each disc was measured after incubation to determine the antimicrobial activity.

A modification of this assay was necessary when assaying purified extracts since the removal of protein and buffer salts decreased the sensitivity of the standard bioassay. Although the seeded plates were prepared in the conventional way, it was necessary to impregnate approximately one-half of the paper disc with 0.1M potassium acid phthalate (pH 5.0) before dipping the disc in the sample to be tested. This adequately buffered the sample and did not in itself limit visible growth. Areas cut from the paper chromatograms were assayed by dipping them into the phthalate buffer and then placing them on the seeded plate.

Media and fermentation

Media. Elliker's broth (Difco) or trypticase soy broth (Difco) was prepared by conventional procedures, dispensed into Erlenmeyer flasks, and sterilized by autoclaving.

Media based on whey were prepared from fresh acid or rennet wheys or from spray-dried Cheddar cheese whey obtained from the University Dairy. The fresh acid or rennet whey was prepared from nonfat dried milk reconstituted to 11% in distilled water. Curd was developed in acid whey by acidification of the milk to pH 4.6 with 1N hydrochloric acid and in rennet whey by addition of rennin. Curd was removed by filtration through cheesecloth and the resultant whey was adjusted to the appropriate pH with 2M ammonium hydroxide. Where yeast extract was used, 0.5% was added to the milk prior to preparation of the whey. The whey media were dispensed into Erlenmeyer flasks and sterilized by autoclaving.

Media were prepared from spray-dried whey by reconstitution of the dried whey to 5% in distilled water and by addition of 0.5% yeast extract (Difco). Media were also prepared using 5 or 10% whey plus citrate, glucose, invert sugar and certain salts in a formulation supplied to us by Lacto-Products, a division of Great Lakes Biochemical, Milwaukee. All media were dispensed in Erlenmeyer flasks and sterilized by autoclaving.

Fermentation. Cooled sterile media were inoculated with 1% by volume of an actively growing culture of *S. diacetilactis* or *L. citrovorum* and subsequently, incubated for 5–10 days at 30°C. Samples were obtained at appropriate time intervals and the fermentation liquors obtained by centrifuging the fermentation media at 20,000 × G for 15 min to remove the cells. The resultant supernatant fluid (fermentation liquor) was adjusted to pH 5.0, autoclaved, and checked for inhibition by the bioassay procedure outlined above.

Properties of fermentation liquors

Location. *S. diacetilactis* DRC-1 or *L. citrovorum* A-3 was grown in the whey broth fortified with yeast extract and adjusted to pH 6.0. Following incubation the cells were removed by centrifugation (20,000 × G for 15 min), washed once with phosphate buffer (pH 7.15) and resuspended in phosphate buffer (pH 4.5). An aliquot of the cell suspension was disintegrated in a Branson model B110 Sonicator for 15 min. The cell suspension, the sonicated cells and the cell-free fermentation liquor were adjusted to pH 4.5 with 0.1N HCl and assayed for antimicrobial activity.

Antimicrobial spectra. Three strains of *S. diacetilactis* (26-2; DRC-1; and M21-35) and two strains of *L. citrovorum* (A-3 and 3036) were

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grown in rennet-whey fortified with yeast extract and adjusted to pH 6.0. The resultant fermentation liquors were tested for activity against *Pseudomonas fragi* #24, *Pseudomonas fluorescens* #23 and *Pseudomonas putrefaciens* #11. The fermentation liquors obtained from *S. diacetilactis* DRC-1 and *L. citrovorum* A-3 were then tested for activity against numerous microorganisms.

pH and antimicrobial activity. The fermentation liquors were adjusted to pH values of 3.6–7.2 using 0.1N HCl or 0.1N NaOH and bioassayed.

Precipitation and solvent extraction of antimicrobials. Attempts were made to salt out the inhibitory substances by adding saturated sodium chloride or ammonium sulfate solutions to the fermentation liquors. Also, attempts were made to extract the antimicrobials with methanol, chloroform, acetone, or acid butanol. 10 ml of the fermentation liquors were extracted twice with 100 ml of each solvent.

Sephadex chromatography. Aliquots of the fermentation liquors were applied to a 2.3 × 55 cm column of Sephadex G-10-fine (Phamacia Fine Chemicals, Inc.) and eluted with distilled water. 5 ml fractions were collected and elution followed by reading absorbance at 210 nm. Fractions corresponding to each peak in absorbance were pooled, concentrated in vacuo, and disc assayed.

To provide an estimation of molecular weight, the Sephadex G-10 column was calibrated with 2 ml samples of lactose (300 daltons), glucose (180 daltons) and lactic acid (90 daltons).

Cation exchange chromatography. The cation exchange resin (CS-101, 20–50 mesh, Duolite) was suspended in five volumes of distilled water and the resultant slurry was poured into a 2.0 × 28 cm column. The column was precycled with, in sequence, 200 ml of 0.5N sodium hydroxide, 400 ml distilled water, 200 ml 0.5N hydrochloric acid and 400 ml distilled water. A dilute sodium hydroxide solution was then washed over the column until the wash approached a neutral pH. The column was then backwashed with 500 ml distilled water. Further washing with dilute sodium hydroxide followed by a distilled water rinse was necessary to equilibrate the column to a pH of 6.5–7.5.

Preliminary experiments indicated that treatment of the fermentation liquors with methanol enhanced the isolation of antimicrobials by cation exchange chromatography. Thus, one part of the liquor was treated with two parts methanol and the precipitated proteins removed by centrifugation. The supernatant fluid was then dried in vacuo, rehydrated, adjusted to pH 6.5 and an aliquot was applied to the cation exchange column. Noncationic materials were washed from the column with 500 ml distilled water and absorbed materials were eluted with 0.15N HCl. 10 ml fractions were collected and elution followed by measuring absorbance at 210 nm. Fractions corresponding to each peak in absorbance were pooled, adjusted to pH 5.0, concentrated in vacuo, and disc assayed. The column was regenerated using the acid, dilute base, backwash, and rinse procedure outlined above for precycling.

Purification of a cationic antimicrobial from *S. diacetilactis*

Based on the chromatography of the fermentation liquor obtained from *S. diacetilactis*, further purification of these antimicrobials seemed feasible. Thus, a scheme for purification was designed based on solvent fractionation followed by cation exchange and sephadex chromatography.

Table 1–Inhibition of Pseudomonas species by strains of S. diacetilactis and L. citrovorum[a]

	Test organism		
Strain	P. fragi #24	P. fluorescens #23	P. putrefaciens #11
S. diacetilactis			
MC 21	–[b]	–	+
DRC-1	–	++	+++
26-2	–	++	+
L. citrovorum			
A 3	+	++	+
3036	+	++	+

[a] Cell-free fermentation liquors were tested for antimicrobial activity using the disc assay.

[b] (–) = no inhibition; (+) = slight but significant inhibition; (++) = definite inhibition; (+++) = high inhibition.

Solvent fractionation. As noted above, a large portion of the protein was precipitated from the fermentation liquor by adding two parts of methanol to one part of fermentation liquor. The precipitated protein was removed by centrifugation, and the resultant supernatant was concentrated to dryness in vacuo. The dried supernatant was rehydrated to 1/4 the original volume and adjusted to a pH of 6.5 to precipitate phosphates. The precipitate was removed by centrifugation at 15,000 × G and the supernatant was dried in vacuo.

Cation exchange chromatography. The CS-101 column was prepared, precycled, and regenerated as outlined above. 4 ml of a 20% solution of the dried methanol extracts was applied to the column, the noncationic materials were washed from the column with distilled water, and the absorbed compounds were eluted with 0.15N HCl. The absorbance peak containing the antimicrobial material was adjusted to pH 5.0, concentrated in vacuo and rehydrated to 4 ml.

Sephadex chromatography. An aliquot of the antimicrobial materials obtained from the cation exchange chromatography was applied to a Sephadex G-10 column prepared as previously outlined. The sample was eluted with distilled water, 5 ml fractions were collected and elution was followed by reading absorbance at 210 nm. Fractions corresponding to each peak in absorbance were pooled, adjusted to pH 5.0, concentrated in vacuo and disc assayed.

Properties of purified antimicrobial

Paper chromatography. Two 5-μl aliquots of the antimicrobial solution obtained from Sephadex chromatography were spotted side by side on a 3 × 30 cm strip of Whatman no. 3 MM filter paper. The spotted paper strips were then subjected to ascending chromatography using 100 ml of either n-butanol/acetic acid/water (4/1/5, v/v) or isopropanol/H_2O (70/30, v/v) as the developing solvent. Glass test tubes (50 × 400 mm), fitted with rubber stoppers through which a slide wire was inserted to suspend the chromatogram, served as chromatography jars. Following chromatography, chromatograms were dried in an oven at 100°C for 30 min. The chromatogram was then cut in half lengthwise and one half was sprayed with 0.2% ninhydrin in n-butanol and heated at 100°C for 10 min to visualize spots. The other half of the chromatogram was cut into zones corresponding to spots developed on the other half. Both ninhydrin positive and negative zones were cut from the chromatogram, dipped into potassium phthalate buffer (pH 5.0) and bioassayed as described above.

Thin layer chromatography. One-dimensional ascending thin layer chromatography was also used to check for purity of the antimicrobial material. Silica gel G (Merck) was used as the absorbant and isopropanol/H_2O (70/30, v/v) was used as the developing solvent. Following development, separated substances were visualized with UV light or by spraying with bromcresol green, diphenylamine or ninhydrin. Areas corresponding to the Rf of reactive materials were scraped from a duplicate unsprayed plate, and the materials were eluted from the silica gel with water and checked for inhibitory activity by the disc assay.

Influence of pH on antimicrobial activity. 2 ml of the eluant obtained from cation exchange chromatography were added to culture tubes containing 50 ml of nutrient broth. The tubes of broth were adjusted to the appropriate pH, autoclaved, and inoculated with 0.2% by volume of a 48-hr culture of *P. fluorescens*. Growth was monitored by reading absorbance at 420 nm. Absorbance readings were converted to dry cell weight using a standard curve of dry cell weight versus absorbance readings.

Molecular weight. The relative molecular weight of the antimicrobial material was determined by elution from a calibrated Sephadex G-10 column.

Hydrolysis. An aliquot of the antimicrobial material was dissolved in 6N HCl and refluxed for 24 hr to achieve hydrolysis. Following refluxing, the HCl was removed in vacuo, and the hydrolysate was adjusted to pH 5.0 and assayed for activity.

Visible and ultraviolet absorbance. The absorbance spectra were determined by scanning from 210–700 nm using an Acta III spectrophotometer (Beckman Inst.).

RESULTS

Screening of strains of *S. diacetilactis and L. citrovorum* for antimicrobial activity against Pseudomonas species.

All strains of *S. diacetilactis* and *L. citrovorum*, which were tested inhibited *P. putrefaciens* (Table 1). However, only two out of the three *S. diacetilactis* strains inhibited *P. fragi* 24. Of the *Pseudomonas* species, *P. fluorescens* was the most sensitive to the antimicrobial materials produced by either *L. citro-*

vorum or *S. diacetilactis* and was thus chosen as the test organism for all other studies. *S. diacetilactis* DRC-1 and *L. citrovorum* A-3 were chosen as the source of antimicrobials for other studies because of the rapid production and high activity of antimicrobial materials.

Production of antimicrobials

S. diacetilactis DRC-1 grew well and produced high antimicrobial activity in Elliker's broth; however, the uninoculated broth, also had antimicrobial activity thus complicating recovery of the antimicrobials produced by *S. diacetilactis*. Uninoculated whey-based media had no antimicrobial activity and thus provided excellent media for study of antimicrobial production. *S. diacetilactis* produced antimicrobials in both rennet and acid whey; although production was slower in rennet whey and activity never reached the levels produced in acid whey. Acid whey at a pH of 4.7 or readjusted to pH 6.0 was suitable for antimicrobial production; however, production was initiated earlier at pH 6.0. The addition of 0.5% yeast extract to the whey substantially increased the total antimicrobial activity. The best overall production was in acid whey readjusted to pH 6.0 and supplemented with 0.5% yeast extract. Antimicrobial activity was present after 18 hr of incubation; but the greatest activity was present after 50–60 hr of incubation.

Media containing 5% of spray-dried Cheddar whey plus 0.5% yeast extract were suitable for antimicrobial production by *S. diacetilactis* or *L. citrovorum*. These media had a natural pH of 6.0 and thus did not require pH adjustment. Highest antimicrobial activity was reached after 24 hr of incubation, and the level of activity did not increase on continued incubation. Addition of numerous other additives or increasing to 10% whey did not increase antimicrobial activity. Thus, the 5% whey plus 0.5% yeast extract was chosen as the media for the remainder of the studies.

Table 2—Spectra of inhibitory activity of Streptococcus diacetilactis and Leuconostoc citrovorum[a]

Testing organism	Source of inhibitor	
	S. diacetilactis	L. citrovorum
Aerobacter aerogenes	+[b]	+
Bacillus cereus	+	+
Brucella abortus	–	–
Clostridium botulinum Type A	–	–
Escherichia coli	+	+
Enteropathogenic *E. coli*	+	+
Lactobacillus casei	–	–
Micrococcus flavus	+	+
Proteus vulgaris	+	+
Pseudomonas aeruginosa 10145	+	+
Pseudomonas fluorescens 23	+	+
Salmonella typhimurium	–	–
Shigella flexnei	+	+
Staphlococcus aureus 100	–	–
Streptococcus faecalis	–	–
Streptococcus pyogenes	–	–

[a] Cell-free fermentation liquors from S. diacetilactis and L. citrovorum were tested for antimicrobial activity by the disc assay.
[b] (+) = definite inhibition; (–) = no inhibition as shown by disc assay.

Table 3—Paper and thin-layer chromatography of isolated antimicrobial materials obtained from Streptococcus diacetilactis

Support	Rf[a]	
	Butanol/acetic acid/H_2O (4/1/5)	Isopropanol/H_2O (70/30)
Paper	0.136	0.531
Silica Gel G	–	0.770

[a] Rf was determined after detection with ninhydrin.

Properties of fermentation liquors

Location. The antimicrobial substances were entirely extracellular when *S. diacetilactis* or *L. citrovorum* were grown in the whey medium. The cell-free fermentation liquor had high antimicrobial activity while neither the suspension of whole cells or the sonicated cells showed any antimicrobial activity.

Spectra of inhibition. The antimicrobial spectra of activity of the fermentation liquors were similar to that reported by Marth and Hussong (1963) (Table 2); however, there was only questionable activity against *Staphylococcus aureus*. Also, activity was found against *Bacillus cereus*, which was not found by Marth and Hussong. In general, Gram negative bacteria were the most sensitive to the antimicrobial materials, particularly *Pseudomonas* species.

Influence of pH on antimicrobial activity. The antimicrobial substances obtained from either *S. diacetilactis* or *L. citrovorum* were most active at low pH values and were inactive above pH 7.0. The increased activity at low pH values was undoubtedly due to an added effect of acid. These results are in agreement with those of Pinheiro et al. (1968a), Mather and Babel (1959) and Marth and Hussong (1963).

Precipitation and solvent extraction. The antimicrobial activity was not precipitated from the fermentation liquors by saturated salt solutions. The activity was soluble in 90% solutions of ethanol, methanol or acetone but was insoluble in chloroform. Small amounts in inhibitory activity were extracted with acidified n-butanol or ethyl ether.

Cation exchange. Major differences existed in the elution pattern of *S. diacetilactis* and *L. citrovorum* fermentation liquors from cation exchange chromatography (Fig. 1). Although most of the inhibitory activity in *S. diacetilactis* liquor was eluted in one large UV absorption peak between pH 4.0 and 1.0, the *L. citrovorum* liquor gave many small peaks with little or no activity. Based on the disc assay, approximately 20–30% of the total antimicrobial activity in the fermentation liquor from *S. diacetilactis* was absorbed and recovered from the cation exchange column; however, less than 5% of the activity was absorbed and recovered from the fermentation liquor from *L. citrovorum*.

Sephadex. The *L. citrovorum* and *S. diacetilactis* liquors also had differing elution patterns from Sephadex G-10 (Fig. 2). The major inhibitory activity in the liquor of *S. diacetilactis* was eluted in fractions 28–33, while in the *L. citrovorum* liquor the activity was eluted in fractions 40–45. In both cases 100% of the total activity was recovered from the column.

Thus it would appear that while *S. diacetilactis* and *L. citrovorum* liquors both contain low molecular weight antimicrobial compounds, these compounds are distinctly different. It is conceivable that lactic or acetic acid accounted for much of the activity of *L. citrovorum* cultures since these two acids were eluted from the Sephadex G-10 column in fractions 35–45.

Purification of antimicrobials from *S. diacetilactis* fermentation liquors

Methanol extraction. The supernatant fluid obtained from extraction with methanol contained 100% of the antimicrobial activity present in the original liquor. This extraction accomplished the removal of large molecular weight proteins from the liquors.

Cation exchange. As shown above, cation exchange chromatography of the methanol extract of the *S. diacetilactis*

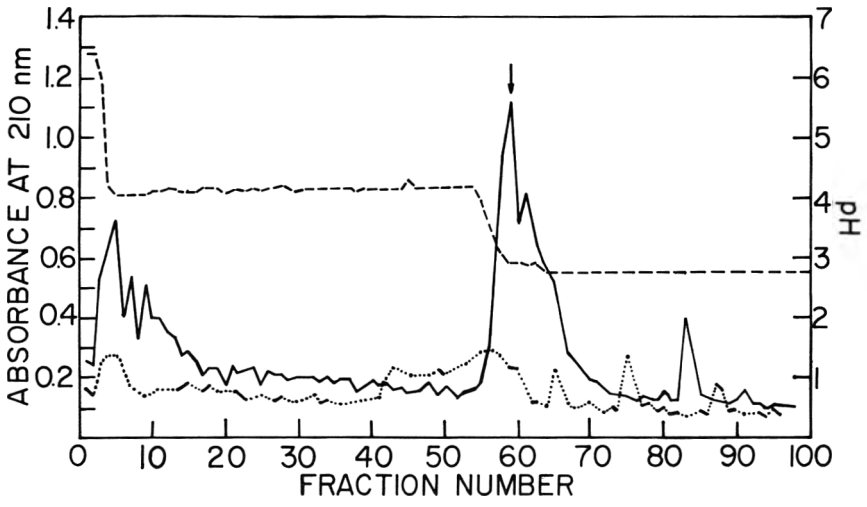

Fig. 1—Elution profile of the Leuconostoc citrovorum and Streptococcus diacetilatis fermentation liquors from CS-101 cation exchange column. (----- = pH; ___ = absorbance at 210 mn of S. diacetilactis liquor; ····· = absorbance at 210 mn of L. citrovorum liquor; sample eluted with 0.15N HCl).

fermentation liquor yielded one major absorbance peak which contained inhibitory activity. Bioassay indicated that these peaks contained 20–30% of the total antimicrobial activity contained in the original fermentation liquor.

Sephadex chromatography. The active material obtained from the cation exchange column gave four UV absorption peaks when eluted from Sephadex G-10 (Fig. 3). Both peaks 3 and 4 contained antimicrobial activity; however, peak 4 contained slightly more activity than peak 3. Paper chromatography indicated that the same compound accounted for the activity in both peaks but that peak 4 was a purer preparation of the antimicrobial material.

Properties of the purified cationic antimicrobial

Purity. Only one antimicrobial spot was detected after paper or thin layer chromatography of the material obtained in peak 4 from Sephadex (Table 3). The material in this spot could be visualized with ninhydrin, bromcresol green or UV light but not with diphenylamine. No other spots were detected on the chromatograms using these visualization techniques.

Visible and UV scan. Scanning absorbance from 210–700 nm of the purified material obtained from Sephadex showed that maximum absorbance occurred at 210 nm with no other distinct peaks.

Antimicrobial spectrum. The isolated material was active against *P. fluorescens, P. fragi, P. putrefaciens* and enteropathogenic *E. coli.*

Molecular weight. Based on the elution of the antimicrobial material from a calibrated Sephadex G-10 column, the material had a molecular weight in the range of 100–300 daltons.

Hydrolysis. Acid hydrolysis of the purified material totally destroyed its inhibitory activity.

Influence of pH and heat on antimicrobial activity. As was indicated in earlier tests with the crude fermentation liquor, the purified antimicrobial material was most active at low pH values. Greatest activity occurred at pH 5.3, although the compound was still active at pH 6.0.

The antimicrobial material withstood autoclaving at a pH of 5.0 without appreciable loss of activity.

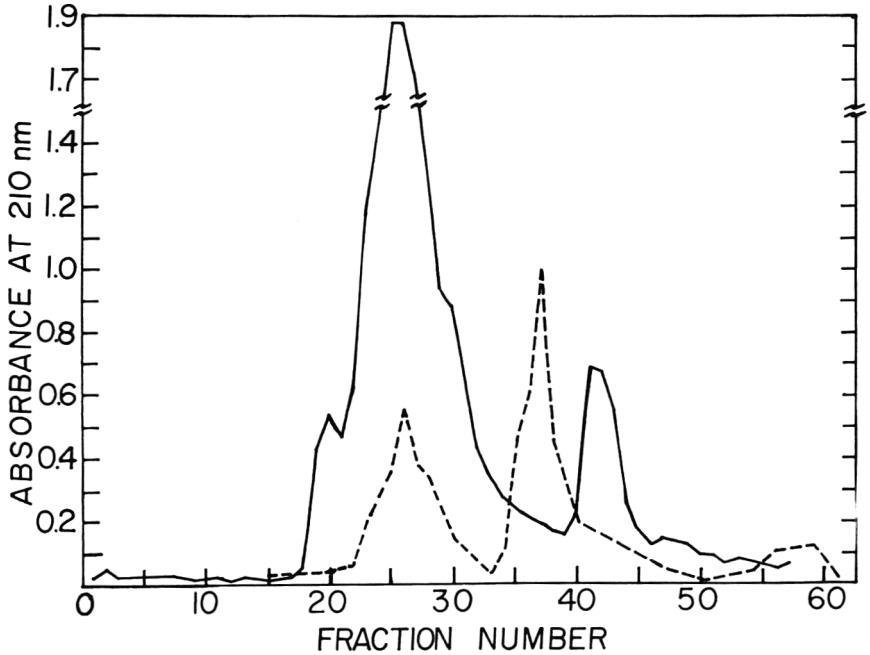

Fig. 2—Elution profile of Streptococcus diacetilactis and Leuconostoc citrovorum fermentation liquors from Sephadex G-10. (___ = S. diacetilactis liquor; ····· = L. citrovorum liquor; sample eluted with distilled H_2O).

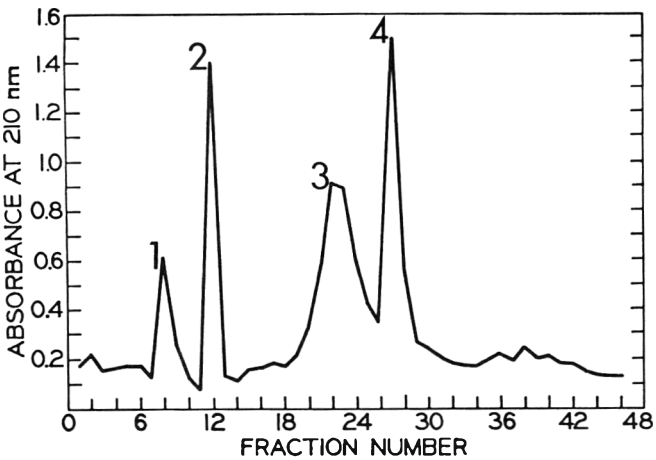

Fig. 3—Elution profile on Sephadex G-10 of the antimicrobial material obtained from cation exchange chromatography of Streptococcus diacetilactis fermentation liquor.

DISCUSSION

A MEDIUM based on whey was found to be excellent for the production of antimicrobial activity by either *L. citrovorum* or *S. diacetilactis*. The medium containing 5% spray-dried whey plus 0.5% yeast extract was inexpensive and easy to reproduce, had no inherent antimicrobial activity and allowed easy purification of the antimicrobial material. The production of antimicrobial materials in this whey medium followed the production of acid by the microorganisms, and although substantial antimicrobial activity was present after 18–24 hr of incubation, maximal activity was present after 50–60 hr.

The antimicrobial materials produced by *S. diacetilactis* and *L. citrovorum* were similar in many respects. The materials from both organisms were extracellular, water soluble, low molecular weight compounds with similar pH optimums and antimicrobial spectrum. However, the elution pattern of the fermentation liquors from cation exchange and Sephadex G-10 chromatography columns indicated major differences in the types of antimicrobials produced by the two organisms. Based on these elution curves and the indicated presence of large amounts of low molecular weight noncationic materials, it appears that as several workers have theorized, organic acids play a major role in the inhibitory effects of *L. citrovorum* and *S. diacetilactis*. Apparently this role is greater in the *L. citrovorum* liquors than it is in the *S. diacetilactis* liquors.

The cationic, low molecular weight material isolated from the *S. diacetilactis* fermentation liquor would appear to have some potential for use in food products. The material, which appears to comprise 20–30% of the total activity of the crude fermentation liuqor, was purified by a relatively simple procedure. Based on paper and thin-layer chromatography, a pure preparation of the material, was obtained via methanol extraction followed by cation exchange and Sephadex chromatography. It appears that this process could be scaled up to commercial size. Both the cation exchange and Sephadex columns were easily prepared and regenerated and the elution patterns were extremely repeatable.

The purified antimicrobial material was heat stable and active towards several *Pseudomonas* species at a pH of 6.0 or lower. Based on the reaction of this material with ninhydrin, its UV spectrum and its loss of activity upon hydrolysis, the material appeared to be a small molecular weight peptide. Numerous other antimicrobial peptides have been isolated from lactic acid bacteria, but this material differs from these peptides in its antimicrobial spectrum, molecular weight, and location within the fermented cultures. While nisin, diplococcin and acidophilin are intracellular low molecular weight peptides which have antimicrobial activity versus Gram positive bacteria (Baribo and Foster, 1951; Oxford, 1944; Vakil and Shahani, 1965) this material is an extremely low molecular weight extracellular compound primarily active against Gram negative microorganisms.

Both the crude fermentation liquors and the purified antimicrobial material would appear to have potential for controlling the growth of *Pseudomonas* species in food products. The use of cell-free material offers obvious advantages over the use of the whole cells as suggested by Daly et al. (1972) and Reddy et al. (1968). There is a high potential for production of off-flavors and texture changes due to the growth of these whole cells when they are added to food products. Preliminary work in our laboratory has shown that the crude fermentation liquors of either *L. citrovorum* or *S. diacetilactis* contribute no off-flavors in themselves and can prevent the growth of *P. fluorescens* and the resulting putrefaction of ground beef at refrigerator temperatures. Undoubtedly, however, the use of these crude fermentation liquors is limited because of the wide variety compounds present in them. Therefore, the use of the purified antimicrobial material would appear to have greater potential. It is conceivable that this heat stable antimicrobial preparation could be added to milk, fish, and meat products, and even to pharmaceutical preparations to extend shelf life. Work is continuing to identify the purified cationic antimicrobial material.

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SOME ADDITIONAL STUDIES ON THE THERMAL-DENATURATION OF LIGHT MEROMYOSIN FRACTION 1

INTRODUCTION

IT HAS BEEN reported that light meromyosin fraction 1 (LMM Fr 1), the tail portion of the myosin molecule prepared by its tryptic cleavage, is the most homogeneous and highly helical subfragment among LMM's prepared by other enzymatic or chemical means (Samejima et al., 1974). Yasui et al. (1971), while studying thermostability of LMM Fr 1 at 65°C in 0.6M KCl (pH 7.0), demonstrated that: (1) LMM Fr 1 depolymerizes into relatively low molecular weight proteins and peptides; (2) A gradual decrease in helical content as well as intrinsic viscosity takes place; and (3) At low ionic strengths during the early stages of denaturation, a rapid loss in paracrystal forming ability occurs. These observations not only supplemented the previous findings of Woods (1969), but also extended them to the point that the heat-induced, subtle conformational change in the protein causes the solubilization of the protein under physiological conditions.

In an earlier attempt to search for the protein which plays a role in determining the binding properties of sausages, Samejima et al. (1969) investigated the heat-gelling properties of muscle contractile proteins and myosin subunits in a saline model system. The authors found that LMM Fr 1 had no effect on the heat-gelling properties of the tested systems, whereas the opposite was true for the parent molecule, myosin. It is, therefore, of interest to study the denaturation of LMM Fr 1 under the various conditions which are used to convert muscle proteins into meat products. From this viewpoint, we further studied the thermal denaturation of LMM Fr 1 in detail and under a variety of conditions.

MATERIALS & METHODS

Preparation of LMM Fr 1

LMM was prepared by treatment of 10 mg/ml solution of myosin in 0.5M KCl at pH 7.0 with trypsin (1:200 wt ratio) freshly dissolved in cold water. LMM was isolated from the resulting solution by the method of Szent-Györgyi et al. (1960) after 10 min of digestion at 25°C. The reaction was stopped by the addition of a 1.5-fold weight excess of soybean trypsin inhibitor freshly dissolved in cold water. LMM Fr 1 was prepared from LMM according to the ethanol precipitation method described by Szent-Györgyi et al. (1960).

Turbidity measurements

Samples (3 ml) of LMM Fr 1 solution (0.4 mg/ml) in M KCl-20 mM phosphate buffer at pH 7.0 were incubated at constant temperatures of 20°C, 45°C or 60°C for 30 min; turbidity measurements were made at 610 nm.

Solubility studies

Samples of LMM Fr 1 solution (1 mg/ml) in 0.6M KCl of different pH values were incubated at various temperatures and the reaction was stopped by the addition of 5 vol of ice-cold buffer solutions containing 20 mM acetate or phosphate. The solutions were left overnight at 0°C and then centrifuged at 10,000 rpm for 20 min. The $A_{280\,nm}^{1\,cm}$ values of the supernatant liquids were determined and taken as the amount of soluble protein unless otherwise noted.

Viscosity measurements

Viscosity measurements were made with Ostwald-type viscometers at 20°C. The flow-times for solvent ranged from 150–180 sec.

Optical rotation measurements

Optical rotatory dispersion measurements were carried out on a JASCO spectropolarimeter model ORD/UV-5 under the same conditions as reported previously by Samejima et al. (1972).

Difference spectrum

The ultraviolet difference spectrum was measured with a Hitachi recording spectrophotometer type ESP-3T as described in an earlier study (Samejima et al., 1972).

Electron microscopy

Aggregates of LMM Fr 1 preparation at pH 5.4 were negatively stained with 1% uranyl acetate solution according to the method of Huxley (1963). Electron microscopy was performed in a Hitachi 11-B electron microscope using an accelerating voltage of 75 kv and a 50μ objective aperture.

RESULTS

Changes in solubility

An interesting finding, the loss of paracrystal forming ability of LMM Fr 1 upon thermal treatment at low ionic strength, has already been reported (Yasui et al., 1971; Samejima et al., 1973). Figure 1 shows that the changes in the turbidity of thermally treated LMM Fr 1 at variable low ionic strength ($\mu <$ 0.2) correspond to the loss of its paracrystal formability, as described in our earlier studies (Yasui et al., 1971; Samejima et al., 1972, 1973). This finding coincides with the changes in solubility of tryptic LMM under the same conditions.

Changes in solubility of LMM Fr 1 in 0.1M KCl as a function of pH are shown in Figure 2a. Protein solutions in 0.6M KCl were heated at various temperatures and diluted to 0.1M after being kept under ice to stop heat-induced changes. The

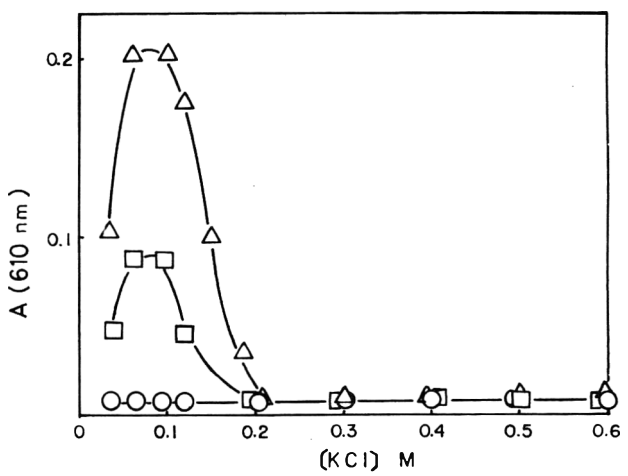

Fig. 1—Changes in turbidity of thermally treated LMM Fr 1. Protein (0.4 mg/ml) dissolved in 0.6M KCl and 20 mM phosphate buffer (pH 7.0) was treated for 30 min at 20°C (△), 45°C (□) and 60°C (○), respectively.

plots reveal that the solubility of LMM Fr 1 increases above pH 6.0, and more remarkably at higher temperatures. Other published work (Woods, 1969; Balint et al., 1970) including our own studies (Samejima et al., 1972) have demonstrated that these solubility changes are somehow related with the proteolytic activity of trypsin. As shown in Figure 2a, the pH dependence of the solubility of LMM Fr 1 heated at 60°C for 120 min, is remarkably consistent with the proteolytic activity data of Sipos and Merkel (1970).

Figure 2b illustrates the changes in solubility of LMM Fr 1 in 0.6M KCl as a function of pH, after performing thermal treatments at 45°, 50° or 60°C for 2 hr. The solvent contained 20 mM acetate buffer of pH 5.4, the value at which LMM Fr 1 precipitates isoelectrically. An electron micrograph of these precipitates (Fig. 3) reveals no similarity with the precipitates formed at low ionic strength and pH 7.0 (Yasui et al., 1971). Therefore, it is fair to assume that the results presented in Figure 2b exhibit the changes in solubility of LMM Fr 1 itself in 0.6M KCl as a consequence of thermal treatment alone, because the proteolytic activity of trypsin is negligible at pH 5.4 (Sipos and Merkel, 1970).

At pH values above 5.4, changes in solubility of thermally treated LMM Fr 1 in 0.6M KCl are more pronounced, and the loss of solubility increases with the increase in the temperature (Fig. 2b). For instance, between pH values of 5.6–7.6, thermal treatment of LMM Fr 1 at 60°C is followed by most drastic changes in the solubility; viz. a decrease of about two-thirds or more than the original value. The highest changes in solubility are observed between pH 5.6 and 6.0.

Kinetics of thermal aggregation

The rates of aggregation during thermal treatment at 40°C between the pH range of 5.5–6.0 were determined (Fig. 4a) using the data presented in Figure 2b. For measurement of the rate of aggregation, samples in 0.6M KCl were subjected to centrifugation. The pH of solutions was varied with the addition of 20 mM acetate or phosphate buffers of corresponding pH values. The data are given in Figure 4b, which indicates that the reaction proceeds according to second-order kinetics. The rate constants calculated from the slopes of the second-order plots of Figure 4b, showed the dependence upon the pH, being 0.85 ml/mg/min at pH 5.6, 0.18 ml/mg/min at pH 5.8 and 0.1 ml/mg/min at pH 5.87, respectively.

Thermodynamics of denaturation in the neutral pH region

By measurement of viscosity, ORD and difference spectrum in the ultraviolet region, it has been established that the structural changes during the course of step-by-step heating of LMM Fr 1, are a typical helix-coil transition (Samejima et al., 1972, 1973). According to the latest investigations of Burk et al. (1973), LMM Fr 1 (as prepared in this study) should have only one transition temperature (Tm) while subjected to thermal treatments. Apparently, it seems that depending upon the specificity of the technique towards the changes incurred in the protein as a result of variable thermal treatment, there are differences in the Tm values calculated from the melting point curves obtained by different methods (Samejima et al. (1972)). In fact, Tm values obtained by the viscosity measurements were always lower than those obtained by the ORD or difference spectrum (Samejima et al., 1972).

Although the thermal denaturation process of tryptic LMM Fr 1 is not reversible (Samejima et al., 1972), it is almost completely reversible in the LMM's obtained by other enzymatic (Samejima et al., 1974) or chemical cleavages of myosin (Samejima et al., 1973). If measurements are made using the same techniques, a comparison of the melting curves of enzymatically obtained LMM's with those of chemical ones, reveals only insignificant differences in Tm values. It is, therefore, justified to calculate thermodynamic parameters by assuming a two-state process i.e., $N \rightleftharpoons D$ equilibrium.

According to classical equations of thermodynamics the changes in free energy, when one mole of native protein is transformed into one mole of denatured moiety, is given as

$$\Delta F = \Delta H - T\Delta S \quad (1)$$

where ΔH is the change in enthalpy of the reaction per mole, and T is the absolute temperature. If K is the equilibrium constant of reaction $N \rightleftharpoons D$ defined by

$$K = [D]/[N]$$

the free energy of denaturation will be given by

$$\Delta F = -RT \ln K \quad (2)$$

where R is the gas constant. This equation can also be written as

$$-\ln K = \Delta H/RT - \Delta S/R \quad (3)$$

which in turn leads to Van't Hoff's equation if it is differentiated with respect to 1/T,

$$-d\ln K/d(1/T) = \Delta H/R \quad (4)$$

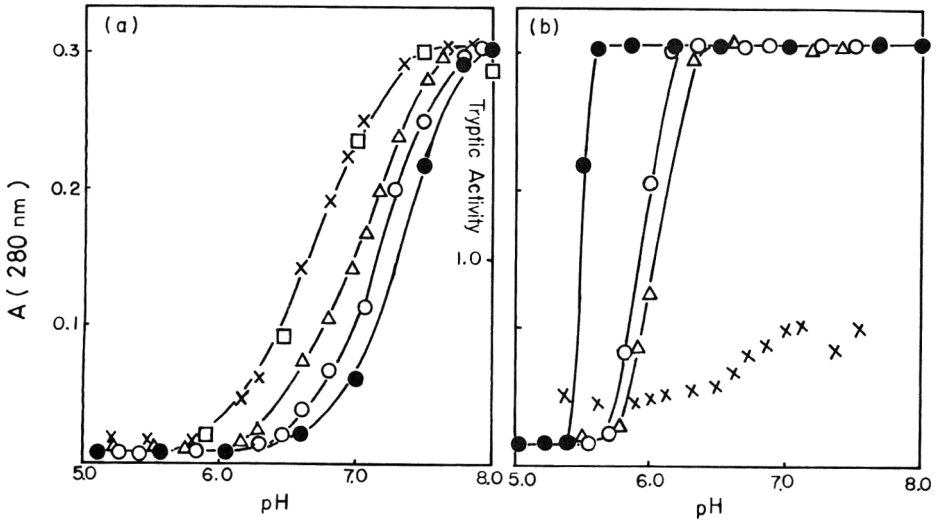

Fig. 2—Effects of temperature on the solubility of thermally treated LMM Fr 1. (a) Changes in solubility of thermally treated LMM Fr 1 in 0.1M KCl. The stock solutions were heated at 45° C (○), 50° C (△) and 60° C (×) for 120 min. Absorbance measurements were carried out as described in Methods. [●: control; □: tryptic activity quoted from data of Sipos and Merkel (1970).] (b) Changes in solubility of thermally treated LMM Fr 1 at pH 5.4 in 0.6M KCl. The pH values of stock solutions were changed after heat treatment at 45° C (○), 50° C (△) and 60° C (×) for 120 min. Absorbance measurements were made the same way as in Fig. 2(a). [●: control]

and if one assumes that ΔH and ΔS are independent of temperature. By incorporating data from one of our earlier studies (Samejima et al., 1972), the plots illustrated in Figure 5 could be made. From the slopes of these plots, ΔH was calculated as 41.5 kCal/mole. Since ΔF must be 0 at Tm (47°C), substitution of the values of ΔF and ΔH into Eq (1) will lead to the value of ΔS being equal to 125 e.u.

Two denaturation products of LMM Fr 1

Woods (1969) and the present authors (Samejima et al., 1972) have demonstrated that one of the most striking features of thermally denatured LMM Fr 1 is the heat induced depolymerization of the molecule, which can easily be followed by viscometry. We measured the changes in viscosity (ηred) after cooling down the sample solutions, which had been incubated for 300 min at various temperatures (20–70°C). These observations are shown in Figure 6, which reveal that the extent of denaturation in terms of ηred reaches its maximum at 60°C. At 70°C, ηred is 0.7 compared to 0.1 at 60°C, thus clearly showing that the maximum decrease occurs at 60°C. From these results, it is assumed that two kinds of denaturation products exist: the one of low viscosity produced at 60°C, and the other, which has a higher viscosity, at 70°C.

To confirm this assumption further studies were carried out. Figure 7 indicates that in phosphate buffer at pH 7.0 and KCl concentration of 0.6M, pre-heating of LMM Fr 1 at 60°C for various times and subsequent transfer to 70°C for 20 min tends to enhance depolymerization during the early stages of pre-treatment (the enhancing effect eventually disappears after prolonged treatment at 60°C). On the other hand, pre-heating at 70°C for various times and subsequent transfer to 60°C for 300 min inhibits the depolymerization over the whole pre-incubation period as is evident from the slight decline in viscosity. This decline is far from the minimum value recorded upon denaturation at 60°C for 300 min. Such behavior suggests convincingly that two distinct kinds of denaturation products exist.

DISCUSSION

THE RESULTS in Figures 1 and 2a further support the suggestion made in our earlier work (Samejima et al., 1972) that thermal treatment of tryptic LMM Fr 1 rapidly destroys its paracrystal forming ability at $\mu = 0.1$. In addition, the data given in Figure 2a lead us to another important possibility that tryptic activity might be involved in this phenomenon, since the changes in solubility as a function of pH are in good agreement with the pH dependence of tryptic activity. Further studies to determine the validity of this suggestion are already in progress and will be reported elsewhere.

In the isoelectric pH range, the effect of heat treatment on LMM Fr 1 should be independent of tryptic effect (if any), because of its negligible activity within this pH range (Sipos and Merkel, 1970). It is, therefore, appropriate to consider the changes in solubility illustrated in Figure 2b, an inherent property of the tail portion of the myosin molecule itself.

Johnson and Rowe (1961) found that mysoin forms insoluble aggregates, slowly obeying the second order law even under conditions with neutral pH at 20°C. Penny (1967) discovered that myosin, when heated at pH 5.3–6.2, lost its adenosine-triphosphatase (ATPase) activity before becoming insoluble, and that both the loss of ATPase activity and solubility were first-

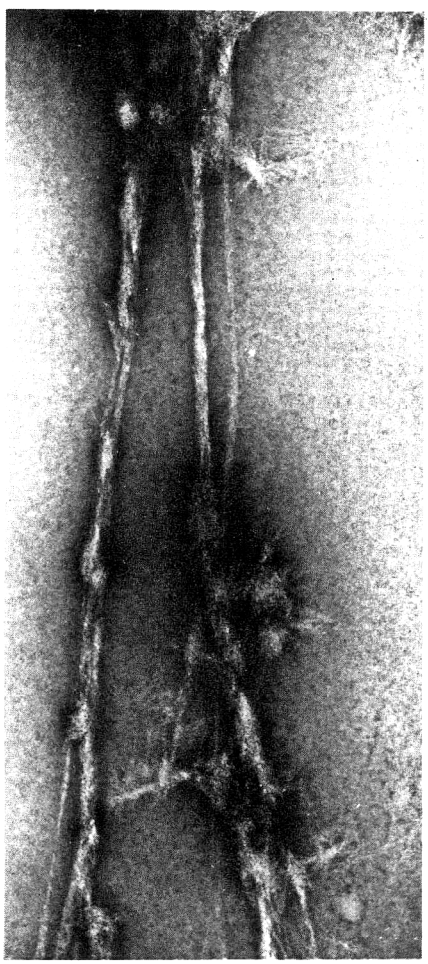

Fig. 3–Electron micrograph of LMM Fr 1 at pH 5.4 in 0.6M KCl. Magnification: 77,000X.

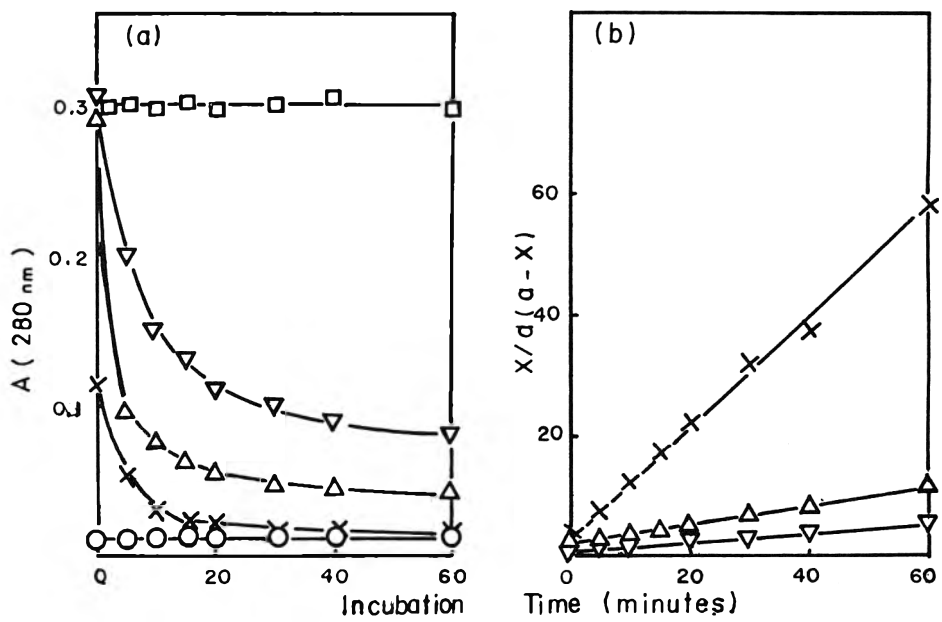

Fig. 4–The rate of aggregation of thermally treated LMM Fr 1 at 40° C. (a) The effect of pH on the rate of aggregation of LMM Fr 1. [□: pH 6.04; △: pH 5.87; △: pH 5.8; ×: pH 5.6; ○: pH 5.4] (b) Second-order plots of the aggregation of LMM Fr 1. [×: pH 5.6; △: pH 5.8; △: pH 5.87; x/a (a–x), where a and x represent the concentration (mg/ml) of the soluble and insoluble LMM Fr 1, respectively.]

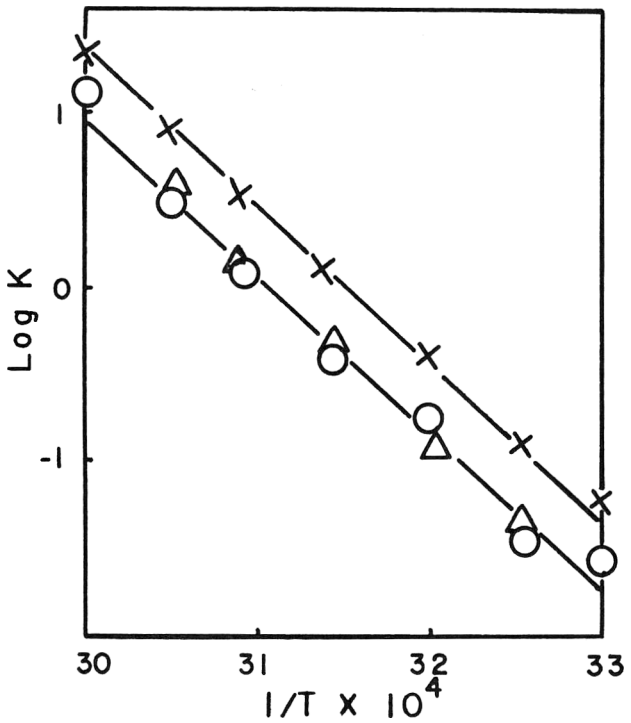

Fig. 5–Arrhenius plot of the effect of temperature on the denaturation of LMM Fr 1 from the melting curves. [×: viscosity; △: ORD; ○: $\triangle A_{285}$]

order and pH-dependent reactions. For similar reasons, myosin in solution is generally regarded as a very unstable molecule. It is of interest that kinetics of its aggregation seem to differ with that of LMM Fr 1. Kinetic data obtained by heat treatment of LMM Fr 1 at 40°C and pH 5.4–6.0 (Fig. 4) clearly demonstrate that the reaction proceeds according to the second-order law, with the rate constants depending upon pH.

The assumption of equilibrium between native and denatured LMM Fr 1 in a neutral solution, enabled the determination of heat of transformation reaction from native to denatured state, as well as the calculation of enthalpy values of 41.5 kCal/mole for ΔH and, 121 e.u. for ΔS respectively. These values are reasonable enough when compared with those listed in the literature (Haurowitz, 1963).

It is evident from the results in Figures 6 and 7 that there are two types of denatured products: one is depolymerizable and the other nondepolymerizable. Taking into account the possible involvement of trypsin in the solubility changes (since highest tryptic activity is recorded within the pH range where maximum solubility changes occur in LMM Fr 1), thermal fragmentation of LMM Fr 1 can be well explained on the basis of following two alternatives: (1) some of the peptide bonds in the helical regions are rapidly split when myosin is digested by trypsin; or (2) the reaction occurs due to dissociation of trypsin-inhibitor complex upon thermal treatment. Withstanding the first possibility, the denaturation product obtained at

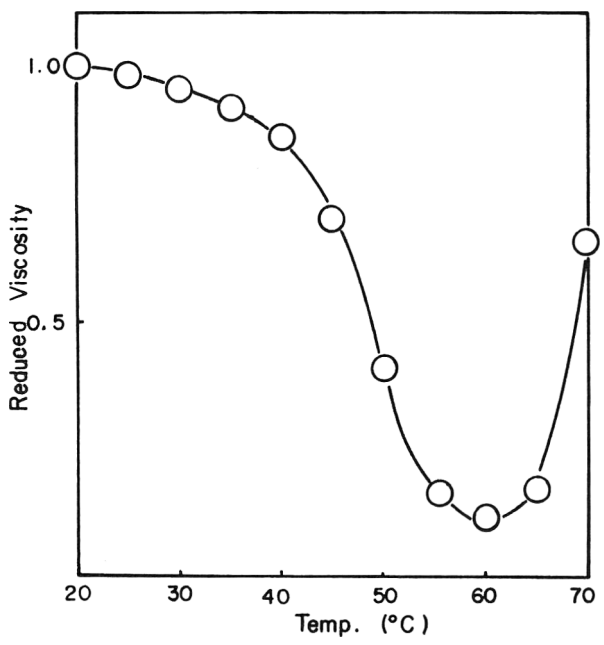

Fig. 6–Changes in the reduced viscosity of LMM Fr 1 after thermal treatment. Proteins (2 mg/ml) in 0.6M KCl and 20 mM phosphate buffer (pH 7.0) were treated for 300 min at various temperatures. Viscosity measurements were carried out at 20° C after each thermal treatment.

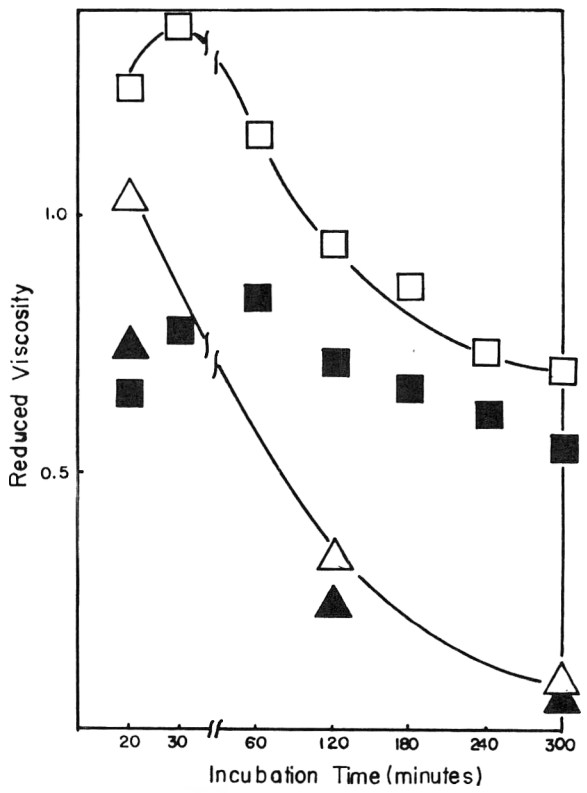

Fig. 7–The effect of pre-heating at 60° C and 70° C on the changes in the reduced viscosity of LMM Fr 1, combined with subsequent heat treatment at 70° C and 60° C, respectively. The viscosity of protein (2 mg/ml) in 0.6M KCl and 20 mM phosphate buffer (pH 7.0) was measured at 20° C after thermal treatment at 60° C (△) or 70° C (□) for various times. Closed squares (■) show viscosity of the LMM Fr 1 treated at 60° C for 300 min, after pre-heating at 70° C for various times. Closed triangles (▲) show the viscosity of the LMM Fr 1 treated at 70° C for 20 min after pre-heating at 60° C for different timings.

70°C should never be found. In case the changes accord with the second possibility, activity of reactivated trypsin should be highest at 60°C and lowest at 70°C. If these two possibilities are ruled out, the only explanation covering two kinds of reaction products, seems to be the state of trypsin absorbed to LMM Fr 1. Such a view requires the existence of only one type of denaturation product, if the LMM's prepared by means other than trypsin are heat treated, such as chemically cleaved LMM's or those obtained by cleaving the myosin with other enzymes. Unfortunately, no experimental data are now available to favor this view.

Our present attempt, and others to follow, are aimed at resolving the controversy of tryptic effect, since the behavior of myosin and its sub-fragments upon heat denaturation still require clarification. Thermal denaturation of the helical tail portion of the myosin molecule, in vitro, proceeds according to second-order law (Fig. 4) at a pH of 5.6, which is in fact the value of meat environment itself. This is still controversial, if the parent molecule also follows the same order, under these conditions. Our choice of LMM as the prime target stems from the fact that it is this portion which also determines the solubility of the myosin molecule.

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FREE RADICALS IN LYSOZYME REACTED WITH PEROXIDIZING METHYL LINOLEATE

INTRODUCTION

INTERACTION of peroxidized lipids with proteins may lead to undesirable changes in nutritional and functional properties of the latter (Roubal and Tappel, 1966a). The possibilities for reactions between peroxidizing lipids and proteins or amino acids are extensive (Karel, 1973).

Proteins are capable of reacting with peroxidizing lipids or with their breakdown products in solution or dispersion (Roubal and Tappel, 1966a), in anhydrous systems (Andrews et al., 1965; Zirlin and Karel, 1969), and in the frozen state (Buttkus, 1967). Similar reactions may be particularly rapid in foods preserved by low water activity; in frozen foods, particularly fish; and in lipoprotein-containing materials.

While the basic characteristics of changes induced in proteins by peroxidizing lipids are fairly well-known (Desai and Tappel, 1963; Roubal and Tappel, 1966b), the mechanisms of the reactions involved have not been fully elucidated. There is potential for reaction between proteins and either: (a) free radicals, (b) hydroperoxides, or (c) breakdown products of hydroperoxides.

Reactions between the breakdown products of peroxides and proteins have received considerable attention, especially the reactions of malonaldehyde, a peroxidation product of some of the fatty acids present in foods. Recently, however, some attention has been given to transient free radicals from lipid peroxidation as a major protein-damaging species. In studies on aqueous solutions of gamma globulin, catalase, serum albumin, hemoglobin and ovalbumin, damage due to lipid oxidation was qualitatively similar to free radical-mediated effects of ionizing radiation (Desai and Tappel, 1963; Roubal and Tappel, 1966a, b). This laboratory has also noted that in lyophilized protein-lipid systems, water exerts effects similar to those observed in irradiated proteins, with low water activities promoting scission, and high water contents promoting crosslinking (Zirlin and Karel, 1969; Takahashi, 1970).

The present study was undertaken to establish whether or not free radical formation in proteins results from reaction with peroxidizing lipids, and to determine the effects of oxidation conditions on the free radical interactions. Electron spin resonance (ESR) was used to study the free radical formation. Although this method has been used extensively in studies on irradiation-induced free radicals in proteins (Shields, 1973), few reports dealing with ESR measurements in proteins exposed to peroxidizing lipids are presently available. Roubal (1970, 1971) concluded that in addition to the central protein resonance ($g \cong 2$) present in his systems, weaker downfield shoulders could be attributed to lipid-free radicals stabilized by the protein matrix. He later proposed (1971) that free radicals are the major source of damage to proteins exposed to oxidizing lipids. However, his results left some doubt about the source of radicals in the system.

This investigation was thus designed to determine: (a) whether protein radicals resulting from protein-lipid reactions can actually be detected with the technique of electron spin resonance spectroscopy; (b) how these radicals compare with those produced by γ-radiation; (c) what conditions are necessary for radical formation in lipid-protein systems; and (d) what specific effects water has on protein radical formation.

MATERIALS & METHODS

Model system preparation

A model system consisting of methyl linoleate (ML) (Hormel Institute) and lysozyme (LYS) (Nutritional Biochemicals, 3x crystallized) in 10:1 molar ratio was emulsified with distilled water by mixing for 5 min in a Sorvall Omni-Mixer, quick-frozen in liquid nitrogen, then lyophilized for 24 hr in a Virtis laboratory freeze drier. Control systems of LYS and water, but no ML were prepared in the same manner.

Treatment

After lyophilization, both experimental and control systems were either oxidized in air following equilibration at 37°C over $CaSO_4$ or over salt solutions in desiccators to water activities of 0.00, 0.07, 0.11, 0.22, 0.32, 0.40 and 0.75 (Gal, 1967); or the systems were exposed to 1 or 2 Mrads γ-radiation.

Samples of about 300 mg were exposed in ampules either evacuated and sealed, or left open to γ-radiation in a Gammacell 60, Cobalt 60 source (Atomic Energy Commission of Canada, Ltd.) at a dose rate of 9.5×10^3 rads/min. To prevent radical decay after irradiation, we held samples in liquid nitrogen until they could be transferred to tubes for ESR measurements.

ESR studies were conducted on incubated samples both with and without extraction of the lipid. Lipid was extracted with a benzene-ethanol azeotrope (32.4:67.6 v/v) solvent by shaking 30 min under nitrogen, and then filtering through a Buchner funnel.

The course of lipid oxidation was followed by iodometric determination of peroxide values (American Oil Chemists' Society, Method AOCS-Cd-8:53) in the extracted lipid.

ESR analyses

ESR spectra were recorded for powder samples in 3.5 mm ID quartz tubes. To avoid interference from the dielectric absorption of energy by water, we dried samples in vacuo over $CaSO_4$ before analyses.

Varian 4502 and E-9 spectrometers operating in X-band with 100 kHz field modulation were used. Microwave power levels (8 and 10 mW, respectively) were chosen for maximum sensitivity without power saturation. Spectra were recorded as first derivatives of the absorption curves, with identical instrumental settings for all samples in each experiment.

Relative spin concentrations were calculated with the equation:

$$N = \frac{A_{pp} H_{pp}^2}{\rho}$$

where A_{pp} and H_{pp} are the peak-to-peak amplitudes and line-widths, respectively, of the derivative spectra, and ρ is the unit sample density in the tubes. Order of magnitude spin populations were determined by comparison of spin concentrations of experimental samples with that of a 2,2-diphenyl-picryl hydrazyl free radical (DPPH) standard, assumed to contain 1.53×10^{21} spins/g.

Spectral g-values were calculated according to the resonance equation:

$$h\nu = g\beta H$$

(h is Planck's constant, ν is the microwave frequency, β is the Bohr

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magneton, and H is the resonant magnetic field). The microwave frequency and resonant magnetic field were measured respectively with a frequency meter and an NMR gaussmeter coupled to an electronic frequency counter.

RESULTS & DISCUSSION

A SUMMARY of qualitative results is presented in Table 1. No signals were evident in either experimental or control systems immediately after lyophilization. Furthermore, no signals were observed in the protein controls (no lipid) exposed to air for up to 40 days. ESR signals developed only in incubated systems which contained lipid oxidized to some extent.

Several lines of evidence indicate that the ESR signals did arise from protein radicals:

(1) ESR signals from native lysozyme exposed to peroxidizing ML were the same as those from lysozyme exposed in air to γ-radiation or high temperatures. The presence of ML during irradiation did not qualitatively change the signal, although radical concentrations were increased.

Table 1—ESR signals in irradiated and peroxidized reaction systems

System	Presence or absence of ESR signal	Presence or absence of principal g-value of 2.0051 ± 0.0005
Lysozyme alone		
lyophilized	–	–
lyophilized, incubated	–	–
irradiated	++	+
heated in air or vacuum, 160°C	+	+
Lipid alone		
ML[a] irradiated	–	–
vegetable oils,[b] irradiated	–	–
ML oxidized in bulk	–	–
ML oxidized on avicel	–	–
ML + LYS		
lyophilized	–	–
lyophilized, incubated	+	+
irradiated, lyophilized or direct-mix	++	+

[a] Methyl linoleate
[b] Peanut oil, safflower oil and Wesson oil

Table 2—Radical quenching by various solvents during the extraction procedure

Solvent	% Reduction of radical concentrations: Lipid systems	Irradiated systems (no lipid)
Chloroform-methanol (3:1)	80	89
Ethanol	72	73
Benzene-ethanol (32.4:67.6)	61	70
Hexane	58	52
Benzene	38	49

(2) The dominant free radical species resulting from lipids are peroxy and alkoxy radicals, which should give signals with g-values of about 2.01–2.02 (Swartz et al., 1972). Such signals did not appear under the experimental conditions reported above.

The ESR signals obtained for oxidative systems and for systems irradiated in air were single lines with g-values of 2.0051 ± 0.0005 and line-widths of 11 ± 3 Gauss (Fig. 1). Under the experimental conditions described above, no downfield "shoulder" signals similar to those noted by Roubal (1970) were observed. Evidence obtained showed that such signals arise from thiyl radicals on the protein rather than from lipid peroxy radicals, and will be discussed in a subsequent paper.

(3) No signals could be obtained from irradiated liquid ML, nor from highly oxidized ML, nor from ML oxidized on a micro-crystalline cellulose matrix. Lück et al. (1963) also reported difficulty obtaining ESR signals from oxidizing lipids and postulated that the half-lives of lipid radicals were too short for total instantaneous concentrations to reach detectable levels. Theoretical considerations of the model systems used suggest that for a peroxide value of 1000, the steady-state ROO · concentrations may be estimated to be in the order of 10^{-13} moles of radicals in the cavity sample volume (10^{-11} moles is the minimum detectable concentration), thus supporting Lück's hypothesis. However, even if detectable lipid radical concentrations were present, it is unlikely that the lipid radicals could be detected at room temperature due to excessive line broadening from very short spin state lifetimes in oxy-type radicals.

We hypothesize that lipid radicals arising during the oxidation are transferred to the protein, thus forming protein radicals. Such a mechanism could explain the reduction in central signal amplitude noted by Roubal (1970) in antioxidant-treated fish samples. Any antioxidant, by decreasing the rate and extent of lipid oxidation, would limit the concentrations of radicals available for transfer to protein sites, thus reducing the numbers of protein radicals formed as well as quenching protein radicals that did form.

Extraction of the lipid prior to ESR analyses results in greater than 50% decrease in signal intensity. Upon first consideration the lipid component seemingly was responsible for a large part of the signal. However, ethanol is known to be a

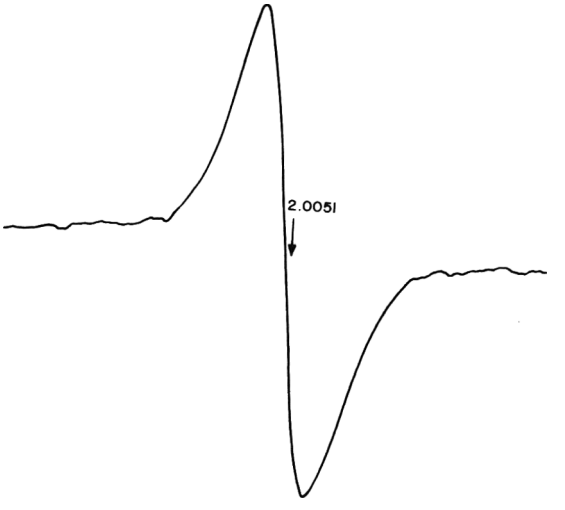

Fig. 1—Typical ESR spectrum of lysozyme exposed to oxidizing linoleate.

Table 3—Lipid oxidation and free radical formation in lyophilized ML/LYS emulsions (Experiment 4)

Days of incubation	Water activity 0.00 PV[a]	0.00 RC[b]	0.07 PV	0.07 RC	0.40 PV	0.40 RC	0.75 PV	0.75 RC
0	126	6.43	15	4.32	50	2.80	75	2.14
1	737	7.87	725	8.23	1700	4.54	1906	2.53
2	1620	8.64	965	7.84	1984	6.71	2025	4.41
3	763	9.60	630	6.77	970	4.29	1647	1.77
5	365	7.63	305	6.70	742	4.11	1072	1.65
8	150	6.06	233	5.87	260	2.87	310	1.59
16	150	5.69	100	5.25	134	1.95	150	1.48

[a] PV = peroxide value of extracted lipid
[b] RC = radical concentration X 10^{15} mg protein

Table 4—Radical concentrations in lyophilized ML/LYS emulsions incubated at specified water activities

Days of incubation	Radical concentrations X 10^{15}/mg protein at specified water activities 0.00	0.11	0.22	0.33
Run 6				
0	0.20	0.11	0.10	0.06
1[a]	0.28	0.11	0.17	0.15
2	0.42	0.28	0.28	0.23
3	1.94	1.85	1.74	0.93
5	2.57	2.09	1.95	1.41
12[a]	2.24	1.58	1.74	1.08
Run 7				
0	0.24	0.24	0.18	0.14
1[a]	0.49	0.64	0.48	0.47
3	1.55	1.31	1.08	0.54
8[a]	1.73	1.74	2.46	1.09
12[a]	2.53	3.37	2.76	1.36
20	2.51	2.14	1.92	1.54
21	2.26	1.89	1.60	1.02
25	3.93	2.63	1.70	1.61

[a] Days when ESR measurements could not be conducted immediately after the usual 24-hr drying period. Samples were stored under vacuum prior to measurement.

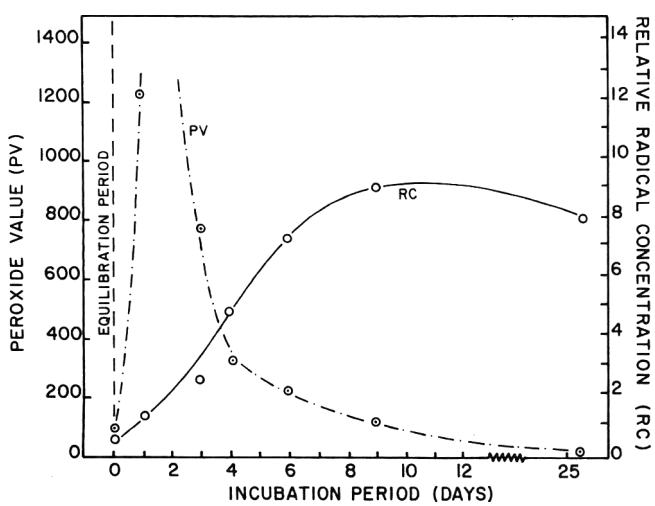

Fig. 2—Relative radical concentration and peroxide value of lipid extracted from the incubated freeze-dried model system containing lysozyme and methyl linoleate.

radical scavenger, as are many organic solvents. Comparison of quenching effects of various solvents on LYS incubated with oxidizing lipid or irradiated in air revealed close similarities, as shown in Table 2. Thus, the reduction of radical concentration due to extraction of the lipid in the model systems may be attributed to direct quenching effects of the solvent rather than to physical removal of the lipid.

Quantitative

Both water activity of the incubated systems and extent of oxidation of the lipid component (linoleate) affected the formation of free radicals in lysozyme.

The course of lipid oxidation, as indicated by peroxide values, and protein radical formation were measured in systems incubated at water activities of ∿0.00, 0.07, 0.41 and 0.75. A general relationship was observed between extent of lipid oxidation and formation of protein radicals. Radicals began to build up along with lipid hydroperoxides, but the most substantial formation of protein-free radicals occurred after lipid peroxides began to break down. This effect is shown in Figure 2 for samples incubated dry.

Although initial formation of protein radicals follows the general build-up of lipid peroxides, radical concentrations do not fall off as rapidly as peroxide values in later stages of oxidation. This may be due to the situation expected from the normal oxidation kinetics, namely that total lipid radical concentrations do not necessarily fall when total peroxide concentrations drop. Accordingly, the lipid radical concentration remains sufficiently high for continued transfer of radicals to protein. Also, once on the protein, the radicals may be stabilized with a corresponding increase in half-life.

The role of lipid peroxides as inducers of protein radicals is further substantiated by higher radical concentrations in experiments where lipid oxidation was higher. Since experimental conditions were identical in all cases, and the extent of lipid oxidation varied in the same order as radical concentrations, lipid-free radicals would seem to be directly responsible for the production of free radicals in protein.

Quenching effects of water, however, appear to be more important than effects of increased lipid oxidation. Whereas peroxide values were greater at high water activities (Table 3), radical concentrations decreased with increasing water activity.

Following the suggestion that the BET monolayer value represents a critical level of water content with respect to lipid oxidation and radiation-induced radical formation (Labuza, 1968; 1971), we incubated lyophilized ML/LYS systems at water activities between 0.00 and 0.30, slightly above the monolayer value (0.20). Results are reported in Table 4. In oxidizing lipid/protein systems, unlike in some irradiated substances, we found radical concentrations to decrease continuously with increasing water content.

In addition to direct effects of water activity on the rate and extent of lipid oxidation (see Labuza, 1971 for discussion), water may also be expected to influence free radical interactions between proteins and oxidizing lipids by influencing the concentrations of initiating radicals present, the degree of contact and mobility of reactants, and the relative importance of radical transfer versus recombination reactions.

ESR cannot provide evidence for large concentrations of radicals which recombine so rapidly that their steady-state concentrations are lower than the sensitivity of the instrument. It may therefore be more appropriate to state that the *stability* of radicals transferred to proteins exposed to oxidizing lipids rather than the concentrations of radicals transferred, increases as the water activity of the system decreases.

The presence of water may "quench" free radicals by either of two mechanisms: (a) promoting radical recombination and crosslinking; or (b) terminating the radical by proton donation.

Polyacrylamide gel electrophoresis of lysozyme reacted

with oxidizing linoleate showed that extensive crosslinking occurred at the higher water activities, e.g., $a_W = 0.40$ and 0.75. Some crosslinking occurred also in low water activity systems ($a_W \sim 0.00$ and 0.07), but not to the same extent.

Crosslinking is not in itself proof for the existence of radical recombination processes, since crosslinking may also result from reaction of the protein with various lipid oxidation products such as malonaldehyde. However, the parallel increases in the extent of crosslinking and peroxide values suggest that radical recombination is responsible for crosslinking. Crosslinking would not be expected to occur if quenching by hydrogen atoms predominated; if malonaldehyde-type crosslinking did occur in this case, it would not develop until later in the incubation period. Although proton quenching cannot entirely be ruled out, it appears that promotion of recombination is the dominant mechanism for direct effects of water on protein radicals.

Implicit in the promotion of radical recombination by water is mobilization of reaction species. Thus, part of the increased crosslinking at high water activities may have been due to increased radical densities, resulting from enhanced radical transfer with increasing degrees of contact between the lipid and protein. A review of literature pertaining to recombination of protein free radicals has been completed by Schaich (1974), and will be published as part of a separate review article.

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ANTIOXIDANT ACTIVITY OF ACETONE EXTRACTS OBTAINED FROM A CARAMELIZATION-TYPE BROWNING REACTION

INTRODUCTION

THERE HAVE BEEN numerous studies which show that antioxidants are formed in various heat-processed food products, through interaction of proteins and carbohydrates present (Griffith and Johnson, 1957; Anderson et al., 1963; Yamaguchi et al., 1964). Evans et al. (1958) isolated amino-hexose reductones from the reaction mixtures of aldohexoses and secondary amines, and demonstrated that these reductones acted as effective antioxidants in soybean, cotton seed and corn oils. Kirigaya et al. (1968) reported that browning reaction mixtures obtained from Maillard-type browning systems showed inhibitory effects on the autoxidation of linoleic acid. Hwang and Kim (1973) also reported that alcohol extracts of a Maillard-type browning reaction mixture exhibited significant antioxidant activity in an edible soybean oil. However, few research works seem to have dealt specifically with the possible antioxidant activity of the products formed in a caramelization-type, or nonenzymatic nonamino sugar browning reaction.

Several research works (Griffith and Johnson, 1957; Yamaguchi et al., 1964; Yamaguchi and Okada, 1968) have been published, which suggest strongly that reductones formed in Maillard-type browning systems were effective in retarding the rancidity development of various substrates. It appears, however, that the nature of the effective antioxidative compounds formed in Maillard-type browning systems have not been fully elucidated. Kirigaya et al. (1968) reported that the antioxidant activity increased in proportion to the color intensity of the reaction solution, and that reductones formed during the browning reaction contributed little to the antioxidant effects. Hwang and Kim (1973), however, reported that the antioxidant activity of the alcohol extracts from a Maillard-type browning reaction mixture did not seem to increase in proportion to the length of reaction time, and that effective antioxidant compounds were already formed in the earlier stages of the browning reaction.

The objective of the present study was firstly to determine whether the acetone extracts of a caramelization-type browning reaction mixture, which did not contain any melanoidin-type nitrogeneous pigments, would act as antioxidant or not. Secondly it was to investigate the relationships between the length of reaction time and the color intensity of the acetone extracts, and also the length of reaction time and the antioxidant activity. The relationships appear to be very important in elucidating the nature of the effective antioxidants formed in either Maillard-type or caramelization-type browning reactions.

MATERIALS & METHODS

Substrate

A refined, deodorized edible soybean oil was used as substrate. The peroxide value, free fatty acid value and iodine value of the oil were respectively 1.0 ± 0.4 meq/kg, 0.21 ± 0.05% and 121 ± 2.0. The peroxide value was determined by a modified method based on Wheeler's (1932). The free fatty acid value was determined by the method described by Tribold and Aurand (1963). The iodine value was determined by the AOAC method (1960).

Browning reaction mixture

180g of glucose was dissolved in 500 ml distilled water. The 2.0M glucose solution was introduced into a 1,000 ml flask fitted with a reflux condensor, and heated at 100°C for a period of 96 hr. 10 ml aliquots of the reaction mixture were withdrawn at intervals of 3, 9, 24, 48, 72 and 96 hr and kept at 4°C before use.

Determination of browning rate

Each aliquot was filtered and absorbance at 470 nm of the filtrate was measured with a Shimadzu QV-50 type photoelectric spectrophotometer. The color development of the browning reaction mixture was also followed visually.

Acetone extracts from the browning reaction mixture

Acetone extracts were prepared by the method described by Yamaguchi and Koyama (1967). 10 ml aliquots of the browning reaction mixture withdrawn at each interval were concentrated to a viscous residue at 40 ± 1.0°C with a rotary vacuum evaporator. Each residue was extracted with 10 ml acetone and the extracts dehydrated with anhydrous sodium sulfate. The acetone extracts were stored in a refrigerator maintained at 4°C, and the precipitated glucose was removed from the acetone extracts by filtration.

Antioxidant activity determination

Each acetone extract of the caramelization-type browning reaction mixture was coded in numerical order. Extract Nos. 1, 2, 3, 4, 5 and 6 were respectively the acetone extracts which had been taken at intervals of 3, 9, 24, 48, 72 and 96 hr. Each acetone extract was added to 70g of the substrate. After mixing the extract with the substrate, the solvent was removed thoroughly from the substrate. 70g of substrate, to which 10 ml acetone was added and then removed, was used as the control. The substrates, to which each acetone extract had been added, were also coded in numerical order. Namely, Nos. 1, 2, 3, 4, 5 and 6 were, respectively, the substrates which contained Extract Nos. 1, 2, 3, 4, 5 and 6. Each substrate was divided into three portions by introducing it evenly into three shallow dishes. All the dishes were incubated at 45 ± 0.7°C for a period of 18 days. Peroxide values of the substrates were determined every 2 days by the method mentioned. The peroxide values were expressed as milliequivalents of peroxides per kg of oil. The antioxidant effects of the acetone extracts were compared on the basis of peroxide value development of the substrates.

RESULTS & DISCUSSION

Interrelationships among length of reaction time, color intensity, and antioxidant activity

The experimental results shown in Table 1 indicate that the color of the caramelization-type browning reaction mixture increased approximately in proportion to the length of reaction time. The linear relationship was similar to that found in the study previously carried out by Hwang and Kim (1973), although the type of browning reaction used was different. The absorbance at 470 nm of the reaction mixture 3 hr after the initiation of the reaction was 0.013 and it increased to 0.029 after 9 hr. It continued to increase nearly in proportion to the length of reaction time, and finally reached a value of 0.537 after 96 hr. The color development in the caramelization-type browning reaction was significantly slower than that found in such a Maillard-type browning reaction as had previously been reported (Hwang and Kim, 1973).

Table 2 summarizes the results of the peroxide value determination. All the substrates containing the acetone extracts were significantly more stable to the rancidity development than the control, indicating that the acetone extracts of the caramelization-type browning reaction mixture possessed significant antioxidant activity. The relative antioxidant effects of the acetone extracts were, in increasing order, Extract No. 1 < Extract No. 2 < Extract No. 3 < Extract No. 4 < Extract No. 5 < Extract No. 6. However, the antioxidant effects of Extract No. 4 and 5 were almost the same as that of Extract No. 6. It seemed very noteworthy that Extract No. 1, which had been obtained only 3 hr after the initiation of the browning reaction, showed considerable antioxidant activity. The control developed a peroxide value of about 25 at the end of 18 days whereas No. 1, 2 and 3 developed peroxide values of about 20, 18 and 17, respectively, at the end of the same period. On the other hand, No. 4, 5 and 6 developed approximately the same peroxide values of about 16 after the 18 days.

These results indicate that the antioxidant activity of the acetone extracts from the browning reaction mixture increased very rapidly at the earlier stages of the browning reaction when the color development was not significant, and then the increase in the antioxidant activity slowed down as the color intensity increased (Fig. 1). It appears, therefore, that the effective antioxidant compounds were already formed at the earlier stages of the browning reaction as had been the case in Hwang and Kim's study (1973).

Reductones vs high molecular weight brown-colored pigments as effective antioxidants

It is well-known that Maillard-type browning reaction systems produce compounds having considerable antioxidant activity. The present study has also shown definitely that a caramelization-type browning reaction system produced effective antioxidant compounds. However, the nature of antioxidants produced in both types of browning reaction systems does not seem to have been elucidated satisfactorily. It appears that the likely antioxidants produced in both types of browning reaction systems are either reductones or brown-colored pigment, or both. It has been assumed by many researchers that reductones probably play an important role in retarding the rancidity development of processed food products (Griffith and Johnson, 1957; Anderson et al., 1963; Yamaguchi et al., 1964; Yamaguchi and Okada, 1968). Evans et al. (1958) stated that the antioxygenic properties of browning reaction

Table 1—Variations of color and absorbance of the caramelization-type browning reaction mixture with reaction time

Sample	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6
Reaction time (hr)	3	9	24	48	72	96
Color	very pale yellow	pale yellow	very light yellow	light yellow	orange	reddish brown
Absorbance[a]	0.013	0.029	0.071	0.216	0.362	0.537

[a] Absorbance at 470 nm was measured directly using a Shimadzu QV-50 type photoelectric spectrophotometer.

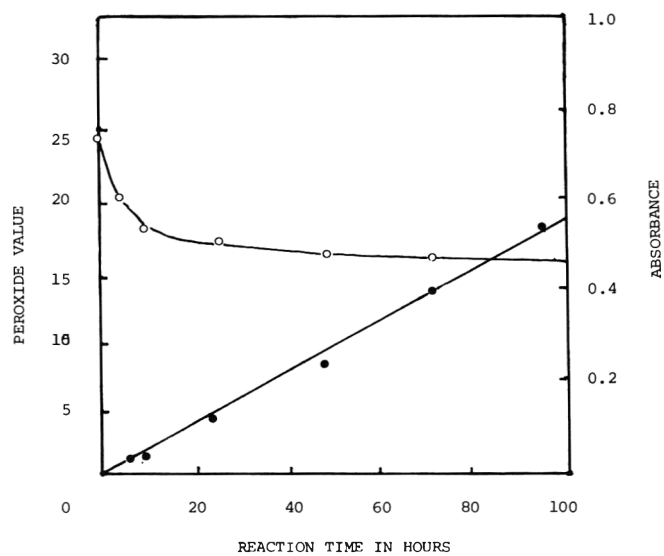

Fig. 1—Relation between the color intensity of browning reaction mixture and reaction time and, also, the antioxidant activity of the acetone extracts and reaction time. [The color intensity, at 470 nm, of the reaction mixture was measured with a spectrophotometer (solid circles). The peroxide values of the acetone extracts were determined on the 18th day of the storage period (open circles). Smaller PVs indicate greater antioxidant activity of the extracts.]

Table 2—Variations of peroxide values[a] of soybean oil[b], containing the same amount of the acetone extracts obtained at successive stages of the caramelization-type browning reaction, with time

Sample no. \ Time (in days)	0	2	4	6	8	10	12	14	16	18
Control	1.0 ± 0.3	1.7 ± 0.7	2.0 ± 0.3	2.8 ± 0.4	3.1[c]	3.7 ± 0.7	5.6 ± 0.5	11.1 ± 0.8	14.0 ± 0.8	24.6 ± 0.9
No. 1	1.0 ± 0.4	1.6 ± 0.5	2.0 ± 0.3	2.2 ± 0.5	3.0 ± 0.6	3.2 ± 0.8	5.4 ± 0.7	7.6[c]	13.0 ± 0.8	20.1 ± 1.0
No. 2	1.1 ± 0.3	1.6[c]	1.9 ± 0.4	2.1 ± 0.7	3.2 ± 0.7	3.9 ± 0.6	5.0 ± 0.8	7.4 ± 0.7	12.0 ± 1.0	18.5 ± 1.1
No. 3	0.9 ± 0.5	1.6 ± 0.3	1.6 ± 0.4	1.8 ± 0.5	2.9 ± 0.6	3.5 ± 0.5	5.3 ± 0.8	7.2 ± 0.8	11.3 ± 1.1	17.3 ± 1.0
No. 4	1.0 ± 0.1	1.5 ± 0.2	1.5 ± 0.7	1.7 ± 0.4	2.9 ± 0.6	3.3[c]	5.5 ± 0.9	6.6 ± 0.9	10.2 ± 1.0	16.4 ± 1.2
No. 5	1.0 ± 0.4	1.5 ± 0.5	1.7 ± 0.5	1.8 ± 0.3	2.8 ± 0.8	3.7 ± 0.7	4.9 ± 1.0	6.5 ± 1.0	9.2 ± 0.8	16.3 ± 1.5
No. 6	1.0 ± 0.3	1.5 ± 0.4	1.5 ± 0.4	1.7 ± 0.3	2.8 ± 0.5	3.3 ± 0.1	4.6 ± 0.7	6.3 ± 0.5	8.8 ± 0.6	16.2 ± 1.0

[a] Peroxide values are expressed as milliequivalents of peroxides per kg of oil.
[b] Each sample was placed in an incubator kept at 45 ± 0.7°C.
[c] Figures without SDs are mean values.

mixtures might be mainly due to the formation of amino reductones in the browning mixtures.

The conclusion drawn by Kirigaya et al. (1968) that the effective antioxidants in the Maillard-type browning reaction mixture were probably high molecular weight brown-colored pigments, i.e., melanoidins, and not reductones appears to be in disagreement with the generally held view. It is also in contradiction with the experimental results of the present study and Hwang and Kim's study (1973). These results indicated clearly that the earlier extracts of the browning reaction mixtures which did not show any significant color development and, hence, were thought to contain little brown-colored pigments exhibited considerable antioxidant activity. Moreover, special emphasis should be put on the fact that the browning reaction used in the present study could not form any melanoidins or melanoidin-like nitrogeneous pigments.

Although high molecular weight brown-colored pigments may play some role in retarding the rancidity development of lipid substrates, their action as antioxidants should be quite different from that of ordinary antioxidants which are usually soluble in fats and oils or at least in fat solvents, for high molecular weight brown-colored pigments do not dissolve in fat solvents, still less in fats and oils. With reference to this, Cooney et al. (1958) reported that the colored substances were bleached by the peroxides; hence the colored substances or their intermediates could exert direct antioxidant action on peroxides.

The results of the present study, together with those of Hwang and Kim (1973), seem to suggest that although high molecular weight brown-colored pigments produced in the later stages of a browning reaction, either Maillard-type or caramelization-type, may have some antioxidant activity, the major antioxidant compounds are probably colorless or almost colorless intermediates such as reductones and dehydro-reductones produced in the fairly earlier stages of the browning reaction.

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FLUOROMETRIC ASSAY FOR TOTAL VITAMIN C USING CONTINUOUS FLOW ANALYSIS

INTRODUCTION

A RAPID and quantitative determination of vitamin C indigenous or supplemented in foods and the determination of the rate of destruction of ascorbic acid during processing and storage is of increasing importance to the food industry. Numerous manual procedures for the determination of vitamin C in foods are available (Freed et al., 1966; Wasa et al., 1961; Stroehecker and Henning, 1965; AOAC, 1970). Titration of acid extracts of food products with 2,6-dichloroindophenol (DCP) and the DCP photometric assay are the most common methods for the determination of reduced ascorbic acid (RAA) in foods. Recently, Egberg et al. (1973) reported a photometric DCP method for RAA in foods which was adapted for continuous flow analysis using Technicon AutoAnalyzer (Technicon Instruments Corp., Tarrytown, NY 10591) equipment. However, the presence of reducing substances, such as sulfhydryl compounds, reductones and reduced iron limit the use of the DCP techniques for RAA in foods and biological fluids.

Of the direct methods for the determination of dehydroascorbic acid (DAA) and total ascorbic acid (TAA), treatment with 2,4-dinitrophenylhydrazine (DNPH) to form hydrazones or osazones have been preferred to those based on reduction of DAA to RAA and determination with DCP (AOAC, 1970; Roe, 1936; Roe and Kuether, 1943). An automated procedure based on the DNPH method has been reported by Pelletier and Brassard (1972). The DNPH reaction, however, has been shown to be affected by the presence of aldoses and glucuronic acid when the temperature of the coupling reaction was above 37°C (Roe, 1961).

Another direct method for the determination of DAA and TAA is a fluorometric procedure based on a condensation reaction between DAA and o-phenylenediamine (PDA) (AOAC, 1970; Deutsch and Weeks, 1964).

The present paper describes a simple, rapid, quantitative method for the continuous flow analysis of DAA and TAA using a modification of the PDA procedure.

EXPERIMENTAL

Reagents and materials

Extracting solutions. HPO_3-HOAc (AOAC 39.052), 6% HPO_3 and 0.5% oxalic acid.

Sodium acetate. 50% NaOAc · $3H_2O$, dissolve 500g NaOAc · $3H_2O$ in distilled H_2O and dilute to 1 liter.

Boric acid-sodium acetate. 3% H_3BO_3-50% NaOAc, dissolve 30g H_3BO_3 in 50% NaOAc solution and make up to 1 liter.

2,6-Dichloroindophenol. 0.2% DCP, follow procedure for indophenol standard solution AOAC 39.052.

Thiourea. 3% H_2NCSNH_2, dissolve 3g of H_2NCSNH_2 in 50% ethanol and make to 100 ml.

Ascorbic standards. Dissolve 10 mg ascorbic acid (preferably U.S.P. Reference Standard) in 6% meta-phosphoric or 0.5% oxalic acid and make to 100 ml. This solution contains 100 μg ascorbic acid per ml. Serial standards ranging from 0–100 μg ascorbic acid per ml were prepared by appropriate dilutions.

o-Phenylenediamine. Dissolve 20 mg o-phenylenediamine-2-HCl (Eastman Kodak Co. No. 678) in a 100 ml volumetric flask and dilute to volume with distilled water.

Analytical system

The following Technicon AutoAnalyzer modules were used to construct the analytical system shown in Figure 1: (1) Sampler II, 50 samples/h; (2) Pump II; (3) Heating bath, 40°C with 15 min delay coil; (4) Fluorometer, 360 nm primary filter and 436 nm secondary filter; and (5) Recorder

Preparation of sample assay solution

Samples were prepared as described in AOAC 39.054 or a modification of this procedure by substituting 6% meta-phosphoric acid or 0.5% oxalic acid as the extracting medium. Sample size and final dilution volumes were adjusted to give a concentration of 20–70 μg TAA per ml of assay solution.

Recovery samples were prepared by adding a known volume of RAA or DAA standard solution to the sample prior to acid extraction. Recovery samples contained 20 μg of added RAA or DAA per ml of assay solution.

Manual procedure

Determination of DAA and TAA was carried out according to the PDA procedure, AOAC 39.061 (1) and a modification of this procedure. The modification involved the substitution of 50 μl of 0.2% DCP for Norit (activated carbon) as the oxidizing agent (AOAC 39.055). Excess DCP was reduced by the addition of 30 μl of 3.0% thiourea. Following the oxidation of RAA to DAA using DCP or Norit, samples were assayed for DAA using the PDA procedure (AOAC 39.061).

Blanks were determined according to the procedure described in (AOAC 39.061).

Automated procedure

Principle. The continuous flow procedure for the determination of DAA and TAA is a modification of the PDA method (AOAC 39.061) as described above.

DAA. As shown in Figure 1, the samples were diluted with 0.5% oxalic acid (6% m-phosphoric acid or m-phosphoric acid-acetic acid mixture) to ensure proper range for vitamin assay. DCP and thiourea were not required for the determination of DAA. Therefore, delivery tubes for these reagents were kept in distilled water. Following addition and mixing of 50% NaOAc the assay mixture was resampled to reduce the volume of PDA reagent required. 0.02% PDA was added to the sample mixture and the assay mixture passed through a 15 min delay coil at 40°C to develop the DAA-PDA fluorophor before entering the fluorometer.

DAA blanks for standards and samples were determined by substituting 3% H_3BO_3-50% NaOAc for 50% NaOAc and passing assay mixture through an 8 min delay coil at 37°C. Boric acid prevented the condensation of PDA to DAA.

The use of elevated temperature following the addition of PDA for ascorbic acid assay and H_3BO_3-NaOAc for blank determinations was a modification of the AOAC procedure, which requires the samples and blanks be held at room temperature for 30 and 15 min, respectively. However, significant reductions in these reaction times were accomplished by using elevated temperatures.

TAA. The presence of DCP in transmission tubing and mixing coils can result in erroneous DAA values. Therefore, the autoanalyzer system should be checked for excess DCP before DAA levels are determined. With manifold properly connected (Fig. 1) water was pumped through all pump tubes and the fluorometer adjusted to give a recorder base line of 5 with sample and reference aperture settings of 2. All reagent delivery tubes were then placed in the appropriate reagents and the system was purged for approximately 20 min before establishing a reagent baseline of 5. Thiourea was introduced into the assay mixture before DCP to prevent discoloration of transmission tubing.

The high RAA standard was then aspirated into the system and the

maximum fluorometric response was adjusted to 95. The sample probe was then placed in water and the system allowed to return to baseline before serial standards and samples were assayed.

An ascorbic acid standard was placed in the sample tray after every 10–15 samples to monitor any recorder drift.

Calculations

Fluorescence was proportional to the concentration of the blue fluorophor, condensation product of DAA and PDA, from 0–100 μg TAA/ml of sample assay solution. High concentrations of ascorbic acid in the assay solution were reduced to the range suggested in AOAC 39.061 by dilution with reagents in the analyzing stream. The range of linear response for TAA concentration was established for the concentration of reagents used in the automated procedure. Blank readings determined the concentration of interfering fluorescent compounds present in the assay mixture. DAA and/or TAA concentrations were calculated from a standard curve or from the following equation:

$$\frac{\text{Fluorescence sample} - \text{blank}}{\text{Fluorescence 1 } \mu\text{g vitamin C/ml}} \times \frac{\text{dilution factor}}{\text{wt. of sample}} \times 100 = \mu\text{gC}/100\text{g}$$

RAA values were calculated from determinations of TAA minus DAA.

RESULTS & DISCUSSION

AUTOMATION of the PDA microfluorometric assay for DAA and TAA required a modification of the oxidation procedure for the conversion of RAA to DAA because of the incompatibility of Norit with the continuous flow system. This required that the modified oxidation procedure be confirmed using the manual PDA technique before adapting the assay to the Auto-Analyzer system.

Data plotted in Figure 2 show the linearity of fluorescent response with ascorbic acid standards from 0.0–0.71 μg DAA/ml in the final reaction mixture, using the modified manual PDA assay. Other parameters that were investigated in the modified oxidation procedure were the effect of DCP and thiourea on the linearity of the assay. As shown by data in Figure 3, reduction of excess DCP in the assay mixture following the oxidation of RAA was required. Loss of linear response was due to the interference of unreduced DCP, which was converted to its salt upon the addition of 50% NaOAc. The addition of 0.03 ml of 3.0% thiourea was sufficient to destroy the excess DCP. A comparison of the data for manual oxidation of RAA using Norit (AOAC 39.061) and the DCP-thiourea procedure showed that thiourea did not affect the linearity of the determination in the range of 0–10 μg RAA/ml of the final reaction mixture (Fig. 4).

The equivalence of the DCP-thiourea oxidation to Norit enabled the PDA assay for DAA and TAA to be adapted to continuous flow analysis.

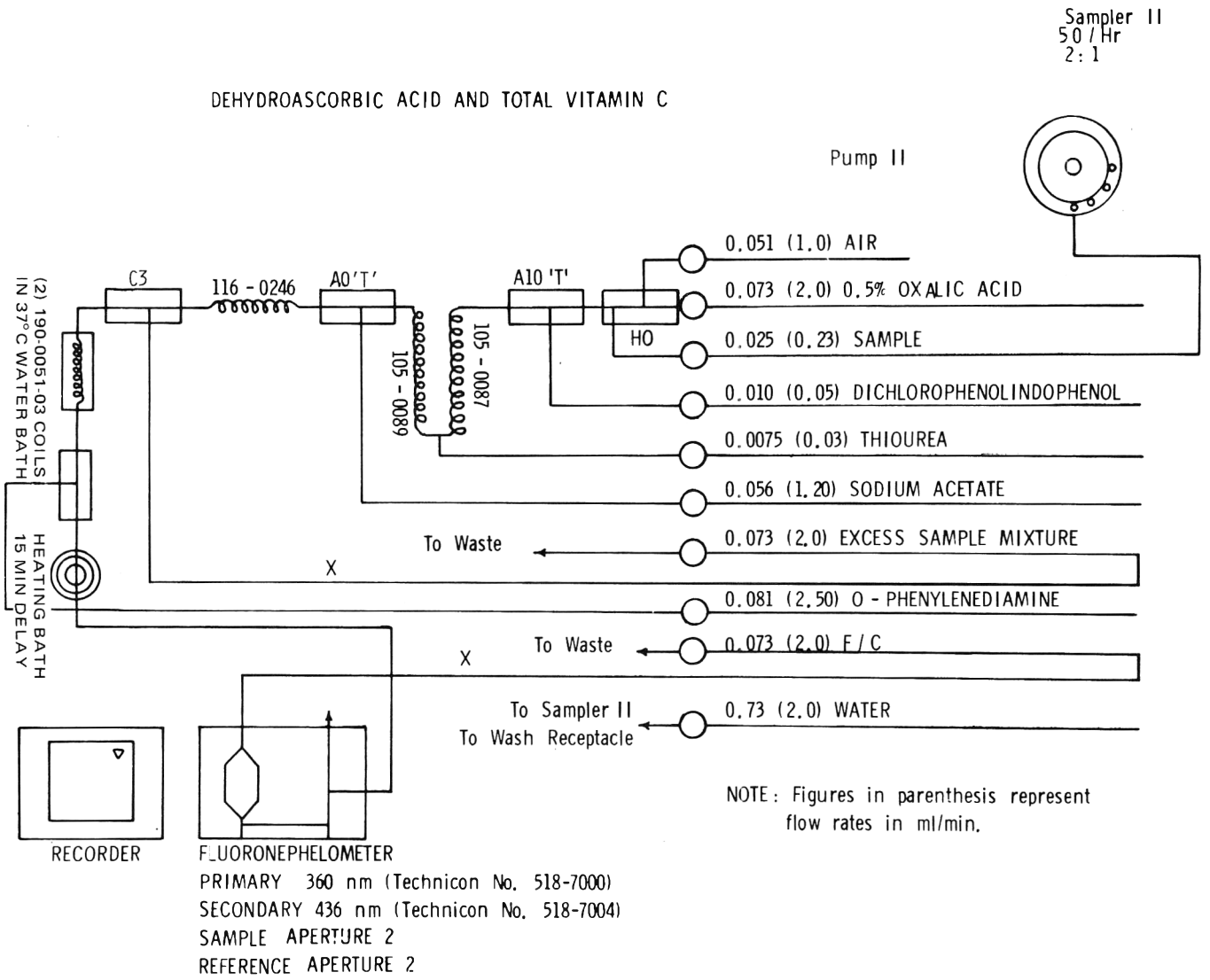

Fig. 1—Continuous flow analytical system for determining dehydroascorbic acid and total ascorbic acid in foods.

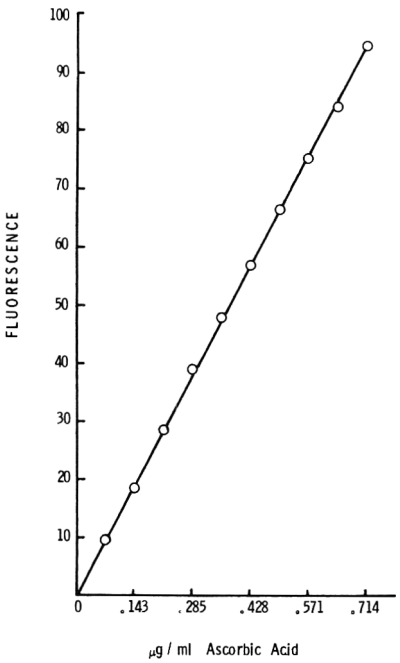

Fig. 2–Linearity of fluorescent response using manual DCP-thiourea oxidation of RAA over a concentration range of 0–0.714 μg RAA/ml in the final reaction mixture.

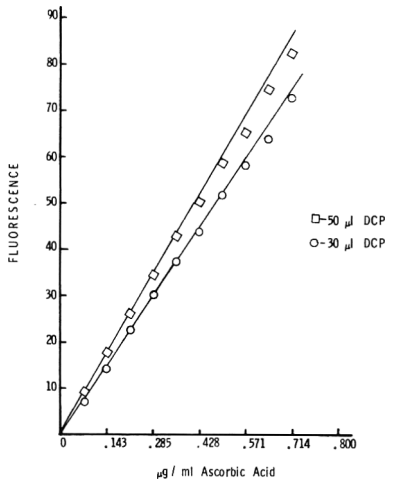

Fig. 3–Linearity of fluorescent response using manual DCP oxidation without thiourea over a concentration range of 0–0.714 μg RAA/ml in the final reaction mixture.

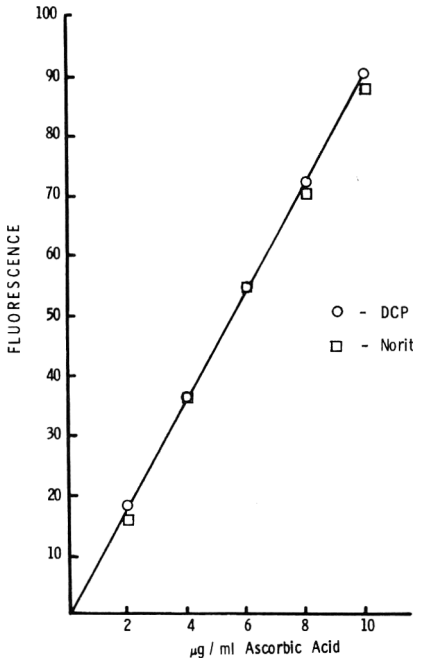

Fig. 4–Correlation between linear fluorescent response oxidized to RAA results by DAA using DCP-thiourea oxidation and by Norit (AOAC 39.061).

Dehydroascorbic acid–Automated

DAA levels in samples were normally determined prior to TAA. Thus, ascorbic acid standards were oxidized with DCP-thiourea manually and DAA determined as described in the experimental procedure.

The manual PDA procedure required a reaction time of 30 min at room temperature for the condensation reaction following the addition of PDA to the reaction mixture (AOAC 39.061). This represented a significant time delay in developing a rapid, continuous flow analytical procedure. In an effort to minimize the time for PDA to condense with DAA the effect of temperature was studied. Experimental data shown in Table 1 indicate that analysis time could be decreased without affecting the sensitivity of the assay procedure by passing the assay mixture containing PDA through a 15 min time delay coil at 40°C. Fluorescence was then determined as previously described. Holding the reaction mixture containing PDA at temperatures above 40°C resulted in the discoloration of the assay mixture and loss of linearity of fluorescent response to increasing concentrations of DAA. However, no discoloration was apparent in the assay mixtures determined manually and treated at 40°C.

An additional decrease in analysis time was accomplished during the determination of DAA blanks by substituting a time delay coil of approximately 8 min at 37°C for the 15 min at room temperature described in AOAC 39.061. Comparison of sample and standard blanks determined by the modified

Table 1–Effect of temperature on fluorescence after addition of o-PDA

Temp (°C)	Time (min)	Fluorescence	
Ambient	30	47.8	
40	15	48.8	
50	15	54.0	degradation
60	15	62.5	to yellow
>65	15	off scale	

Table 2–Comparison of automated PDA procedure with AOAC microfluorometric procedure for TAA

	Autoanalyzer–PDA	Manual[a]–PDA
Sample	TAA (μg/ml)	TAA (μg/ml)
Orange juice	394	404
Cereal (corn)	630	650
Cereal (corn)	3090	3416
Milk	53	52
Tomatoes (canned)	179	134

[a] AOAC 39.061

Table 3–Comparison of automated PDA procedure with AOAC DCP titration procedure for RAA in orange juice

Sample	TAA (μg/ml)	Recovery (%)	DAA (μg/ml)	RAA (TAA-DAA)	Manual RAA (μg/ml)
1	457	102	82	374	382
2	446	102	87	358	359
3	459	98	77	382	379
4	437	100	93	344	347

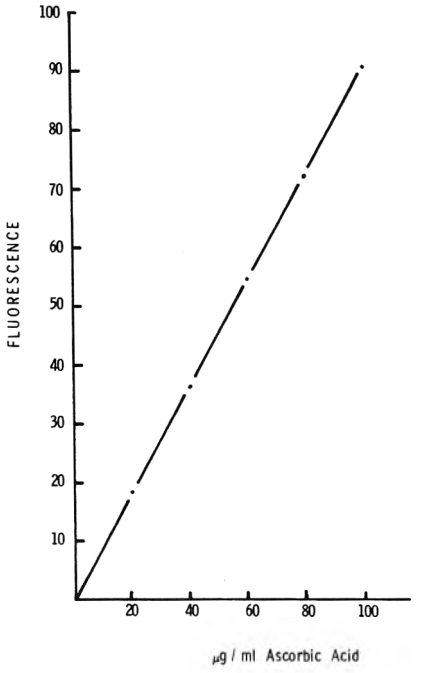

Fig. 5–Linearity of response using automated fluorometric assay for total ascorbic acid over a concentration range of 0–100 µg ascorbic acid/ml of 6% meta-phosphoric acid assay extract.

procedure, and the standard AOAC procedure showed no difference in fluorometric response.

Total ascorbic acid

Following treatment with DCP-thiourea to oxidize RAA to DAA, TAA values and blanks were determined in standards and samples as described for DAA. The validity of this procedure was previously discussed (c.f. Fig. 4).

The precision of the automated fluorometric assay for TAA is shown by a plot of mean fluorescent response for 13 separate sets of ascorbic acid standards selected at random (Fig. 5). The standard deviations for these mean values ranged from ± 0.685 to ± 1.01.

Ascorbic acid in foods

The accuracy of the automated PDA procedure for the determination of DAA and TAA in foods was determined by comparing the analytical results obtained by the continuous flow PDA procedure with the AOAC manual PDA technique. Mean TAA values determined for orange juice, milk, dry breakfast cereal and canned tomatoes during various experimental studies indicated good agreement between the two methods (Table 2). The reason for the higher TAA values for canned tomatoes using the continuous flow procedure is not known.

Additional analyses for vitamin C content in orange juice indicated that the continuous flow fluorometric procedure could be used to obtain RAA values. Table 3 shows the mean RAA values for four different orange juice samples obtained by the DCP manual titration (AOAC 39.055) and the automated PDA procedure. Comparison of these two methods showed less than 1% difference in RAA values.

The results of this study indicate that the continuous flow PDA procedure is a simple, rapid and quantitative method for the determination of TAA and DAA. The automated procedure allowed for the analysis of 60–80 samples (plus blanks) per day for TAA and DAA, which represents a substantial decrease in analytical time without affecting the accuracy and precision of the AOAC PDA assay.

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EFFECT OF CALCIUM, MAGNESIUM AND WHEY PROTEINS ON THE ACTIVITY OF β-GALACTOSIDASE (A. niger) IMMOBILIZED ON COLLAGEN

INTRODUCTION

THE APPLICATION of an immobilized enzyme for the hydrolysis of lactose in various milk products has been the subject of several recent publications. Woychik and Wondolowski (1973) reported the hydrolysis of acid whey by a fungal lactase bound to glass beads. Okos and Harper (1974) reported on the activity of glass-bound lactase in acid whey, as did Wierzbicki et al. (1973, 1974). Olson and Stanley (1973) reported on the hydrolysis of reconstituted nonfat dry milk by lactase bound to a phenol-formaldehyde resin. Giacin et al. (1974) evaluated the hydrolysis of acid whey by collagen-bound lactase.

A comparison of the performance of bound lactase preparations showed an inhibition in activity in acid whey as compared to aqueous lactose (Okos and Harper, 1974; Wierzbicki et al., 1974; Woychik and Wondolowski, 1973; Giacin et al., 1974). The cause of this inhibition is not fully understood, although several rationales have been proposed (Woychik and Wondolowski, 1973; Okos and Harper, 1974; Giacin et al., 1974).

In an attempt to elucidate the mechanism of this inhibition, the effect of specific metal ions and whey protein on the activity of collagen-bound lactase has been investigated. The present paper describes the results of this study.

EXPERIMENTAL

Reagents

A sample of the β-galactosidase of *A. niger* (Lactase LP) was donated by the Wallerstein Corp., Morton Grove, Ill. and was used without further purification. The enzyme was bound to cattle-hide collagen obtained from the USDA Eastern Regional Research Center.

Analytical grade reagents and distilled water were used throughout this work. Other materials used in this work were glucose oxidase, peroxidase, and 3,3′-dimethoxybenzidine, Sigma Chemical Company; all other chemicals, Fisher Scientific.

All lactose solutions (w/v) were prepared by dissolution of α-lactose powder in 0.1M sodium acetate buffer, pH 4.0.

Whey was obtained from the cottage cheese processing plant of Lehigh Valley Dairy, Allentown, Pa. The whey was deproteinized by ultrafiltration using an Amicon Model 402 ultrafiltration apparatus. A pm-10 membrane (cutoff limit, 10,000 molecular weight) was employed.

Preparation of the active membrane

5g of freeze-dried hide collagen was suspended in 480 ml of distilled water, pH 3.5 (Hockstadt et al., 1960). The final solids content was between 0.8–1.0%. The pH was adjusted by the addition of 4N HCl.

The dispersion was homogenized with a blender until viscous. Lactase LP (1g) was then added and the dispersion was again homogenized. Degassing was done under vacuo.

The dispersion was cast on a plastic sheet (Mylar®) and air dried at room temperature for 48 hr. After drying, the membrane was peeled from the Mylar, washed with 10 liters of distilled water and dried at room temperature. The membrane thickness was 3.8 mil, measured with a micrometer (Testing Machines, Inc. Model 549).

In some experiments, the dried film was tanned with glutaraldehyde. The film was soaked for 3 min in 0.2% glutaraldehyde solution, pH 7.6. After tanning, the film was washed with 10 liters distilled water to remove residual glutaraldehyde.

Preparation of module

Dried films were layered onto a supporting material of polyethylene (Vexar®) and coiled about a central core element to form a module. This cartridge was then fitted into a plastic cylinder to form a reactor.

Analytical

Soluble and bound lactase activity was determined in 5% lactose (w/v) and acid whey as substrate.

Soluble lactase activity was determined by pipetting a 1.0 ml aliquot of enzyme solution (5 mg/ml, in 0.1M sodium acetate buffer, pH 4.0) into 200 ml of substrate solution maintained at 37°C, and removing aliquots for assay at different time intervals. The reaction was stopped by heating the aliquots at 100°C for 2–3 min, after which they were analyzed for glucose. Glucose concentration was determined by the glucose oxidase-chromogen procedure supplied by Sigma Chemical Co. A unit of activity was defined as μmole of glucose produced/min/mg of enzyme.

Collagen-bound lactase activity was assessed by utilizing the complex in a convenient spiral reactor configuration (Giacin et al., 1974) as a batch recycle reactor. Prior to hydrolysis of lactose in whey, the reactor was stabilized to steady limit activity with 5% lactose (w/v). The stable limit activity of the biocatalytic reactor was established by determining the activity as a function of repeat contacts (runs) until the activity of the reactor remained constant over a number of reactor volume replacements (Giacin et al., 1974). In a typical run, the substrate volume, containing 200 ml of substrate solution, was maintained at 37°C. The biocatalytic reactor was immersed in a 37°C constant-temperature water batch and the substrate was recirculated through the reactor for 10 min, at a flow rate of 100 ml/min by a peristaltic pump. The change in glucose concentration of the reservoir was followed by removing an aliquot (usually 0.2 ml) for assay as a function of time. This aliquot was diluted to 0.5 ml by the addition of 0.3 ml of pH 4.0 sodium acetate buffer before assay. To determine the concentration of glucose in the sample, 2 ml of Sigma glucostat mixture was added followed by 2.5 ml of 6N HCl after 15 min. Units of activity were defined as μmole of glucose produced/min/g of complex.

For both soluble and bound enzyme, initial rates were calculated from a minimum of six samples taken at different times, within the first 10 min of reaction.

Analysis for magnesium and calcium ion concentration in acid whey was conducted using an atomic absorption spectrophotometer (Perkin Elmer Model No. 290). Protein determination was carried out by a modified biuret method (Koch and Putnam, 1971).

RESULTS & DISCUSSION

A NUMBER of studies have been reported describing the effect of specific metal ions on the activity of soluble lactase preparations (Reithel and Kim, 1966; Hill and Huber, 1971; Strom et al., 1971) and on bound microbial β-galactosidase (Hustad et al , 1973). Hustad et al. (1973) reported a 96% increase in activity for bound lactase (*E. coli* K-12) when magnesium ion (10^{-3} M) was added to the substrate solution. The work of Hill and Huber (1971) showed the effect of metal ions (Na^+, Mg^{++}) on soluble enzyme activity to be a function of ion concentration.

It has been reported (Wallerstein Co. Technical Bulletin, 1970) that at a level of 10^{-4} M magnesium ion was without detectable effect on the activity of Lactase LP. Activity was determined using o-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. Since the concentration of magnesium and calcium ions in acid whey is considerably higher (Table 1) the effect of these cations on the activity of the bound lactase was investigated. The effect of whey proteins was also considered.

Effect of magnesium ions on an untanned membrane

The effect of magnesium ions on the activity of an un-

tanned collagen-lactase complex is shown in Figure 1. The activity of the immobilized enzyme is plotted as a function of repeat contacts (runs).

As shown in Figure 1, the biocatalytic reactor attained steady-state activity after five runs. The reactor was subsequently contacted with 0.01M magnesium citrate solution under the standard experimental conditions and experienced a partial irreversible inhibition upon exposure to the magnesium ions. This is illustrated graphically in Figure 1. This inhibition is of the same order of magnitude as that recorded when an untanned film was contacted with ultrafiltered acid whey, being 41% and 33%, respectively (Giacin et al., 1974). When 5% lactose dissolved in buffered solutions containing 10^{-4}M and 10^{-6}M Mg^{++} ion, respectively, was contacted with the biocatalytic reactor, no further inhibitory effect was observed (Fig. 1). Thus, it can be concluded that magnesium ions, at levels of 0.01M and above, have a deleterious effect on the activity of untanned collagen-lactase complexes.

Effect of magnesium and calcium ions on a tanned membrane

In the course of this study, it was determined that tanning the collagen-lactase membrane with glutaraldehyde afforded a tenfold increase in steady-state activity as compared to an untanned film. The steady-state activities in 5% lactose were 130 and 13 μmole glucose/min/g complex, respectively.

The effect of magnesium and calcium ions on the activity of a tanned collagen-lactase complex was evaluated by contacting a reactor with 5% lactose solutions containing the same molar concentrations of Mg^{++} and Ca^{++} ions as found in acid whey. In these experiments, the reactors were brought to steady-state activity with 5% lactose as substrate, prior to contacting with Mg^{++} and Ca^{++} ions. The results (Table 2) show that magnesium and calcium ions had no effect on the activity of the biocatalytic reactors. A possible explanation for these results is that the tanning process alters the structure of the complex, and therefore its diffusional properties (Lieberman et al., 1972), to the extent that the cations (Mg^{++}, Ca^{++}) cannot participate in an inhibitory manner.

Since inhibition in acid whey was observed for biocatalytic reactors constructed from both tanned and untanned collagen-lactase membranes (Table 3), it is apparent that other mechanisms of inhibition are operative in these systems, which do not involve the Mg^{++} and Ca^{++} ions.

Effect of whey proteins on bound lactase

The reuseability of a biocatalytic reactor constructed from a glutaraldehyde-tanned membrane over a number of reactor contracts (runs) with ultrafiltered (UF) acid whey is shown in Figure 2. The activity of the reactor is plotted as a function of repeat contacts (runs) with UF acid whey and lactose. The reactor was washed with two liters of water between runs.

As shown in Figure 2, the glutaraldehyde tanned membrane experienced a 35% decrease in activity upon initial contacting with UF acid whey. Subsequent contacting with 5% lactose resulted in the partial recovery of activity. However, a gradual decline in activity (5% lactose as substrate) approaching the activity level in UF acid whey was observed upon prolonged contacting with the whey substrate. These results were in contrast to the activity profile of an untanned collagen-lactase membrane which showed a partial, irreversible inhibition upon exposure to UF acid whey (Giacin et al., 1974).

The catalytic potency of the fungal lactase in the soluble and bound state was determined with UF acid whey and acid whey as substrate to evaluate the effect of whey proteins on enzyme activity (Table 3). The activity of the glutaraldehyde tanned membrane in UF acid whey was approximately 65% of

Table 1–Proximate analysis of acid whey

Constituent	Concentration	
	Jakubowski et al.[a]	Feeley et al. (1972)
pH	4.4	–
Titratable acidity	0.48	–
Total solids	7.06%	4.8%
Protein	0.90%	0.85%
Lactose	4.75%	–
Ash	0.62%	–
Magnesium	5.24×10^{-3} M	3.7×10^{-3} M
Calcium	2.35×10^{-2} M	2.44×10^{-2} M

[a] Proximate analysis of acid whey evaluated in these studies

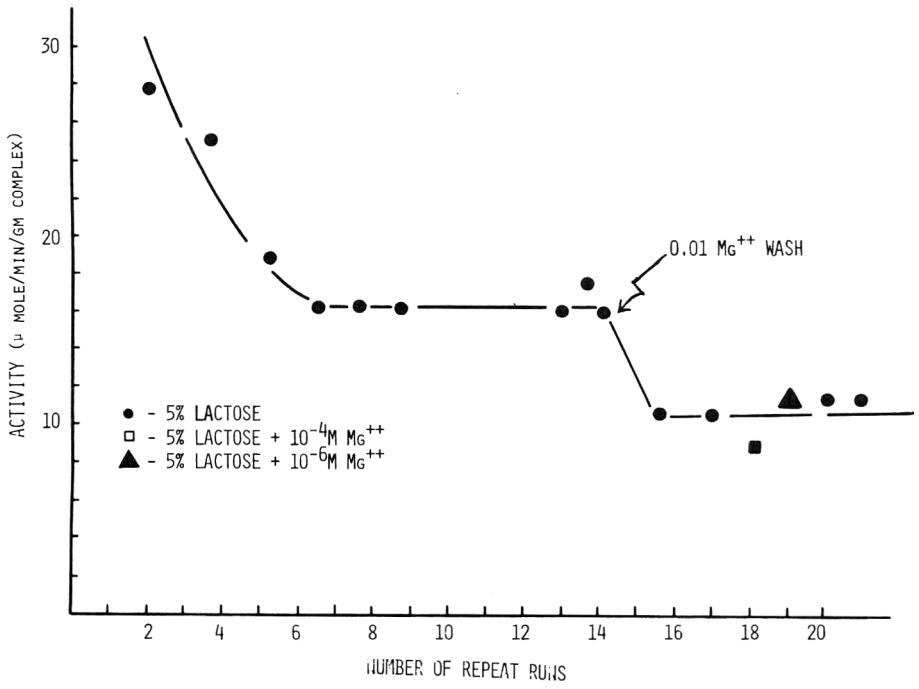

Fig. 1–The effect of magnesium ion on the activity of a biocatalytic reactor fabricated from an untanned collagen-lactase membrane.

Table 2—Influence of magnesium and calcium on the activities of glutaraldehyde-tanned catalytic modules

Run no.	Substrate	Activity[a]	Relative activity (%)
	Reactor A		
1	5% Lactose (L)	131	
2	L	131	100
3	L + 5.24 X 10^{-3} M Mg	136	109
4	L + 5.24 X 10^{-3} M Mg	125	92
5	L	125	92
6	L	112	82
	Reactor B		
1	L	84	
2	L	87	
3	L	84	100
4	L + 2.36 X 10^{-2} M Ca	82	97.5
5	L + 2.36 X 10^{-2} M Ca	–	–
6	L	96	117
7	L	87	107

[a] Activity: μmoles glucose X min^{-1} X grams $complex^{-1}$

Table 3—Effect of whey proteins on β-galactosidase activity

Enzyme state		% Relative activity	% Inhibition
Soluble[a]	5% Lactose	100	–
	Acid Whey	44.4	55.6
	Ultrafiltered Acid Whey	43.8	56.2
Bound[b]	5% Lactose	100.0	–
Untanned	Acid Whey	59.0	41.0
Membrane	Ultrafiltered Acid Whey	69.7	30.3
Bound[b]	5% Lactose	100	–
Tanned	Acid Whey	50.0	50.0
Membrane	Ultrafiltered Acid Whey	65.0	35.0

[a] Assay conditions: 37°C, pH = 4.5, 200 ml substrate
[b] Assay conditions: 37°C, pH = 4.5, 200 ml substrate, recycle at 100 ml/min

the activity in lactose, while only 50% of the initial activity (5% lactose) was found with acid whey as substrate. The catalytic potency of the untanned membrane showed similar responses.

The results of these studies indicate that whey proteins of molecular weight above 10,000 are, in part, responsible for the observed inhibition of collagen-bound lactase activity for both tanned and untanned membranes. While these results represent preliminary findings, the data are suggestive of the general inhibitory action of whey proteins. The observed inhibition of the collagen-lactase preparations in UF acid whey (Table 3), may then be attributed, in part, to low molecular weight proteins (<10,000) not removed by ultrafiltration. The effect of whey proteins on the activity of bound lactase and a correlation with molecular weight warrant further investigation. It is interesting to note that when the enzyme is in the soluble state the extent of inhibition is not affected by removal of low molecular weight proteins (Table 3).

While whey proteins appear to be responsible, in part, for the inhibitory action of acid whey on collagen-bound lactase, the mechanism of inhibition of the tanned lactase complex in UF acid whey (Fig. 2) is not fully understood. However, one possible explanation is the modification of the diffusional properties of the film resulting from cross-linking with glutaraldehyde (Lieberman et al., 1972).

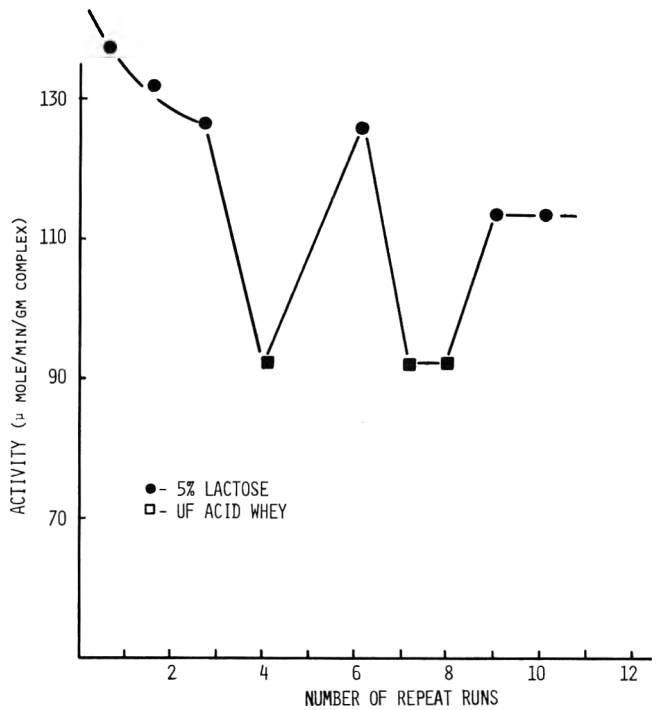

Fig. 2—Reuseability of a biocatalytic reactor fabricated from a tanned collagen-lactase membrane utilizing lactose (5%) and ultrafiltered acid whey as substrate.

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FUNGAL FERMENTATION OF PEANUT FLOUR: EFFECTS ON CHEMICAL COMPOSITION AND NUTRITIVE VALUE

INTRODUCTION

THE PREPARATION and consumption of fermented foods are common practices in Oriental and Indonesian lands. Ontjom is a fermented peanut press cake very popular in Western Java (van Veen et al., 1968). In the preparation of ontjom, oil is first extracted from peanuts and, after soaking and steaming, the press cake is mixed with ontjom from a previous batch and packed in banana or other suitable leaves to facilitate opening of the packages for aeration and cooling if necessary. *Neurospora sitophila* is primarily used to produce a red or orange ontjom while *Rhizopus oligosporus* is used less often to produce a white ontjom (Hesseltine, 1965; van Veen et al., 1968; van Veen and Steinkraus, 1970).

Fermentation of grains and oilseeds is disputably said to result in increased nutritional value and wholesomeness over the starting material (Hackler et al., 1964; Smith et al., 1964; Rajalakshmi and Vanaja, 1967; Wang et al., 1968; van Veen and Steinkraus, 1970; Rao and Rao, 1972). Fermentation also contributes to the masking or destroying of undesirable odors and flavors, while imparting desirable flavors to the finished product. Most importantly, fermentation is reported to enhance the digestibility of starting materials (Hesseltine and Wang, 1970) by breaking down complex protein structures to peptides of varying lengths and free amino acids. Changes in vitamin levels may also occur as a result of fermentation (Roelofsen and Talens, 1964; Rajalakshmi and Vanaja, 1967; van Veen et al., 1968; van Veen and Steinkraus, 1970). Numerous studies involving the fermentation of soybeans have been reported; however, less information is available relating chemical and nutritional property changes resulting from peanut fermentation.

The primary objective of this study was to determine changes taking place in defatted peanut flour as a result of fungal fermentation. The molds chosen for the study are already employed in traditional and commercial oilseed fermentations. Changes in proximate composition were investigated to lend insight into chemical alterations which may occur during the fermentation of a peanut flour substrate. Other parameters examined included amino acid analyses, fatty acid profiles and vitamin levels, namely, riboflavin, thiamin, niacin and pantothenate. Protein efficiency ratios (PER) and fatty liver infiltration were determined in order to assess effects of fermentation on the nutritional value of peanut flour.

EXPERIMENTAL

Fungal fermentation

Substrate. 40g of solvent-defatted peanut flour (SDPF, Gold Kist, Inc., Graceville, Fla.) were supplemented with tapioca (0.5% by weight), NaCl (0.8%) and citric acid (2.0%). The mixture was combined with 160 ml tap water in a cotton-stoppered 500-ml Erlenmeyer flask and sterilized at 15 lb pressure (121°C) for 15 min. The resultant substrate acquired a consistency similar to a thick, lumpy pudding.

Cultures. The fungal strains used in this study were obtained from Dr. C.W. Hesseltine (USDA, ARS, Peoria, Ill.) and were identified as *Actinomucor elegans* NRRL 3104, *Aspergillus oryzae* NRRL 1988, *Mucor hiemalis* NRRL 3103, *Neurospora sitophila* NRRL 2884 and *Rhizopus oligosporus* NRRL 2710.

Potato dextrose agar (PDA) slants were inoculated with the appropriate fungus and incubated for 4–6 days at 31°C. The resultant spores, along with some hyphae, were harvested by washing the PDA slants with sterile 0.001% Tween-80 solution. 20 ml of diluted suspensions of each of the five fungi served as inocula. 48 samples were fermented by each fungus.

Fermentation. The inoculated substrates were placed on a gyratory shaker (New Brunswick Scientific Company, New Brunswick, N.J.) and fermented for 4 days at 28°C while rotating at a medium speed. The 48 fermented samples for each fungus were then combined, lyophilized, pulverized through a Wiley Mill (40 mesh screen), and stored in sealed glass containers at 4°C prior to laboratory examination.

The experimental design included eight test materials:

Control 1	SDPF as received from Gold Kist, Inc.
Control 2	SDPF supplemented with tapioca, citric acid, NaCl, water; autoclaved, immediately frozen without incubation, lyophilized and pulverized
Control 3	SDPF supplemented with tapioca, citric acid, NaCl, water; autoclaved, incubated at 28°C for 4 days, frozen, lyophilized and pulverized
Ferments	Five (one composite sample of each of five fungal strains); SDPF supplemented with tapioca, citric acid, NaCl, water; autoclaved, inoculated and fermented at 28°C for 4 days, frozen, lyophilized and pulverized

Chemical analyses

Proximate composition. Moisture, ash, pH and Kjeldahl nitrogen were determined in triplicate (AOAC, 1970). Crude protein was calculated using the appropriate conversion factor of 5.46 (Breese, 1931). Crude fiber content was determined in duplicate by a modified method from Triebold and Aurand (1963). Hydrolysates were filtered through Whatman filter paper (No. 541) with the aid of a suction flask rather than through the recommended filtering cloth. Total lipids were determined in triplicate by a 12-hr diethyl ether extraction procedure using a Goldfisch extractor. Carbohydrates were calculated by difference.

Amino acid analysis. Individual samples were prepared in triplicate for amino acid analysis by hydrolyzing in 6N HCl (2 mg crude protein/ml acid) under nitrogen for 24 hr at 110°C. Alkaline hydrolysates were prepared for tryptophan determinations according to a method described by Hugli and Moore (1972). 40-ml samples were hydrolyzed under nitrogen in 6 ml of 4.2N NaOH at 110°C for 24 hr. One or two drops of 0.001% Antifoam B (Technicon Instruments Corporation, Tarrytown, N.Y.) were added to each solution to prevent excessive foaming during the oxygen evacuation step. After hydrolysis, the solutions were adjusted to pH 4.25 with cold 6N HCl, brought to volume with distilled water, and centrifuged. Amino acids were determined according to the method of Spackman et al. (1958) on a Durrum Auto Analyzer Model D-500 equipped with a Digital PDP 8 m computer (Durrum Instrument Co., Palo Alto, Calif.) as described by Moore (1972).

The percent protein was calculated as g of total amino acids per 100g of dry matter (Coleman, 1972).

Fatty acid identification. Fatty acid methyl esters were prepared in triplicate from extracted lipids with a 2:1 (v/v) mixture of methanol benzene containing 3% sulfuric acid as described by Worthington et al. (1972). Extent of esterification was checked on 0.5 mm silica gel G thin-layer plates. After developing in a solvent system of hexane-diethyl ether-acetic acid 90:10:1 (v/v/v), the plates were sprayed with a dye

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solution containing rhodamine B and 2,7-dichloroflurescein (Jones et al., 1966) and observed under ultraviolet light.

Fatty acid methyl esters were analyzed using a MicroTek Model 220 gas liquid chromatograph equipped with a dual flame ionization detector and an Infotronics electronic integrator. Two 1.85 m × 4 mm i.d. glass columns packed with 70/80 mesh chromosorb W (AW)(DMCS) and coated with either 10% ECNSS-S or 3% OV-225 were used. The helium flow rate was 100 ml/min and the ECNSS-S and OV-225 columns were run isothermally at 195 and 200°C, respectively. Peak identifications were made by comparison of retention times on each column with standards (Hormel Inst., Austin, Minn.) run under identical operating conditions. Respective peak areas were then converted to relative percentages.

Nutritional evaluation

Vitamin determinations. Pantothenate and niacin (nicotinic acid and nicotinamide) were determined microbiologically as described in *Difco Supplementary Literature* (Anonymous, 1972) using Bacto-pantothenate AOAC medium and Bacto-niacin assay medium, respectively. Turbidimetric measurements were made in both vitamin assay procedures, using a Bausch and Lomb Spectronic 20 spectrophotometer.

The procedure used for thiamin quantitation was adapted from Conner and Straub's thiochrome method (1941) and riboflavin was determined by a modification of the method of Peterson et al. (1943). These revised methods are described in "Southern Cooperative Series Bulletin No. 10" (Anonymous, 1951).

Protein efficiency ratios. Weanling Sprague-Dawley male rats (Charles River Breeding Laboratories, Wilmington, Mass.) were fed eight different diets, each containing 15.5% protein as calculated from previous moisture and protein content (amino acid analysis) determinations. Test materials used to supply protein were vitamin-free casein (Nutritional Biochemical Corp., Cleveland, Ohio), SDPF as received from Gold Kist, Inc. (Control 1), a mixture of Controls 2 and 3 (1:1, w/w), and the five fermented samples.

In addition to the 15.5 protein supplied by the test material, each diet contained, in percent: salt mixture P-H, 4.0; vitamin fortification mixture, 2.2; dextrin, 40; and cellulose, 1.5 (all obtained from Nutritional Biochemicals Corp., Cleveland, Ohio); and corn oil, 5.0. The casein diet was supplemented with 0.2% l-methionine while the remaining diets were supplemented with 0.25% l-lysine, 0.4% methionine, and 0.13% l-tryptophan. Sucrose was added to 100%.

Each test group consisted of eight rats fed for three consecutive weeks after a 4-day adjustment period during which they were fed a commercial diet. The rats were housed individually, receiving diets and deionized water ad libitum.

Liver analysis. Upon termination of PER studies, the animals were anesthetized with ether and sacrificed by exsanguination.

Liver was removed and freeze-dried. Two composite liver samples from animals on each diet were obtained by combining 0.5g of liver from the even-numbered rats and 0.5g from odd-numbered rats, respectively. Lipid was extracted from the samples by the Bligh and Dyer (1959) procedure, including a second extraction with chloroform for complete removal of phospholipids. After mixing, samples were centrifuged for 15 min at 13,200 × G at 0 to 5°C. The chloroform extract was filtered through anhydrous sodium sulfate which had previously been wet with chloroform. Chloroform was evaporated and the lipid was taken up in hexane. An aliquot was transferred to a tared vial and the lipid content was measured gravimetrically after the solvent was evaporated.

Toxicological evaluation. Chloroform-ethanol extracts of fermented and nonfermented samples and PDA slant cultures of the five fungi included in the investigation were sent to Dr. N.D. Davis at Auburn University Agricultural Experiment Station, Auburn, Ala., for examination of potential toxic characteristics. Evaluations were made using brine shrimp and chick embryo bioassays as modified from methods reported by Harwig and Scott (1971) and Verrett et al. (1964), respectively.

Statistical analyses. Statistical analyses were by analysis of variance and sample means were compared using Duncan's (1955) new multiple range test at the 5% level of significance ($P \leq 0.05$).

RESULTS & DISCUSSION

Fungal fermentation

After autoclaving, the SDPF substrate took on the appearance of a thick porridge. The consistency of the substrate and the low oxygen tension caused by autoclaving may have hindered hyphal penetration and development as evidenced by growth on the surface of the medium in the early stages of fermentation. However, during the later stages of fermentation, mycelial development was apparent throughout the medium. The peanut flour substrate exhibited some of the problems associated with solid state fermentations as enumerated by Hesseltine (1972). Fermentation variables, such as the spore inocula, moisture levels, substrate size, vessel shape, aeration and time were not extensively investigated. Therefore, it cannot be stated that optimal conditions were provided for studying various changes during fermentation of SDPF. It should also be noted that the ferments analyzed in this study were semi-solid in consistency, unlike the more solid texture associated with ontjom.

Preliminary experiments indicated that citric acid, which lowered the acidity from pH 6.3 to 5.0, and tapioca starch enhanced the growth of fungi selected for examination in this study (Beuchat and Worthington, 1974). A carbohydrate source such as tapioca, potato, or potato peels, may be used in the preparation of ontjom and is reported to promote growth of *N. sitophila* on peanut press cake acidified to a pH of 4.5–5.5 (van Veen et al., 1968). Hesseltine et al. (1967) also reported tapicca to promote good growth of *Rhizopus* spp. on peanuts. Added sodium chloride apparently enhanced maximal growth of the five fungi. Apparently sodium chloride, as well as some other ionizable salts, promotes the release of mycelium-bound proteinase (Wang, 1967) and therefore increases proteinase activity. Salt may exert an osmotic or ionic effect on the fungal mycelia, whereby extracellular enzymes are more readily freed to act upon substrate constituents (Beuchat and Worthington, 1974; Wang and Hesseltine, 1970).

Chemical analyses

Proximate composition. The results of proximate analysis of control and fermented SDPF are shown in Table 1. It should be noted that reported values are averages of at least duplicate analyses and are based on the dry weight of the samples. The percent lipid in the ferments was relatively unchanged from that of the autoclaved controls. The difference in percent lipid between Control 1 and Controls 2 and 3 may be attributed to the presence of nonlipid diethyl ether-extractable material in Control 1, which is apparently tied up, volatilized, or physically altered in some manner during the autoclaving process so that it is no longer extractable. Crude fiber increased slightly, except in the case of *N. sitophila*. Increases in crude protein, ash and in some cases crude fiber in ferments may be explained by losses of volatiles during fermentation.

Table 1—Proximate composition of nonfermented and fermented peanut flour

	g per 100g freeze-dried sample				
Sample	Ash	Crude fiber	Lipid[a]	Crude protein[b]	Carbohydrate[c]
Control 1	4.3	3.8	1.2	55.6	35.1
Control 2	4.9	4.1	0.5	54.7	35.8
Control 3	5.0	4.1	0.4	54.8	35.7
A. elegans	5.7	4.9	0.3	57.4	31.7
A. oryzae	5.8	5.0	0.6	59.3	29.3
M. hiemalis	5.6	4.7	0.4	57.8	31.5
N. sitophila	6.2	3.9	0.6	64.5	24.8
R. oligosporus	5.7	4.3	0.4	56.4	33.2

[a] Diethyl ether-extractable material
[b] Kjeldahl N × 5.46
[c] By difference

Table 2—Amino acid composition of nonfermented and fermented peanut flour expressed as g per 100g protein

	g per 100g protein							
	Control			Ferment				
Amino acid	1	2	3	Actinomucor elegans	Aspergillus oryzae	Mucor hiemalis	Neurospora sitophila	Rhizopus oligosporus
Aspartic acid	12.59	12.71	12.60	12.71	12.74	12.56	12.74	12.83
Threonine	2.80	2.84	2.81	2.83	2.85	2.90	2.99	2.99
Serine	5.25	5.18	5.09	5.11	4.95	4.97	5.17	5.27
Glutamic acid	21.69	21.73	21.68	21.59	21.68	21.48	21.71	21.19
Proline	4.35	3.75	3.86	3.56	3.43	3.48	2.96	2.97
Glycine	6.13	6.11	6.16	6.50	6.45	6.43	6.38	6.42
Alanine	4.09	4.14	4.12	4.41	4.36	4.31	4.44	4.41
Cysteine	0.26	0.21	0.18	0.21	0.23	0.19	0.21	0.22
Valine	4.35	4.54	4.52	4.71	4.74	4.83	4.73	4.49
Methionine	1.06	1.06	1.05	1.17	1.12	1.24	1.17	1.12
Isoleucine	3.53	3.59	3.73	3.81	3.81	3.86	3.89	3.74
Leucine	6.60	6.81	6.92	6.86	6.73	7.15	7.13	6.92
Tyrosine	3.80	3.78	3.65	3.79	3.77	3.67	3.94	3.85
Phenylalanine	6.19	6.43	6.28	6.31	6.37	6.76	6.41	6.73
Histidine	2.35	2.27	2.38	2.45	2.41	2.51	2.39	2.43
Lysine	3.15	3.12	3.17	3.26	3.21	3.28	3.30	3.37
Arginine	11.77	11.73	11.81	10.83	11.09	10.43	10.49	10.85
Tryptophan	0.34	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Protein[a]	51.05	52.87	50.46	53.09	52.73	51.76	58.17	53.47

[a] g protein per 100g freeze-dried sample as calculated from amino acid analysis

These volatiles are probably low molecular weight acids, carbon dioxide, esters, aldehydes, ketones and other aromatics evolved as by-products of fungal metabolism during the 4 days of fermentation. It would be interesting to analyze these volatiles in order to better understand the origins of aroma development and apparent dry weight losses during fermentation. Our findings coincide with those of Murata et al. (1967), except these authors found a decrease in crude fat after 72 hr tempeh fermentation. Our results reflect the findings of Wang et al. (1968), who showed that an increase in percent protein in fermented wheat and soybeans was due to decreases in other constituents. The changes in proximate composition reported in Table 1 also agree closely with the findings of van Veen et al. (1968), who found increases in crude protein and ash accompanied by decreases in carbohydrate and an essentially unchanged lipid content in ontjom. Worthington and Beuchat (1974) reported that *N. sitophila* essentially metabolized sucrose as well as raffinose and stachyose, the intestinal gas-forming sugars, during the first 21 hr of peanut fermentation. These authors also reported *A. oryzae* to be active in hydrolyzing and utilizing these sugars, while *R. oligosporus* utilized only small amounts of stachyose but did not utilize raffinose or sucrose. *M. hiemalis* was reported to utilize sucrose early in fermentation but failed to use stachyose and raffinose, while *A. elegans* slowly hydrolyzed stachyose, which resulted in an apparent temporary increase in raffinose and sucrose levels. These findings may explain, in part, the decreased carbohydrate levels in fermented SDPF.

Amino acid analysis. Table 2 reports g amino acid per 100g protein. Protein contents were calculated as described by Coleman (1972) and are more representative of the true protein content than are the crude protein figures (Kjeldahl N × 5.46) shown in Table 1. As shown in Table 2, there appears to be a slight decrease in arginine and proline while glycine and alanine seem to be slightly increased due to fungal fermentation. Contrary to reports of decreases in lysine and methionine during long oilseed fermentations (Hesseltine, 1965), slight increases in these two amino acids were noted in this study. Since slight differences in amino acid content do not account for the differences in percent protein between the controls and the ferments, the previously cited explanation of weight losses in the form of nonnitrogenous volatiles during fermentation probably accounts for most increases in percent protein in dried ferments.

Fatty acid identification. The results of fatty acid analysis expressed as relative percentages of total fatty acids are shown in Table 3. *A. oryzae* and *N. sitophila* belong to the class Ascomycetes while *R. oligosporus*, *M. hiemalis* and *A. elegans* belong to the class Phycomycetes. Shaw (1966) reviewed the fatty acids commonly found in mycelial lipid of classes of fungi as well as in individual genera and species. According to Shaw (1966), fungi in the class Ascomycetes are generally low in polyunsaturates except for linoleic acid (18:2), which is usually a major component and may be present in quantities up to 50% of the total fatty acids. This would account for the substantial increase in linoleic acid in the SDPF fermented by *A. oryzae* and *N. sitophila*. Shaw also reported some Ascomycetes to contain α-linolenic (18:3) acid, an acid which appeared in *A. oryzae* and *N. sitophila* ferments. In this study, the Ascomycetes ferments also yielded slight increases in palmitic (16:0) and stearic acids (18:0) accompanied by decreases in oleic (18:1), eicosanoic (20:0), eicosenoic (20:1), docosanoic (22:0) and tetracosanoic (24:0) acids. These data tend to reflect relative percentages of fatty acids reported to be normally present in Ascomycetes mycelia (Shaw, 1966).

In the Phycomycetes ferments, increases in palmitic, stearic, and eicosanoic acids, accompanied by decreases in oleic and tetracosanoic acids, were observed. The remaining fatty acids of SDPF were relatively unchanged by fermentation with *A. elegans*, *M. hiemalis*, and *R. oligosporus*.

Since the initial fatty acid content of the nonfermented SDPF substrate was low, the fatty acid profiles of both Asco-

Table 3–Fatty acid composition of nonfermented and fermented peanut flour

Sample	g per 100g total fatty acids										
	14:0	16:0	18:0	18:1	18:2	18:3	20:0	20:1	22:0	24:0	Other
Control 1	0.90	11.09	2.74	50.59	24.93	ND[a]	1.01	0.98	2.65	1.50	3.21
Control 2	0.40	11.02	2.94	51.09	23.07	ND[a]	1.19	1.13	2.91	2.09	5.16
Control 3	0.20	10.39	3.44	51.59	22.29	ND[a]	1.30	1.44	2.96	2.10	4.29
A. elegans	0.45	12.76	4.72	46.46	24.61	ND[a]	3.98	0.80	2.15	1.50	2.57
A. oryzae	0.15	11.81	4.40	38.05	37.21	0.85	0.44	0.27	1.12	1.28	4.55
M. hiemalis	1.61	21.86	5.79	37.83	21.23	ND[a]	6.67	0.33	1.29	1.31	2.28
N. sitophila	0.27	14.24	3.60	47.97	27.09	0.72	0.47	0.41	1.11	1.11	3.01
R. oligosporus	0.33	14.96	5.11	45.13	24.47	ND[a]	2.88	1.89	2.00	1.59	1.64

[a] None detected

mycetes and Phycomycetes ferments undoubtedly reflect an intermediary effect of the fungal fatty acid composition on the substrate fatty acid profile.

Nutritional evaluation

Vitamin determinations. Table 4 shows the results of vitamin assays. Some thiamin was destroyed during autoclaving. In contrast to reports of thiamin levels being decreased or unchanged (Roelofsen and Talens, 1964; van Veen et al., 1968; van Veen and Steinkraus, 1970) during oilseed and grain fermentation, thiamin increased significantly in all SDPF ferments except *M. hiemalis*. Rajalakshmi and Vanaja (1967) reported increased thiamin in idli, a fermented product prepared from milled rice and dehusked black gram. Riboflavin increased remarkably during SDPF fermentation, trebling in the case of *M. hiemalis* and *N. sitophila*, thus agreeing with previous reports (Roelofsen and Talens, 1964; van Veen et al., 1968; van Veen and Steinkraus, 1970; Rajalakshmi and Vanaja, 1967). Niacin increased significantly in the *N. sitophila* ferment as compared to the controls and to the *A. oryzae* and *A. elegans* ferments. van Veen and Steinkraus (1970) and Roelofsen and Talens (1964) showed an increase in niacin during tempeh fermentation. Pantothenate did not change significantly in SDPF as a result of fungal fermentation.

As cited by Roelofsen and Talens (1964), the reason for increases in thiamin, riboflavin and niacin during fermentation is due to the autotrophicity of the molds with respect to these vitamins (Robbins and Kavanagh, 1942; Peltier and Borchers, 1947). Increases in thiamin and riboflavin as a result of fungal fermentation have important nutritional implications in cultures where fermented foods are part of a predominately rice diet.

Protein efficiency ratios. 15% protein was incorporated into the rat diets because of the rather low biological value of peanut protein. Without supplementation, the calculated amounts of lysine, methionine and tryptophan present in the diet would hardly provide maintenance levels for the rat, even at the 15.5% protein level (National Academy of Sciences, 1972). Therefore, methionine, lysine and tryptophan were added to provide about 75% of the required level for growth (National Academy of Sciences, 1972). This level includes amino acids initially present in the substrates as determined from amino acid analysis. It was thought that if the limiting amino acids were made more available to the rat due to fermentation, better growth would occur.

All eight diets were acceptable to the rats and promoted rapid growth (Table 5). The PER of the controls were not significantly different from that of the ferments. The casein diet resulted in a PER significantly higher than Control 2 + 3 (blend) and the ferments, but was not significantly higher than Control 1. Thus, the nutritive value of peanut flour as measured by PER for rats did not change significantly during fermentation and is consistent with findings reported by van Veen et al. (1968) and van Veen and Steinkraus (1970). However, it is possible that supplementation of the diets to rela-

Table 4–Thiamin, riboflavin, niacin and pantothenate levels in nonfermented and fermented peanut flour

Sample	μg per g freeze-dried sample[a]			
	Thiamin	Riboflavin	Niacin	Pantothenate
Control 1	6.87b	4.16a	99ab	33a
Control 2	6.02a	3.98a	100ab	32a
Control 3	5.83a	3.93a	104ab	33a
A. elegans	7.54c	6.28b	95ab	35a
A. oryzae	10.63f	10.24c	62a	36a
M. hiemalis	7.05bc	18.32d	136bc	36a
N. sitophila	9.94e	10.90d	174c	31a
R. oligosporus	8.63d	7.82b	144bc	37a

[a] Values in a column followed by the same letter are not significantly different at $P \leq 0.05$.

Table 5–Nutritive value of nonfermented and fermented peanut flour

Sample[a]	Avg wt gain (g)[b]	Avg protein intake (g)[b]	PER[c]	Avg liver fat (%)
Casein	170.1	63.5	2.68a	3.7
Control 1	176.7	69.9	2.53ab	4.3
Control 2 + 3[d]	164.6	65.9	2.50b	4.0
A. elegans	162.3	66.7	2.43b	4.3
A. oryzae	159.0	65.8	2.42b	4.1
M. hiemalis	150.6	63.4	2.37b	4.1
N. sitophila	163.6	67.5	2.42b	4.1
R. oligosporus	163.4	67.2	2.44b	4.1

[a] Sample provides 15.5% protein. Casein diet supplemented with 0.2% methionine; remaining diets supplemented with 0.4% methionine, 0.25% lysine, 0.13% tryptophan

[b] Average of eight rats fed over a 3-wk period

[c] Values followed by the same letter are not significantly different at $P \leq 0.05$.

[d] Blend of Controls 2 and 3

tively high levels of lysine, methionine and tryptophan may have masked differences in nutritive values of the SPDF ferments. In addition to PER studies at lower amino acid supplementation levels, further nutritional characterization of fermented and nonfermented SDPF in the form of nitrogen balance and digestibility studies would provide valuable information.

Liver analysis. Since an increase in liver fat, mostly as neutral lipid, is frequently noted in cases of protein deficiency and amino acid imbalance, fat content of the rat livers was determined. Data in Table 5 show little difference among the diets in liver lipid content of the rats used in this study, thus implying an absence of protein deficiency or amino acid imbalance in the diets as used. There is nothing in the literature, to our knowledge, concerning the capacity of ontjom or tempeh to prevent fatty infiltration of the liver.

Toxicological evaluation. Extracts of the fermented samples and the controls were nontoxic to fertile chicken egg embryos (Davis, private communication, 1974). Since the extraction procedure involves relatively small amounts of substrate, subcultures of each fungus were also evaluated using both brine shrimp and chick egg embryo assays. *A. elegans, M. hiemalis* and *N. sitophila* each gave an overall rating of zero on a toxicity scale of zero to six (0—nontoxic; 6—extremely toxic). *A. oryzae* and *R oligosporus* gave readings of three and two, respectively, which is generally interpreted as possibly slightly toxic. In summary, none of the cultures was classified as toxin producers although *A. oryzae* approaches that denotation. As was shown in the rat-feeding studies, there was no apparent toxicity over a 3-wk period.

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FUNCTIONAL PROPERTY CHANGES RESULTING FROM FUNGAL FERMENTATION OF PEANUT FLOUR

INTRODUCTION

PROTEINS are structural components which play a major role in the texture of foods through their functional behavior (Wuhrmann, 1972). However, the suitability of proteins for specific uses, such as food ingredients or additives, is difficult to define in terms of structure, composition and functional properties in the same manner that amino acid content is related to nutritive value (Smith et al., 1959). Desirable physicochemical properties attributed to proteins in product formulation systems which contain other food components, such as fats and carbohydrates, depend in part upon the molecular size and structure of the proteins. Origin and physical treatment of proteins also greatly effect their functionality.

Recently, a tremendous amount of research effort has been made to develop nutritionally and functionally acceptable protein sources for use in fortifying food products. The most extensive efforts have been made in the area of soybean-derived products. These products do not always display the functionality required by food and beverage manufacturers, since flavor, viscosity and solubility characteristics are restricted to those inherent in the soybean (Mattil, 1973). Oilseed products presently described as having the highest potential as possible alternative protein sources to soybean products include peanut flour, degossypolized cottonseed protein concentrate, glandless cottonseed flour and rapeseed, sesame and sunflower meals (Mattil, 1973; Lin et al., 1974).

Commercial utilization of fungal enzymes for tenderizing meat, processing fish and producing bakery items such as breads, crackers, sugar wafers, waffles, pancakes and pizza dough is well established. It is likely that enzymes released by fungi during their growth on a substrate such as peanut flour could have profound effects on the functional properties of that flour. To test this possibility, each of five fungi presently used in traditional oilseed fermentation processes were cultured on solvent defatted peanut flour (SDPF) for 4 days. Functional properties of lyophilized ferments and nonfermented SDPF (controls) were characterized by nitrogen solubility, viscosity, emulsifying capacity, water and oil retention and equilibrium moisture adsorption isotherms.

EXPERIMENTAL

Fermentation

Solvent defatted peanut flour (Gold Kist, Inc., Graceville, Fla.) was fermented with *Actinomucor elegans* NRRL 3104, *Mucor hiemalis* NRRL 3103, *Rhizopus oligosporus* NRRL 2710, *Aspergillus oryzae* NRRL 1988, and *Neurospora sitophila* NRRL 2884 according to the procedures described in a related report (Quinn et al., 1975).

The experimental design included seven samples.

Control 1	SDPF as it was received from Gold Kist, Inc.
Control 2	SDPF supplemented with 0.5% tapioca, 2.0% citric acid, 0.8% NaCl and water; autoclaved 15 min at 121°C, immediately frozen, lyophilized and pulverized.
Ferments	Five (one each of five test cultures); SDPF supplemented with tapioca, citric acid, NaCl and water; autoclaved, inoculated with test culture and fermented at 28°C for 4 days, frozen, lyophilized and pulverized. Each ferment consisted of a composite of 48 separately fermented samples.

Nitrogen solubility profiles

Nitrogen solubility of control and fermented samples was determined over a pH range of 2.0–11.0 by a modification of a procedure reported by Hagenmaier (1972). Each sample was added to distilled water to make a 2% suspension and the desired pH was maintained by the addition of HCl or NaOH over a 45-min period of constant agitation. The suspension was then centrifuged at 10,000 × G for 10 min at room temperature and soluble nitrogen in the supernate was determined using the Kjeldahl procedure (AOAC, 1970). The percentage of soluble nitrogen in each sample was calculated and plotted against corresponding pH values.

Apparent viscosities

A Brookfield (Model RVT) viscometer equipped with a No. 1 spindle was used to measure the apparent viscosity of the soluble fraction of 8% sample suspensions (w/v) in water and in 4% NaCl at 24°C. Each suspension was mixed in a 1-liter Erlenmeyer flask for 2 min and the pH was determined. The suspension was blended in an Omni-mixer at speed eight for 2 min and then at speed ten for 1.5 min followed by centrifugation at 14,600 × G for 20 min. The supernatant fluid was decanted into a 600-ml beaker and the viscosity was measured at 100 rpm after 30 sec rotation. Apparent viscosity in centipoises was reported as the average of four readings. A subsample of the supernatant fluid was analyzed for nitrogen by the Kjeldahl method and soluble nitrogen was reported as mg/ml.

Emulsion capacities

A procedure for determining emulsion capacities was adapted from those described by Carpenter and Saffle (1964) and Inklaar and Fortuin (1969). Each sample was added to a 4% NaCl solution to attain an 8% suspension and blended in an Osterizer blender for 30 sec at low speed. (Low speed was used throughout the procedure.) 25 ml of Gold Kist Ravo peanut oil were added to the suspension and the mixture was blended for an additional 30 sec. A partially clamped tubing allowed restricted flow of peanut oil from a buret to an improvised glass blender jar. Flow of oil was stopped when the breakpoint (emulsion coalescence breaks to liquid separation and loses thick consistency) of the emulsion was subjectively viewed through the side of the jar. Milliliters of oil emulsified per g dry sample were calculated.

After an identical mixing procedure involving an 8% sample suspension in 4% NaCl, the suspension was centrifuged and the soluble protein in the supernatant fluid was measured by the Lowry et al. (1951) method prior to the addition of oil.

Moisture adsorption isotherms

Equilibrium moisture contents (EMC) of control and fermented samples at various equilibrium relative humidities (ERH) were determined at 8, 21, and 36°C using a method similar to that described by Kilara et al. (1972). ERH values of 14, 33, 54, 75 and 97 ± 2% were maintained in closed desiccators by using saturated solutions of LiCl, $MgCl_2$, $Mg(NO_3)_2$, NaCl and K_2SO_4, respectively, as described by Rockland (1960). 2g of each sample were placed in tared aluminum cups, placed on a shelf above the saturated salt solution in the desiccator, and stored at a constant temperature. Samples were weighed after various equilibration times to determine the length of time necessary to attain equilibrium. A period of 11 days was usually necessary to achieve equilibrium. The percent EMC [(g water gained/g dry wt sample) × 100] was plotted against corresponding ERH to determine the moisture adsorption isotherms.

Water and oil retention

Water and oil retention were measured by a modified method of Lin

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et al. (1974). A 10% suspension (2-g sample in 20 ml deionized water or 20 ml Gold Kist Ravo peanut oil) was stirred in a centrifuge tube using a glass rod for 2 min at room temperature. After 20 min the suspension was centrifuged for 20 min at 4340 × G at room temperature. The freed water or oil was decanted into a graduated cylinder and the volume was recorded. Milliliters of water or oil retained per g sample were then calculated.

Statistical analyses

All statistical analyses of data in this paper are analysis of variance. Significance in the F-tests are in the 95% confidence interval. Sample means were compared using Duncan's (1955) new multiple range test at the 5% level of significance ($P \leq 0.05$).

RESULTS & DISCUSSION

Fermentation

The consistency, hue and aroma of the fermented SDPF varied among the five fungal strains examined. *M. hiemalis* changed the thick, lumpy characteristics of the substrate to a thin, more uniform consistency which rendered the ferment easily pourable. A sweet, ethanol-like aroma was noted. *A. oryzae* fermentation left the substrate almost as lumpy as it was prior to inoculation and an unpleasant, slightly putrid odor was detected. *N. sitophila* changed the substrate to a darker shade of brown as compared to the controls and produced a fragrance reminiscent of fruity, sweet esters and acids. The fermented substrate also appeared more homogeneous and liquefied than the controls. *A. elegans* liquefied the substrate and produced an ester-like odor similar to that of baker's yeast, while the *R. oligosporus* ferment was not as liquefied as *A. elegans* and produced a pleasant odor similar to apple cider or pineapple juice. It should be noted that the consistencies of these semi-solid ferments are unlike that of ontjom, which is a solid-state fermentation product.

Nitrogen solubility

The data on percent soluble nitrogen in water over a range of pH for unfermented and fermented samples are presented in Figure 1. Control 1 was relatively insoluble between pH 3.5 and 5.0, which encompasses the isoelectric point of most peanut proteins and coincides with the protein solubility curve reported by Lawhon et al. (1972) and Ayres et al. (1974). Apparently, autoclaving had an adverse effect on the nitrogen solubility of the SDPF. The low nitrogen solubility of Control 2 is undoubtedly due to extreme protein denaturation and coagulation induced by the heat treatment. Each of the fungi studied increased the nitrogen solubility of the substrate over nonheated, nonfermented SDPF (Control 1) in the pH range of about 3.0–6.0. *M. hiemalis*, in particular, increased the nitrogen solubility at pH 4.0–5.0 from less than 5% in the nonheated control to about 34% in the ferment. The increased nitrogen solubility in this pH range is due to protein hydrolysis by fungal acid proteases to form peptides and free amino acids. In a related study (Beuchat et al., 1975) it was shown that as high as 12.7% of the amino acids of peanuts fermented with *A. elegans* for 98 hr were free. The extent of protein solubility, however, could not be directly correlated to free amino acid levels. Data presented in this paper and from previous work suggest the possibility of following peanut protein breakdown during fermentation by using column chromatographic and gel electrophoretic techniques to determine the rates and extents of arachin and conarachin hydrolysis. Further studies characterizing the effects of enzymatic hydrolysis of peanut protein on protein solubility are warranted.

Apparent viscosities and emulsion capacities

Data showing protein and nitrogen solubilities, emulsion capacities and apparent viscosities of control and fermented samples are reported in Table 1. It should be pointed out that the Lowry method for measuring protein depends upon tyrosine and phenylalanine in a dye-binding reaction. In these studies, the protein solubility should be cautiously examined since varying percentages of tyrosine and phenylalanine may be present in the soluble peptides and free amino acids of the ferments. These percentages may be influenced by the ratio of fungal versus SDPF protein present in the soluble fraction. It should also be noted that Lowry protein was determined from a standard curve using bovine serum albumin as the protein source.

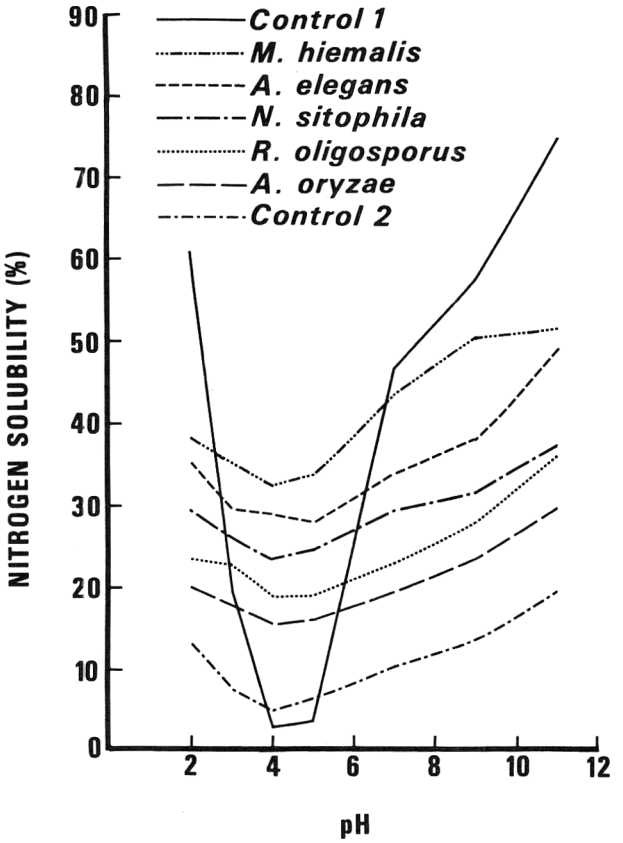

Fig. 1—Nitrogen solubility of nonfermented and fermented peanut flour in water in the pH range 2.0–11.0.

Table 1—Protein and nitrogen solubility, emulsion capacity and apparent viscosity of nonfermented and fermented peanut flour

Sample	Solvent	pH	Solubility (mg/ml) Kjeldahl nitrogen	Solubility (mg/ml) Lowry protein	Apparent viscosity (cps)	Emulsion capacity (mg oil/g sample)
Control 1	H_2O	6.28	1.64		12.8	
	NaCl[a]	5.90	2.23	15.8	13.4	38.5
Control 2	H_2O	5.05	0.61		12.8	
	NaCl	5.00	1.02	6.3	13.6	0
A. elegans	H_2O	5.50	2.38		12.6	
	NaCl	5.30	2.57	8.9	13.0	21.6
A. oryzae	H_2O	5.30	1.62		12.4	
	NaCl	5.15	1.79	6.2	12.8	18.6
M. hiemalis	H_2O	5.18	1.81		12.8	
	NaCl	5.01	2.00	15.3	13.3	26.7
N. sitophila	H_2O	5.70	2.02		13.0	
	NaCl	5.50	2.24	9.6	13.4	27.0
R. oligosporus	H_2O	5.30	1.58		12.0	
	NaCl	5.10	1.71	10.0	13.0	26.0

[a] 4% NaCl (w/v)

Since dispersions in 4% NaCl consistently exhibited higher apparent viscosities, higher nitrogen solubilities, and more acidic pH than did dispersions in water, it was decided to examine the emulsion capacity of each sample using 4% NaCl as the continuous phase. The autoclaving procedure adversely affected emulsion capacity, as well as protein and nitrogen solubility. This is probably due to heat denaturation of protein as described by Wu and Inglett (1974). Moisture in peanuts exposed to thermal treatment also greatly affects functional characteristics of paste prepared from the peanuts (McWatters and Heaton, 1974). According to Carpenter and Saffle (1964), the soluble protein fraction is the fraction most responsible for emulsifying oil, the amount as well as the source of soluble protein having important consequences. Since emulsion studies were performed using portions of the entire substrate in suspension rather than a protein isolate, carbohydrates and other sample components undoubtedly affected emulsion capacities. In general, fermentation increased the emulsion capacity, nitrogen solubility and protein solubility of SDPF over that of Control 2. However, the emulsion capacity of Control 1 was not completely restored during fermentation.

The dependence of nitrogen solubility on pH generally coincides with the nitrogen solubility profiles in water as previously discussed.

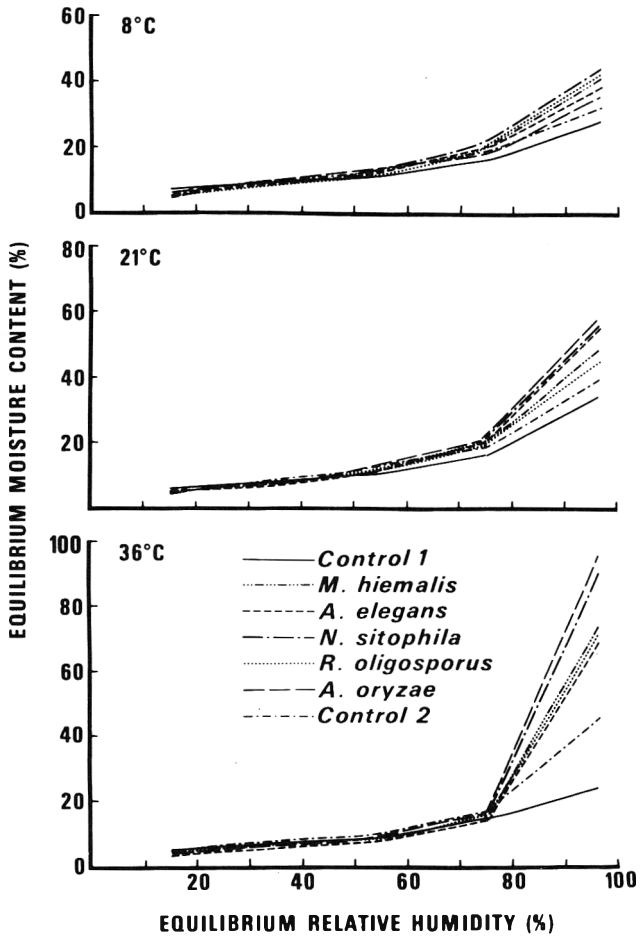

Fig. 2–Equilibrium moisture content of nonfermented and fermented peanut flour over a range of equilibrium relative humidities of 8, 21, and 36° C.

Table 2–Water and oil retention properties of nonfermented and fermented peanut flour

Sample[a]	ml retained/g sample[b]	
	Water	Peanut oil
Control 1	2.1a	1.5a
Control 2	3.5b	2.0b
A. elegans	3.1c	1.7c
A. oryzae	3.4bc	2.2d
M. hiemalis	2.1a	1.8e
N. sitophila	2.8d	1.9be
R. oligosporus	2.2a	1.8e

[a] 10% dispersion
[b] Values in a column followed by the same letter are not significantly different at $P \leq 0.05$.

Moisture adsorption isotherms

The moisture adsorption isotherms for control and fermented samples at 8, 21, and 36°C are presented in Figure 2. Since fungal growth was visually apparent at 36°C at an ERH of 75% or higher, samples may not have reached true equilibrium moisture content. The 8 and 21°C isotherms are more representative of moisture adsorption characteristics. Little difference was noted between moisture contents of samples equilibrated at relative humidities ranging from 14–75%. However, marked changes were noted above 75% ERH. The control samples did not absorb as much moisture at high ERH as did the ferments. These differences may be due to the ratio of exposed hydrophilic to hydrophobic groups resulting from fermentation. As expected, the extent of moisture adsorption appears to be directly related to increased sample temperature. *A. oryzae* and *N. sitophila* ferments generally adsorbed more moisture, especially at higher ERH and temperatures. This may reflect more extensive growth of the Ascomycetes. High moisture levels, and correspondingly high water activities (a_w), are known to be required for the growth of certain Mucorales (Wang et al., 1974), including *A. elegans, M. hiemalis* and *R. oligosporus.*

Water and oil retention

Data relating changes in water and oil retention properties of SDPF as a result of fungal fermentation are shown in Table 2. The samples were consistently more hydrophilic than lipophilic. The mechanism of water and oil retention may be more physical than chemical as evidenced by the amounts of water and oil retained by the autoclaved (Control 2) compared to the nonheated SDPF (Control 1). Wu and Inglett (1974) suggested that as nitrogen solubility of soy flour decreases, water absorption first increases and then decreases with further decreasing nitrogen solubility. A similar relationship between nitrogen and water retention may be exemplified in this study by the low nitrogen solubility of Control 2 and its high water retention property.

Theoretically, the technique used to measure water and oil retention is a physical method, employing centrifugal force to exert a squeezing effect on the test sample. Highly liquefied ferments result from enzymatic breakdown of peanut cell membranes, proteins, carbohydrates and lipids, whereas the substrates retaining their original consistency may also retain more of their original structural integrity. Failure of developing hyphae to fragment during fermentation might also contribute to less liquefaction. Results listed in Table 2 may be related to observations pertaining to the consistency of substrates after fermentation. For example, *M. hiemalis* strongly liquefied the peanut substrate, which resulted in very low water and oil retention values, while *A. oryzae* fermentation left the substrate as thick as Control 2. Both the *A. oryzae* and Control 2 samples have high water and oil retention values. The *A. elegans* ferment, on the other hand, was somewhat liquefied and has a relatively high water retention value accompanied by a rather low oil retention value. These observations suggest the implication of physical entrapment of water

and oil in contributing to liquid retention. Quantity and distribution of hydrophilic and lipophilic groups may also be reflected in retention characteristics.

Whether the dried fermented peanut flour described in this study has practical application in the formulation of protein-fortified foods and beverages remains to be demonstrated. Although the ferments were demonstrated to be nontoxic (Quinn et al., 1975), there are special regulatory problems associated with the incorporation of microbially-derived or modified ingredients into food products. Further studies are needed to fully characterize and describe the potentially valuable functional properties associated with fermented peanut flour.

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COMPOSITIONAL DIFFERENCES IN WHEY SYSTEMS

INTRODUCTION

CONSIDERABLE ATTENTION is being paid to the utilization of cheese whey as a solution to the whey waste problem. Ultrafiltration and reverse osmosis membrane processing have been introduced to manufacture whey protein concentrates for use in food formulations but the processes have not yet achieved theoretical expectations. It appears that problems in stability of whey protein concentrates may, in part, be related to the distinctive but variable composition and properties of sweet and cottage cheese wheys. Results on the macro composition of sweet and acid whey and whey products are available (Mavropoulou and Kosikowski, 1973a; Cerbulis et al., 1972; Vaughn, 1970; Roeper, 1971; and Whey Products Institute, personal communication). However, a comprehensive comparative study of sweet and acid cheese wheys with control whey systems prepared from the same whole or skim milk has not been made. It is thought that minor changes in protein and nonprotein nitrogenous components (Mavropoulou and Kosikowski, 1973b) and multivalent minerals and citric acid of whey during cheese making might contribute to subsequent behavior during membrane processing and in final product stability.

Therefore, a study was initiated to evaluate compositional and whey protein property differences resulting from cheese making practices. Cheddar and cottage wheys and pH 4.6 acidified wheys were prepared in our laboratory from the same whole or skim milk and compared for differences in nitrogen distribution, lactose, total and nondialyzable multivalent components (citric acid, calcium, magnesium and phosphorus), and whey protein properties.

Concurrently, commercial ultrafiltration and spray-dried processed sweet cheese whey protein concentrates were similarly analyzed and the data compared to those for the laboratory prepared fresh Cheddar whey.

EXPERIMENTAL

CHEDDAR CHEESE whey was prepared by the standard 4.5 hr cheese manufacturing procedure and cottage cheese whey by the short-set process from pooled Grade A whole milk and skim milk, respectively. Control wheys were prepared from the same pasteurized milk by acidification to pH 4.6 with 1N HCl. All wheys were centrifuged at 1000 × G for 30 min and sediments were discarded. Supernatant samples were stored at 0–5°C or frozen for future analysis. Ultrafiltration (UF) spray-dried processed sweet cheese whey protein concentrates were obtained from a commercial source.

Standard AOAC procedures (1970) were used for determination of Kjeldahl nitrogen, moisture, ash and lactose (polarimetry). Lactose was also determined by the phenol-sulfuric acid method (Dubois et al., 1956). Lactic acid was measured by a modification of the Ling procedure (Harper and Randolph, 1960). 12% trichloroacetic acid soluble nitrogen values for the whey systems were obtained by the method of Rowland (1938). Mineral analyses were run for calcium and magnesium (Ntaillianas and Whitney, 1964) and phosphorus (Fiske and Subbarow, 1925). Citric acid was measured by the method of Marier and Boulet (1958). The FDNB method of Booth (1971) was used for determining available lysine in the spray-dried UF sweet whey powders.

Alkaline urea starch gel electrophoresis (SGE) (Morr, 1971), alkaline disc polyacrylamide gel electrophoresis (PAGE) (Davis, 1964), and Sephadex G-100 filtration chromatography (Morr and Josephson, 1968) were used to evaluate the whey systems for process induced whey protein changes.

Table 1—Composition of whey systems[a]

	Cheddar whey	Cottage whey	pH 4.6 Whole milk whey	pH 4.6 Skim milk whey
Total solids (%)	6.5	5.2	6.4	5.7
Nondialyzable	0.54	0.56	0.58	0.54
pH	6.3	4.6	4.6	4.6
Ash (%)	0.57	0.46	0.65	0.60
Lactose[b] (%)	4.9	4.3	5.0	5.0
Total nitrogen (mg/100 ml)				
Range	116–146	82–105	129–137	109–119
Mean	132	96	133	115
12% TCA soluble Nitrogen (mg/100 ml)				
Range	29–30	23–29	19.5–20	22–25
Mean	29.7	26.3	19.8	23.3
12% TCA soluble nitrogen (% of Total N)	22.0	27.3	15.0	20.3

[a] Mean of duplicate determinations for three trials
[b] Determined by polarimetry.

RESULTS & DISCUSSION

Composition and properties of fresh Cheddar and cottage wheys and pH 4.6 acidified wheys

Compositional data for the experimental Cheddar and cottage cheese wheys and pH 4.6 acidified wheys prepared from the same whole milk or skim milk are presented in Tables 1 and 2. Total solids, pH, ash, lactose and total nitrogen showed some variation between trials but results were within normally expected ranges for the respective wheys. Cheddar whey had the highest pH and solids, but was lowest in calcium, magnesium, phosphorus and ash. Cottage whey was lowest in total nitrogen, citrate and lactose, the reduction of the latter two components apparently resulting from starter activity. Both Cheddar and cottage wheys were higher in 12% TCA-soluble nitrogen than their comparable pH 4.6 wheys which is most likely the result of secondary rennin and microbial proteolysis. Residual proteolytic activity as measured by an agar-casein diffusion method was, in fact, detected in a concurrent study in Cheddar whey samples and to a lesser extent in cottage wheys. These results concur with a recent report (Mavropoulou and Kosikowski, 1973b) of large but different concentrations of free amino acids and soluble peptides in commercial sweet and acid whey powders. Dialysis of the wheys (Table 2) revealed differences in nondialyzable multivalent components. Cheddar whey was highest in nondialyzable calcium and lowest in nondialyzable phosphorus and citrate, although the differences from cottage whey were small. As expected, the differences in total and nondialyzable multivalent minerals and citrate between the pH 4.6 whole milk and skim milk wheys were slight.

The above results indicate that cheese making processes had a significant and varied effect on increasing the amount of nonprotein nitrogen and changing the composition and balance of multivalent minerals and citric acid in the whey systems. These differences in the composition and balance of low molecular weight components of Cheddar and cottage cheese wheys must certainly be considered as potential factors affecting the ultimate functional properties and stability of cheese wheys in general.

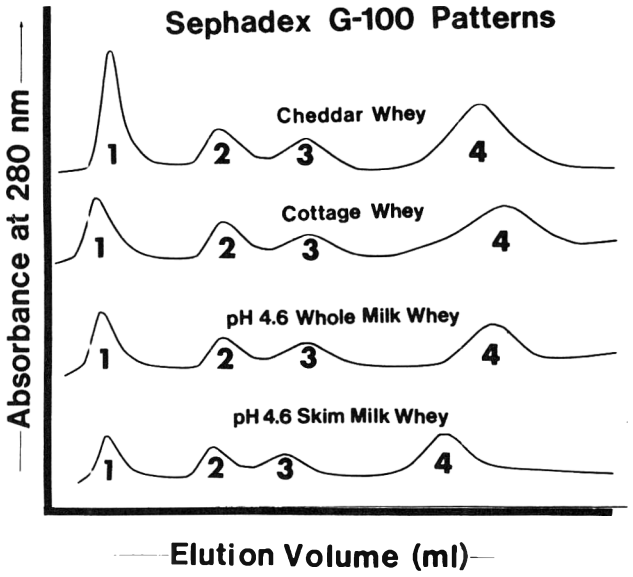

Fig. 1—Sephadex G-100 elution patterns of fresh Cheddar whey and pH 4.6 whole milk whey prepared from the same whole milk and cottage cheese whey and pH 4.6 skim milk whey prepared from the same skim milk. Identification of pattern peak components: Peak 1—immunoglobulins, bovine serum albumin and residual casein; Peak 2—β-lactoglobulin; Peak 3—α-lactalbumin; and Peak 4—low molecular weight nitrogenous components, lactose and minerals.

Table 2—Multivalent component analyses of whey systems[a]

Component (mg/100 ml)	Cheddar whey	Cottage whey	pH 4.6 Whole milk whey	pH 4.6 Skim milk whey
Citric acid				
Total				
Range	150–165	130–135	158–175	155–195
Mean	157	133	166	169
Nondialyzable				
Mean	4.6	5.0	5.0	5.0
Calcium				
Total				
Range	30–41	77–110	118–119	111–121
Mean	36	92	118.5	115
Nondialyzable				
Mean	3.4	2.7	1.5	1.0
Magnesium				
Total				
Range	7.6–9.5	7.3–10.8	11.4–11.8	10.2–12.5
Mean	8.3	8.5	11.6	11.3
Nondialyzable[b]	–	–	–	–
Phosphorus				
Total				
Range	42–59	68–86	90–96	52–94
Mean	49	75	93	70
Nondialyzable	5.5	6.2	8.0	8.0

[a] Results from duplicate determinations for three trials
[b] None detected.

All wheys exhibited typical Sephadex G-100 gel filtration (Fig. 1, only one pattern shown) and alkaline gel electrophoresis (Fig. 2) patterns for undenatured whey proteins except that Cheddar wheys showed a slightly larger void volume peak (Fig. 1). Most likely, the larger peak size was the result of a larger amount of residual casein rather than denatured whey protein. It would appear that cheese making causes no gross changes in the native whey proteins.

Composition and properties of commercial UF spray-dried sweet whey protein concentrates

The composition of five lots of UF-processed spray-dried sweet whey powders are shown in Table 3. The results agree, generally, with those of Morr et al. (1973). With the exception of lactose and lactic content, the values for the other components were relatively similar for the five lots. The differences which did exist appeared to be within the limits which can be expected as a result of variations in milk composition, cheese making procedures, and whey processing treatment.

With respect to lactose content, this is frequently determined for commercial whey powders by difference calculations or analysis using the Dubois or polarimetry methods. The results in Table 3 show that lactose values differed considerably between lots of whey powders and methods of lactose estimation. In our study, the lactose contents as determined by the Dubois method ranged from 9.3–28.7% and by polarimetry from 6.6–21.4%. Morr et al. (1973) using the Dubois method found lactose contents in three samples of UF-treated whey powders ranging from 15.5–40.2%.

One reason for the apparent discrepancy between methods of lactose estimation in the whey powders may have been the lactic acid content (Table 3). A wide range of lactic acid values was obtained, with lot A having the highest (8.1%) and lot B the lowest (0.8%). Lactic acid from lactose fermentation can be optically active or inactive depending upon the relative proportion of dextrarotatory and levarotatory forms produced by lactic acid bacteria (Foster et al., 1957). The identity of the starter culture used in the production of these UF-processed sweet whey systems is unknown, so it is not possible to predict the relative concentration of optical form(s) present. However, no matter which form of lactic acid was in the whey systems or the relative concentration of the two, unless equal, the presence of lactic acid would lower polarimetry readings as the respective specific optical rotations of the two forms are:

$$D(-)\text{ lactic acid} = [\alpha]\ \frac{21.5}{546} = -\ 2.6^\circ\ (H_2O)$$

and

$$L(+)\text{ lactic acid} = [\alpha]\ \frac{21-22}{546.1} = +\ 2.6^\circ\ (H_2O)$$

as compared to $[\alpha]\ \frac{20}{D} = +\ 52.3^\circ\ (H_2O)$ for mutarotated α- and β-D lactose. This could explain the low polarimetric lactose values for lots A and C but not those values for lots D and E which were slightly higher than the calculated difference values. Neither, would it explain the low polarimetric lactose value for lot B which had the lowest lactic acid content among the lots tested.

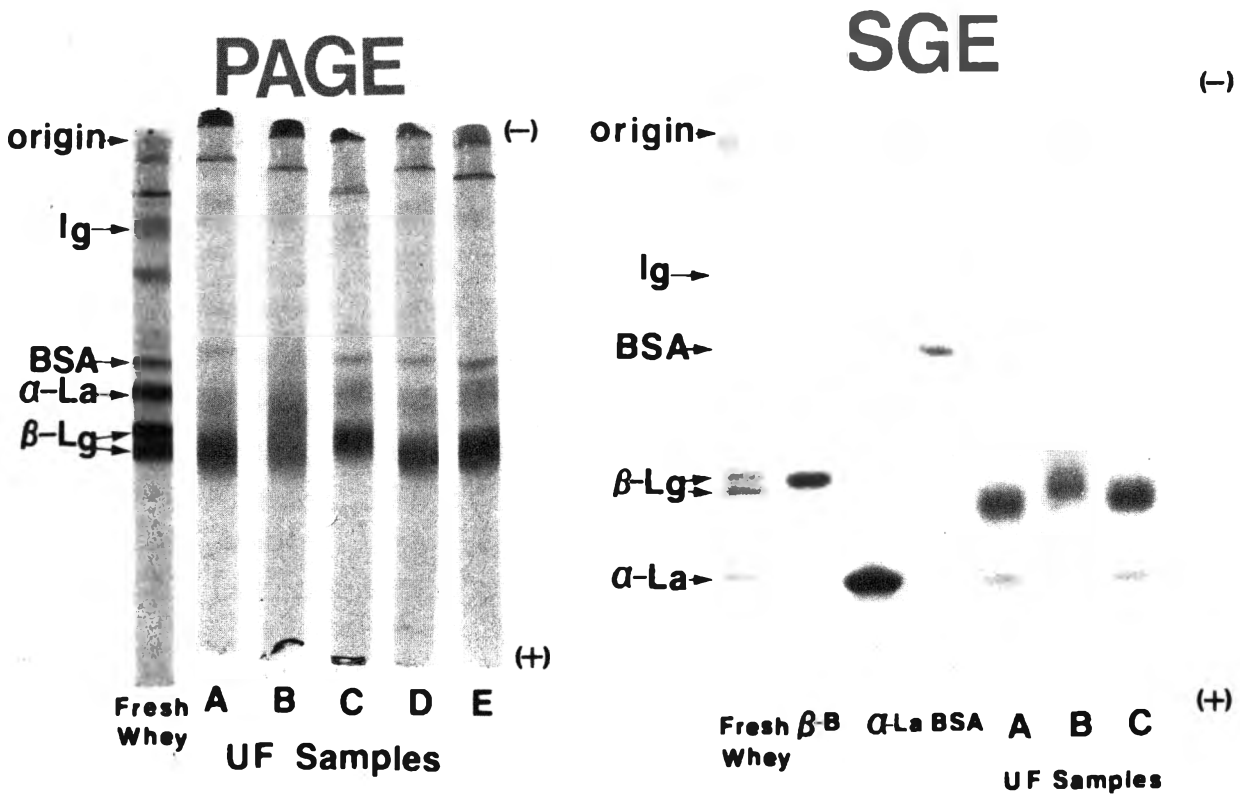

Fig. 2–Alkaline disc polyacrylamide gel electrophoresis (PAGE) and alkaline urea starch gel electrophoresis (SGE) patterns of fresh Cheddar whey selected whey protein standards (SGE pattern only) and selected lots of UF spray-dried sweet whey powders. Whey protein identification: β-Lg (β-lactoglobulin); α-La (α-lactalbumin); BSA (bovine serum albumin); and Ig (immunoglobulins).

Table 3—Composition of UF sweet whey powders[a]

Analyses	Lot identification					
	A	B	C	D	E	Mean
Moisture (%)	5.5	5.5	5.5	5.1	5.5	5.4
Crude protein (%)						
(% total N X 6.38)	61.9	65.4	61.4	58.5	59.7	61.4
Lactose (%)						
(Polarimetry)	7.2	6.6	10.9	21.4	20.5	13.3
(Dubois)	(9.3)	(13.3)	(18.0)	(28.7)	(23.3)	(18.7)
(By difference)[b]	(10.6)	(15.9)	(14.7)	(19.3)	(19.2)	(15.9)
Lactic acid (%)	8.1	0.8	3.9	3.0	1.9	3.5
Fat (%)	7.9	7.8	9.0	8.7	7.7	8.2
Ash (%)	4.7	3.3	3.6	3.4	4.5	3.9
Citric acid (%)	1.25	1.31	1.88	1.95	1.50	1.58
Calcium (%)	1.60	0.74	0.69	0.61	1.11	0.95
Magnesium (%)	0.08	0.08	0.08	0.09	0.08	0.08
Phosphorus (%)	0.44	0.42	0.25	0.44	0.41	0.39
Available lysine						
(g/100g protein)[c]	7.1	8.0	8.1	7.5	7.7	7.7

[a] Mean of duplicate determinations
[b] Computed by subtracting values for moisture, protein, fat, ash, lactic acid and citric acid from 100
[c] Single determinations for FDNB method of Booth (1971) using 1.05 as the correction factor

Lactic acid would also be expected to increase lactose values by Dubois, although its relative reactivity with phenol-sulfuric acid has not been reported (Dubois et al., 1956). The contribution of lactic acid, however, is hard to explain given, for example, the high lactic acid (8.1%) and low Dubois (9.3%) values for lot A and the lower lactic acid (3.0%) yet very high Dubois (28.7%) values for lot D. The fact that lactose values by Dubois were consistently higher than by polarimetry may be attributed to the reactivity of lactic acid with phenol-sulfuric acid and the negative effect of optically active lactic acid on polarimetry. Furthermore, the possibility of increased Dubois values as a result of the presence of glycoproteins in the whey powders should not be overlooked.

Another possible cause for differences in lactose values could have been from excessive heat treatment during processing of these lots and the occurrence of Maillard reactions. To establish this possibility the available lysine content of the five lots of whey powder was determined (Table 3). The range in lysine content was from 7.1–8.1g/100g protein with lot A having the lowest content and lots B and C the highest. This would indicate that the Maillard reaction may or may not have occurred to a varying degree in the whey powders and may or may not have had an effect on lactose determinations. Lysine values for whey prior to heat processing would need to be established to provide conclusive evidence. In either case, it would appear that the reasons for the low lactose values of lot B are still unknown. Other process induced physical/chemical alterations of lactose may occur during whey treatment which influence its analytical determination. The elucidation of this question would appear important for the determination of the true composition and functional properties of UF-processed whey powders.

A comparison was made of the average composition of the UF sweet whey powders to that of fresh Cheddar whey on an equivalent solids basis (Table 4). The results reveal important changes in whey as a result of membrane processing. The UF product is considerably higher in protein but lower in all low molecular weight components with the exception of calcium. The higher calcium content could be indicative of the calcium ion binding capacity of the higher protein levels in the UF product. These changes in composition of sweet cheese whey as a result of ultrafiltration most likely contribute significantly to the stability and functional properties of the UF processed whey systems.

Alkaline polyacrylamide gel electrophoretic patterns (Fig. 2) and Sephadex G-100 gel filtration patterns (Fig. 3) of the UF powders showed significant changes in whey proteins. β-lactoglobulin and α-lactalbumin electrophoretic bands were diffuse and smeared and considerable protein remained in the sample gel (PAGE pattern, Fig. 2). These observations are evidence of protein denaturation and aggregation. The electrophoretic bands for UF samples in the presence of urea (SGE pattern, Fig. 2) were also smeared in comparison to the distinct bands for whey protein standards, indicating that the protein changes observed are not similar to those caused by urea denaturation of the whey proteins. The G-100 patterns (Fig. 3) showed larger void volume peaks (Peak 1), which are indicative of whey protein aggregation, and smaller nonprotein nitrogen peaks (Peak 4) as a result of removal by the UF

Table 4—Comparison of fresh Cheddar whey and UF whey powder compositions

	Cheddar whey (Avg 3 trials)	UF whey powder[a] (Avg 5 lots)
Total solids (%)	6.5	6.5
Ash (%)	0.57	0.25
Calcium[b]	35.9	44.4
Magnesium[b]	8.3	5.2
Phosphorus[b]	49.3	28.0
Citric acid[b]	157.0	102.7
Lactose (%)	4.85	(0.43–1.4)
Total N[b]	132.0	629.0
12% TCA soluble[b]	29.7	41.5
12% TCA soluble (%)	22.0	6.8

[a] Data on 6.5% solids basis for comparison to Cheddar whey
[b] Units are reported as mg/100 ml (Cheddar whey) or mg/6.5% solids (UF whey powder)

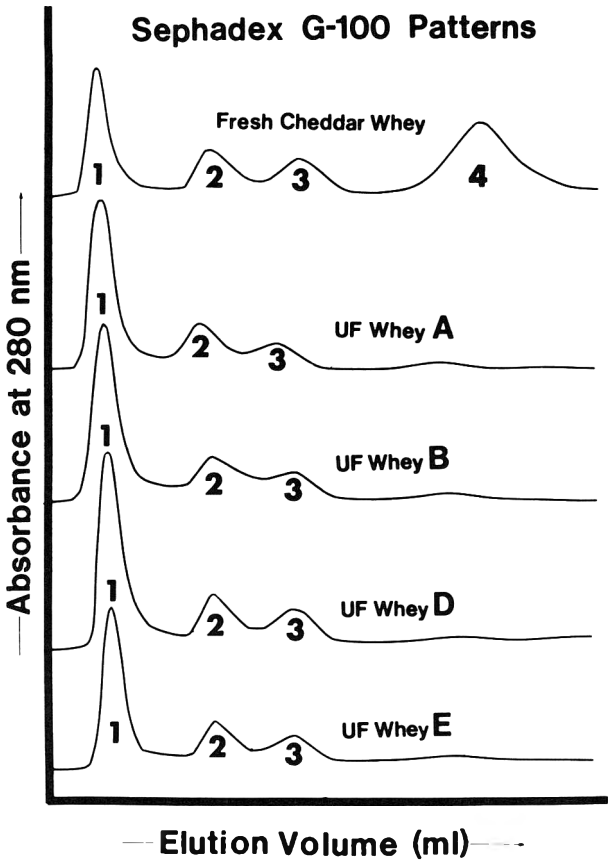

Fig. 3–Sephadex G-100 elution patterns of UF spray-dried sweet whey powders (lots A, B, D and E) and fresh Cheddar whey.

process. Changes in Peak 4 could also reflect loss of small proteose peptone (Kolar and Brunner, 1970) components 5 (molecular weight ≈ 14,300) and 8 (molecular weights ≈ 4,100 and 9,900) although this is yet to be proved. Reichert et al. (1974) have also observed a high degree of whey protein denaturation (44.2% of total protein) in ultrafiltration whey protein concentrates on the basis of solubility differences at pH 4.6. Their urea SGE patterns were somewhat different than those for the UF products studied here (Fig. 2) in that two additional atypical unidentified protein zones were also found. However, both studies confirm that there have been significant changes in the physical/chemical properties of native whey proteins as a result of UF and spray-drying processes.

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FORMATION OF N-NITROSOPYRROLIDINE FROM PROLINE AND COLLAGEN

INTRODUCTION

TRACE AMOUNTS of dimethylnitrosamine (DMN) have been demonstrated in certain types of cured meat products (Crosby et al., 1972; Fazio et al., 1971; and Panalaks et al., 1973). Recently, much attention has focused on the presence of N-nitrosopyrrolidine (N-Pyr) and its potential precursors in raw and cooked bacon. Fazio et al. (1973) found N-Pyr in pan fried bacon but not in raw bacon. They isolated 10–108 ppb of N-Pyr on cooking eight commercial brands of bacon and confirmed its presence by mass spectrometry. These results confirm the findings of Crosby et al. (1972) who showed that conventional cooking of Danish bacon produced levels of N-Pyr as high as 16–40 ppb.

Herring (1973) investigated the effects of level of sodium nitrite and cooking method on the formation of N-Pyr in bacon. The concentration of the N-nitrosamine increased with increasing nitrite levels. Pan-fried samples had the most and microwave-cooked samples the least N-Pyr. All uncooked samples contained no N-Pyr. The variation in the amounts of N-Pyr formed in bacon by the different cooking procedures was also demonstrated by Pensabene et al. (1974). They concluded that N-Pyr formation in bacon is primarily dependent on frying temperature. A model system study of the decarboxylation of N-nitrosoproline showed this precursor was maximally converted to N-Pyr at 185°C, near the recommended temperature for frying. Sen et al. (1973), using a specific thin-layer chromatographic technique, analyzed for N-nitrosamine content in 16 samples of bacon (fried and unfried), five samples of baby foods containing bacon as one of the ingredients and six other meat products. Eight samples gave a positive test for either DMN or N-Pyr. Raw bacon did not contain N-Pyr.

At present there is considerable doubt as to the actual mode of N-pyr formation in cooked bacon. Lijinsky and Epstein (1970) have proposed that it may arise either through the formation of N-nitrosoproline from proline and sodium nitrite with subsequent decarboxylation to N-Pyr or by direct interaction of pyrrolidine which arises from proline or putrescine and nitrite. Using a heating system simulating the physical conditions present in a system such as bacon being fried at a pan temperature of 170°C, Bills et al. (1973) were able to produce N-Pyr from N-nitrosoproline, pyrrolidine, spermidine, proline and putrescine in yields of 2.6, 1.0, 1.0, 0.4 and 0.04% theoretical, respectively. The formation of N-Pyr from spermidine and sodium nitrite was also demonstrated by Ferguson et al. (1973).

Ender and Ceh (1971) showed that N-Pyr could be produced in a rice starch medium containing proline and sodium nitrite. Production increased from 3.47 mg at 130°C to 72.5 mg N-Pyr/g proline at 170°C. Dry samples of L-glycyl L-proline, L-prolyglycine and pyrrolidine also produced N-Pyr when heated with sodium nitrite at 170°C for 2 hr (Huxel et al., 1973). These authors also examined the formation of N-Pyr from collagen heated with sodium nitrite in buffered solution at pH 4.6, 6.2 and 9.0 and dried at temperatures ranging from 120–195°C.

Proline is a natural component of many foods and is especially abundant in connective tissue. This present study was undertaken to ascertain whether N-Pyr could be produced from these precursors in the presence of nitrite and under simulated conditions of pan-frying bacon. Potential precursors investigated included proline, commercial collagen and connective tissue isolated from cured, smoked ham.

EXPERIMENTAL

Production of N-Pyr from proline and sodium nitrite in a low moisture carboxymethylcellulose (CMC) system

A system containing 8.7 mM proline, 26.1 mM $NaNO_2$, 10g CMC 7LF (Hercules, Inc.) and 250 ml biphthalate buffer (pH 4.0) was prepared as described previously (Gray and Dugan, 1974). The mixture was freeze dried for 24 hr in a Virtis RePP Model No. 42 sublimator at a pressure of 5μ and a platen temperature of 24°C. Residual moisture content was approximately 3%. The dried system was macerated to a fine powder and doubly wrapped in aluminum foil before heating in an oven at the required temperature for 2 hr. Temperatures of 80, 100, 120, 140, 160, 180 and 200°C were investigated.

Production of N-Pyr from collagen

The production of N-Pyr from collagen (Nutritional Biochemical Corp.) was investigated in three systems:

(1) A dry system containing collagen (1g), $NaNO_2$ (2g), CMC 7LF (10g) and biphthalate buffer (250 ml, pH 4.0) was treated in a manner similar to that described above for proline.
(2) Collagen (1g) was hydrolyzed 24 hr at 105°C by refluxing with 6M HCl. On cooling, the pH was adjusted to 4.0 with NaOH so that the final volume was 250 ml. CMC 7LF (10g) and $NaNO_2$ (2g) were added to the hydrolysate which was then freeze dried as before. Only a temperature of 160°C was used in this study.
(3) A system, similar to that of Bills et al. (1973), was used to simulate the heating stresses undergone by bacon during pan-frying. Mazola corn oil (90g), collagen (0.3g), $NaNO_2$ (0.3g), water (1 ml) and a boiling chip were introduced into a two-necked, 500 ml round-bottom flask fitted with a reflux condenser and thermometer. The flask was heated in a bath of silicone oil on a hot plate. The contents of the flask were brought to the required temperature in about 10 min and held at this temperature for an additional 10 min. Temperatures of 120, 140, 160 and 178°C were investigated. The effect of adding 0, 5 and 10 ml of water to the oil was also investigated.

Production of N-Pyr from connective tissue of cured, smoked ham

The connective tissue used was from epimysium located on the biceps femoris. This was combined with the tendon from the femur-ischium joint. The wet tissue (6g), dissected free from adhering fat and muscle was frozen in liquid nitrogen and quickly ground to a fine powder. One 2-g sample was heated for 10 min at 175°C in the oil-water system, a warm-up time of 10 min being used. Another 2-g sample was treated in a similar manner except that 0.5g $NaNO_2$ was added. The last 2-g sample was added to 350 ml 3M NaOH and distilled under vacuum.

Distillation and extraction

At the completion of the reaction periods, the products were distilled under reduced pressure (13 mm Hg, 35°C) after the addition of 350 ml 3M NaOH. The receiver flask was cooled in a water-alcohol/dry ice mixture at −8°C until 250 ml distillate was collected. This was extracted with 3 × 100 ml aliquots of redistilled dichloromethane after the addition of K_2CO_3 (10g). The extract was dried over anhydrous Na_2SO_4 and concentrated to a volume of 2.5 ml in a Kuderna-Danish apparatus equipped with a Snyder column. Hexane (1 ml) was added

and the extract was further concentrated under a stream of nitrogen to a volume of 1 ml (for the proline study) and 0.2 ml (for the collagen study).

Isolation of N-Pyr (collagen study)

The dichloromethane extract was streaked on a 20 × 20 cm silica gel G (Woelm) thin layer chromatographic plate (500μ layer) and developed in a solvent system containing hexane, diethyl ether and dichloromethane (4:3:2). The band containing N-Pyr was monitored by spotting 5 μg of standard N-Pyr at the edge of the plate and developing simultaneously with the band. The band having the same R_F value as standard N-Pyr was removed from the plate. The N-nitrosamine was removed from the silica gel by steam distillation (Eisenbrand et al., 1970) and extraction of the distillate (75 ml) with 2 × 50 ml aliquots of dichloromethane.

Quantitation of N-Pyr produced from collagen

0.1, 0.5 and 1.0 mg aliquots of N-Pyr were subjected to the same experimental procedures as the collagen extracts to determine the percentage losses. Losses can occur during vacuum distillation, extraction, concentration and by incomplete removal from silica gel.

Gas chromatographic analysis

A Beckman GC-5 dual column gas chromatograph equipped with flame ionization detectors and a stainless steel column (6 ft × 1/8 in. o.d.) packed with 3% OV 210 on 100–120 mesh Supelcoport was used for analysis. The chromatograph was operated isothermally at 110°C with gas flows of 25.6, 18.0 and 300 ml/min for nitrogen, hydrogen and compressed air, respectively. Temperatures of the detector and injection port were 240 and 185°C respectively.

Gas chromatography-mass spectrometry

Mass spectra were obtained using a combined GLC-mass spectrometer LKB 9000 equipped with a glass column (6 ft × 1/8 in. o.d.) of 3% OV 210, with an ionizing electron energy of 70 eV; the flash heater was set 20° above the GLC column temperature (110°C), molecular separator at 230°C and the ion source at 290°C. The spectra were reported as bar graphs by means of an on-line data acquisition and processing program (Sweeley et al., 1970).

RESULTS & DISCUSSION

RECENT REPORTS have implicated proline in the formation of N-Pyr in bacon and other fried foods (Bills et al., 1973; Huxel et al., 1973; and Pensabene et al., 1974). This may be a serious problem since proline is a natural component of many foods. Schweigert and Payne (1956) showed that proline constitutes 4.6% of the crude protein in pork. The amino acid composition of connective tissue protein is markedly different from that of muscle and organ meats in that it contains relatively large amounts of proline and hydroxyproline. Crevasse et al. (1969) reported values of 15.04 and 13.10% for proline and hydroxyproline in acid soluble collagen from the epimysium of normal pork muscle. Values of 12.55 and 14.3% for proline and hydroxyproline were recorded by Crevasse (1967) in acid soluble pig skin tropocollagen.

The formation of N-nitrosamines in low moisture CMC systems containing secondary amines and sodium nitrite has been recently demonstrated by Gray and Dugan (1974). Incorporation of proline as the nitrosatable amino source into a similar system and heating at elevated temperatures for 2 hr in the presence of sodium nitrite yielded N-Pyr (Fig. 1). Little or no N-Pyr was formed at 80°C and maximum production occurred at 180°C. This is in agreement with the data of Pensabene et al. (1974) which showed that the decarboxylation of N-nitrosoproline in silicone oil was maximal at 185°C. It is very probable, however, that the observed decrease in N-Pyr formation at temperatures above 185°C is due to its expulsion from the systems. Since the boiling point of N-Pyr is 214°C, it would be expected that some volatilization of the N-Pyr produced would occur around 200°C. Because of the potentially hazardous nature of this compound, the heating oven was enclosed in a well ventilated fume cupboard and rubber gloves were worn at all times when working with these substances.

The percentage conversion of proline to N-Pyr in the low moisture CMC system at 180°C was 6.5% of the theoretical value. Incorporation of ascorbic acid, sodium bisulfite and tannic acid into the system prior to freeze drying almost completely (99%) inhibited the formation of the N-nitrosamine (Gray and Dugan, unpublished data). When 4-hydroxy-L-proline was used in the system, no 4-hydroxy-N-nitrosopyrrolidine was separated or detected under the gas chromatographic conditions employed.

A similar treatment of collagen in the low moisture CMC system failed to produce N-Pyr in the temperature range 80–180°C. At 200°C, a yield of 0.009 mg N-Pyr/g collagen was recorded. This substantiates the findings of Huxel et al. (1973) who showed that dry collagen samples produced N-Pyr at 195°C, but not at 120, 145 and 170°C although Huxel et al. did report that collagen heated with sodium nitrite in buffered solution at pH 5.2 produced N-Pyr at temperatures of 120°C and above.

Hydrolysis of collagen in 6M HCl for 24 hr at 105°C produced sufficient proline to react with nitrite and produce 0.17 mg N-Pyr at 160°C. Crevasse et al. (1969) showed that chymotrypsin and elastase treatment caused significant changes in the protein subunits of calf skin tropocollagen, whereas, a pepsin-treated sample showed little or no change from the control sample. However, it is very unlikely that any substantial hydrolysis of collagen will occur in the mildly acidic condi-

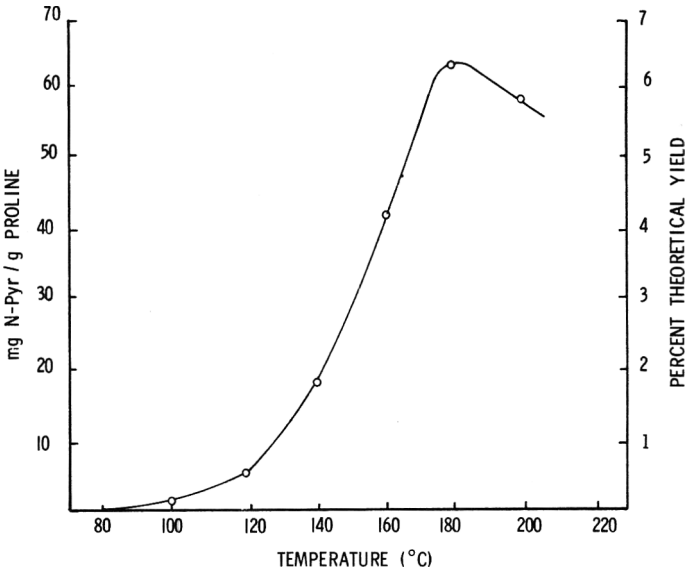

Fig. 1—Production of N-nitrosopyrrolidine from proline and $NaNO_2$ in a low moisture CMC system.

Table 1—Production of N-nitrosopyrrolidine from collagen and $NaNO_2$ in an oil-water system

Temp of frying system (°C)	mg N-Pyr produced /0.3g collagen[a]
120	0.010
140	0.020
160	0.040
178	0.068

[a] Average of three determinations of three samples each

tions of the stomach, even in the presence of pepsin. This indicates the improbability of N-Pyr formation in the stomach from ingested collagen.

Collagen when heated with nitrite in an oil-water system simulating conditions encountered in pan-frying bacon, produced varying amounts of N-Pyr. Table 1 shows that N-Pyr formation depends on the temperature of the heating system. This temperature dependence was also indicated by Pensabene et al. (1974) who found that frying bacon at 99°C for 105 min produced no N-Pyr, while 6 min at 176.7°C produced 10 ppb N-Pyr. The identity of the N-Pyr produced from collagen was confirmed by TLC analysis (Griess and ninhydrin tests, Sen et al., 1969), GLC retention time and by GLC-mass spectrometry. The fragmentation patterns of the isolated N-Pyr and the authentic N-Pyr standard are very similar (Fig. 2) and both show very strong peaks at m/e 100 (M^+).

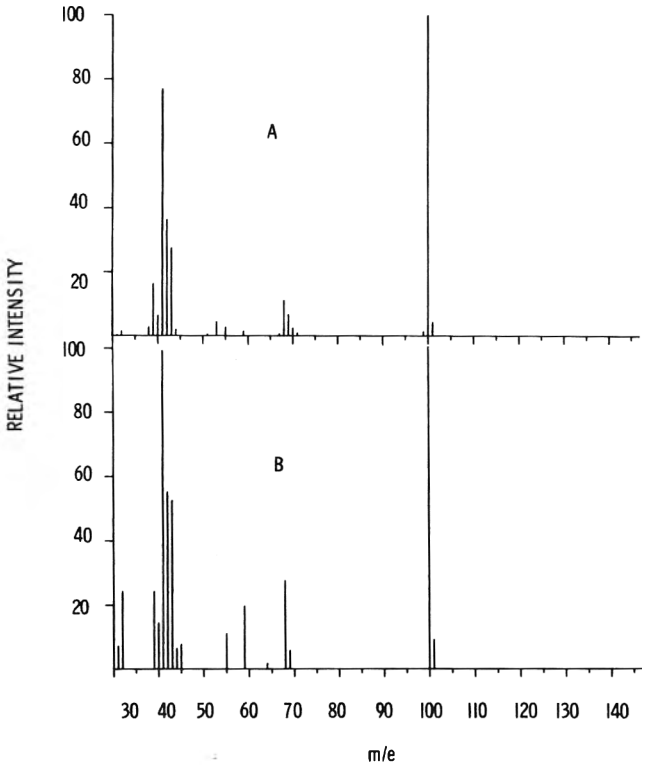

Fig. 2—Mass spectra of N-nitrosopyrrolidine: (A) Standard N-Pyr (Aldrich Chemical Co.); (B) N-Pyr isolated from collagen sample heated in the oil-water system.

Table 2—Production of N-nitrosopyrrolidine from connective tissue of a cured ham and with/without added nitrite at 175°C

Sample no.	Treatment	Nitrite added (g)	mg N-Pyr[a]
1	175°C for 10 min	0	0.001[b]
2	175°C for 10 min	0.5	0.015[c]
3	none	0	N.D.[d]

[a] Average of three samples
[b] Tentatively identified by GLC (2 columns)
[c] Confirmed by mass spectrometry
[d] N.D.—none detected

Table 3—Effect of water in the oil-water system on N-nitrosopyrrolidine formation from collagen

ml H_2O in system	Max. temp obtained °C	mg N-Pyr /0.3g collagen[a]
0	200	0.021
5	122	0.030
10	116	0.033

[a] Average from three samples

Connective tissue from a smoked cured ham was also analyzed as a potential precursor of N-Pyr. The pumping brine used in the curing process contained salt (6.67 lb), sugar (3.33 lb), $NaNO_2$ (12.5g), sodium ascorbate (37g) and water (44.25 lb). The ham was pumped to give a 10% increase in weight, then immersed in a cover brine of the same composition for 12 days. The ham was soaked for 3 hr in cold tap water, held overnight, and smoked to an internal temperature of 61°C. The cured ham had a residual nitrite content of 190 ppm. The connective tissue, dissected free from adhering fat and muscle also gave a positive color reaction with Griess reagent, indicating the presence of nitrite. This tissue was treated at 175°C in a manner similar to that for the commercial collagen. As expected, treatment with added nitrite resulted in a substantial increase in N-Pyr produced (Table 2). The dichloromethane extract of the sample which was heated without added nitrite was analyzed only by GLC using two columns (OV-210 and Carbowax 20M). The suspected compound only can be called "apparent" N-Pyr since confirmation by mass spectrometry was not achieved. A similar sample of the connective tissue was not heated in the oil-water system and no trace of N-Pyr was observed under the GLC conditions employed in the study. This is in agreement with reports that N-Pyr has been found in cooked but not in raw bacon (Fazio et al., 1973).

The effect of water on N-Pyr formation from collagen and nitrite was not very conclusive. Table 3 indicates that the presence of water in the system increased the amount of N-Pyr produced. The added water had a very noticeable effect on the final temperature reached in the system. Heating for 20 min in a silicone oil bath at 210°C produced temperatures of 124°C in the sample containing 5 ml of water and 115°C in the sample containing 10 ml of water. It appears that the presence of water in the system increases the degree of hydrolysis of collagen and consequently the amount of available proline in spite of the lowering of the temperature. It could be speculated that phosphate treated bacon (to hold more water in the system) might be a greater source of N-Pyr than bacon not so prepared.

To show that hydrolysis of collagen and nitrosation of the released proline did not occur during vacuum distillation from an alkaline medium, a sample of the commercial collagen and sodium nitrite was distilled. No N-Pyr was detected in the solvent extract of the distillate.

Two techniques were investigated for eluting the suspected N-Pyr from the silica gel scraped from the TLC plate. Using water-saturated diethyl ether (Sen et al., 1969) and stirring for 6 hr gave only a 41% recovery. Steam distillation of the scraped-off sorbent resulted in a 85% recovery. Eisenbrand et al. (1970) using a similar technique obtained recoveries of 96.5, 98.0 and 98.0% for dimethyl-, diethyl- and di-n-amylnitrosamines, respectively, from Kieselgel PF_{254}.

Although most of the proline in meat is found within protein molecules, this study has shown that hydrolysis or pyrolysis of protein can occur as a result of heating stresses to produce free proline. Collagen, on the basis of this study can be considered to be a potential precursor of N-Pyr. In the majority of samples, high levels of nitrite were used to study the formation of N-Pyr. However, N-Pyr was tentatively identified as being present in a sample of connective tissue from a cured

ham and where only the legal limit of nitrite was used in the curing process. This study also confirmed reports that heating (cooking) is required for N-Pyr formation in bacon.

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EFFECT OF SODIUM NITRITE AND NITRATE ON Clostridium botulinum GROWTH AND TOXIN PRODUCTION IN A SUMMER STYLE SAUSAGE

INTRODUCTION

THIS STUDY on the effect of nitrite on *C. botulinum* growth in fermented sausage is one part of a series undertaken cooperatively by the American Meat Institute, the Food & Drug Administration, and the United States Department of Agriculture. The series was conducted to determine minimum levels of sodium nitrite required for consumer acceptance and botulinal protection in perishable cured meats. Because differences in formulation and processing techniques might affect the impact of nitrite, a variety of cured meats were included in the overall study. Previous studies in this series have shown that increased nitrite levels decreased the probability of botulinal toxin formation in a canned perishable cured meat (Christiansen et al., 1973), wieners (Hustad et al., 1973) and in bacon (Christiansen et al., 1974).

The formulation and processing, particularly the fermentation process, used in the production of fermented sausage makes this product considerably different from the above three products. This paper is concerned only with the effect of nitrite on *C. botulinum* growth in the sausage. Other phases of the fermented sausage study dealing with the effects of nitrite and nitrate on organoleptic properties of the sausage and on nitrosamine formation will be reported separately.

MATERIALS & METHODS

Experimental design

Two experiments were conducted. The variables and processing conditions used in the first experiment are given in Table 1. The experimental design was an incomplete factorial of the given treatments. The experimental design for Experiment 2 is shown in Table 2.

Inoculum

The *C. botulinum* inoculum for both experiments consisted of a mixture of five type A and five type B strains prepared as previously described (Christiansen et al., 1973). The composite spore suspension was heat-shocked at 80°C for 15 min, appropriately diluted, and added to the sausage mix during formulation.

In both experiments, a commercial starter culture (Lactacel MC, Merck & Co., Inc., Rahway, N.J.), containing *Lactobacillus plantarum* and *Pediococcus cerevisiae* was added to most variables at formulation at a level of 0.125% (Tables 1 and 2).

Preparation of sausage

In Experiment 1, primal cuts of beef were ground, the ground meat divided into appropriate sized batches, and frozen until needed. In Experiment 2, the beef cuts were surface pasteurized by dipping in hot water for 15 sec before being ground and frozen. Prior to formulation the meat was thawed overnight at 10°C.

The sausage generally was formulated to contain 2.5% sodium chloride, 2.0% dextrose, and 0.35% spices (certain variables in Experiment 2 were formulated without dextrose as shown in Table 2). These ingredients were added dry and mechanically mixed into the meat. Solutions of the various concentrations of nitrite and nitrate and suspensions of *C. botulinum* spores and starter culture were added in a total of 3% added water.

The formulated sausage was stuffed into 60 mm × 460 mm Brechteen collagen casings (The Brechteen Co., Mt. Clemens, Mich.) for further processing. In Experiment 1, the sausage was fermented as outlined in Table 1, then heated to an internal temperature of 58.3°C. In Experiment 2, the fermentation was omitted and the stuffed sausage

Table 1—Formulation and processing variables for experiment 1

Nitrite levels:	0, 75, 150 and 300 μg per g of meat
Nitrate levels:	0 and 1,500 μg per g of meat
C. botulinum levels:	100 and 10,000 spores per g of meat

Fermentation conditions

With starter culture

1. Stuffed raw mix held 2 days at 10°C, then held at 32.2°C for 4 hr before being heated to an internal temperature of 58.3°C.
2. Stuffed raw mix heated to 32.2°C and held for 4 hr, then heated to 58.3°C.
3. Stuffed raw mix held at 32.2°C until pH reached 5.3–5.4 (18–24 hr). Product then heated to 58.3°C.

Without starter culture

1. Stuffed raw mix held at 32.2°C for 18 hr, then heated to 58.3°C

Table 2—Formulation variables for experiment 2

Variable No.	Starter culture	Dextrose	Nitrite level (ppm) 0	50	100	150
1	X	X	X			
2	X	X		X		
3	X	X				X
4		X	X			
5		X		X		
6		X			X	
7		X				X
8	X		X			
9	X			X		
10	X				X	
11	X					X
12			X			
13				X		
14						X

Table 3–Development of pH in sausage during processing and storage

	Before processing	After processing	1 wk at 27°C	2 wk at 27°C	16 wk at 27°C
Formulated with dextrose					
Range	5.45–5.75	5.70–6.10	4.45–5.08	4.32–4.50	4.00–4.35
Average	5.63	5.97	4.68	4.38	4.22
Formulated without dextrose					
Range	5.45–5.65	5.80–5.95	5.65–5.80	5.60–6.10	*
Average	5.58	5.87	5.74	5.86	

* No samples remained in incubation.

heated immediately to 58.3°C. This insured that all variables would have approximately the same pH when placed in incubation at 27°C. Also, the product formed without a fermentation step would be representative of a variety of sausages which are not fermented or sausage which has undergone a poor fermentation. After the final heat process, the sausage was cooled to 10°C, then cut into 100g pieces, and the pieces vacuum packaged in Curpolene 200 (Curwood, Inc., New London, Wisc.) pouches. Pouches were stored at 27°C. Nonswollen packages were randomly selected at predetermined intervals for analysis. Swollen packages were analyzed at time of swell.

Analysis of samples

Samples were blended in a Waring Blendor with an equal weight of gelatin phosphate buffer (pH 6.2) and analyzed for botulinal toxin and levels of *C. botulinum* as previously described (Christiansen et al., 1973).

Total aerobic counts

The blended sample was diluted and plated in APT agar (Difco). Plates were counted after 2 days at 32°C.

pH determinations

Measurements of pH were made on sausage minced with distilled water at a ratio of approximately 1:1.

RESULTS & DISCUSSION

THE FERMENTATION conditions used in Experiment 1 (Table 1) provided product at two pH levels. Following heat processing, sausage formulated with starter culture and fermented according to condition 3 had a pH of 5.2. All other fermentation variables had a pH of 5.8 after heat processing. Toxin formation did not occur under any of the fermentation conditions. Five samples of each nitrite-nitrate variable tested after the different fermentation procedures were nontoxic.

All product held at 27°C subsequent to fermentation had pH values ranging from 4.6–4.9 within 8 days independent of the fermentation conditions or nitrite-nitrate levels. Toxin was not produced at these low pH levels. Five samples of each variable tested after 14 and 28 days of storage at 27°C were nontoxic.

Results of the initial test suggested that nitrate would have little inhibitory effect in fermented sausage. A previous study (Christiansen et al., 1973) suggested that nitrate must be converted to nitrite to affect inhibition. The release of nitrite from nitrate is too slow to be effective in a product which undergoes a rapid inhibitory drop in pH. Thus, a second test was designed to investigate the relative effect of nitrite, starter culture and dextrose on *C. botulinum* growth in the event these materials are accidentally omitted from the sausage formulation and a normal fermentation does not occur.

The final pH of product in Experiment 2 was controlled effectively by formulating samples with and without dextrose (Table 3). The pH was determined on samples of the different variables before and after processing and after 1 wk, 2 wk, and 16 wk of storage at 27°C. Determinations at these intervals showed no effect of starter culture or nitrite levels on pH development. Although the pH was essentially the same in

Table 4–Botulinum toxin developed in sausage held at 27°C

Nitrite level (μg/g meat)	Starter culture	Dextrose	Number toxic/Number tested at: (Days)							
			7	14	21	28	49	56	112	Total
0	X	X	0/3	0/3	0/3	0/5		1/5	1/6	2/25
50	X	X	0/3	0/3		0/5		0/5	0/9	0/25
150	X	X	0/3	0/3		0/5		0/5	0/7	0/23
0		X	1/3	0/3	2/3	3/5		0/5	2/6	8/25
50		X	0/3	0/3		0/5		0/5	0/9	0/25
100		X	0/3	0/3		0/5		0/5	0/9	0/25
150		X	0/3	0/3		0/5		0/5	0/8	0/24
0	X		0/3	22/22[a]						22/25
50	X		0/3	22/22[a]						22/25
100	X		0/3	0/3	0/4	2/5	2/10[a]			4/25
150	X		0/3	0/3	1/3	5/5	6/11[a]			15/25
0			0/3	1/3	19/19[a]					20/25
50			0/3	3/3	18/19[a]					21/25
150			0/3	0/3	2/2	3/5	9/12[a]			14/25

[a] Swelled

Table 5—C. botulinum counts (MPN) in sausage during processing and storage

	Before processing		After processing		After 112 days storage at 27°C	
	Nonheat shocked	Heat shocked	Nonheat shocked	Heat shocked	Nonheat shocked	Heat shocked
Range	4.3×10^2 – 1.1×10^4/g	2.3×10^2 – 1.1×10^4/g	2.3×10^2 – 1.1×10^4/g	9.3×10^1 – 2.4×10^3/g	1.7×10^3 – 2.4×10^4/g	2.3×10^1 – 2.4×10^4/g
Average	1.7×10^3/g	7.4×10^2/g	9.1×10^2/g	4.6×10^2/g	5.3×10^3/g	1.5×10^3/g

samples formulated with and without dextrose before and after processing, the pH in the two types of samples after storage at 27°C differed markedly. After 1 wk of storage, samples with dextrose had an average pH of 4.68, which decreased to 4.38 at 2 wk and 4.22 at 16 wk. Samples formulated without dextrose had an average pH of 5.74 and 5.86 after storage for 1 and 2 wk, respectively. No samples formulated without dextrose remained in storage for the 16-wk period.

The pH drop which occurred during storage in the dextrose containing samples clearly affected, but did not completely prevent, growth and toxin production by *C. botulinum* (Table 4). Only 10/50 samples formulated with dextrose but without nitrite became botulinogenic.

The addition of 50 μg or more of nitrite/g of meat to samples containing dextrose prevented toxin production (0/122 samples) throughout the 112 days of storage at 27°C.

A majority (115/175) of the samples formulated without dextrose became toxic (Table 4). Increasing the nitrite level in these variables up to 150 μg of nitrite/g of meat tended to reduce the rate of toxin development but did not totally prevent it. Most of these toxic samples were swollen and all were putrid.

The use of commercial starter culture had some effect on the growth of *C. botulinum* in samples formulated with dextrose but without nitrite. Only 2/25 of these samples with starter culture became toxic; whereas, toxin was detected in 8/25 of the same type samples formulated without starter culture (Table 4). Initial total aerobic counts in samples with and without starter culture were approximately the same; the $\log_{10}$ average total count in the two types of samples was 7.5 $\times 10^6$/g and 4.9 $\times 10^6$/g, respectively. Processing to an internal temperature of 58.3°C affected the indigenous flora more than the starter culture. After processing, counts in samples without starter culture averaged 2.3 $\times 10^2$/g; whereas, counts in samples with added starter culture averaged 2.7 $\times 10^5$/g.

Counts of *C. botulinum* in nontoxic samples stored at 27°C for the 112-day period were essentially the same as initial levels (Table 5), suggesting that neither growth nor death had occurred in these samples. The geometric means in samples before processing were 1.7×10^3 and 7.4×10^2/g in nonheat-shocked and heat-shocked aliquots, respectively. After processing, the average count in nonheat-shocked samples was 9.1 $\times 10^2$/g and in heat-shocked samples the count was 4.6×10^2/g. Two samples of each variable remaining in storage after 112 days were selected at random for determination of *C. botulinum* levels. The average count was 5.3×10^3/g in nonheat-shocked samples and 1.5×10^3/g in heat-shocked samples (Table 5).

The above results for Experiment 2 show that the inhibitory effect of nitrite on *C. botulinum* growth in sausage was markedly influenced by pH. Only 50 μg of nitrite per g of meat was necessary to inhibit botulinal growth in samples formulated with dextrose in which the pH dropped during 27°C storage. For samples (formulated without dextrose) in which the pH remained at the initial level, formulated nitrite levels up to 150μg per g of meat did not prevent toxin production. The effect of starter culture in the samples formulated with dextrose but without nitrite gives further evidence of the importance of pH on nitrite inhibition in sausage. Although the pH in samples with and without starter culture was the same when first measured after 1 wk of storage, fewer of the samples with starter culture became botulinogenic. Presumably the starter culture lowered the pH faster than did the indigenous flora.

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EFFECT OF ADDED SODIUM NITRITE AND SODIUM NITRATE ON SENSORY QUALITY AND NITROSAMINE FORMATION IN THURINGER SAUSAGE

INTRODUCTION

NITRITES AND NITRATES have long been used in the curing of meat and poultry, such use pre-dating the Food Additives Amendment of 1958. At the present time, the use of nitrites and nitrates, insofar as nitrates are converted to nitrites, is technologically necessary in maintaining flavor, color and texture characteristics associated with and expected of certain staple food items (Fine, 1972).

Haldane (1901) and Brooks et al. (1940) related cured meat color to nitrosohemoglobin formation, brought about by the combination of nitrite with hemoglobin present in meat, blood and muscle tissue. The reaction between nitrite and reduced hemoglobin is rapid at pH and salt concentrations obtained in muscle during curing, the time required for color fixation proportionate to the time necessary for adequate nitrite diffusion. Kerr et al. (1926) attributed incomplete color fixation to insufficient nitrite penetration into the tissue; given complete change to nitrosohemoglobin, depth of color depended on tissue hemoglobin concentration. Unsatisfactory color was traced to an unusually low hemoglobin content. Wasserman and Talley (1972) associated an unpleasant gray color with unsmoked frankfurters prepared without sodium nitrite in the cure. When nonnitrite frankfurters were smoked as well as cooked, a browned surface but gray interior resulted. A red pigment, identified as nitrosomyoglobin, occurred at the smoked skin and gray underlying meat interface. Nitrosomyoglobin formation was attributed to certain oxides of nitrogen within the smoke which penetrated the sausage casing and reacted with the myoglobin of the meat.

The distinctive flavor of cured meat is its most outstanding organoleptic feature. Brooks et al. (1940) ascribed characteristic bacon and ham cured flavor to the reaction of nitrite with constituents of muscle tissue during curing or cooking. A satisfactory bacon was made by using only sodium nitrite and sodium chloride. Although no taste panel data were presented, these authors stated that nitrite cured ham and bacon were preferred to non-nitrite cured. Cho and Bratzler's (1970) untrained panelists found a flavor difference between pork roasts cured in nitrite and nonnitrite water solutions and more cured flavor in the nitrite cured variation. Flavor distinctions were also made when smoke was included in the curing process, more cured flavor occurring in nitrite-containing roasts. Wasserman and Talley (1972), through triangle flavor difference evaluations, tested the effect of nitrite concentration on detection of frankfurter flavor differences. Flavor differences were established between unsmoked franks with and without sodium nitrite added to the cure and between smoked franks with and without nitrite. No difference was determined between smoked frankfurters containing 100% and 50% normally added nitrite amounts, however. Simon et al. (1973), with limited panelists, correlated preference scoring and nitrite level. Preference differences occurred between nonnitrite and 39, 78 or 156 ppm nitrite-containing all-meat frankfurter formulations, and between 39 and 156 ppm level extremes. Butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), nor smoke could compensate for the elimination of nitrite. Acceptable tasting frankfurters, during a 3-wk display case storage, were obtained only when full cure was included. Preference scoring of all-meat and all-beef frank formulations, vacuum and bulk packaged, decreased with storage regardless of nitrite addition, however. Hustad et al. (1973) found, through expert panel evaluations, flavor quality of weiners containing 50–300 μg of nitrite per g higher than weiners made without nitrite.

Recent research reported by Skjelkvale and Tjaberg (1974) on ripened salami sausage was not in agreement with previous findings. A series of trained panel triangle difference tests revealed no detectable flavor difference among nonnitrite, 82 ppm nitrite, and 164 ppm nitrite levels, no difference between 5% glucono-delta-lactone (GDL) ± 164 ppm nitrite treatments, nor between salami containing starter culture (Duplofermente) ± 164 ppm nitrite. After 3 months storage at 20°C, flavor distinctions were made, but between the nonnitrite and 164 ppm added nitrite salami treatments only. However, if one considers the individual triangle comparisons reported as simple inputs to answer the broad question of whether there is a flavor difference between salami containing nitrite and salami containing no nitrite, the reported results do, indeed, reveal a significant difference at a 0.05 level. This applies both to products after ripening and after 3 months storage. In addition, the authors alluded to the possibility of a heavy smoke and spice flavor masking effect which could, in itself, restrict sample differentiation.

This present investigation was conducted to determine the effect of nitrite, nitrate, combinations of nitrite and nitrate, fermentation temperature and storage temperature-time on fermented thuringer sausage sensory properties, nitrite residuals, and nitrosamine formation. Thuringers were evaluated in sliced fresh, fried, and baked pizza topping forms.

EXPERIMENTAL

Product formulation

Thuringer was prepared from an all-beef formulation. Lean and fat components were controlled to produce a final thuringer product containing 25% fat. Meat was ground through a 1/8 in. plate, blended to assure uniformity, and sub-divided into 40-lb lots.

Formula base

Ingredient	Amount/100 pounds meat
Salt	2.5%
Dextrose	2.0%
Blended Spices	0.35%
Merck & Co., Inc. Lactacel MC Starter Culture	0.125%
Water	3.0%

0, 50, 100 and 150 ppm sodium nitrite and 0, 500 and 1500 ppm sodium nitrate levels were added. Reblended individual batches were stuffed into 45 mm Tee-Pak fibrous casings to form approximate 2-pound chubs. Sausages were fermented at both 32°C and 10°C to a 5.2 pH level, then cooked with heavy smoke under controlled humidity and temperature conditions to a 57°C internal temperature. Approximately 3 hr cooking and smoking were required. Processed sausages were then chilled to a 10°C internal temperature.

Holding conditions

Thuringer for fresh and fried panel evaluations was held 1 wk at 7.5°C. Thuringer for pizza topping was sliced into 3/16-in. pieces and 1/2-lb amounts placed on commercial 10-in. diameter frozen cheese pizzas. Pizzas were loosely covered with a polyethylene wrap, repackaged in original containers, and subjected to 1 wk, −18°C storage.

Nitrite and nitrate concentrations were established immediately after thuringer formulation. Nitrite, nitrate, fat, moisture and pH measurements were performed on the product after fermentation. Thuringer for residual nitrite and nitrate chemical analyses was stored at 7.5°C for intervals of 1 and 4 wk, and at 27°C for intervals of 2 and 8 wk.

Thuringer submitted for fresh and fried product nitrosamine determinations was stored at 7.5°C for 2 and 4 wk intervals. Additional thuringer for fresh product analyses was held at 7.5°C for 1 wk plus 2 days at 27°C. Pizza, topped with thuringer and held frozen (−18°C), was baked at 2- and 4-wk storage intervals. The cooked thuringer topping only was supplied for nitrosamine assays.

Chemical analyses

Nitrite and nitrate concentrations were determined spectrophotometrically by using modifications of methods 24.014 and 24.011 of the Association of Official Analytical Chemists (1970). The nitrite procedure involved the separate additions of sulfanilic acid and alpha-naphthylamine to the sample. For nitrate analysis, the sample was treated with urea in acid solution to destroy the nitrite. The nitrate was then reacted with metaxylenol, the reaction product was distilled off, and the distillate was diluted for analysis. Samples with various combinations of nitrite and nitrate levels were quantitatively analyzed for 14 volatile nitrosamines by using the Food and Drug Administration's multidetection system (Fazio et al., 1971).

Residual nitrite and nitrate chemical analyses were performed by Swift's Analytical Chemistry Section; nitrosamine analyses were performed by the Division of Chemistry & Physics, Food & Drug Administration, Washington, D.C.

Sensory panel evaluations

All thuringer variations were panel tested in sliced fresh form. Non-nitrite plus 0 and 500 ppm nitrate, 50 ppm nitrite plus 0 and 500 ppm nitrate, 100 ppm nitrite plus 0 nitrate, and 150 ppm nitrite plus 0 and 500 ppm nitrate variations were evaluated fried and as baked pizza topping (Table 1). Fried thuringer evaluations involved cooking 3/16-in. thick sausage slices in 172°C electric skillets, 2–3 min per side. Pizzas, for baked thuringer evaluations, were baked at 218°C for 20 min. Cooked thuringer slices were removed and evaluated.

To facilitate comparison of numerous thuringer treatments and to increase panelists' sample discrimination ability, Balanced Incomplete Block sample distribution methods were applied within flavor evaluations; a completely randomized design was used within appearance evaluations. Thus, the comparison of 15 sliced fresh product variations was performed by 15 trained panelists, while fried and pizza topping comparisons of seven thuringer variations were each performed by 12 trained panelists. A pool of 30 trained panelists, persons screened for sensitivity to rancid meat off-flavors and having previous panel experience in evaluating cured meat products, was used within the panel series. Fresh, fried and baked thuringer evaluation criteria included appearance quality, rancid off-flavor intensity and flavor quality. Rancid off-flavor intensity ratings were given relative to a 6-point quantitative scale. Appearance and flavor quality responses were based on a 10-point rating scale. Appearance quality evaluations regarded presence, uniformity, and intensity of cure color; flavor quality pertained to cure flavor characteristics and detectable off-flavor. Prior to fresh, fried, and baked product evaluation series, panelists openly discussed and rated a representative thuringer sausage, the 32°C fermented, 150 ppm nitrite plus 500 ppm nitrate variation, to establish baseline ratings for subsequent sample evaluations.

Flavor evaluations were conducted in individual panel booths and, to assure appearance similarity among thuringer samples presented, under subdued red fluorescent lighting. Appearance evaluations succeeded flavor and were conducted in a separate daylight lighting area.

Statistical analysis and design

The data from the panels were analyzed statistically via analysis of variance and regression analysis. The experimental design (Table 1) allowed analysis of variance of the data in factorial groupings. For fresh thuringer, the designs were a 4 × 3 (nitrite × nitrate) factorial at 32°C fermentation and a 2 × 3 (fermentation temperature × nitrate) factorial at no nitrite. For fried and baked thuringer fermented at 32°C, the design was a 3 × 2 (nitrite × nitrate) factorial with one added nitrite point at no added nitrate.

RESULTS

Sensory panel evaluation

Fresh thuringer. In Table 2 is shown the effect of nitrite and nitrate addition on fresh thuringer off-flavor development and resulting flavor quality. The addition of nitrite in the cure

Table 1—Experimental design, added nitrite and nitrate in thuringer sausage

Treatment variation code no.	Nitrite added (ppm)	Nitrate added (ppm)	Fermentation Temp. (°C)
1[a]	0	0	32
2[a]	0	500	32
3	0	1500	32
4[a]	50	0	32
5[a]	50	500	32
6	50	1500	32
7[a]	100	0	32
8	100	500	32
9	100	1500	32
10[a]	150	0	32
11[a]	150	500	32
12	150	1500	32
13	0	0	10
14	0	500	10
15	0	1500	10

[a] Denotes thuringer treatments involved in fried and baked pizza topping chemical and sensory evaluation series; all 15 thuringer variations were evaluated fresh.

Table 2—Trained sensory panel evaluation of fresh thuringer

	32°C Fermentation temp				10°C Fermentation temp
Nitrate level	Nitrite level				Nitrite level
	0 ppm	50 ppm	100 ppm	150 ppm	0 ppm
	Off-flavor/rancidity intensity mean scores[a]				
0 ppm	3.25	1.56	1.50	1.44	5.00
500 ppm	1.88	1.38	1.50	1.19	3.88
1500 ppm	2.00	1.69	1.13	1.50	4.25
	Flavor quality mean scores[b]				
0 ppm	4.00	6.19	6.63	6.50	1.81
500 ppm	6.00	6.63	6.06	6.81	3.00
1500 ppm	5.94	6.19	6.81	6.00	2.50
	Appearance quality mean scores[b]				
0 ppm	1.47	7.10	6.93	7.03	1.27
500 ppm	7.53	7.17	6.97	7.20	7.13
1500 ppm	7.33	7.43	7.17	7.27	6.80

[a] Off-flavor/rancidity intensity scale: 1.00 = none; 2.00 = very little; 3.00 = little; 4.00 = moderate; 5.00 = much; 6.00 = very much

[b] Flavor and appearance quality rating scales: 1.00 = repulsive; 2.00–3.00 = poor; 4.00–5.00 = fair; 6.00–7.00 = good; 8.00–9.00 = very good, 10.00 = excellent.

Table 3–Fermentation temperature effects on fresh thuringer containing added nitrate[a] only

Fermentation temp	Off-flavor/rancidity intensity mean scores[b]	Flavor quality mean scores[c]
10°C	4.38	2.23
32°C	2.38	5.31

[a] Off-flavor and flavor quality scores represent fresh thuringer treatments containing 500 and 1500 ppm added sodium nitrate and no added sodium nitrate.
[b] See footnote a, Table 2
[c] See footnote b, Table 2

reduced off-flavor development and improved flavor quality. Nitrite addition beyond the 50 ppm level yielded highest quality and lowest off-flavor scores. Although rancidity was most apparent in nonnitrite treatments, regardless of nitrate addition, thuringers containing nitrate only were judged less off-flavored and of better flavor quality than nonnitrate. Thuringer containing neither nitrite nor nitrate was judged most rancid or off-flavored and poorest in flavor quality ($\alpha = 0.05$).

Fermentation temperature affected the flavor quality of thuringer treatments containing added nitrate only (Tables 2 and 3). Poorer flavor quality, more intensely off-flavored product resulted from 10°C than from 32°C fermentation.

Fresh thuringer appearance quality was affected by the addition of both nitrite and nitrate (Table 2). Whenever nitrite, nitrate, or a combination of nitrite and nitrate were present in the formulation, regardless of difference in fermentation temperature, appearance quality was improved; in the absence of either or both, appearance quality was poor. Panelists' descriptions of the nonnitrite, nonnitrate thuringer included "gray, brown, very poor, no cure color, etc."

It should be noted that the nonnitrite, nonnitrate thuringer evaluated was not a typical fermented product, thus it is not surprising that significant ($\alpha = 0.05$) flavor and appearance improvement would result from the addition of nitrite or nitrate in the curing process. Within this study it was consistently shown that nitrite addition at a minimal level of 50 ppm did improve nonnitrite, nonnitrate sausage product quality; however, at either 100 or 150 ppm addition levels, effects were more apparent.

Fried thuringer. Reduced off-flavor and increased flavor quality occurred with nitrite addition (Table 4). The addition of 50 ppm nitrite, however, did not optimally improve thuringer flavor quality; higher flavor quality and lower off-flavor scores occurred in 100 and 150 ppm nitrite variations ($\alpha = 0.05$). The addition of nitrate up to 500 ppm neither reduced off-flavor intensity nor improved flavor quality ($\alpha = 0.05$).

Table 4–Trained sensory panel evaluation of fried thuringer

Nitrate level	Nitrite level			
	0 ppm	50 ppm	100 ppm	150 ppm
	Off-flavor/rancidity intensity mean scores[a]			
0 ppm	3.30	2.00	1.60	1.60
500 ppm	2.08	1.92	–	1.50
	Flavor quality mean scores[b]			
0 ppm	3.58	4.75	5.30	5.58
500 ppm	4.83	4.83	–	5.16
	Appearance quality mean scores[b]			
0 ppm	1.19	4.86	6.19	6.10
500 ppm	3.76	5.38	–	6.52

[a] See footnote a, Table 2
[b] See footnote b, Table 2

Table 5–Trained sensory panel evaluation of baked pizza topping thuringer

Nitrate level	Nitrite level			
	0 ppm	50 ppm	100 ppm	150 ppm
	Off-flavor/rancidity intensity mean scores[a]			
0 ppm	2.30	1.16	1.30	1.16
500 ppm	1.50	1.42	–	1.08
	Flavor quality mean scores[b]			
0 ppm	4.00	5.83	6.08	6.25
500 ppm	5.42	5.60	–	5.92
	Appearance quality mean scores[b]			
0 ppm	1.48	5.81	6.53	6.86
500 ppm	6.91	6.10	–	5.76

[a] See footnote a, Table 2
[b] See footnote b, Table 2

Table 6–Residual levels of nitrite and nitrate in thuringer sausage at various stages of 7.5°C storage

	Treatment identification			Residual nitrite levels				Residual nitrate levels			
Code no.	Added nitrite (ppm)	Added nitrate (ppm)	Fermentation temp (°C)	Raw emulsion (ppm)	0 wk storage (ppm)	1 wk storage (ppm)	4 wk storage (ppm)	Raw emulsion (ppm)	0 wk storage (ppm)	1 wk storage (ppm)	4 wk storage (ppm)
1	0	0	32	0	0	0	0	14	18	14	33
2	0	500	32	0	39	12	9	480	255	262	256
3	0	1500	32	0	62	17	14	536	1082	1092	1084
4	50	0	32	31	10	8	10	34	14	42	14
5	50	500	32	28	17	8	7	536	391	430	126
6	50	1500	32	28	18	9	8	1550	1276	1298	1304
7	100	0	32	66	12	7	10	44	20	37	90
8	100	500	32	57	18	8	10	520	412	413	373
9	100	1500	32	57	24	12	11	1496	1276	1298	1384
10	150	0	32	75	15	10	1[illegible]	40	48	47	58
11	150	500	32	85	17	4	10	520	456	454	451
12	150	1500	32	84	36	12	12	1566	1210	1346	1554

Table 7—Residual levels of nitrite and nitrate in thuringer sausage at various stages of 27°C storage

Code (no.)	Treatment identification: Added nitrite (ppm)	Added nitrate (ppm)	Fermentation temp °C	Residual nitrite levels: Raw emulsion (ppm)	0 wk storage (ppm)	2 wk storage (ppm)	8 wk storage (ppm)	Residual nitrate levels: Raw emulsion (ppm)	0 wk storage (ppm)	2 wk storage (ppm)	8 wk storage (ppm)
1	0	0	32	0	0	1	1	14	18	30	7
2	0	500	32	0	39	4	18	480	255	250	134
3	0	1500	32	0	62	5	43	536	1082	1206	924
4	50	0	32	31	10	3	14	34	14	25	16
5	50	500	32	28	17	6	35	536	391	1200	731
6	50	1500	32	28	18	4	36	1550	1276	1306	974
7	100	0	32	66	12	4	16	44	20	21	62
8	100	500	32	57	18	5	23	520	412	411	67
9	100	1500	32	57	24	6	23	1496	1276	1346	264
10	150	0	32	75	15	5	13	40	48	40	18
11	150	500	32	85	17	2	20	520	456	458	27
12	150	1500	32	84	36	4	132	1566	1210	1420	174

Depending on level added, appearance quality improvement occurred when nitrite was included within the formulation (Table 4). At least 100 ppm nitrite was necessary to differentiate nitrite and nonnitrite treatment scores. Based on a significant ($\alpha = 0.05$) nitrite × nitrate interaction, the effectiveness of added nitrate depended on nitrite's presence. Appearance quality of thuringer containing 500 ppm nitrate improved with increased nitrite. Gray, green, dark, brown, dark center, off-colors characterized nonnitrite thuringer appearance.

Baked pizza topping thuringer. Nitrite, regardless of level added or presence of nitrate, reduced rancidity intensity and improved flavor quality of frozen and baked pizza topping thuringer (Table 5). At 100 and 150 ppm added nitrite levels, flavor effects were most obvious. Intensity and quality effects due to nitrate depended on the presence of nitrite. Nitrate reduced off-flavor development in nonnitrite thuringer but did not appreciably improve flavor quality.

Higher appearance quality scores resulted from nitrite addition, regardless of level or presence of nitrate (Table 5). A significant nitrite × nitrate interaction was observed ($\alpha = 0.05$). Thuringer treatments containing nitrite, nitrate, or a combination of nitrite and nitrate received higher appearance quality ratings than nonnitrite, nonnitrate. Nitrate's effectiveness was most apparent in nonnitrite product; the influence of nitrate decreased as nitrite level increased. "Gray, brown, spoiled, green" appearance descriptions characterized the nonnitrite, nonnitrate thuringer variation; "red or dull red" appearances were associated with nitrite or nitrate-containing treatments.

Residual nitrite and nitrate determinations

In Tables 6 and 7 residual levels of nitrite and nitrate are shown at various stages of product storage, at storage temperatures of 7.5°C and 27°C. A difference between nitrite and nitrate concentrations added and amounts analytically determined before fermentation, i.e., within the raw meat emulsion, was observed. Nitrite concentrations were less but nitrate levels were relatively unaffected.

Residual nitrite and nitrate concentrations diminished during product storage, reduction rate depending on storage temperature and length of storage time. Nitrate depletion was more pronounced at 27°C than at 7.5°C; nitrite concentration decreased rapidly regardless of storage temperature. Nitrite, due to the generation of nitrite from nitrate during product fermentation, cooking, or storage, was detected in thuringer containing added nitrate only and in treatments containing neither added nitrite nor nitrate. Small amounts of inherent nitrate were detected in thuringer to which neither nitrite nor nitrate was added. Unaccounted for, although not uncommon, fluctuations in residual nitrate concentration occurred during product storage. Previously, Christiansen et al. (1974) and Herring (1973) noted nitrate formation in cured bacon to which nitrite only was added. Data reported revealed nitrate formation during pumping, processing, and storage, the concentration of accumulated nitrate a function of added nitrite. Herring ascribed nitrate formation during storage to possible disproportionation of nitrous acid to give nitric oxide and nitrate and from the oxidation of nitric acid by oxygen to give nitrite which then reacted with water to give nitrate and nitrite.

Nitrosamine determinations

In Table 8 is shown the effect of initially added nitrite and nitrate on the detection of nitrosamines under normal and abused storage conditions. Regardless of nitrite or nitrate concentration, storage treatment, or serving form, no nitrosamines were detected.

Table 8—Effect of added nitrite and nitrate on detection of 14 volatile nitrosamines in stored thuringer sausage[a]

Added nitrite level[c]	Nitrosamines detected[b]: Added nitrate level 0 ppm	Added nitrate level 500 ppm	Added nitrate level 1500 ppm
0 ppm	negative	negative	negative
50 ppm	negative	negative	negative
100 ppm	negative	negative	negative
150 ppm	negative	negative	negative

[a] Each stored thuringer sausage tested as: (1) product held 2 and 4 wk at 7.5°C; (2) product held 1 wk at 7.5°C plus 2 days at 27°C; (3) product held 2 and 4 wk at 7.5°C before frying; (4) product held 2 and 4 wk at −18°C before baking as pizza topping.

[b] The analyses, sensitive to 10 ng, included the following volatile N-nitrosamines: dimethylamine, methylethylamine, diethylamine, methylpropylamine, ethylpropylamine, dipropylamine, ethylbutylamine, propylbutylamine, methylamylamine, dibutylamine, piperidine, pyrrolidine, morpholine and diamylamine.

[c] The 0 ppm nitrite series was fermented at 10°C and 32°C; the 50, 100 and 150 ppm series were fermented at 32°C only.

CONCLUSION

IN THIS INVESTIGATION, the addition of nitrite at a minimum level of 50 ppm was necessary to achieve reasonably typical thuringer flavor and appearance characteristics in sliced fresh and baked pizza topping products. At least 100 ppm added nitrite was necessary to produce these effects in fried thuringer. It was shown that more desirable product, i.e., thuringer receiving higher appearance and flavor quality scores, resulted from the addition of 100 ppm or more sodium nitrite in the cure. The effect of added nitrate was negligible. Fresh, fried, or baked thuringer containing neither nitrite nor nitrate was judged most rancid and of poorest flavor and appearance quality.

Nitrite and nitrate concentrations decreased with product storage. Rapid nitrate depletion occurred at 27°C. Nitrate was apparently converted to nitrite, possibly during product fermentation, cooking, or storage. Residual nitrite diminished rapidly at both 7.5°C and 27°C.

No nitrosamines were detected in thuringer regardless of initial nitrite or nitrate concentration, storage condition, or kitchen preparation method.

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PERSISTENCE OF STAPHYLOCOCCUS. Bruised Tissue Microenvironment Affecting Persistence of Staphylococcus aureus

INTRODUCTION

POULTRY BRUISES harbor large numbers and various kinds of bacteria of which *Staphylococcus aureus* is predominant (McCarthy et al., 1963). These staphylococci are able to combat host defense mechanisms and persist in the bruised tissue for more than 18 days (Hamdy and Barton, 1965). Poultry bruises were implicated as a source of the staphylococcal infections that commonly occur on the hands, arms, chest and abdomen of poultry processing-line workers (Roskey and Hamdy, 1972). Again Hamdy and his co-workers (Hamdy et al., 1957; McCarthy et al., 1963; Brown and Hamdy, 1965a, b; Hamdy, 1969) described many of the physical and biochemical changes occurring in the microenvironment of the bruise and related these changes to the growth and the persistence of *S. aureus* in these tissues. McCarthy et al. (1963) showed that bruising increased tissue permeability thereby facilitating microbial invasion. Hamdy et al. (1957) demonstrated that the concentration of extrastromal hemoglobin increased following trauma. Brown and Hamdy (1965b) reported that bruising elicited the release of many hydrolytic enzymes such as β-galactosidase, β-glucuronidase, acid phosphatase, deoxyribonuclease, ribonuclease as well as other enzymes in tissues. McKee and Braun (1962) and Braun and Firshein (1967) reported that the degradation products resulting from the action of deoxyribonuclease on deoxyribonucleic acid stimulated the growth of coagulase positive staphylococci.

Hamdy and Carpenter (1974) showed that the persistence of Salmonella after invasion of animal tissue appeared to be related to the phase of growth, virulence of culture used and the number of organisms ingested. Also, the act of bruising and the accumulation of blood and fluid within the chicken muscles, played an important role in the rate of growth and proliferation of *S. aureus* and *S. epidermidis*. Therefore, the present investigation was directed toward examining the effects of poultry bruises, at different stages during healing, on growth, respiration and virulence factors produced by *S. aureus* in vitro. The effect of hemoglobin on the production of enterotoxin B as well as on the respiration of this pathogenic bacteria was also studied.

MATERIALS & METHODS

Cultures

Three strains of *S. aureus* were used. The first, designated as "A-10," was originally isolated from infected lacerations of poultry workers (Roskey and Hamdy, 1972) and exhibited the following characteristics: Formed golden pigmented colonies on Staphylococcus medium 110 (Difco), actively produced coagulase, deoxyribonuclease, gelatinase and lysozyme enzymes, fermented mannitol and exhibited the phage type 83A/85. The second strain, "A-3," was also isolated from an infected cut on a poultry worker, had the same morphological and biochemical properties as "A-10" strain but had the phage type 29/52/52A/79/80/6/42E/47/53/54/75/77/81 and actively formed hyaluronidase. The third *S. aureus*, "S-6," obtained from Natick Labs, was employed as a standard enterotoxin B producer. All three strains were maintained on nutrient agar slants and stored at 4°C. Prior to use they were activated by three successive inoculations in (Difco) brain heart infusion (BHI) and incubated at 37°C for 24 hr. Flasks of BHI were inoculated with the active test strain and incubated for 24 hr at 37°C. Cells were then harvested by centrifugation, washed twice with sterile saline then resuspended in saline to the desired concentration. The latter was checked by plating appropriate dilutions on (Difco) mannitol-salt-agar (MSA) (Hamdy and Barton, 1965).

Experimental animals

Apparently healthy, normal white Leghorn chickens (mixed sex), 8–10 wk old, weighing 3–4 lb and kept in a constant temperature room (22°C) were used. The birds were maintained in batteries and were offered nonmedicated rations and water ad libitum.

Trauma and sampling

The feathers over the breast muscle of each live bird were clipped and the pectoralis major muscle was contused using the standard technique previously described by Hamdy et al. (1961a). Slight trauma was elicited by two blows and severe trauma by five blows. Symmetrically located areas on the same and/or different normal birds served as controls (nonbruised). At daily intervals post-trauma the birds were sacrificed by cervical separation, the tissue (3–6g) immediately excised aseptically, and placed in sterile pre-cooled petri dishes kept at 4°C. The tissues were minced and 3-g samples were homogenized at 4°C in 27 ml cold 0.066M phosphate buffer (pH 7.4) for 2 min using a Sorvall Omnimixer (16.000 rev/min). Each homogenate was centrifuged at 10,500 × G for 20 min at 4°C and the supernate (tissue extract) sterilized by filtration (0.22μ). An aliquot of each extract was lyophilized, stored at −40°C and rehydrated to original volume when used.

Measurement of growth and virulence factors

Flasks containing 15 ml of sterile rehydrated bruised or normal tissue extracts were inoculated with 0.2 ml (3×10^6 cells) saline-suspension of *S. aureus* "A-10," incubated for 18 hr at 37°C and the number of cells/ml extract was determined by plating appropriate dilutions on MSA. The cells in the tissue extract were removed by centrifugation (10,500 × G, 30 min, 4°C) and the supernates assayed for both free coagulase and beta hemolysin activities. The titer of coagulase was determined using the technique of Yotis and Eksted (1959) and the beta hemolysin was estimated by the procedure of Chesboro et al. (1969). Hyaluronidase was assayed using the turbidimetric procedure of Tolksdorf et al. (1949). Flasks containing 3 ml of sterile tissue extracts were inoculated with 0.2 ml (10^3 cells) of saline suspension of *S. aureus* "A-3" and incubated with shaking at 37°C for 12 hr. The cells were then harvested by centrifugation, washed twice and resuspended in saline. One-tenth ml aliquots of saline-cell suspensions were then used to inoculate 10 ml nutrient broth (Difco) containing 1% glucose and the flasks were then incubated with shaking for 15 hr at 37°C. Assay for hyaluronidase was always performed on the culture supernate.

Assay of enterotoxin B

Lyophilized, pooled, extracts of normal and of severely bruised tissue of various ages post-bruising, were rehydrated in 0.02M phosphate buffer (pH 7.4), and sterilized by filtration through a 0.22μ filter. Flasks containing 8 ml sterile filtrates were inoculated with 1.0 ml containing 10^6 cells of saline suspension of *S. aureus* "S-6" and incubated with shaking at 100 cycles/min for 24 hr at 25°C. Enterotoxin B was assayed in the culture supernate by the single gel-diffusion technique described by Weirether et al. (1966). The effect of hemoglobin on enterotoxin B production was ascertained by growing *S. aureus* "S-6" in saline-buffered BHI (pH 7.4) containing 0, 0.0004, 0.0017 and 0.0035 μM hemoglobin/ml of media.

Respiration

Oxygen consumption was measured by standard Warburg techniques using a Gilson differential respirometer. Reaction vessels contained: 1.5 ml of a 0.066M phosphate buffer (pH 7.4), which had 2×10^8 cells of *S. aureus* "A-10" in the flask; 1.5 ml of sterile bruised or normal tissue

extract in the side arm; and 0.1 ml of 40% KOH and a folded filter paper wick in the center well. The reaction vessels were allowed to equilibrate for 15 min at 37°C and then respirometer measurements were obtained at intervals over a 60 min incubation period. The effect of hemoglobin on glucose oxidation was determined by preparing reaction vessels to contain: 0.1 ml of 0.1M glucose, 0.3 ml of 0.066M phosphate buffer (pH 7.4); 2.6 ml of saline-cell suspension, which had 2 × 10^8 cells; 0.3 ml of hemoglobin solution (0.0035 μM/ml) in side arm; and 0.1 ml of 40% KOH in the center well.

RESULTS & DISCUSSION

THE DATA comparing the growth of staphylococci in normal tissue extract and in extracts prepared from slight and severe bruises of various ages post-bruise are shown in Table 1. It can be seen that growth of staphylococci in extracts of slight bruises obtained at any time, 1st to 9th day, post-trauma was much more than that noted in normal tissue extract. In extracts prepared from 1–4 day old slight bruises, the average increase above the growth level in the normal (control) tissue extract was 192%. The average growth in the tissue extract prepared from the 5-day old bruise was only 20% higher than that noted in normal tissue extract. In tissue extracts of 6, 7, 8 and 9-day old bruises, staphylococcal growth was also greater than that observed in the normal tissue extract. However, in these extracts the average percent increase above the level attained in the control was only 70%. Maximal growth of *S. aureus* cells occurred in tissue extract prepared from the 3-day post-bruise.

Growth of staphylococci in extracts of severe bruises prepared from tissues secured during the 1st to the 8th day post-trauma was also superior to that noted in normal tissue extract. On the other hand, growth in the extract of the 9-day old severe bruise was slightly less than control. Microbial growth in the 1–3 day post-bruise extracts averaged 124% above control while growth in 6–8 day post-bruise extracts averaged 184% above control level. Growth of staphylococci in extracts prepared from 4 and 5-day old bruises exceeded the control level by 15% and 27%, respectively. It should be pointed out that *S. aureus* cells grew best in the 6-day post-bruise extracts of severely damaged tissue where they exceeded the control level by 222%. The increase in microbial growth in bruised tissue extracts may be due to either the presence of a growth stimulating substance(s) or the inactivation of bacteriostatic and/or bactericidal components present in normal tissue of the birds. Increased growth rates in bruised tissue extracts compared to that in normal tissue extracts may be attributed to the increase in concentration of nucleic acid fragments resulting from the release of lysosomal nucleases at the site of trauma. The concentration of lysosomal nucleases in tissues increased in concentration in bruised tissues reaching a maximal between the 3rd and 5th day post-bruise and then declined toward normal levels as healing progressed (Brown and Hamdy, 1965b). The enhanced growth rate observed in bruised tissue extracts above control, particularly during 1–4 days post-trauma, may also be due to the increase of the extrastromal hemoglobin concentration in these tissues. McCarthy et al. (1963) showed that hemoglobin stimulated the growth of *S. aureus* while acid and alkaline hematin as well as biliverdin inhibited the growth of this organism in vitro. The growth of staphylococci in bruise tissue extracts may also be governed by a wide variety of other metabolites. In fact, the biphasic pattern of growth stimulatory activity, in extracts of both severe and slight bruise tissues, suggests that the rate of growth in these extracts is governed by a complex series of biochemical events that vary as healing progresses. However, the results of this study point out that the effect of these biochemical alterations apparently stimulates the growth of *S. aureus* throughout the healing process. An accelerated growth rate in the bruised tissue creates a greater opportunity for selection of those staphylococcal cells that are most resistant to natural tissue bacteriostatic and bactericidal substances and may therefore account for the prolonged persistence of viable staphylococci in bruised tissue.

Table 1–Comparative growth of S. aureus "A-10" in traumatized tissues during healing[a]

Time post-trauma (days)	Percent increase above control	
	Slight trauma	Severe trauma
1	151[b]	150
2	193	163
3	218	60
4	205	15
5	20	27
6	57	222
7	70	189
8	111	140
9	42	–7

[a] 15 ml of sterile bruised or normal tissue extract in 125 ml flasks were inoculated with 0.2 ml (3 X 10^6 cells) saline suspension of S. aureus "A-10" at time zero.
[b] Each data represent the average of six birds.

Respiration

Respiratory activity of staphylococcal cells in bruised tissue extracts was found to be correlated to both severity and time post-trauma. For example, oxygen uptake of cells incubated in severe bruises prepared immediately following trauma (0 day) was much lower than those kept in normal tissue extract (Fig. 1A). In extracts prepared from bruised tissue after 1 and 4 days post-trauma, the oxygen uptake (compared to normal) was much higher, particularly after 30 min incubation. Slightly higher oxygen uptake was also noted in tissue extract of 4-day old bruises compared to 1-day old bruises, especially during early incubation (<30 min). Afterwards, no significant differences in oxygen uptake ($P < 0.05$) was observed between 1 and 4-day old bruised tissue extracts. Slight differences in oxygen uptake were noted between staphylococcal cells incubated in extracts of 5, 7 or 9 day post-bruise and in normal tissue extracts. The patterns of oxygen uptake in slight bruises (Fig. 1B) were similar to those of severe bruises, but of lesser magnitude. It was noted that the oxygen uptake of staphylococcal cells incubated in 0, 1 and 4-day old bruised tissue extracts was much lower compared to those of severe bruises.

Studies were also performed to ascertain the effect of hemoglobin on the oxidation of endogenous substrates and the results revealed that the addition of hemoglobin (0.0035 μM/ml of reaction mixture) inhibited the bacterial oxidation of endogenous substrates. In the presence of hemoglobin, oxygen consumption by endogenously respiring cells was nearly 30% less than control after 60 min incubation period. It was also noted that the pattern of endogenous oxygen consumption was not affected by the presence of 3.3 mM glucose per ml reaction mixture.

The data from these experiments indicated that during the early stages of healing (1–4 days post-bruise) the ability of the staphylococci to oxidize mixed substrates is greater in the traumatized than in the normal tissue extracts. Stimulation of respiratory activity may be an important factor in the establishment of staphylococcal infection in the traumatized tissue. Ivler (1965) reported that virulent staphylococci consumed oxygen at a more rapid rate than did avirulent staphylococci. Similar results were reported by Beining and Kennedy (1963) who demonstrated that when virulence was increased by animal passage the respiratory activity of the staphylococcal cells increased. The metabolic energy obtained through increased respiratory activity may be utilized for the synthesis, repair of

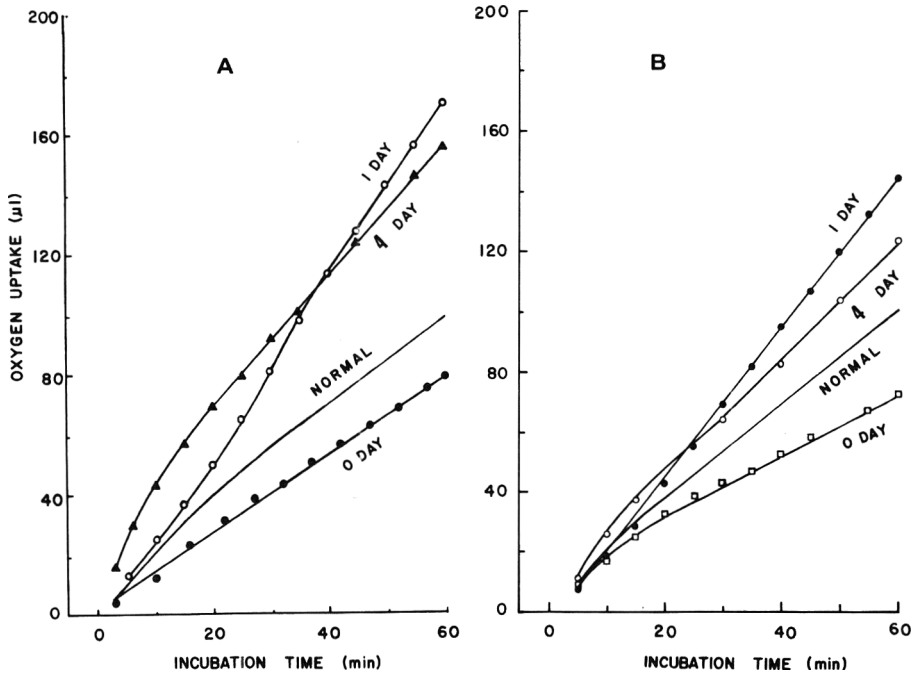

Fig. 1—Oxygen uptake by staphylococcal cell suspension in tissue extracts of normal and of 0, 1 and 4 days severe bruises (A) and slight bruises (B). Respirometer vessels contained 1.5 ml of normal or bruised tissue extract and 1.5 ml of a 0.066M pH 7.4 phosphate buffer suspension of S. aureus "A-10" (2 × 10^6 cells). Values plotted represent the mean of five replicates.

cellular compounds or structures that enable the staphylococci to withstand the onslaught of host defense mechanisms and thus persist in the bruised tissue.

Coagulase

Growth of *S. aureus* in bruised tissue extracts for 18 hr at 37°C effectuated an inhibition of coagulase production. The coagulase titer of the supernate obtained from the staphylococcal culture grown in the normal tissue extract was 1:16. When *S. aureus* cells were grown in tissue extracts of slight or of severe bruises, the coagulase titer was only 1:2 except in tissue extract prepared from the 9-day old slight bruise where the titer slightly increased to 1:4. The reduction in coagulase activity occurred in spite of the enhanced growth rate observed in the bruised tissue extracts as indicated from data presented in Table 1.

Beta hemolysin

The elaboration of beta hemolysin was also inhibited by growth in extracts of bruised tissue. The beta hemolysin titer of the supernate obtained from the staphylococcal culture grown in the normal tissue extract was 1:8. When *S. aureus* cells were allowed to grow in bruised tissue extracts the titer of beta hemolysin in the culture supernate was only 1:2. The production of beta hemolysin was inhibited even further when cells of the test culture were grown in extracts prepared from bruised tissue obtained after 5 days post-trauma of slight or severe bruises.

Hyaluronidase

The relationship between staphylococcal hyaluronidase activities and time post-trauma of slight and of severe bruises is shown in Table 2. Growth in bruised tissue extracts inhibited

Table 2—Effect of severity and time post-trauma on hyaluronidase (expressed as turbidity reducing units—TRU/ml extract) and on enterotoxin B production (µg/ml) in tissues (average of six tissues are reported)

Time post-trauma (days)	Hyaluronidase (TRU/ml)[a]		Enterotoxin B (µg/ml)[b]	
	Slight trauma	Severe trauma	Slight trauma	Severe trauma
1	1.25	0.84	13.5	11.0
2	1.20	0.84	14.0	11.0
3	1.25	1.02	14.0	12.0
4	1.20	1.02	15.5	11.0
5	1.37	1.43	18.5	18.5
6	1.52	1.39	18.0	18.0
7	1.37	1.52	20.0	18.0
8	–	–	18.5	18.5
Normal (nonbruised)	1.48		20.0	

[a] The extracts of each traumatized and control (normal) tissue were inoculated with 10^3 S. aureus "A-3" cells and incubated for 12 hr at 37°C. After incubation cells were harvested, washed and inoculated into glucose-nutrient broth. After incubation (15 hr 37°C) cells were removed by centrifugation and the supernates examined for hyaluronidase activity.

[b] Sterile tissue extracts were inoculated with 10^6 cells of S. aureus "S-6" and incubated for 24 hr at 25°C. Cells were removed by centrifugation and the supernates assayed for enterotoxin B.

Table 3–Effect of the presence of hemoglobin in media on production of enterotoxin B. Results are reported as average of three experiments[a]

Hemoglobin (10^{-3} μM/ml)	Enterotoxin B (μg/ml)
0	37
0.4	35
1.7	20
3.5	13

[a] Hemoglobin was added to brain heart infusion and the media inoculated with 2.5×10^3 cells of S. aureus "S-6." After incubation at 25°C for 24 hr, the cells were removed by centrifugation and the supernates assayed for enterotoxin B.

the production of staphylococcal hyaluronidase. However, inhibition resulted only after the rapid growth noted in tissue extracts prepared from bruises obtained during the early stages of healing (1–4 days) but not during the latter stages (4–8 days). It should be pointed out that the pattern of growth of *S. aureus* "S-6" in slight and severe traumatized tissue extracts followed the same general behavior of strain "A-10" in these extracts. When staphylococcus cells were grown in 1–4 day old slight bruised tissue extracts the subsequent elaboration of hyaluronidase was inhibited by nearly 20%. Increasing the severity of trauma caused further inhibition during that time. When staphylococcus cells were grown in 1–4 day old severe bruise tissue extracts the subsequent elaboration of hyaluronidase was inhibited by nearly 40%. This inhibition decreased as healing progressed and the hyaluronidase activities in slight and in severe trauma approached the level noted in normal tissue extract.

Enterotoxin B

The data depicting the relationship between enterotoxin B production in extracts of slight and of severe trauma as a function of time during healing are also shown in Table 2. The growth of *S. aureus* "S-6" cells in extracts of bruised tissue prepared from bruised birds on the 1st, 2nd, 3rd and 4th day post-bruise resulted in decreased enterotoxin production. When these staphylococci were grown in extracts of slight bruises, toxin production was approximately 30% less than that noted in normal tissue extracts; whereas, when they were grown in extracts of severe bruises their production of enterotoxin was diminished to nearly 50% of that produced in normal tissue extracts. Again, the level of enterotoxin B increased toward normalcy as healing progressed.

The data on the effect of hemoglobin on enterotoxin production of *S. aureus* "S-6" cells in brain heart infusion media is shown in Table 3. The presence of hemoglobin in the media inhibited enterotoxin B production. Increasing the concentration of hemoglobin from 0.0004 μM/ml, the level in normal tissue, to 0.0035 μM/ml, the level in 2-day old bruises (Hamdy et al., 1961b), caused a decrease in the production of enterotoxin. Again, this hemoglobin level was noted by McCarthy et al. (1963) to stimulate the growth of *S. aureus*.

The data obtained in this investigation suggest that the persistence of *S. aureus* in bruised poultry tissue is primarily due to biochemical alterations in the bruised tissue microenvironment which favors increased growth and respiration of the staphylococci at the expense of the production of extracellular proteins such as coagulase, beta hemolysin, hyaluronidase and enterotoxin, particularly during the early stages of healing. Enhanced microbial growth leading to the rapid establishment of a large population of cells, each with inherent, subtle variations, increases the probability that some cells will be present in the tissue to withstand the natural defense mechanism and persist in traumatized tissue for a longer time. The data obtained also indicated that these organisms regain some of their virulence factors after 6–8 days post-trauma.

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COMPARISON OF SARCOMERE LENGTH MEASUREMENT OF COOKED CHICKEN PECTORALIS MUSCLE BY LASER DIFFRACTION AND OIL IMMERSION MICROSCOPY

INTRODUCTION

SINCE THE REPORT by Locker (1960) that tenderness in beef is influenced by the degree of muscular contraction in the postmortem muscle, a number of workers have investigated the correlation betweer sarcomere length, as a measure of the contractile state and tenderness. The influence of contractile state on texture is demonstrated in the phenomenon of 'cold shortening' (Locker and Hagyard, 1963; Marsh and Leet, 1966) and in the effect of the configuration of the carcass during rigor mortis on a number of muscles (Herring et al., 1965; Hostetler et al., 1970).

Since a direct correlation exists between the contractile state of the muscle and its quality as meat, it is clearly desirable to develop a simple and reliable method for measuring sarcomere length. Many methods have been developed such as the microscopic examination of thin stained sections of embedded tissue. The space between adjacent Z-discs is then measured against a calibrated eye-piece graticule. Oil-immersion and phase-contrast microscopy of unstained sections also reveal the detailed striation pattern of the muscle fiber. These methods are accurate but slow.

A more convenient method is to prepare a suspension of myofibrils and to examine it by phase-contrast microscopy but mechanical disruption of the fibers tends to produce a shortening of the sarcomere, probably in the I-band region (Rome, 1967).

A method in use at the Meat Research Institute, England, (Voyle, 1971) derives initially from early observations that a striated muscle acts as a transmission grating when placed in a beam of light. Diffraction patterns are formed on a screen, the separation of the lateral orders being determined by the contractile state of the muscle. A recent development in this technique is the use of a gas laser as a source of coherent monochromatic light. This has been described by Rome (1967) and Cleworth and Edman (1969).

It was the purpose of the work reported herein to compare the laser diffraction method of sarcomere length determination with conventional microscopic measurements on samples of chicken broiler muscle cooked at various postmortem aging times.

MATERIALS & METHODS

Muscle preparation

One Pectoralis major muscle was excised from each of three randomly selected 6 wk-old broiler chickens at approximately 10 min, 3 hr and 24 hr postmortem, respectively. The excised muscle was clamped between two aluminum plates spaced 0.7 cm apart and cooked for 10 min in boiling water (deFremery and Pool, 1960). When cooking was completed, samples were cooled in running tap water for 5 min. After removal from the plates, samples were individually wrapped in Saran wrap until needed.

Oil immersion microscopy

Small bundles of fibers were dissected out from eight areas of the cooked muscle, laid out on a glass slide and covered with paraffin oil. Single fibers or small fiber bundles were teased out and measurements of sarcomere lengths were made using a conventional microscope with an oil immersion objective (Wild, n.a. = 1.25 mm, Magnification 1250). Sarcomere length was calculated using a calibrated eyepiece micrometer. Ten measurements per area were made and the results were averaged.

Laser diffraction studies

The apparatus consisted of a 1-mW helium-neon laser with a wavelength of 632.8 nm, mounted on an optics bench with a specimen-holding device and a screen. The screen consisted of a vertically mounted white card bearing a central millimeter scale.

A small piece of tissue from the Pectoralis major muscle was cut with known orientation of the fibers. Single fibers or small fiber bundles were teased out and mounted between two glass cover slips using a drop of buffered sucrose solution. The sample was then placed vertically in relation to the laser beam to give a horizontal array of diffraction bands on the screen. An example of this is shown in Figure 1. Sarcomere lengths were calculated using the following formula:

$$d = \frac{(632.8 \times 10^{-3})D}{S} \mu m$$

where d is equal to the sarcomere length; (632.8×10^{-3}) is the wavelength of radiation in microns; D is the distance in millimeters between the specimen-holding device and the screen; S is the separation between

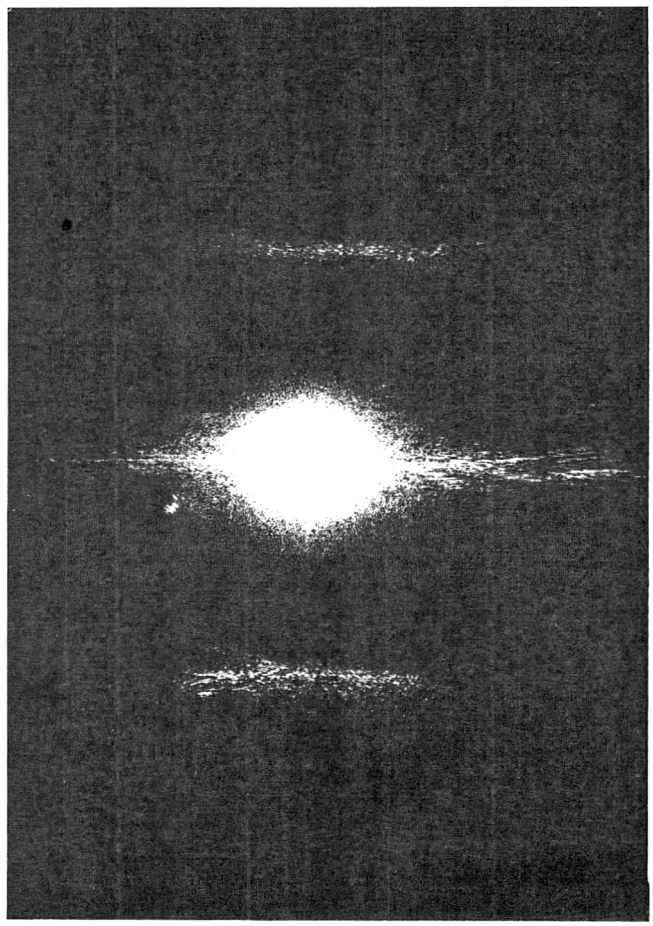

Fig. 1—Diffraction pattern obtained from muscle fiber sarcomere length = 1.68 μm.

the 0th and the nth order diffraction band. (Throughout this experiment D had a constant value of 84 mm). Ten measurements were made in each of the eight areas of the muscle and the results averaged.

RESULTS

THE MEAN SARCOMERE lengths obtained using the two methods of measurement were paired according to the area and the postmortem aging time of the muscle.

The results of simple regression analysis performed on the data obtained using the two methods of measurement for each of the three aged muscles and for the combined data are presented in Table 1 and Figure 2. Lines CC′ and DD′ represent the extremes of the slope found among the three muscles studied.

Sarcomere length is shown to decrease as the muscle passes from the pre-rigor state (aged 10 min) to the in-rigor state (aged 3 hr). This is followed by an increase to maximum length in post-rigor muscle (aged for 24 hr). Some differences in sarcomere length were found within the muscle tissues but both methods of measurement are shown in Table 1 to be well correlated especially in the pre-rigor and in-rigor muscles (r = 0.85 and 0.82, respectively). Greater differences were found within the postrigor muscle than in the other two samples. In this case, the correlation coefficient for the two methods was lower (r = 0.68). Analysis of the combined data revealed a strong and significant correlation between the two methods of measurement used on cooked muscle aged between 10 min and 24 hr postmortem (Table 1).

Table 1—Results of simple regression analysis of laser diffraction versus a microscopic method of measuring sarcomere length

	All	Pre-rigor	In-rigor	Post-rigor
Mean sarcomere length (μm)	–	1.86	1.54	2.29
Standard error of estimate	0.09	0.03	0.04	0.06
Slope	1.08	0.69	2.15	0.58
Y-intercept	−0.27	0.41	−1.94	0.95
Correlation coefficient (r)	0.96***	0.85***	0.82**	0.68*
n	24	8	8	8

* $P < 0.1$
** $P < 0.02$
*** $P < 0.01$

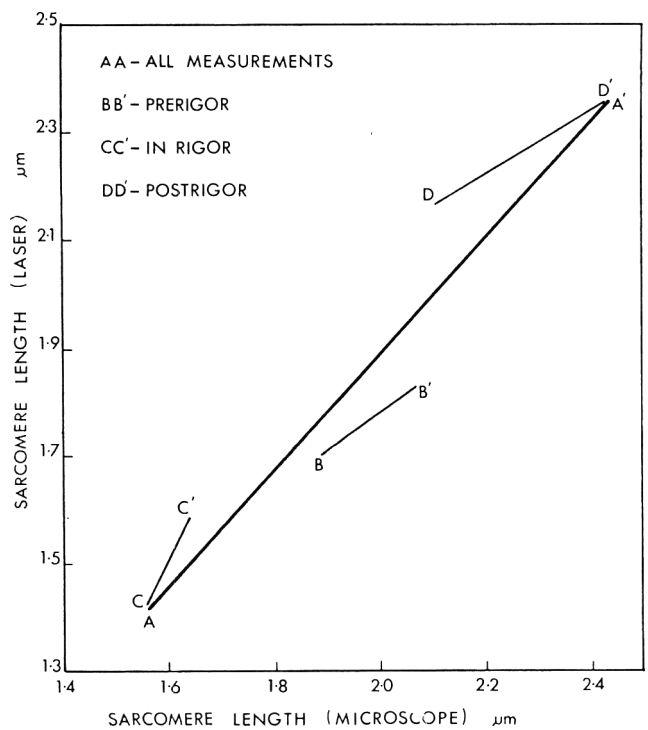

Fig. 2—Simple regression analysis of laser diffraction on a microscopic method of measuring sarcomere length.

DISCUSSION

FROM RESULTS obtained, it is apparent that both methods of measuring sarcomere length give similar values. Sources of error, however, exist for both methods.

When the bundle of fibers was placed vertically in relation to the laser beam, the horizontal array of diffraction bands which appeared on the screen gave an average of all the unit spacings illuminated by the beam. The diameter of the beam was about 2 mm. In some instances, the bands were diffuse showing that a large range of unit spacings existed within the fiber. Because such wide, diffuse diffraction bands occurred in some cases, it was necessary to set a standard for taking measurements such that the center of the 0th and the nth order diffraction bands were used as the upper and lower limits of the measurement. Some error may have resulted from inaccurate measurements in such instances. Another potential source of error may have been inadequate sample preparation. Ordered diffraction patterns were not obtained if the fibers in the teased bundle were in disarray. In this experiment, every care was taken to ensure that single fibers were teased out and mounted in the path of the laser beam.

Preparation of the muscle fibers for measurement using the microscope was undertaken in a similar manner to that used for laser diffraction samples with every attempt made to ensure that single fibers were teased out. Micrometer measurements were a definite source of error using this method as was the establishment of the true limits of the sarcomere. While the laser diffraction method gave an average of all the unit spacings illuminated by the beam, measurement using the microscope gave the length of one sarcomere only making this method much slower and much more prone to errors than the laser diffraction method.

CONCLUSION

THE LASER diffraction method appears to be of particular value when the mean sarcomere length is required on a large number of samples. The apparatus is simple to operate and greater accuracy can be obtained when making measurements using this method compared to microscopic methods. It provides only limited information, however, about the morphology of the fibers. For this, an examination of sections by conventional methods of microscopy is necessary.

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QUALITY CHARACTERISTICS OF SOY-SUBSTITUTED GROUND BEEF, PORK AND TURKEY MEAT LOAVES

INTRODUCTION

THE UTILIZATION of soy products has increased significantly in recent years. On February 22, 1971, the USDA's Food & Nutrition Service issued Notice 219, which permitted the use of up to 30% rehydrated textured vegetable products to replace up to 30% of the meat or meat alternate portion in the Class A school lunch menu. Approximately 23 million pounds of textured vegetable protein (hydrated weight) were used in the school lunch program during the 1971–1972 school year; this figure doubled the following year (Butz, 1974). Since this approval of the use of textured vegetable products, there has also been a tremendous increase in use in hospitals and other institutional food services and more recently hamburger-soy mixtures have become available in the retail market.

A major drawback with the use of soy in meat systems is the consumer acceptance of the finished product. Soy companies advertise that the use of soy in a meat system will cause an increase in juiciness and flavor and better shape retention (Rakosky, 1974). Soy products, however, often have a characteristic off-flavor, which has been described as "beany" or "cereal-like." When the meat formulation is prepared with insufficient seasonings to mask the off-flavors, consumers often find these foods objectionable.

Textured soy products are engineered to look, feel and taste like ground meat and its use in beef systems is widely accepted. However, its use in other systems such as ground pork or ground turkey, should be investigated more thoroughly.

Many hospitals use textured soy in their ground meat systems. With the use of 30% rehydrated soy in a ground beef product there is almost a 30% reduction in the amount of fat present in the raw product. One might expect a similar decrease in the amount of fat in the cooked product, thereby reducing both the amount of fat and the number of calories. However, two major functional properties of soy proteins are their ability to retain fat and moisture, and these functionalities moderate the amount of fat and moisture lost from soy-substituted meat systems. Data are needed to see what effects the incorporation of textured soy protein in ground meat systems would have on the retention of lipids and moisture after cooking.

A major problem in using cooked meat which has been stored at either refrigeration or freezing temperatures is the development of off-flavors and off-odors which have been attributed to lipid autoxidation. Numerous studies on lipid autoxidation have been intensively reviewed by Love and Pearson (1971) and Sato and Herring (1973). Various meat systems, such as beef, pork and turkey, undergo lipid oxidation at different rates when stored under the same conditions.

The addition of soy to meat systems has been shown to exhibit varying degrees of antioxidant ability. Pratt (1972) noted potent antioxidant activity in lipid-aqueous systems and postulated that this ability was due to flavonoid components that occur naturally in soybeans. Sato et al. (1973) noted that various vegetable protein products decreased the development of warmed-over-flavor (WOF) in cooked ground meat and attributed this decrease to the production of various browning reaction products which exhibit antioxidant activity.

The objectives of this study were to investigate the effect of inclusion of rehydrated textured soy on the quality characteristics of ground beef, pork and turkey loaves. These characteristics included sensory evaluations; the effect of soy addition on cooking losses; and the percentages of lipid and moisture. The rate of malonaldehyde accumulation which occurred in these soy-substituted meat systems stored under refrigeration and freezing temperatures for short periods of time as would be used in school lunch or hospital cycle menus was also determined.

EXPERIMENTAL

Preparation

Three ground meat systems using 0% or 30% soy-substitutions were investigated in this study. The basic formulas are adapted from various recipes which were investigated during preliminary research and are presented in Table 1.

Meat loaves for five replications of each variable were prepared at one time. The ham and turkey thighs were ground separately through a Hobart Food Cutter, Model 84181D, using a 4.7 cm plate. The textured soy which had been rehydrated in cold water for 5 min and ground meat were mixed for approximately 1 min in a Hobart Mixer, Model K-200, in order to thoroughly mix the two ingredients. The remaining ingredients were divided into thirds, each portion was then added separately and mixed into the meat for approximately 30 sec. Finally, both the 0% and 30% soy-substituted meatloaf mixtures were then reground through the Hobart Food Cutter to insure uniformity.

Approximately 1500g of meatloaf mixture were placed in a 10 × 4 × 3-1/2-in. ham loaf press and held under pressure for 5 min in order to obtain loaves of the same degree of compactness. Each loaf was then wrapped first in Reynolon® Food Service Film, rewrapped in alumi-

Table 1—Amount of ingredients (grams) used in the preparation of 0% and 30% soy-substituted ground meat systems

	Meat system		
Ingredient	Beef[a]	Turkey[b]	Pork[c]
Ground meat[d]	1703	1703	1703
Bread crumbs	207	207	207
Dried onions	38	38	38
Salt	10	10	10
Poultry seasoning		10	
Bar-B-Q sauce	75		
Catsup	187		

[a] Ground beef (20% fat)

[b] Ground from thighs containing a natural portion of skin

[c] 50:50 mixture of ground Pullman ham and pork was used.

[d] For the 30% soy-substituted loaves, 1192g of ground meat was used with 170g of texturized soy protein (Temptein, Miles Laboratories, Elkhart, Ind.) and 340g of cold water.

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Table 2—Means and standard deviations[a] of total cooking loss, drip loss and volatile loss of soy-substituted meat systems

Meat system	% Soy	Cooking losses		
		Total (%)	Drip (%)	Volatile (%)
Beef	0	13.3 ± 1.4	2.9 ± 0.3	10.4 ± 1.2
	30	9.8 ± 1.8*	0.4 ± 0.1***	9.4 ± 1.7
Pork	0	15.8 ± 1.4	5.9 ± 1.4	10.0 ± 0.2
	30	12.2 ± 2.3**	0.4 ± 0.1***	11.8 ± 2.1
Turkey	0	16.6 ± 0.7	0.5 ± 0.1	16.0 ± 0.7
	30	15.6 ± 1.5	0.1 ± 0.1*	15.5 ± 1.5

[a] Based on five replications
* 0 and 30% soy-substituted meat loaf means differ significantly at the 5% level of probability.
** 0 and 30% soy-substituted meat loaf means differ significantly at the 1% level of probability.
*** 0 and 30% soy-substituted meat loaf means differ significantly at the 0.1% level of probability.

num foil and stored at −11°C for a period of 5 wk or less. The loaves were removed from the freezer and thawed at 5°C for 14 hr prior to baking.

Since ham is susceptible to increased rancidity upon freezing, the pork loaves were prepared on the day of baking from ground pork purchased in a common lot and frozen at −11°C and ham which was held at 5°C. The procedure was the same as that used for the beef and turkey, except that a Kitchen Aid Mixer, Model K-5A was used to mix the ingredients.

The 0% and 30% soy-substituted loaves were randomly assigned as two preparations for any one "baking" day. The loaves, which were supported on racks in 9 × 12-in. baking pans lined with aluminum foil, were baked in a General Electric, 30-in. Compact oven, Model CN16, with the damper half closed and the grid set at medium. The oven temperature was maintained at 177 ± 1°C by a Versatronik controller. Loaves were baked to an internal temperature of 77°C, which was determined by an iron constantan thermocouple lead inserted in the center of the loaf. Upon removal from the ovens the loaves were allowed to stand for 5 min before total, volatile and drip losses were determined according to the method outlined by Funk et al. (1966).

The middle third of each loaf was used for sensory evaluation. The end slices were removed and discarded. Three consecutive 1/2-in. slices were taken from each end portion for the 2-thiobarbituric acid tests. These slices were then wrapped in aluminum foil and stored at either 5°C for 0, 2, or 4 days or −11°C for 1, 2 or 3 wk. Assignment of slices was rotated so that each slice was used the same number of times for the TBA determinations at any one temperature and storage time. The remaining loaf was ground, mixed thoroughly and used for moisture and lipid analyses.

Analyses

Samples were assigned random numbers and served warm to an 11-member trained taste panel. Flavor, juiciness, mouthfeel and overall acceptability were evaluated using a descriptive score card, with scores of 7 being optimum.

Duplicate determinations were made for each chemical analysis. Raw and cooked ground meatloaf mixtures were used for all analyses except for the TBA determinations, which were carried out on cooked samples only. Moisture in the raw and cooked meatloaf mixtures was determined by drying 2-g samples, weighed to the nearest 0.001g, for 6 hr at 90°C under vacuum.

A chloroform-methanol extraction was used to determine percentage total lipid (Yadrick et al., 1971). Percentage total lipid was calculated on a dry weight basis in order to compare raw and cooked lipid values.

The 2-thiobarbituric acid test was used to determine the development of oxidative rancidity during short-term storage at refrigeration (5°C) or freezing (−11°C) temperatures. TBA values for beef and turkey samples were determined using slight modifications of Tarladgis and Watts (1960) distillation method (Whipple, 1974). Acetic acid was refluxed with 2g TBA/100 ml acetic acid for 3 hr and then distilled to remove any interfering carbonyl compounds. The TBA method as outlined by Zipser and Watts (1962) was used to analyze the ham samples, since nitrite was found to reduce TBA "numbers." TBA values are reported as mg of malonaldehyde/1000g of meat sample based on the distillation constant (K = 14.09) as determined by Witte et al. (1970).

Data were analyzed for variance and Duncan's Multiple Range Test (1957) was used to pinpoint significant differences revealed by these analyses.

RESULTS & DISCUSSION

TOTAL, drip and volatile cooking loss data are presented in Table 2. The 30% soy-substitution significantly decreased total cooking losses in both the beef ($P < 0.05$) and pork ($P < 0.01$) systems. Soy-substitution decreased drip loss significantly in all three systems; nevertheless, no differences were found in volatile losses between the 0% and 30% soy-substitution in any of the three systems.

Drip losses consist of fat that has melted out during the cooking process whereas volatile losses are a result of the evaporation of water and other volatile compounds (Paul and Palmer, 1972). Since two of the important functional properties of soy proteins are their ability to bind fat and to retain moisture (Wolf, 1970) it had been expected that the incorporation of texturized soy protein would result in a decrease in total cooking losses, a subsequent decrease in cooking drip and a possible decrease in volatile losses.

The sensory panel results are presented in Table 3. The taste panelists scored the 30% soy-substituted beef and turkey systems slightly lower than the corresponding nonsoy-substituted systems, but all scores were above 4.6 on a 7-point scale and none of these differences was significant. However, taste

Table 3—Means and standard deviations[a] of sensory scores[b] of soy-substituted meat systems

Meat system	% Soy	Sensory evaluation			
		Flavor	Juiciness	Mouthfeel	Overall
Beef	0	5.7 ± 0.2	5.6 ± 0.3	5.6 ± 0.3	5.6 ± 0.3
	30	5.3 ± 0.8	5.2 ± 0.2	5.1 ± 0.5	4.6 ± 0.7
Pork	0	6.4 ± 0.2	5.9 ± 0.4	6.0 ± 0.2	5.8 ± 0.3
	30	4.6 ± 1.1**	4.8 ± 0.5**	5.2 ± 0.9	4.1 ± 1.0**
Turkey	0	6.0 ± 0.4	5.6 ± 0.2	5.7 ± 0.2	5.3 ± 0.5
	30	5.3 ± 0.6	5.5 ± 0.3	5.8 ± 0.5	4.7 ± 0.5

[a] Based on five replications
[b] Scale of 1–7, with 7 being optimum
** 0 and 30% soy-substituted meat loaf means differ significantly at the 1% level of probability.

panelists scored the 30% soy-substituted pork system significantly lower ($P < 0.01$) than the nonsoy-substituted pork system for all quality characteristics except mouthfeel. Since the textured soy protein used in this study was formulated for use with beef, it was expected to blend well in this system. The caramel-colored product also blended well with the turkey; however, color differences were obvious between the 0% and 30% soy-substituted ham loaf and may have contributed to the lower scores.

The moisture contents of the three meat systems are presented in Table 4. The percentage moisture of the cooked 0% soy-substituted beef and turkey system and for all three cooked 30% soy-substituted systems was significantly less ($P < 0.001$) than that of the respective raw meat loaves. Soy proteins are hydrophilic and would therefore be expected to absorb and retain water (Wolf, 1970). Nevertheless, moisture contents were quite similar for the nonsoy-substituted and 30% soy-substituted meat systems.

The values for total lipid in the raw and cooked 0 and 30% soy-substituted meat loaves are presented in Table 4. Due to dilution by the addition of a low fat product, the raw 30% soy-substituted beef, turkey and pork systems had significantly less ($P < 0.001$) fat than did corresponding 0% soy-substituted meat systems. The 30% soy-substituted systems, however, retained more of the lipids present during cooking than did the 0% soy-substituted systems, therefore there was no significant difference between the total lipid contents of the cooked 0% and 30% soy-substituted beef and pork systems. Thus, consuming 30% soy-substituted beef or pork loaves would not appreciably decrease total number of calories or the amount of fat in one's diet. The 30% soy-substituted cooked turkey loaves did possess less total lipids than the nonsoy-substituted cooked turkey loaves.

The TBA values for the three meat systems are shown in Figures 1, 2 and 3. Thiobarbituric acid has been used to quantitatively determine the malonaldehyde concentration as a means of measuring the degree of oxidation in the meat system. The TBA values indicated that the 30% soy-substituted systems were initially slightly more rancid, but at some point during either refrigerated or freezer storage the rate of oxidation for the 0% soy-substituted pork and turkey increased more rapidly so that their TBA values were higher than those of the 30% soy-substituted meat systems at the close of the storage period.

The slow increase in oxidation of ground beef under both refrigerated and frozen storage can be seen in Figure 1. The only significant difference that occurred between the TBA values of 0% and 30% soy-substituted beef was for day 2. There was no significant increase in TBA reactive compound for either the 0% or 30% soy-substituted beef stored under refrigerated or frozen storage. Cooked beef stored in the refrigerator would be expected to oxidize more rapidly than frozen cooked beef (Chang et al., 1961). The results from this study indicate that there was not a significant increase in TBA reactive compounds, in either the 0% or 30% soy-substituted beef loaves during 4 days of storage at 5°C. Malonaldehyde concentration increased at a much slower rate in frozen beef as ex-

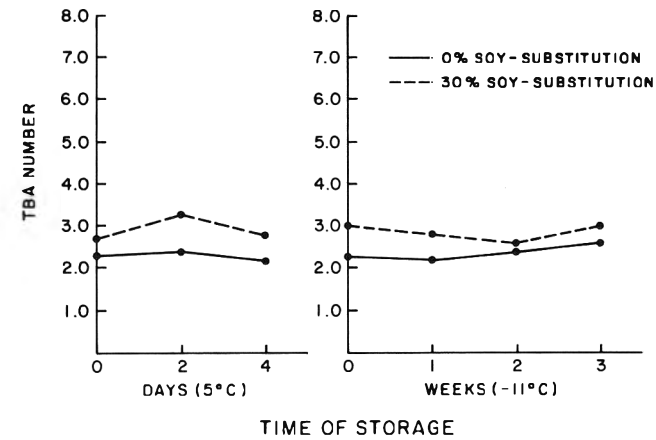

Fig. 1—TBA numbers for 0% and 30% soy-substituted ground beef stored at 5°C and −11°C.

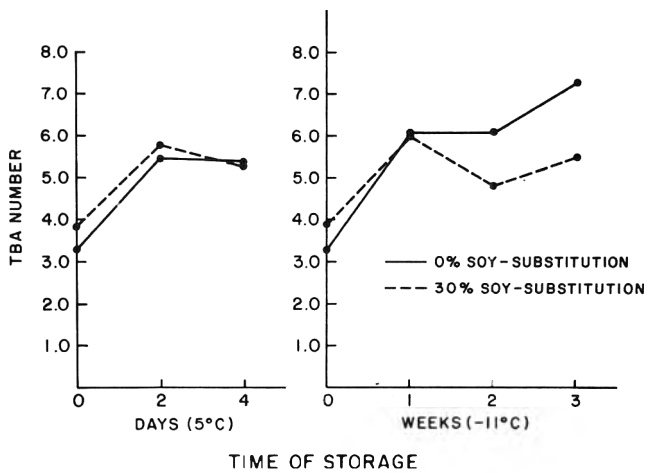

Fig. 2—TBA numbers for 0% and 30% soy-substituted ground pork stored at 5°C and −11°C.

Table 4—Mean values and standard deviations of percentage moisture and percentage total lipid of raw and cooked soy-substituted meat systems

Meat system	State	Moisture		Total lipid	
		0%	30%	0%	30%
Beef	Raw	58.4 ± 1.0[a]	58.7 ± 0.6	25.5 ± 1.8	17.8 ± 0.5
	Cooked	54.4 ± 1.6	53.1 ± 1.5	17.5 ± 2.3	15.7 ± 1.6
Pork	Raw	54.9 ± 1.4	57.3 ± 1.0	30.7 ± 2.4	22.2 ± 4.4
	Cooked	53.6 ± 1.8	52.1 ± 1.5	21.8 ± 1.7	19.7 ± 1.0
Turkey	Raw	62.9 ± 0.9	61.8 ± 0.6	20.3 ± 0.9	13.1 ± 0.5
	Cooked	57.4 ± 0.8	55.7 ± 0.4	17.9 ± 3.2	12.8 ± 1.4

[a] Based on five replications

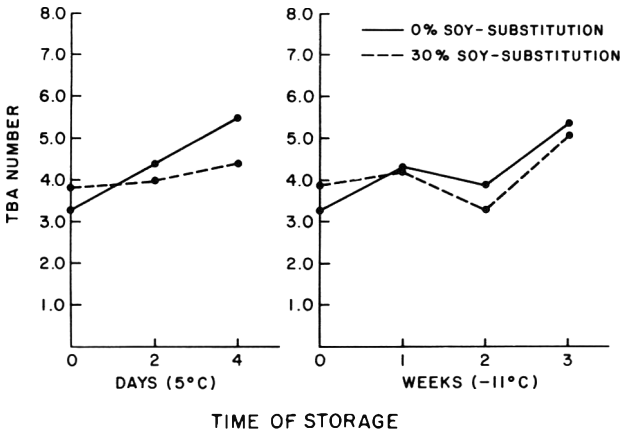

Fig. 3—TBA numbers for 0% and 30% soy-substituted ground turkey stored at 5° C and −11° C.

pected. Soy, however, did not significantly affect the level of TBA values in the frozen beef. To show the effects of soy on the rate of oxidation in a frozen beef system, a longer storage period would be required.

There appeared to be a substantial increase, followed by a leveling off of TBA values for the refrigerated pork loaves (Fig. 2). This increase in TBA values was very highly significant ($P < 0.001$); however, soy substitution did not significantly affect the TBA values.

The frozen pork systems contained higher malonaldehyde concentrations (Fig. 2) than did the beef systems frozen for the same period of time. TBA values for the nonsoy-substituted frozen pork loaves stored for 1, 2, or 3 wk were significantly higher ($P < 0.05$) than the values for day 0. Cured meats can be held longer at refrigerated temperatures than if held at freezer temperatures (Younathan and Watts, 1959). The stability of the refrigerated cured meats towards lipid oxidation is also high compared to the stability of uncured meats held under the same conditions (Zipser et al., 1964).

In Figure 3, the TBA values of 0% soy-substituted turkey system shows a steady increase during the refrigerated storage, whereas the TBA values of 30% soy-substituted turkey loaves shows a much slower rate of increase. The TBA values for the 0% soy-substituted turkey at day 4 were significantly higher ($P < 0.001$) than the values for day 0. There was a significant ($P < 0.05$) decrease in TBA values between the 0% and 30% soy-substituted turkey for day 4, with the 30% soy-substituted turkey loaves possessing lower TBA values. Neither soy-substitution nor length of storage significantly affected the TBA values of the frozen turkey loaves. Poultry meat is much more highly unsaturated than beef or pork (Hilditch et al., 1934; Chang and Watts, 1952) and would be expected to show a marked increase in lipid oxidation under refrigerated storage, as was shown by the increase in TBA values in this study (Fig. 3).

In conclusion, the use of 30% soy-substitution did not seem to adversely affect the quality characteristics of ground beef and turkey systems. There were no differences found in the total lipid content of the cooked 0% and 30% soy-substituted meat systems. Although 30% soy-substituted meat appeared to have a slightly lower TBA value during refrigerated and frozen storage, soy does not appear to prevent the accumulation of TBA reactive compounds in cooked meats; however, a great deal more work with soy-lipid model systems and work on methods of determining oxidation in soy-meat systems needs to be conducted.

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A COMPARISON OF HYDROGEN SULFIDE EVOLUTION FROM COOKED LAMB AND OTHER MEATS

INTRODUCTION

CURRENT RESEARCH on meat flavor has substantially increased our knowledge of the meaty flavor and aroma but has done little to elucidate our understanding of the species flavor and aroma. In the case of lamb, it appears that the meaty aroma comes from the lean portion while the species specific notes originate in or are deposited in the fat portion (Batcher et al., 1969; Pearson et al., 1973; Wasserman and Talley, 1968; Wasserman and Spinelli, 1972).

In our laboratory we have been primarily concerned with analysis of the lamb or mutton flavor. This has become an increasingly important problem with the possible use of mechanical deboning equipment for red meat and the subsequent increased utilization of this meat in processed products.

Preliminary odor panel studies on the volatile compounds evolving from lamb and beef roasts suggested differences in these volatiles. Unpublished results from our laboratory showed panel members had no difficulty identifying the beef and lamb volatiles when given known standards for comparison. Informal discussion of the results of this study attributed part of the odor of the volatiles escaping from the roasts as that of hydrogen sulfide (H_2S). Hydrogen sulfide is not unknown as a flavor precursor in meats (Bouthilet, 1951; Klose et al., 1966; Minor et al., 1965; Pippen and Eyring, 1957). Mecchi et al. (1964) observed that glutathione gives off H_2S about 180 times as fast as does muscle protein. These workers also felt that the rate of H_2S evolution from heated muscle could be approximately predicted from its cystine content. Macy et al. (1964a) found glutathione in the water extract of lamb but not in beef or pork. In a second report, Macy et al. (1964b) found higher concentrations of cystine in water extracts of lamb than in beef or pork. By refluxing beef adipose tissue in water, Pepper and Pearson (1969) demonstrated the evolution of significant quantities of H_2S. Thus, with H_2S already implicated in meat flavors and its evolution from beef fat demonstrated, the first objective of the present study in lamb flavor and aroma analysis was to determine the amount of H_2S evolved from the cooked meat. Since the major precursors of H_2S, glutathione and cystine, were demonstrated to be present in lamb tissue and since subjective observation indicated adequate quantities of this gas evolving from cooked loaves, it was felt quantitative differences could be observed in various cooked meats. Rather than subject the meats to rigorous cooking conditions to maximize H_2S evolution, cooking time and temperature as close to those used for preparation of the meats for home consumption were chosen.

MATERIALS & METHODS

Sample preparation

One 2-yr-old Hereford steer, one 1-yr-old white faced lamb, one 7-month-old barrow and one 7-month-old gilt carcasses from the University of Wyoming meat laboratory provided the beef, lamb and pork samples for this study. The game meats were procured from the Wyoming Game and Fish Laboratory. Samples were prepared for each species and the meats ground through a 1/2-inch plate and reground through a 1/8-inch plate. The ground meat was divided into 400g loaves for subsequent cooking and analysis. Fat and lean samples were prepared by physically separating the two components and grinding as noted for the meat samples. Small subsamples were homogenized in a Virtus laboratory homogenizer for ether extraction, Kjeldahl and dry matter analysis by standard AOAC methods (1970).

Hydrogen sulfide determinations

The washing procedure was a modification of the extensive method of Pepper and Pearson (1969). Essentially the procedure was doubled, resulting in the following washes for all glassware: chloroform, acetone, chloroform, acetone, tap water, 6N HCl, tap water, 6N HCl, hot alconox detergent, tap water, hot alconox detergent, tap water, deionized water and 2X with triply glass distilled water as prepared by Mecchi et al. (1964). The methylene blue method of Sands et al. (1949) as modified by Prince (1955) was used for actual H_2S analysis. Hydrogen sulfide standards were prepared weekly and a standard curve run with each meat analysis. The cooking apparatus was essentially the same as that described by Mecchi et al. (1964) except a 500 ml resin reaction flask equipped with heating mantle was used. The 400g ground meat samples were allowed to warm to room temperature, placed in the 500 ml resin reaction flask and shaped to the contour of the bottom of the flask. This helped to insure equal heat penetration into all loaves. The flask was placed into the mantle, the condenser and mantle were turned on (0 min) and nitrogen, previously washed through 2% $KMnO_4$ and 3% $HgCl_2$ solutions (Pepper and Pearson, 1969) was bubbled into the flask and through the trap at a pressure differential of 70 mm of water. The traps containing 5 ml of 20% zinc acetate and 45 ml of water were changed every 15 min for 1 hr. In the longer term studies (2 1/3 hr) the traps were changed every 20 min. The bubbler was withdrawn from the cylinder and 50 ml of distilled water rinsed through the bubbler and into the cylinder. The contents of the cylinder were mixed and an appropriate aliquot removed for analysis. Upon completion of the hour long cooking procedure, the internal temperature was 78°C and the resulting loaves were rated medium well done, juicy and exhibited typical meat loaf characteristics.

RESULTS & DISCUSSION

WHEN COMPARED on the basis of 10g of meat, the ground beef and lamb samples containing 19% fat produced significantly ($P < 0.05$) different amounts of H_2S when cooked for 1 hr (Table 1). A comparison of leaner ground samples in the same manner showed more H_2S produced by the lamb as compared to beef but this difference was not significant. When H_2S evolution was calculated on the basis of 10g of fat, protein and dry matter, very little difference was noted. No significant differences were noted in H_2S evolution between pork and the other two meats. However, further analysis of the data pointed to an interesting aspect of the H_2S evolution. The leaner samples, although producing less total H_2S, yielded the gas sooner than the higher fat samples. The 19% fat ground beef and lamb samples gave off 11 and 15%, respectively, of the total amount of H_2S evolved in the first half hour. However, the leaner beef and lamb samples gave off 17% and 22%, respectively, of the total H_2S evolved in the first half hour. Pepper and Pearson (1969) found adequate quantities of H_2S given off by various fractions of beef adipose tissue. Therefore, an attempt was made to look at the role fat and lean play in H_2S evolution under our cooking conditions. Although the beef and lamb adipose tissue were of similar composition

(Table 2) the amount of H_2S evolved over 2 1/3 hr was significantly different ($P < 0.05$) when viewed on a 10g meat basis. When the H_2S evolution was placed on a fat, protein or dry matter basis, the lamb always evolved more H_2S; however, these differences were not significant. The same situation existed when beef and lamb lean were compared. Large differences were noted in the amount of H_2S produced between adipose and lean and in the pattern of gas evolution (Fig. 1). Both lean samples gave off H_2S after only 20 min of cooking, peaked after 1 hr and remained constant or declined thereafter. The adipose samples issued no H_2S during the first 20 min period but then dramatically increased their H_2S evolution. After 1 hr the beef adipose declined in rate of H_2S evolution but the lamb adipose continued to increase its rate of H_2S evolution. These factors could be important in the cooking of lamb. As noted in the Materials & Methods section, under the conditions described, the ground roasts were considered done after 1 hr. Further cooking of beef past the point would result in less H_2S being given off and subsequently this gas would play a lesser role in the aroma of the cooked meat. However, in the case of lamb, the continued increase in rate of H_2S evolution from the adipose tissue would suggest that lamb loaves which are overcooked would give off even larger amounts of H_2S. This evolved gas could play an important role in the aroma of the meat as well as the aroma permeating the cooking and serving area. The problem would be accentuated in fatty lamb loaves.

Beef is an excellent standard to judge lamb by since beef is so widely accepted as a delightfully flavored food. However, it was also of interest to compare lamb with some less desirable meats. Table 3 compares the evolution of H_2S from ground game meats. Since these meats are naturally very low in fat the lamb lean (3% fat) was used for comparison. The amount of H_2S evolved from lamb was not significantly different from the amount of H_2S given off from elk. The amount of H_2S

Table 1—Least-squares means of H_2S evolution from cooked beef, lamb and pork[a]

Meat	μg H_2S/ 10g meat	μg H_2S/ 10g fat	μg H_2S/ 10g protein	μg H_2S/ 10g dry matter
Ground beef (19.0% fat) (17.7% protein) (37.3% dry matter)	2.76 a	14.55	15.61	7.41 a
Ground lamb (19.0% fat) (20.6% protein) (39.4% dry matter)	3.94 b	20.76	19.14	10.00 b
Ground beef (8.2% fat) (20.3% protein) (31.1% dry matter)	3.46 ab	42.21	17.05	11.13 b
Ground lamb (14.3% fat) (22.3% protein) (36.4% dry matter)	3.63 b	25.42	16.30	9.99 b
Ground pork (11.0% fat) (22.1% protein) (33.5% dry matter)	3.29 ab	29.88	14.87	9.81 b

[a] Beef and lamb sample means of seven determinations; composite pork sample means of five determinations. Means with differing letters within columns are different ($P < 0.05$).

Table 2—Least-squares means of H_2S evolution from fat and lean[a]

Sample	μg H_2S/ 10g tissue	μg H_2S/ 10g fat	μg H_2S/ 10g protein	μg H_2S/ 10g dry matter
Beef adipose tissue (78.0% fat) (3.7% protein) (81.9% dry matter)	6.35 c	8.14 a	171.51 b	7.75 a
Lamb adipose tissue (76.2% fat) (4.2% protein) (82.0% dry matter)	7.47 d	9.81 a	177.92 b	9.11 a
Beef lean tissue (2.0% fat) (22.8% protein) (25.9% dry matter)	1.76 a	87.90 ab	7.71 a	6.79 a
Lamb lean tissue (3.0% fat) (22.3% protein) (25.7% dry matter)	4.36 b	145.29 b	19.55 a	16.96 b

[a] Adipose tissue means of five determinations; lean tissue means of two determinations. Means with differing letters within columns are different ($P < 0.05$).

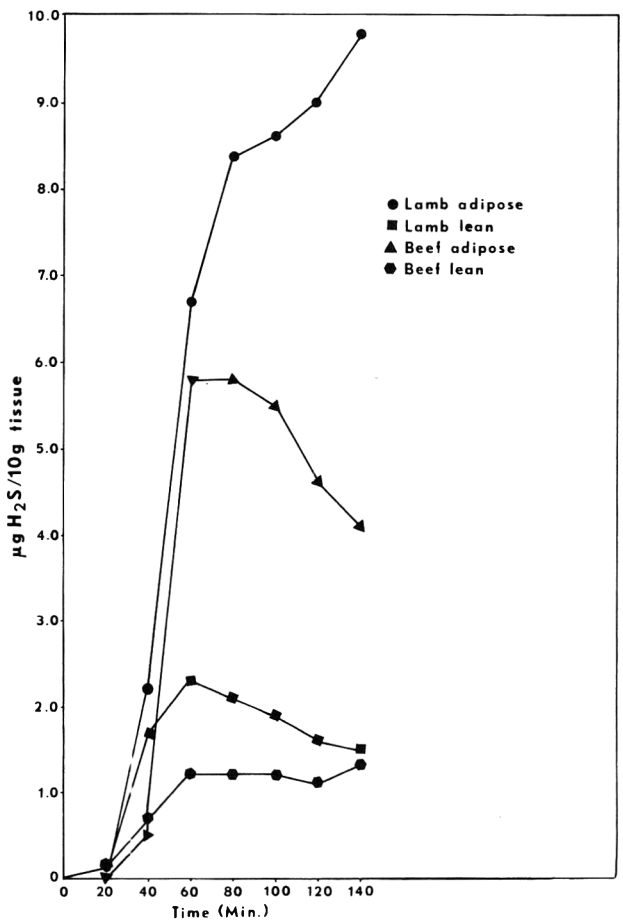

Fig. 1—H_2O produced by adipose and lean tissue of beef and lamb cooked for 2 1/3 hr as noted in text.

Table 3—Least-squares means of H_2S evolution from lamb and game meat[a]

Meat	μg H_2S 10g tissue
Lamb lean tissue (3.0% fat) (22.3% protein) (25.7% dry matter)	4.36 b
Moose tissue (0.7% fat) (24.1% protein) (26.8% dry matter)	2.90 a
Elk tissue (1.8% fat) (22.7% protein) (26.4% dry matter)	4.65 bc
Deer tissue (1.2% fat) (23.9% protein) (26.9% dry matter)	5.17 bc
Antelope tissue (2.2% fat) (22.8% protein) (26.6% dry matter)	5.42 c

[a] Lamb sample means of two determinations; elk and deer sample means of three determinations; moose and antelope sample means of four determinations. Means with differing letters within columns are different ($P < 0.05$).

evolved from antelope was significantly higher ($P < 0.05$) than for lamb and the amount of H_2S from moose was significantly lower ($P < 0.05$) than lamb. It is interesting to note that taste panel analysis of game meats placed antelope at the very bottom while moose was near the top and is usually described as being "beef like" (Field, personal communication).

In conclusion, several factors seem apparent. Under our cooking conditions lamb gave off more H_2S than beef. This situation seems to be the case for both the adipose tissue and lean tissue. However, the adipose tissue portion contributes more H_2S than the lean tissue. The smaller contribution of the lean to the H_2S evolved comes earlier in the cooking process, whereas, the larger percentage of H_2S which comes from the adipose tissue is evolved later in the cooking period. In fact excessive cooking of lamb results in copious quantities of H_2S being released.

Finally, at present it is difficult to ascertain the extent of the role H_2S plays in lamb flavor and aroma. However, the fact that the lamb gives off more of this odoriferous, volatile gas than beef and since H_2S in combination with other compounds has been implicated in meat flavor (Pippen and Mecchi, 1969) further study of H_2S evolution from lamb and mutton seem warranted. Presently an analysis of other compounds isolated from the volatiles trapped from beef and lamb loaves is being undertaken. Thus, the various ways in which H_2S can contribute to lamb flavor and aroma directly, or in combination with other compounds can be ascertained.

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ULTRASTRUCTURE OF THAW RIGOR BOVINE MUSCLE

INTRODUCTION

VERY LITTLE investigation has been made on the ultrastructural changes occurring in striated muscle fibers during the phenomenon of thaw rigor. In their studies on thaw rigor in lamb, Marsh and Thompson (1958) observed no structural differences between pre-rigor frozen muscle prevented from thaw shortening and muscle which was frozen after rigor-onset, then thawed with or without restraint. They, however, noted extensive structural disturbances resulting in complete loss of striation in muscle strips which freely shortened during freezing or during subsequent thawing. Cassens et al. (1963) in their studies on porcine longissimus muscle suggested that the formation of irregular banding pattern characteristic of thaw rigor and other violent treatments are of myofibrillar origin. Menz and Luyet (1965), further showed that in the formation of these bands, a number of adjacent sarcomeres converge forming a width of about 1 micron. Herring et al. (1964) were apparently unable to observe similar extreme structural disorganizations in bovine psoas and semitendinosus after thaw rigor.

The present studies were carried out to study further the ultrastructural changes which accompany the incidence of thaw rigor in bovine striated muscle. Furthermore, an attempt has been made to simulate several levels of contraction and stretching to which individual muscles are likely to be subjected during conventional processing with a view to understanding the effect of thaw rigor on intact bovine carcasses.

EXPERIMENTAL

ALL SAMPLES used in these studies were obtained from beef sternomandibularis muscles. All animals supplying the experimental muscles were slaughtered at the Meat Division abbatoir, Cornell University.

Directly after exsanguination, the neck muscles were rapidly excised. A subjective rest length was rapidly established for each muscle by attaching a 55g weighted clamp to one end and allowing it to hang freely while the length of the muscle was measured. This was taken as the reference length. A second clamp was attached to the other end and both clamps were fastened to a steel frame. The investing fat, connective tissue and nerves were carefully removed with minimum damage to individual fibers. Each muscle was dissected into five approximately equal strips along the long axis. Two of these strips were stretched to 150% of the rest length and tied to the steel frame. The remaining strips were similarly restrained at the rest length. All samples were transferred into a −29°C gravity air flow sharp freezer within 30 min of exsanguination. After complete freezing, the strips were wrapped individually in aluminum foil to prevent freezer burn on the surface and stored at the freezing temperature until subsequently used.

After at least 1 day but usually not more than 3 days, the five strips from each muscle were thawed at room temperature for 2 hr during which individual strips were allowed to remain at or contract to either 150, 125, 100, 75 or 50% of the rest length. They were again restrained and further stored at 3°C for another 18–22 hr.

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Light microscopy

Small sections approximately 1 cm × 0.5 cm² were obtained from the thaw rigor samples and fixed in 10% formalin in 0.2m phosphate buffer (pH 7.2) for at least 1 wk at 3°C. Smaller sections from the fixed tissue samples were homogenized at 3°C briefly with water to separate intact muscle fibers using a Sorval omni-mixer with inverted cup and reversed blades. Sarcomere lengths and fiber diameters were determined on 20 randomly selected fibers from each sample using a Nikon Phase Contrast microscope (Model SUR-Ke) equipped with an eye piece micrometer.

Simultaneous studies were also carried out on stained muscle sections. Fixed tissues were dehydrated in graded alcohol to absolute alcohol, cleared with xylene infiltrated with paraffin and sectioned 10μ in thickness. Mounted sections were deparaffinized, taken down through the alcohol series to water and stained with Harris hematoxylin for 5 min. They were rinsed repeatedly in tap water for 3–4 min, then differentiated in acid alcohol until appropriate color development. They were again rinsed in tap water before staining for 15 sec with eosin B. The sections were rinsed in three changes of 65% absolute alcohol each lasting for 3 min, cleared with xylene and mounted with "permount." Micrographs were taken with the above microscope and a Polaroid MP-3 land camera.

Electron microscopy

Three tissue samples (1.0 mm² × 3.5 cm) were obtained from each muscle as follows: (1) directly after the death of the animal while still in the relaxed state; (2) in the frozen state from the strip at rest length; and (3) at 24 hr post-thawing from the five strips in the various contractile or stretched states. All samples were fixed in 6% gluteraldehyde in 0.1M phosphate buffer (pH 7.3) overnight at 2°C. Both pre-rigor and frozen strips were tied to glass rods prior to immersion in gluteraldehyde to prevent subsequent contraction. Since thaw rigor readily occurred in the frozen strips even within the short period necessary for the fixative to penetrate the tissue, the fixative was stored at −29°C till it was almost frozen. The glass rods to which the frozen muscle strips were tied were then introduced into the fixative which was subsequently frozen completely. Under this condition, infiltration and fixation during subsequent slow thawing at 2°C occurred continuously as the solid ice phase receded from the surface of the muscle strips to the center.

The fixed samples were cured in 0.1m phosphate buffer (pH 7.3) for about a month at 3°C, post fixed in cold 1% osmium tetroxide for 1 hr, then dehydrated with graded alcohol to absolute alcohol. They were washed twice in propylene oxide, each for 30 min. Embedding was carried out in an epon-araldite mixture.

Sections were cut and mounted on carbon coated formvar copper grids. They were stained successively with uranyl acetate and lead citrate. Electron micrographs on these sections were taken on an RCA EMU-3G electron microscope operated at 50 or 100 kV.

RESULTS

Light microscopic observations

Phase contrast microscopic observations indicate that muscle strips which were maintained in the stretched or contracted state within 50% of the rest length suffered extensive structural damage in most of the fibers following thaw rigor. The alternate bands of supercontraction and superstretching similar to the irregular transverse bands observed in porcine longissimus muscle which were permitted to go into thaw rigor by

Cassens et al. (1963) are evident. The contracture bands shown in Figure 1 may or may not extend across the width of the fibers. Although there was no consistent patterns, the contracted muscle strips appeared to exhibit longer and thicker transverse bands than the stretched muscle strips.

Other sections of the fibers show I bands which are characteristic of relaxed or superstretched sarcomeres. The A bands and Z lines while apparently maintaining their integrity suffered longitudinal displacements which placed the parallel array of cross striations out of register. Those fibers exhibiting the alternate banding pattern also showed some peculiar flocculations which were dispersed throughout the stretched zones, and probably arose from the denaturation of sarcoplasmic proteins by the drastic change in pH during thaw rigor.

Histograms of sarcomere lengths based on phase microscope measurements of fixed fibers are shown in Figure 2. Since the individual fibers exhibited varying degrees of contraction and stretching, the histograms are representative of distributions of sarcomeres not exhibiting extreme contraction. As evident from Table 1, the mean sarcomere lengths differ significantly ($P < 0.005$) with the different degrees of gross muscle contraction or stretching during thaw rigor. Studies on whole sections stained with Harris hematoxylin and electron micrographs of thaw rigor muscle strips which contracted to 50 or 75% of the rest length suggested that certain sarcomeres in these sections had longer sarcomere lengths.

The fiber diameter also varied significantly ($P < 0.005$) with the degree of stretch or contraction and appeared to be related to the tendency of muscle fibers to suffer from the compressional effects of thaw shortening. In contrast to normal rigor shortening, the severe rupturing of the fiber contents may itself cause a differential decrease or increase in fiber diameter. The sarcomere lengths in the transverse supercontracted bands varied between $1.09 \pm 0.09\mu$ and $1.13 \pm 0.21\mu$ irrespective of the gross muscle length during thaw rigor.

Table 1—The effect of contractile state during thaw rigor on the sarcomere length and fiber diameter of bovine sternomandibularis

	Muscle length at percent of rest length				
	50	75	100	125	150
Sarcomere Length[a]	1.13 (0.21 [c]	1.13 (0.19)	1.09 (0.09)	1.10 (0.11)	1.11 (0.13)
Sarcomere Length[b]	1.18 (0.14	1.56 (0.22)	2.26 (0.47)	3.28 (0.53)	3.97 (0.30)
Fiber Diameter[b]	59.32 (11.72)	49.05 (10.55)	40.71 (7.49)	28.24 (7.25)	25.78 (6.58)

[a] Measurements in microns made on zones of supercontraction on intact fibers
[b] Measurements in microns made on zones other than those contracting maximally
[c] Standard deviations from the means

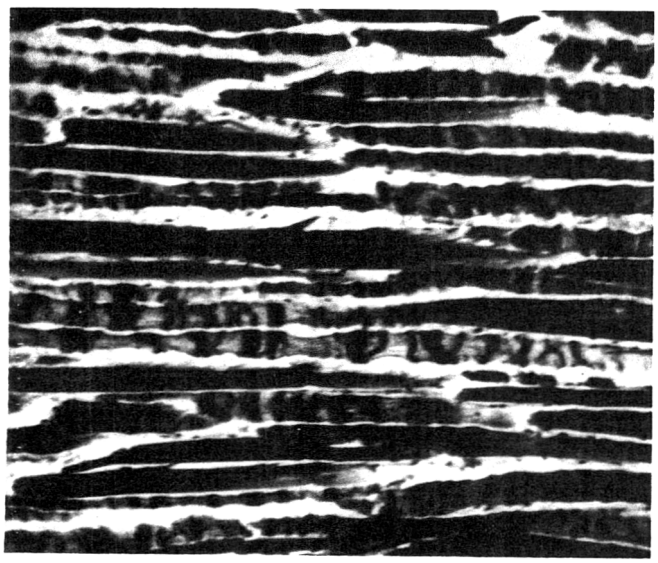

Fig. 1—Bovine sternomandibularis held isometrically during thaw rigor (100X).

Electronmicroscopic observations

Figure 3 shows a micrograph of bovine muscle fixed very shortly after death. The ultrastructural features are typical of relaxed striated muscle. The characteristic banding patterns of the A, I and Z bands are evident. The A bands are flanked by moderately wide I bands which are bisected by the sharp, well defined Z lines. Both the thick and thin filaments maintained their structural integrity. The H zones remained broad and the M lines can be seen as narrow dense stripes down the middle of each H zone. Elements of the sarcoplasmic reticulum are observable; however, their distribution is not extensive. Although variable in different fibers, fairly abundant granules of glycogen are distributed between the myofilaments in the region of the I band and more sparsely within the A band. Mitochon-

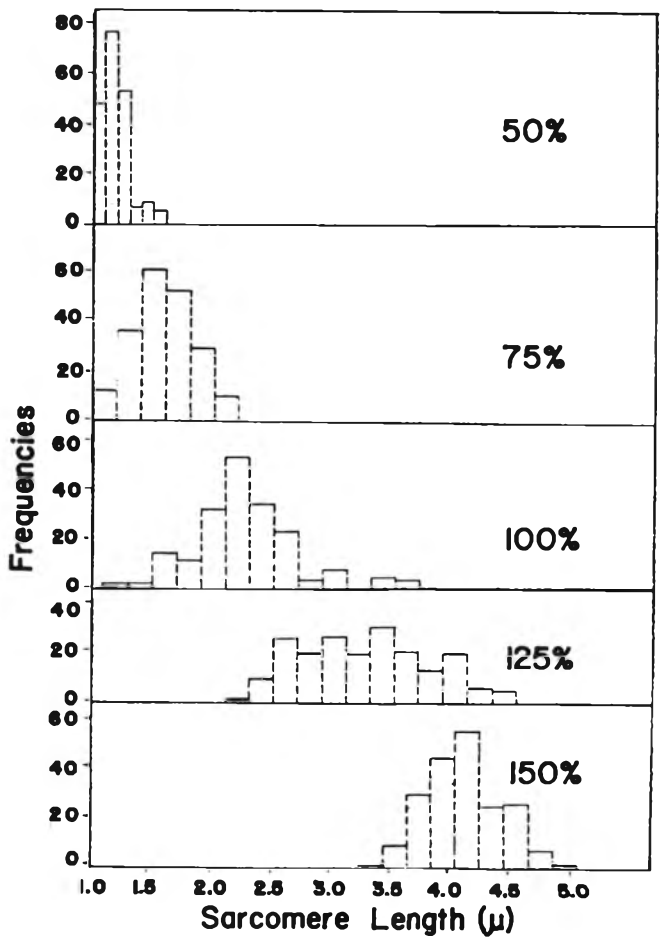

Fig. 2—Histograms of sarcomere length distribution in bovine sternomandibularis maintained at: 50%; 75%; 100%; 125% and 150% of rest length during thaw rigor.

dria are localized within the I bands and between the myofibrils.

Electron micrograph (Fig. 4) of pre-rigor frozen muscle fixed from the frozen state shows features similar to those of the fresh muscle. Apart from the presence of ice crystal pockets between individual myofibrils, the myofibrillar contents exhibited no structural damage. From this observation it appears that freezing per se was not the direct cause of structural disintegration although it served as an efficient potentiator of the ensuing biochemical and physical activity in the thaw rigor muscle fibers.

Since thaw rigor muscles exhibited the same ultrastructural features irrespective of their final length, the same description is applicable to all. Figure 5 shows the ultrastructure of a pre-rigor frozen muscle strip which was permitted to contract to 75% of its rest length. Morphologically, two types of fibers can be identified. Active fibers, typified by the two fibers in the upper part of the micrograph, are characterized by sarcomeres of various lengths. The zones of supercontraction failed to exhibit the extreme convergence of sarcomeres observed in frog sartorius muscle by Menz and Luyet (1965). The zones of extreme contraction alternated with zones of superstretching.

Fig. 3—Electron micrograms of myofibrils from bovine sternomandibularis sampled at death (23,200X): A — A band; I — I band; H — H zone; m — m line; Z — Z line; Mi — Mitochondria.

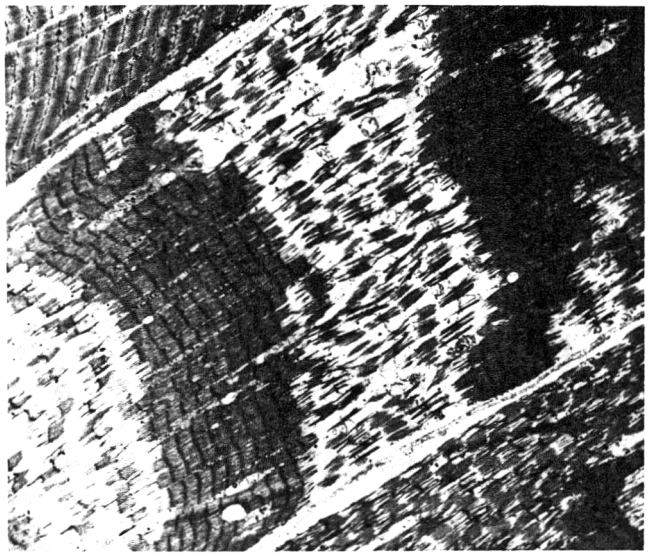

Fig. 5—Electron micrograph of bovine sternomandibularis after thaw rigor contracture. The alternating pattern of transverse zones of supercontracted and superstretched sarcomeres is evident in the central fiber, the lower adjacent fiber remained intact and relaxed (4,500X).

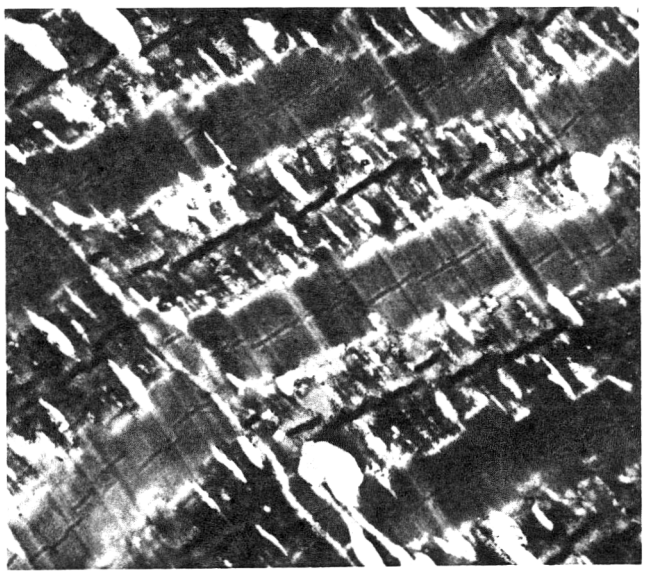

Fig. 4—Electron micrograph of bovine sternomandibularis frozen pre-rigor and fixed in the frozen state without undergoing thaw rigor (17,000X).

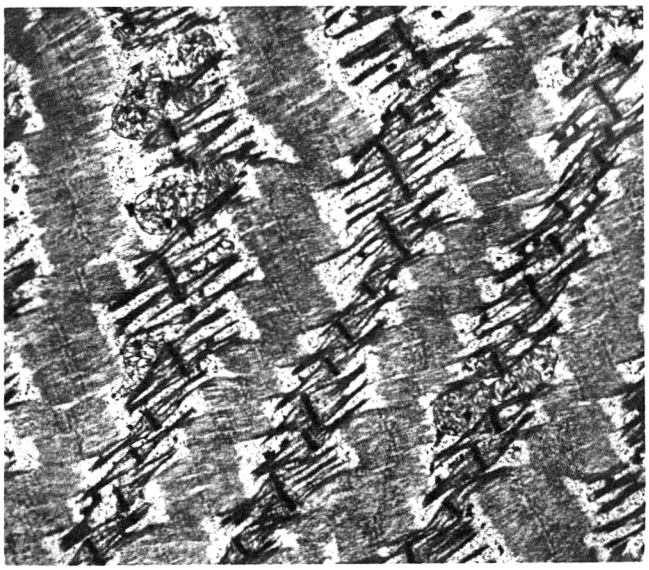

Fig. 6—Electron micrograph of bovine sternomandibularis after thaw rigor at 125% of rest length (17,000X).

On an intact muscle, these zones may be visible as microswelling alternating with zones of small depressions at points of myofibrillar rupturing. The endomysial sheath remained intact although there was a likely presence of micropunctures arising from mechanical damage by ice crystals. The lower fiber shown in the same figure typified the passive fibers which remained virtually in an intact state. The paucity of mitochondria in this fiber is evident. Both the actin and myosin filaments exhibit uniform overlap and contraction bands are conspicuously absent. Thick fibers appeared to survive structural disintegration more frequently than fibers with small diameters. This might suggest differences in the susceptibility of the fibers to the trauma of thaw rigor.

A stretched fiber which was later passively shortened by an adjacent actively contracting fiber is shown in the electron micrograph (Fig. 6) from a muscle strip maintained at 125% of the rest length during thaw rigor. The waviness of the fibers resulted in a steplike orientation of the Z lines and reflects the ease with which some adjacent myofibrils slide relative to one another. In contrast, the electron micrograph from a muscle strip after thaw rigor contracted to 75% of the rest length (Fig. 7) shows a section of a kinky fiber in which the filaments of the sarcomeres have been locked in rigor bonds before the affected myofibrils suffered a compressional effect. Sarcomeres in the irregular transverse bands appeared similar to those reported in barnacle muscle by Hoyle et al. (1965). Figure 8 shows the ultrastructure of sections of supercontracted myofibrils. The M lines, H zones and I bands have completely disappeared and the Z lines have assumed a rather diffuse profile. The myosin filaments appeared to be grossly compressed between the Z discs. The estimate of sarcomere length showed that the sarcomeres have contracted terminally to a width of $1.1 \pm 0.2\mu$. Since the normal A band width in mammalian striated muscle is 1.5μ (Huxley, 1953), it is not clear whether the reduced sarcomere length was due to the compression alone or whether the myosin filaments actually penetrated the Z disc. Possibly the two mechanisms may have occurred. Thus the myosin filaments remained fairly well aligned longitudinally, the doubling back of the myofilament on itself occurred only at points of impingement of the Z disc.

On the other hand, some light areas of the diffuse Z lines do not indicate any crumpling of the thick filaments. Rather, they appeared to have passed straight through the Z disc. Voyle (1969) made similar observations on cold shortened bovine neck muscle. Stromer and Goll (1967) observed what appeared to be gaps in the Z line of at-death KCl extracted myofibrils through which thick filaments appeared to have penetrated. More recently, Hagopian (1970) also demonstrated that in glycerol extracted chicken pectoral muscle, penetration of the Z line by myosin filaments from adjacent sarcomeres occur at sarcomere lengths between 1.3 and 1.5μ. In extreme contraction, the myofilaments suffer severe contortional disorientation (Fig. 9) resulting in violent distortion at the level of the Z line.

Figure 10 shows the electron micrograph of myofibrils with moderately contracted sarcomeres [A]. The I band is greatly reduced in width. The M as well as the Z lines are very evident. However, there is a broad dark band at the center of the A band which appeared to coincide with the area of overlap between actin filaments from opposite ends of the sarcomeres. The adjacent sarcomere [B] demonstrates clearly that both supercontraction and superstretching occur in individual sarcomeres and contradicts the suggestion (Hoyle et al., 1965) that the sarcomere contracts as a unit. The present observation may be attributed to differential release of calcium from the sarcoplasmic reticulum at the two A-I junctions of each sarcomere resulting in the contraction of one end of the sarcomere while permitting the other end to be stretched.

The ultrastructure of myofibrils which were stretched during thaw rigor is presented in the electron micrograph (Fig. 11) taken from a muscle strip maintained at rest length during thaw rigor. The large clear spaces are ice pockets. All the characteristic features of the sarcomeres are evident. However, the H zones are obliterated. The thin filaments appear to be clumped together like a wet feather while maintaining their parallel orientation relative to the thick filaments. This characteristic is demonstrated in cross section in Figure 12 from a thaw rigor muscle. It contrasts with a similar cross section (Fig. 13) from an at-death muscle strip. Although the same orientation is maintained in most extremely superstretched sarcomeres, infrequently, some sarcomeres exhibit the drastic disorganization shown in Figure 14 in which the Z lines suffer frequent breakages and the myofilaments lose their parallel array characteristics.

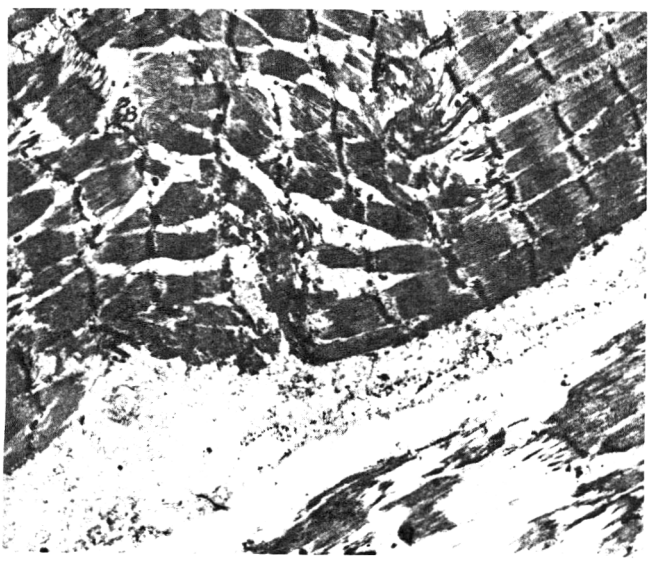

Fig. 7–Electron micrograph of bovine sternomandibularis after thaw rigor contracture to 75% of rest length (11,500X).

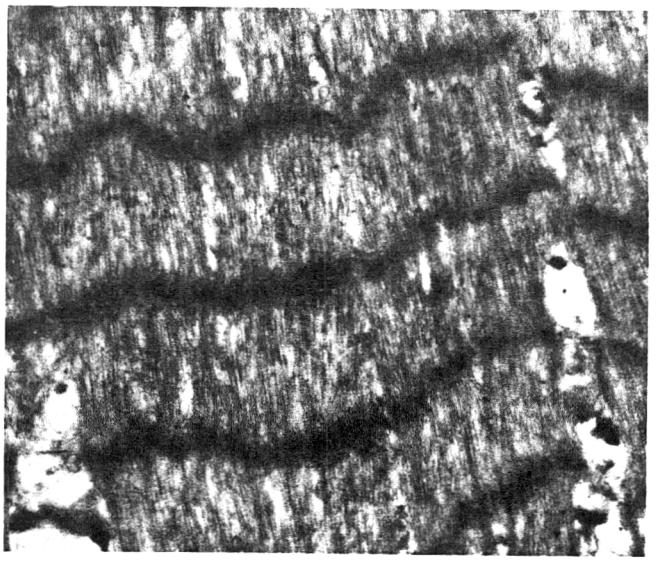

Fig. 8–Electron micrograph of bovine sternomandibularis maintained at 125% of rest length after thaw rigor (45,000X).

Estimates of sarcomere lengths, A band width and Z line width determined on randomly selected micrographs are presented in Table 2 and show the upper and lower limits of the measured dimensions only. Considerable variations were noted in these estimates. There appears to be some disagreement between the two estimates determined by electron and phase contrast microscopy. These differences may be due in part to the fact that only small areas are observable with electron microscopy as suggested by Hegarty et al. (1973). Furthermore, the homogenization procedure used in the phase contrast observations may have disrupted atypical sarcomeres in each fiber section leaving only zones with fairly uniform sarcomeres.

Elements of the sarcoplasmic reticulum were not readily identifiable in the 24 hr post thaw rigor micrographs. This contrasts with previous observations by Stromer and Goll (1967) who observed these membranous structures in bovine

Table 2—Sarcomere dimensions of myofibrils fixed in the fresh, pre-rigor frozen or post thaw rigor state[a]

			Treatments[b]				
Band	Fresh	Frozen	50	75	100	125	150
			Minimum values				
Sarcomere	2.97	2.78	0.76	1.26	1.48	1.00	2.70
A band	1.47	1.47	0.76	1.18	1.19	1.00	0.70
Z line	0.09	0.09	0.10	0.12	0.10	0.19	0.16
			Maximum values				
Sarcomere	3.19	3.01	4.29	4.47	4.53	4.47	4.35
A band	1.61	1.60	1.59	1.59	1.67	1.62	1.77
Z line	0.12	0.12	0.24	0.30	0.29	0.24	0.27

[a] Dimensions in microns were obtained from randomly selected electron micrographs.
[b] Muscle length after thaw rigor as percent of rest length.

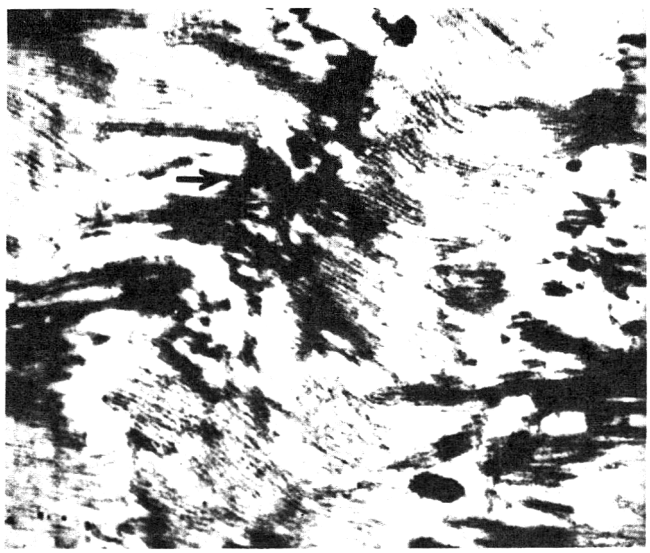

Fig. 9—Electron micrograph of bovine sternomandibularis after thaw rigor contracture. Note Z line distortion (→) which was seen infrequently in the electron micrographs (31,000X).

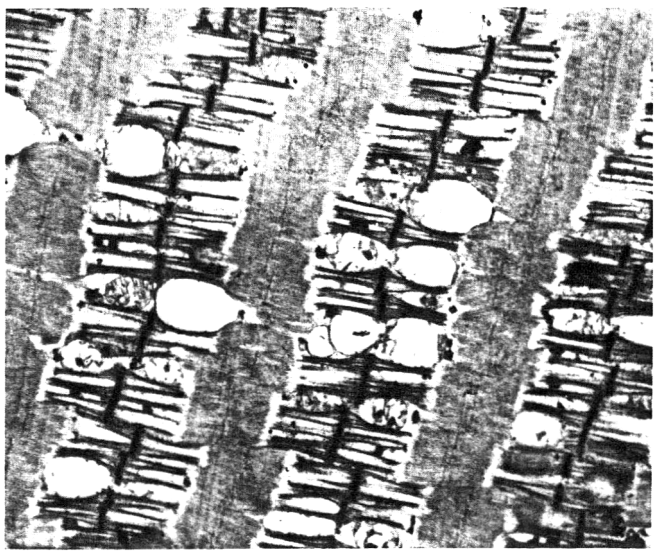

Fig. 11—Electron micrograph of bovine sternomandibularis maintained at rest length after thaw rigor (17,000X).

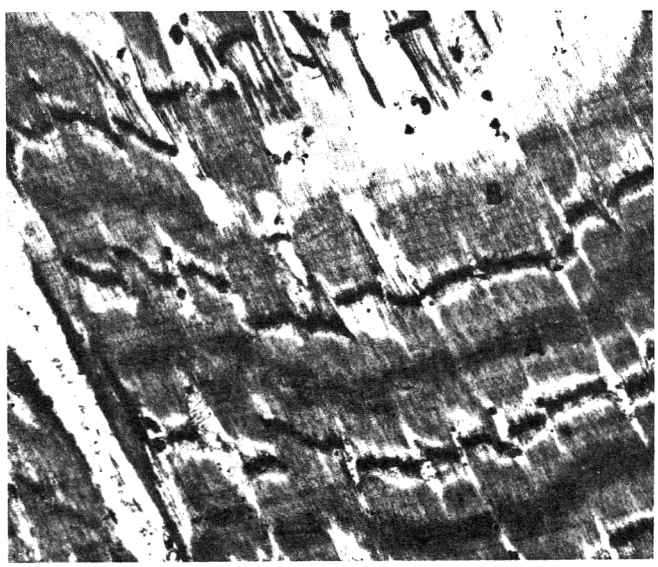

Fig. 10—Electron micrograph of myofibrils illustrating that supercontraction (A) and superstretching (B) occur in adjacent sarcomeres (17,000X).

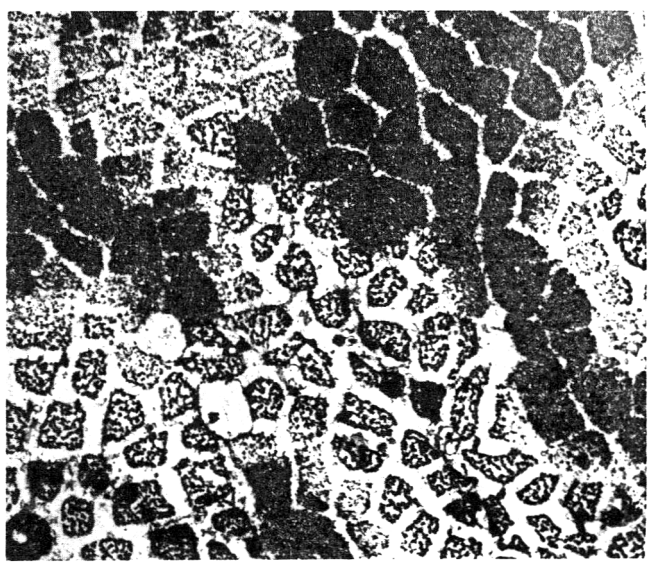

Fig. 12—Electron micrograph of cross sectional view of bovine sternomandibularis sampled after thaw rigor (17,000X).

semitendinosus after storage for 312 hr at 16°C. The degradation of the sarcoplasmic reticulum and the triad system may in part explain the ease with which each myofibril separates from adjacent myofibrils, thus enabling each of them to contract or stretch independently.

Some dark granular precipitates are distributed in relaxed or stretched myofibrils from thaw rigor muscles at the level of the A-I junction (Fig. 6 and 11). In the supercontracted zones (Fig. 7 and 10) the granular precipitates are located between the myofibrils. Such precipitates were not observable in the pre-rigor muscles. A comparison of electron micrographs from at death muscle (Fig. 3 and 13) with thaw rigor muscle (Fig. 11 and 12) demonstrates a complete absence of glycogen granules in the bovine muscle after thaw rigor. This supports earlier observation that during thaw rigor, metabolic changes are greatly accelerated (Bendall, 1960).

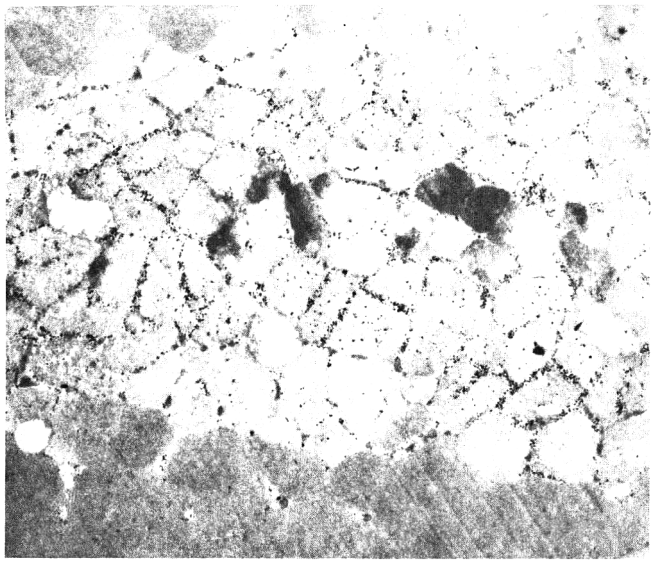

Fig. 13—Electron micrograph of a cross sectional view of bovine sternomandibularis sampled at death (23,200X).

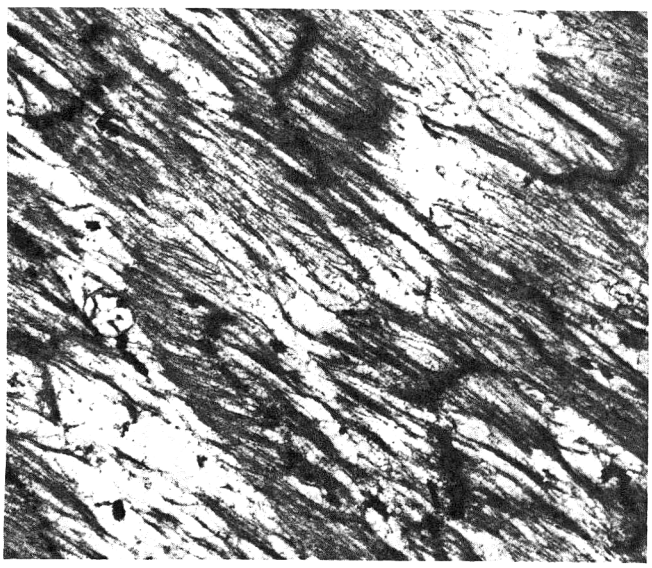

Fig. 14—Electron micrograph of bovine sternomandibularis after thaw rigor showing sarcomeres in superstretched disoriented state (31,000X).

DISCUSSION

THE PRECEDING OBSERVATIONS clearly show that extensive disorganization of the myofibrillar content of bovine muscle fibers are caused when pre-rigor frozen muscle strips in various states of stretch or contraction are thawed at elevated temperatures. This poses some grave implications on muscle tenderness since in the superstretched sections of the muscle fibers, the myofibrillar density was greatly reduced per unit cross sectional area of the muscle tissue. The connective tissue can then assume a greater proportion per unit cross sectional area and thereby afford a greater resistance to shear across the fiber direction. Under this condition, the background tenderness may therefore become a very important factor in determining the ultimate meat tenderness of the thaw rigor meat. This is particularly so in the limited depth of muscle which Howard and Lawrie (1956) suggested could be frozen in the pre-rigor state in freshly slaughtered bovine carcasses subjected to various forms of conventional freezing techniques.

Furthermore, the differential superstretching and supercontraction of sarcomeres from isometrically stretched muscle during thaw rigor does give a strong credence for the partial extensibility of muscle in rigor. Evidence for this is based on the fact that in superstretched sarcomeres, the actin filaments were completely pulled out of the adjacent myosin filaments during thaw rigor resulting in large increases in sarcomere lengths. The occurrence was ostensibly due to the tremendous strain placed upon the stretched sarcomeres by the adjacent contracting sarcomeres in much the same way as occurs during normal rigor if the muscle strips were subjected to a sufficient external load. If thaw rigor muscle were inextensible, myofibrillar rupturing should occur by breakage at the level of the Z line or at the level of the I band, the bulk of the actin filaments should remain attached rigidly to the myosin cross bridges. Only stumps of the thin filaments should remain attached to the Z line, but such observation was not found in this study.

It may be hypothesized therefore, that the superstretching of sarcomeres during thaw rigor involves the breakage of stable crosslinks initially formed between actin and myosin cross bridges. This allows the actin filaments some degree of slippage that is consistent with macroscopic and molecular extensibility. At the same time, more permanent cross bridge linkages are formed in the adjacent supercontracted sarcomeres. This theory is consistent with the sliding filament theory of Huxley and Niedergerke (1954). An a priori conclusion from the extensibility theory is that thaw rigor muscle strips will first develop tension and as a result of the drastic extensibility, will also suffer an accelerated decline in thaw tension.

As to the actual mechanism of events leading to thaw rigor, it has been suggested that the 'Marsh factor' responsible for maintaining the relaxation of muscle fibers is temporarily inhibited as a result of freezing and thawing (Bendall, 1960). Phospholipase C, present in muscle is known to inhibit the ability of the relaxing factor to induce muscle relaxation, and indeed, of the sarcoplasmic reticulum itself to accumulate calcium ions. Consequently, calcium ions are released repeatedly from the sarcoplasmic reticulum, the free calcium ions themselves probably inducing the release of more calcium ions as suggested for skinned skeletal muscle fibers (Endo et al., 1970). At calcium concentrations above 10^{-7} M myofibrillar ATPase became greatly activated resulting in the severe contraction and syneresis which are normally associated with thaw rigor. Differential release of calcium ions at locations along the entire length of the fiber may also account for the fact that both superstretched and supercontracted zones are found within the same fibers after thaw rigor.

It was earlier observed that in thaw rigor muscle some dark granular precipitates were located at the level of the A-I junctions of superstretched sarcomeres and between the myofibrils in the supercontracted zones probably within remnants of the sarcoplasmic reticulum. Similar granulations were not seen in the fresh muscle sections and may well be a feature of muscle in rigor generally since they are also observable in electron micrographs of pig biceps femoris after normal rigor (Hegarty et al., 1973). Further complications arose from the observation that similar precipitates were found in many of the degenerated and necrotic mitochondria and were probably accumulated during the early phase of thaw rigor. As shown by Trump (1971), degenerating mitochondria do accumulate abnormal amounts of insoluble inorganic compounds (e.g., calcium oxalate or calcium phosphate) in the presence of rapidly accumulating inorganic phosphate and a fastly decreasing ATP/ADP ratio. Both of these conditions occur simultaneously during the development of thaw rigor. Many of the degenerated mitochondria were in various stages of contraction or swelling. However, other healthy intact mitochondria were observable with the integrity of their cristae unimpaired.

The conspicuous absence of glycogen granules in the 24-hr post-thaw samples, irrespective of the stretched or contractile states of the muscle, contrasts sharply with a previous observation by Stromer et al. (1967) of large amounts of glycogen granules in bovine semitendinosus muscle stored for a period of 312 hr at 16°C. This may therefore give further proof of accelerated glycolysis during this phenomenon suggested by Bendall (1960) and Scopes and Newbold (1968) probably through increased activities of phosphorylase and phosphofructokinase.

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EFFECT OF STORAGE TIME AND TEMPERATURE ON HISTAMINE CONTENT AND HISTIDINE DECARBOXYLASE ACTIVITY OF AQUATIC SPECIES

INTRODUCTION

HISTAMINE has been reported to be one of the principle compounds leading to scombroid poisoning. The syndrome is classically characterized by nausea, vomiting, facial flushing, intense headache, epigastric pain, burning sensation in the throat, dysphagia, thirst, swelling of the lips and urticaria. Recently, attention has been focused on histamine formation in food due to the February 1973 outbreak of scombroid poisoning involving 254 cases and associated with consumption of canned tuna fish (CDC, 1973b). Preliminary reports from the FDA indicated histamine concentrations ranging from 76–280 mg histamine per 100g of fish muscle (CDC, 1973a). Concentrations of histamine exceeding 100 mg/100g fish muscle are usually thought to result in clinical illness (Halstead and Courville, 1967).

Scombroid poisoning is generally associated with consumption of spiney finned fish of the suborder Scombroidei which includes tuna, bonito, skipjack, mackerel and albacore. Histamine build-up is the result of growth of histidine decarboxylase positive bacteria under conditions favorable for enzyme synthesis and activity. Bacteria known to produce histidine decarboxylase include species of *Proteus, Salmonella, Shigella, Clostridium* and *Escherichia* (Kimata, 1961).

Several aquatic species have been examined for their ability to support histamine formation. Dabrowski et al. (1968) found no histamine in sterile Baltic herring muscle, but histamine was formed in muscle samples containing the normal microflora when stored at temperatures between 0–2°C. Takagi et al. (1971) reported that histamine was not formed in muscle of squid or octopus. In an examination of ocean mackerel, Gheorghe et al. (1970a) found that histamine concentration remained below toxic levels when the fish were stored at 8°C for 11 days or for 8 months at −8°C. Gheorghe et al. (1970b) also reported that histamine never exceeded 6 mg/100g when the fish were stored at −10°C for 8 months. Schmidtsdorf (1970) found approximately 800 ppm histamine in herring being processed to fish protein concentrate after it was held 2 days at 20°C. When formic acid was added as a preservative, 500 ppm histamine was found after 2 days of hydrolysis.

Takagi et al. (1969) investigated histamine formation in 21 aquatic species. After 24 and 48 hr of storage at 25°C highest histamine concentrations were found in Pacific Saury (263 mg/100g after 48 hr). Pacific mackerel reached a level of 246 mg/100g after the same storage period, and three other species reached concentrations exceeding 700 ppm. The remaining species never exceeded 10 mg/100g and seven species were reported to remain histamine free. These researchers concluded that the degree of histamine formation in marine products tended to be governed by the muscle histidine content but was not proportional to the loss of histidine.

Hughes (1959) found that the free histidine content of herring varied with the season with highest levels occurring in the summer months. He reported that upon storage at ambient temperature the free histidine content dropped to zero after an initial slight increase. Histamine concentrations greater than 3 mg/100g muscle developed 40–50 hr after death and ultimately reached 30–40 mg/100g.

Many species have not been investigated for their potential to support histamine formation and little information is available concerning the relationship of histidine decarboxylase formation during storage to histamine build-up. The purpose of this study was to determine the effect of storage time and temperature of histamine content and histidine decarboxylase activity in several species commercially important in Georgia. The species examined were Spanish mackerel (*Scomberomorus maculatus*), common mullet (*Mugil cephalus*), speckled trout (*Cynoscion nebulosus*), white shrimp (*Penaeus setiferus*) and channel catfish (*Ictalurus punctatus*).

EXPERIMENTAL

Sample preparation and storage

Spanish mackerel, speckled trout, white shrimp, common mullet and channel catfish were purchased from a local wholesale fish distributor on days fresh shipments were received. No information was available as to how long the various species were out of the water, but all were judged to be of high organoleptic quality. All fish were eviscerated by the distributor at the time of purchase. Shrimp were deveined prior to analysis. In all instances, initial histamine concentrations were low (< 2 μg/g muscle).

Filleted sections of from 3–4 fish were stored in plastic bags at 4°C and at ambient temperature (24 ± 2°C) for varying times depending on temperature

Histamine analysis

Histamine was determined by the method of Shore (1971). 5g of fish muscle were homogenized in 45 ml of 0.4N perchloric acid using a Sorvall homogenizer. The homogenate was allowed to stand at room temperature for several minutes and was then centrifuged. A 4-ml aliquot of the protein-free supernatant fluid was transferred to a centrifuge tube containing 10 ml of n-butanol, 0.5 ml 5N NaOH, and 2.0g for NaCl. The tube was shaken for 5 min and centrifuged. To remove any free histidine, the butanol layer was transferred to a second tube containing 5 ml of NaCl-saturated 0.1N NaOH, shaken for 1 min and centrifuged. A 8-ml aliquot of the washed butanol extract was transferred to a third tube containing 4 ml of 0.1N HCl and 15 ml of n-heptane. The tube was shaken for 1 min, centrifuged and the organic phase removed by aspiration.

To 2 ml of the acid phase containing histamine was added 0.4 ml of 1N NaOH followed by 0.1 ml of O-phthalaldehyde (OPT) (10 mg/ml in methanol). After 4 min at room temperature, 0.2 ml of 3N HCl was added. The contents of the reaction tube was mixed using a Genie Vortex mixer following each addition.

The fluorescence at 450 nm resulting from activation at 360 nm was measured on a Turner Model 430 Spectrofluorometer. Histamine concentration was determined from a standard curve based on varying concentrations of free histamine which were carried through the extraction procedure and OPT reaction. Reagent blanks were run to correct any native fluorescence.

Histidine decarboxylase assay

Histidine decarboxylase activity in the muscle was determined by an isotopic decarboxylase assay method adopted from the procedure of Levine and Watts (1966). To 0.5 ml of a 10% muscle homogenate

prepared by homogenizing 1g of muscle in 9 ml of ice cold deionized water in a glass tissue grinder, was added 2.4 ml of 1.0M sodium acetate buffer, pH 5.5, and 100 µl of a 5 × 10^{-3}M solution of L-histidine in 10^{-4}N HCl containing approximately 0.125 µCi of ^{14}C-carboxyl labeled L-histidine (Cal-Atomic, Los Angeles, Calif.). After incubation at 25°C for 1 hr in an air tight reaction flask, 2 ml of 1.2N perchloric acid was injected into the flask to stop the reaction and force the liberation of dissolved $^{14}CO_2$. The liberated $^{14}CO_2$ was trapped on filter paper (3 MM Whatman, 1 × 3 cm rolled cylinders) saturated with Phenethylamine (Packard, Downers Grove, Ill.) that was suspended over the reaction solution by a paper clip inserted into a No. 2 rubber stopper. After shaking for 30 min at 25°C in a Dubnoff Metabolic Shaker to insure complete liberation and entrapment of dissolved $^{14}CO_2$, the filter paper was transferred to a polyethylene scintillation vial containing 10 ml of a toluene scintillation fluor and counted in a Beckman Model LD – 100C Liquid Scintillation Counter. Controls consisted of reaction mixtures containing muscle homogenate added after addition of the perchloric acid. Activity was expressed as nmoles histamine/min/g muscle.

Statistical analysis

The data were analyzed using analysis of variance. The data were normalized by (ln x + 1). Comparisons were made between species within temperature, temperature within species and time within species. Significant ($P < 0.05$) differences between means were determined by Least Significant Difference.

RESULTS & DISCUSSION

HISTAMINE FORMATION in the five species stored at 4°C is shown in Figure 1. The plotted values are the average of three storage trials and represent samples purchased over a 6-month period. The histamine content after 14 days storage did not increase "appreciably" in any of the species although psychrophilic spoilage had occurred extensively as judged by appearance and off-odor development. Statistical comparison of the mean histamine concentration at the different storage times showed that while the increased histamine levels were small they were significant at the 0.05 level in trout, mullet and catfish muscle between 1 and 2 wk of storage (Table 1). The highest level observed was 3.40 µg/g muscle in channel catfish. Histamine content of mackerel did not increase significantly ($P < 0.05$). Comparison of the overall species means (0, 7 and 14 day observations)/(no. of observations) within the 4°C temperature indicated significant difference between species. Histamine levels in mackerel, shrimp and catfish were statistically higher than those in the trout. Mullet levels did not differ significantly from those of the trout or the other three species.

Figure 2 shows histamine development in the five species stored at ambient temperature (24 ± 2°C). The histamine levels were significantly ($P < 0.05$) higher in mackerel and mullet muscle than in channel catfish, speckled trout and white shrimp. The mean histamine level for mackerel muscle after 24 hr storage was 17.8 µg/g and 238 µg/g after 48 hr. For mullet, average histamine concentrations were 24.7 µg/g and 27.1 µg/g after the 24 and 48 hr storage periods, respectively. Significant increases at the 0.05 level were noted in mackerel between the 0 and 24, and the 24 and 48 hr storage periods and in mullet, trout and catfish between the initial and 24 hr storage period. Histamine concentrations in white shrimp muscle remained constant at initial levels over the 48 hr storage period. Statistically higher histamine levels occurred at ambient storage than at 4°C for mackerel, trout and mullet. There was no difference in histamine levels for shrimp and catfish between the two temperatures.

It was noted that the histamine levels observed in the mackerel stored at ambient temperature varied greatly between storage trials. The histamine concentrations found in mackerel muscle for the 3 trials ranged from 69 µg/g to 333 µg/g after 48 hr storage. It is evident from the literature (Ienistea, 1971) that histamine formation is the result of bacterial histidine decarboxylation. Ienistea (1971) also reported that histamine formation is not directly related to the total number of bacteria present but that it is somewhat influenced by the number of bacteria within a given microflora that are capable of synthesizing histidine decarboxylase. Ferencik

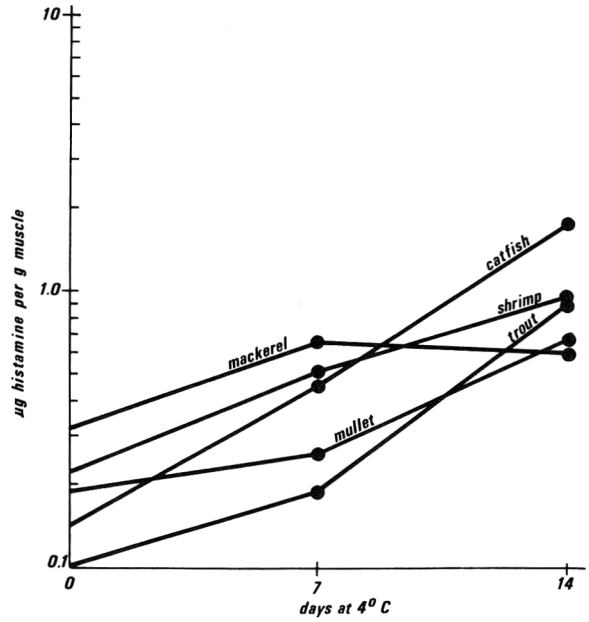

Fig. 1–Histamine formation at 4°C.

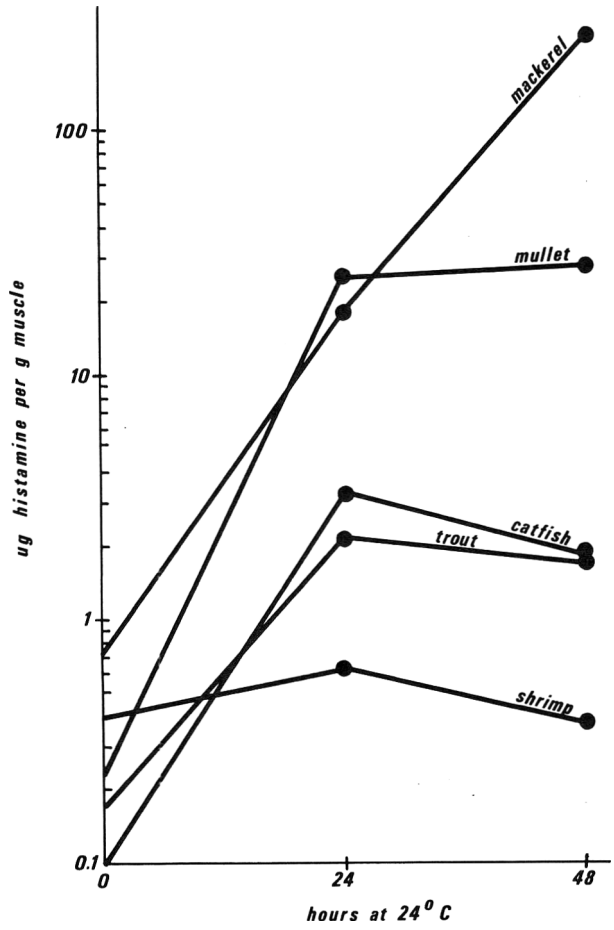

Fig. 2–Histamine formation at ambient temperature (24 ± 2°C).

Table 1—Mean value histamine levels (μg/g) for the examined species[a]

Species	Storage time at 4°C			Storage time at ambient temp		
	0	7 days	14 days	0	1 day	2 days
Spanish mackerel	0.32c	0.65c	0.60c	0.73c	17.77b	237.97a
Speckled trout	0.08f	0.19f	0.90e	0.17f	2.13d	1.67d
Common mullet	0.19i	0.26i	0.67h	0.23i	24.70g	27.13g
White shrimp	0.22k	0.52jk	0.96j	0.40jk	0.63j	0.37jk
Channel catfish	0.15m	0.46m	1.74l	0.10m	3.23l	1.80l

[a] Means in same row bearing different letters are significantly ($P < 0.05$) different.

Table 2—Mean value histidine decarboxylase activities (nmoles/min/g muscle) for the examined species[a]

Species	Storage time at 4°C			Storage time at ambient temp		
	0	7 days	14 days	0	1 day	2 days
Spanish mackerel	2.63c	2.35c	0.96c	6.73b	135.60	79.73a
Speckled trout	0.63f	0.00f	0.00f	5.47e	8.23de	21.10d
Common mullet	6.91g	3.28g	1.41g	3.30g	4.70g	10.83g
White shrimp	0.00h	0.42h	0.10h	1.06h	1.13h	2.13h
Channel catfish	7.05j	1.92j	0.48j	0.90j	40.43i	61.83i

[a] Means in same row bearing different letters are significantly ($P < 0.05$) different.

(1970) concluded from a study of histamine formation in several marine fish species that histamine build-up depends primarily on the histidine decarboxylase activity of the contaminant microorganisms and on the free histidine content of the muscle.

In order to determine if the presence of histidine decarboxylase in muscle during storage was a limiting factor in the formation of histamine in the various species, histidine decarboxylase activities were determined after storage at 4°C and ambient temperature. Figure 3 shows the histamine levels and histidine decarboxylase activities present in the mackerel muscle during the three storage trials at ambient temperature. In each of the trials, initial histamine content was low (< 2.0 μg/g) and histidine decarboxylase activity ranged from 3.5–12.2 nmoles histamine/min/g muscle. After 24 hr storage, the enzyme activity in the muscle increased (significant at 0.05 level) to 47, 253 and 107 nmoles/min/g in Trial 1, 2 and 3, respectively. Histamine content rose slightly (significant at 0.05 level) during this time interval to 9, 12 and 33 μg/g in Trials 1, 2 and 3, respectively. After 48 hr storage, a decrease (not significant at 0.05 level) occurred in decarboxylase activity while histamine increased (significant at 0.05 level) in each of the trials. The largest histamine concentration noted in the study was 333 μg/g after 48 hr in the mackerel in the second storage trial. The mackerel in this trial also developed the greatest decarboxylase activity. Mackerel muscle in Trial 1 possessed the lowest decarboxylase activity after both 24 and 48 hr and reached only 69 μg histamine/g.

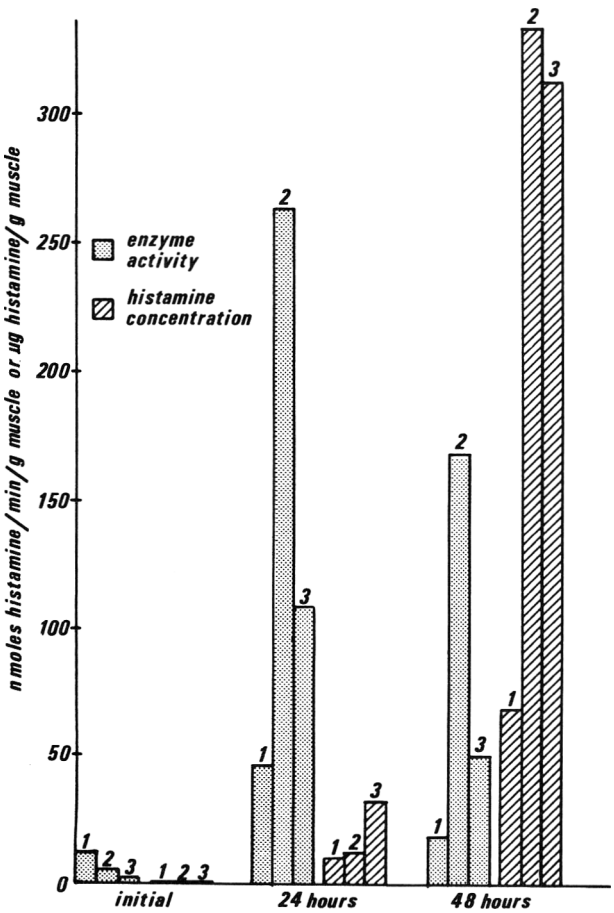

Fig. 3—Comparison of histamine content and histidine decarboxylase activity of mackerel muscle during three storage trials at ambient temperature.

Table 2 gives the average histidine decarboxylase activities present in the species after the 4°C and ambient temperature storage trials. Each value was calculated from three trials. A trend of decreasing decarboxylase activity with storage time was observed in all species except shrimp during the 4°C storage, but no significant change was noted for any of the species. The highest decarboxylase activity observed in any of the 4°C storage trials was 19.37 nmoles/min/g muscle in channel catfish. Mullet enzyme activity was statistically ($P < 0.05$) higher than that observed in trout muscle while the activities found in the other species were statistically the same as those in mullet and trout muscle.

Examination of the histidine decarboxylase activities found in the muscle of the five species held at ambient temperature (Table 2) indicated that enzyme activity in the mackerel muscle was higher ($P < 0.05$) than that observed in the other species. Likewise, decarboxylase activity in catfish muscle was higher at the 0.05 level than that observed in the remaining species except trout. Trout muscle decarboxylase activity was higher than that found in the shrimp but the same as that found in mullet. Mullet and shrimp activities were statistically the same. Mackerel showed a significant increase in activity between 0 and 24 hr. The decrease in histidine decarboxylase activity was observed between 24 and 48 hr in each of the mackerel storage trials (Fig. 3) and although the decrease was not significant ($P < 0.05$), this may indicate a change in the microflora and/or build-up of inhibitors in the muscle. Mullet and shrimp showed no significant changes in enzyme activity, and catfish showed significantly ($P < 0.05$) different activities between 0 and 24 hr with no significant difference occurring between 24 and 48 hr storage. Trout did not show a significant increase between 0 and 24 hr or between 24 hr and 48 hr storage, but the 48 hr activity was significantly higher than the initial activity.

Mackerel, trout and catfish showed statistically higher enzyme activities at ambient storage than at 4°C; whereas mullet and trout showed no significant differences with temperature. Although there were significant increases in decarboxylase activity for the catfish and trout when stored for 2 days at ambient temperature, the increase in histamine contents were slight and occurred between 0 and 24 hr storage. The initial increases were followed by decreases (although not significant) in the histamine content, indicating that increasing levels of enzyme activity do not necessarily lead to increasing histamine build-up. This is further supported by the fact that although mullet showed no significant change in enzyme activity with storage temperature, histamine levels were significantly higher in samples stored at ambient temperatures. While there were significantly higher enzyme activities in catfish stored at ambient temperature, histamine levels showed no significant difference between the two storage temperatures. It is also apparent from the literature (Ienistea, 1971) that diamine oxidase formation by histaminolytic bacteria can influence final histamine levels.

It is evident from this study that microflora developing on species such as the catfish and trout under ambient temperature conditions will synthesize histidine decarboxylase even though the free histidine content of the muscle is low as is the case in fresh water fish, crustaceans and most marine fish (Lukton and Olcott, 1958). In mackerel muscle, the higher enzymatic activities observed at ambient temperature storage are probably the result of enzyme induction resulting from the relatively large amounts of free histidine characteristically present in scombroid species. The results of the study support conclusions of other investigators that the potential for development of high levels of histamine in a specific species rests primarily with the free histidine level of the muscle (Takagi et al., 1969; Fçrencik, 1970; Ienistea, 1971). The low levels of histidine decarboxylase noted during the 4°C storage trials shows that the psychrophilic microflora developing on the species included in this study would not readily decarboxylate free histidine.

There is little chance that speckled trout, common mullet, white shrimp, or channel catfish would develop sufficient histamine levels during spoilage to lead to histamine intoxication. Even though the highest histamine level observed in this study for mackerel muscle (333 μg/g) was far from the recognized toxic level (1000 μg/g), mackerel would have to be considered as potentially capable of developing sufficient histamine to produce intoxication symptoms. The data do seem to indicate that mackerel muscle would have to reach an advanced stage of decomposition before histamine concentrations would reach toxic levels.

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LIPID-PROTEIN INTERACTION DURING AQUEOUS EXTRACTION OF FISH PROTEIN: FISH ACTIN PREPARATION AND PURIFICATION

INTRODUCTION

FISH ACTIN was reported as more stable than myosin during aging (Connell, 1960) and freezing (Connell, 1968). Molecular weight studies by Rees and Young (1967) indicated that rabbit globular-actin (G-actin) is close to 46,000g per mole and is constructed from a single covalently linked polypeptide chain. Adelstein and Kuehl (1970) stated that rabbit G-actin is a single polypeptide with molecular weight of 45,000 dalton. Elzinga (1970, 1971) reported that there is a single 3-methyl-histidine among the 420 amino acids forming the polypeptide of the G-actin. Lusty and Fasold (1969) indicated that actin contains five cysteine residues, but only two are involved in the polymerization reaction. Drabikowski et al. (1968) showed that part of tropomyosin is very tightly bound to actin, and the other loosely bound part becomes tightly bound with actin in the presence of troponines. Ebashi and Maruyama (1965) reported a procedure for eliminating β-actinine from the actin preparation by repeated polymerization-depolymerization, combined with sedimentation at high-speed centrifugation. This paper describes the method of preparation and amino acid analysis of pure actin from fish, while the following paper will describe the properties of fish actin and its interactions with lipids.

MATERIAL & METHODS

Actin preparation and purification

The procedure consists of four steps:

Preparation of acetone powder. Acetone powder from rockfish muscle (*Sebastes auriculatus*) was prepared essentially as described by Carstens and Mommaerts (1963). 300g ground muscle was extracted in the cold room, first with 3 volumes of 0.1M KCl and then with 3 volumes of 0.05M $NaHCO_3$. Each extraction, with continuous stirring, lasted for 30 min. The mince was then washed with 10 volumes of water. All extractions or washings were followed by filtration through several layers of guaze. The residue was then extracted twice with 2 volumes of precooled acetone (−20°C) for a few seconds in a Waring Blendor. The fibrous mass was filtered through a Buchner funnel and air-dried overnight at room temperature.

Preparation of G-actin. A modification of Adelstein and Kuehl's (1970) method was used. The dried acetone powder was treated three times with 5 volumes of chloroform precooled to −20°C and air-dried. G-actin was extracted from the powder by stirring for 30 min at 5°C, using 500 ml buffer solution consisting of 0.5 mM ATP, 0.1 mM $CaCl_2$, and 0.75 mM 2-mercaptoethanol, pH 7.5 (ATP-ME-$CaCl_2$ buffer). The muscle residue was separated by centrifugation at 10,000 × G for 1/2 hr, and the supernatant was clarified by positive pressure filtration (nitrogen 20 psi) through a 0.45μ Millipore® filter, as stated by Rees and Young (1967).

Purification by polymerization. The crude preparation of G-actin was transformed into fibrous-actin (F-actin) by addition of KCl and $MgCl_2$ to 0.1M and 1 mM concentrations, respectively. The solution was placed at room temperature for 2 hr, then placed in a cold room at 4°C for several hr. Centrifugation was done at 20,000 rpm overnight (Spinco Rotor No. 21). The supernatant was decanted and the pellets suspended in 100 ml cold ATP-ME-$CaCl_2$ buffer solutions. This gave a solution of G-actin which was clarified by centrifugation at 40,000 rpm for 2 hr (Spinco Rotor No. 40). KCl and $MgCl_2$ were added to 0.1M and 1 mM respectively, and the repolymerization cycle was repeated. The pellets from the second polymerization were homogenized in 40 ml of Tris-ATP-Ascorbate buffer (2 mM Tris-HCl, 0.2 mM ATP, 0.2 mM

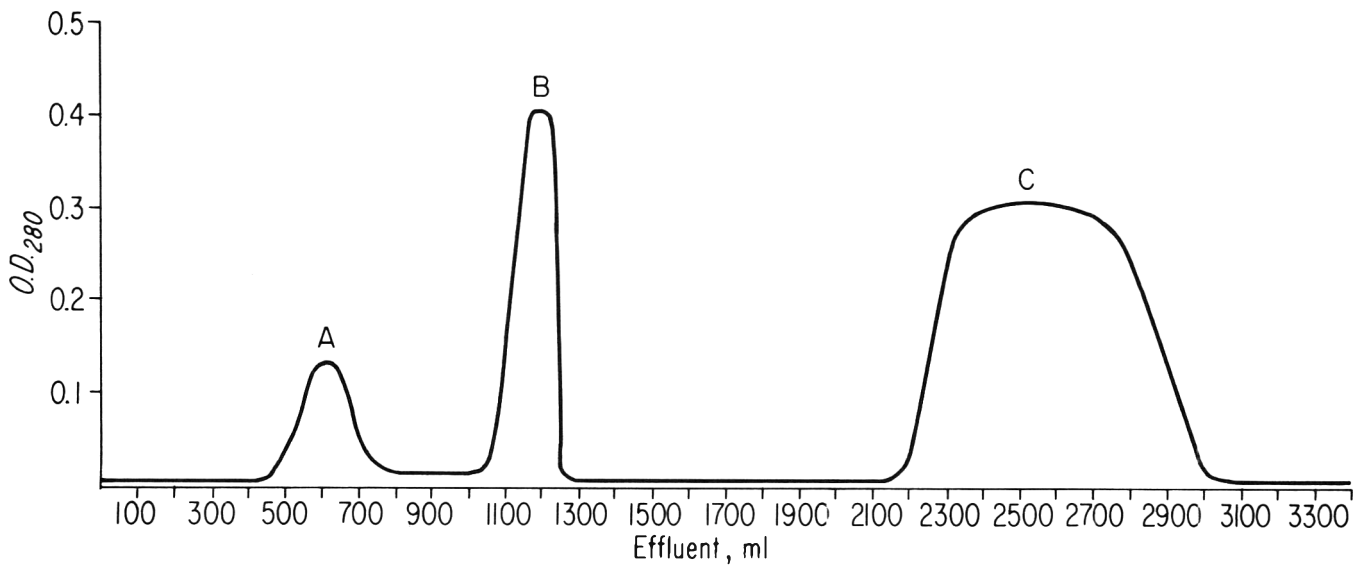

Fig. 1—Elution profile of fish actin prep.-I on Sephadex G-200 column. 30 ml actin solution (15 mg/ml) were applied to a column 5 × 100 cm, packed with Sephadex G-200, with the lowest 10 cm of the column packed with glass beads 6 mm in diameter. The column was equilibrated at 4° C with Tris-ATP-Ascorbate buffer, pH 7.5. Upward flow rate was 20 ml/hr. (B) is the pure actin fraction.

Ascorbate, pH 7.5) and dialyzed overnight against the same buffer at 4°C, followed by centrifugation at 40,000 rpm for 2 hr. The supernatant was lyophilized to yield actin preparation-I used in some experimental work.

Purified G-actin. The actin preparation I was further purified by passing through Sephadex G-200 column as described by Adelstein et al. (1963). 10 cm of the 100 × 5 cm column was packed with glass beads 6 mm in diameter to achieve a higher flow rate. The column was equilibrated with Tris-ATP-Ascorbate buffer, and the upward flow was maintained at 20 ml/hr. The elution profile was monitored spectrophotometrically at 280 nm, and the actin peak (fraction #2) was then lyophilized to yield pure G-actin.

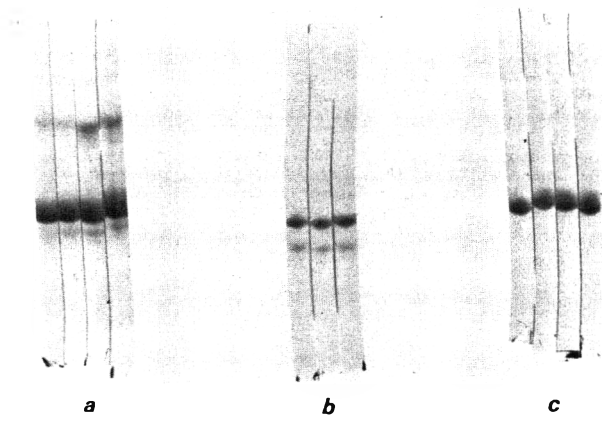

Fig. 2–Electrophorogram showing the main bands on sodium dodecyl sulfate polyacrylamide gel electrophoresis of fish actin. Method described in text. (a) actin prep.-I (purified actin by polymerization-depolymerization-before Sephadex treatment). The protein bands listed from top to bottom are actinins, G-actin, and tropomyosin. (b) is the first elution peak on Sephadex G-200 column (peak A in Fig. 1). The top band is actin and the bottom band is tropomyosin. (c) is the second elution peak on Sephadex (peak B in Fig. 1) which is pure actin.

Table 1–Amino acid composition of actin (as residues/46,000 gm protein)

Residues	Rockfish	Rabbit	
		A[b]	B[b]
Lys	22	19	24
His	10	9	9
Arg	21	18	21
Asp	39	34	40
Thr	30	28	30
Ser	27	22	25
Glu	47	39	46
Pro	21	19	21
Gly	39	28	32
Ala	36	29	34
Val	24	21	20
Met	15	16	16–17
Ilu	31	29	29
Leu	31	26	31
Tyr	19	16	17
Phe	14	12	13
Trp	–	4	5
Cys	–	5	5
3-Me-His	0	1	1

[a] Cited from Dayhoff (1973)
[b] Cited from Elzinga (1970)

SDS-polyacrylamide gel electrophoresis

Sample preparation. The method described by Weber and Osborn (1969) was employed. The protein was incubated at 37°C for 2 hr in a solubilizing solution consisting of 0.01M sodium phosphate buffer, 1% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol (M.E.) and 8M urea, pH 7.0. The solubilized protein was next dialyzed overnight at room temperature against 0.01M sodium phosphate buffer containing 0.1% SDS, 0.1% M.E., pH 7.0. For each 1.5 ml dialyzed protein sample, an equal volume of the dialyzing buffer, 0.06 ml bromo-phenol-blue (0.05%), 0.1 ml M.E. and 10 drops glycerol were added and mixed thoroughly. 0.20 ml of this mixture was used per gel for electrophoresis.

The polyacrylamide gel was prepared basically as described by Weber and Osborn (1969), except for altering the acrylamide percentage to 5%.

After the electrophoretic run, the gels were first fixed in 25% trichloroacetic acid, as recommended by Chrambach et al. (1967) for 1/2 hr. Staining with 0.1% Coomassie blue in 25% TCA for 6 hr and destaining with 7% acetic acid was employed.

Amino acid analysis

Amino acid analysis was carried out according to Moore and Stein (1963). Analysis was done on a Beckman 120-B amino acid analyzer. The pure actin samples were dialyzed extensively before analysis to a relatively salt-free solution, as recommended by Smyth and Elliott (1964). Several times of hydrolysis (24, 48 and 72 hr) were used to permit accurate correction for those residues which were partially destroyed or incompletely released during the acid hydrolysis. The results were calculated by calibration against internal standards.

RESULTS & DISCUSSION

THE ELUTION profile for the actin preparation (Actin-I) on a Sephadex G-200 column is shown in Figure 1. Each peak, as well as the applied actin (Actin-I), was identified on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The actin preparation (Actin-I) showed a major actin band (the top major band on Figure 2-a) and a tropomyosin band below the G-actin. Traces of actins as faint bands at the top of the gel were also detected. Scanning the gel in a densitometer at wavelength 550 nm indicated that the tropomyosin impurities amounted to 12% in the actin preparation (Actin-I).

The major peak (B) from the sephadex column was pure G-actin, as demonstrated by a single band on the SDS-PAGE. The first peak (peak A) was identified on the gel as a mixture of G-actin and tropomyosin in a ratio of 2:1. Actinin traces in Actin-I were lost on the column. The last elution peak (peak C) was a protein-free fraction as tested by the Lowry method and by SDS-PAGE. Spectrophotometric scanning of this peak over the range 240–300 nm indicated that it consisted of nucleotides derived mainly from the effluent buffer used.

The amino acid analysis of the purified G-actin is presented in Table 1. The data show a close similarity in amino acid composition between rabbit and fish actin. Table 1 also shows high amounts of the basic and acidic amino acids (Glu, Asp, Lys, and Arg) which mounts up to about 30% of the residues. The amino acid analysis shows the absence of 3-methylhistidine in fish actin. 3-methylhistidine has been detected in both rabbit myosin and actin by several workers (Huszar and Elzinga, 1971; Tsayer et al., 1968; Johnson and Perry, 1970). Johnson et al. (1967) cited that the 3-methylhistidine in rabbit myosin was localized in subfragment-1, which represents the globular part of the myosin molecules and contains both the ATP-ase and actin combining site of myosin. Thus, there was a belief that the 3-methylhistidine was the site of recognition or interaction during cross-bridge formation between actin and myosin, although there was no evidence that this methylated amino acid was directly involved in this interaction. However, Kuehl and Adelstein (1970) reported the absence of the 3-methylhistidine in red cardiac and fetal myosin. Again, our results report the absence of this amino acid in fish actin.

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LIPID-PROTEIN INTERACTION DURING AQUEOUS EXTRACTION OF FISH PROTEIN: ACTIN-LIPID INTERACTION

INTRODUCTION

IN 1961, the term fish protein concentrate (FPC) was adopted by the FAO to replace the earlier name of "fish flour." In 1972, Bon proposed a classification of concentrated fish protein based on the proximate analysis. However, the various methods and techniques applied in producing a concentrated protein from fish can be roughly classified as illustrated in Figure 1.

Each technique still faces major technological problems. For example, the enzymatic techniques suffer mainly from the cost of enzymes and/or the bitter taste in the product, which is probably due to the presence of low molecular weight peptides (Minamiura et al., 1972; Yamashita et al., 1969; Solms, 1969). The highly desired functional properties of the chemically modified protein isolated by acylation, i.e., succinylation, as reported by Oppenheimer et al. (1967) and Groninger (1973), do not overcome the loss in the nutritional value of the product. The loss of functional properties in the organic solvent extraction procedures, the fire hazard involved and the need for expensive equipment (Moorjani and Vasantha, 1973; Smith et al., 1973; Spinelli et al., 1972; Bass and Caul, 1972; Ernst, 1971) are still considered as the major problems in this technique. And, finally, the major obstacle with the aqueous extraction procedures is the high lipid content (Meinke et al., 1972; Chu and Pigott, 1973), which leads to instability and deterioration in functional properties and loss in nutritional value (Varma, 1967; Gamage and Matsushita, 1973).

Since all the research work published concerning reduced lipid content in the aqueously extracted FPC was empirically done, there is a need for research which will furnish the basic information regarding the high residual lipid. Information is needed about the nature of the residual lipids; whether they exist in bound and/or free state(s); the factors which affect the transformation from one state to another and, consequently, increase or decrease the lipid complex formation; the type of components that are tied to the lipids; the type and degree of binding between the lipid and other components in these lipid complexes. Also, the feasibility, technologic and economic, of breaking down these lipid complexes and freeing the product from such lipids without a deleterious effect on protein functionality and without the use of other techniques, such as organic solvents or enzyme hydrolysis, needs investigating. This type of information should prove or disprove the aqueous techniques as useful methods in FPC production.

As depicted in Figure 2, the general scheme for aqueous techniques starts by washing the ground fish with water and thereby eliminating the majority of the sarcoplasmic proteins. Thus it is logical to assume the involvement of the myofibrillar proteins in lipid complex formation. Actin, which is the second major component of the myofibrillar proteins (Lowey, 1972) was used in this study.

From our previous work on myosin-lipid interaction (Shenouda and Pigott, 1974), the aqueous extraction of FPC does not significantly alter the type or proportion of the residual lipids from the original fish lipids. The main changes observed were a decrease in the monoglycerides, hydrocarbons and phosphatidylethanolamine. The residual fish lipids in aqueously extracted FPC, as well as the original fish lipid from yellow perch, consist of monoglycerides, diglycerides, triglycerides, hydrocarbons, two other unidentified neutral lipids, phosphatidyl choline, phosphatidyl ethanolamine, lysophosphati-

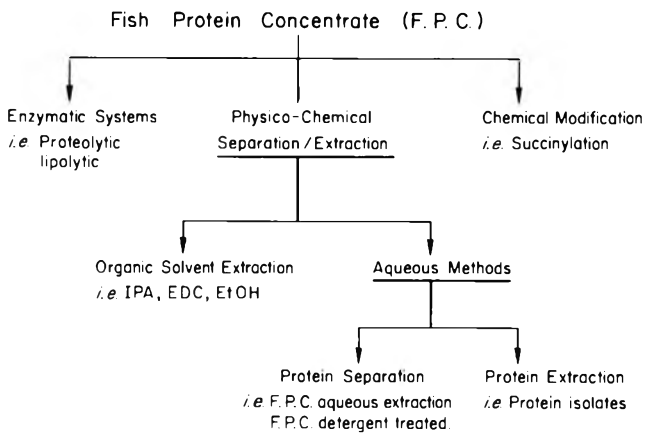

Fig. 1—Classification of fish protein concentrate (FPC) according to the method of preparation.

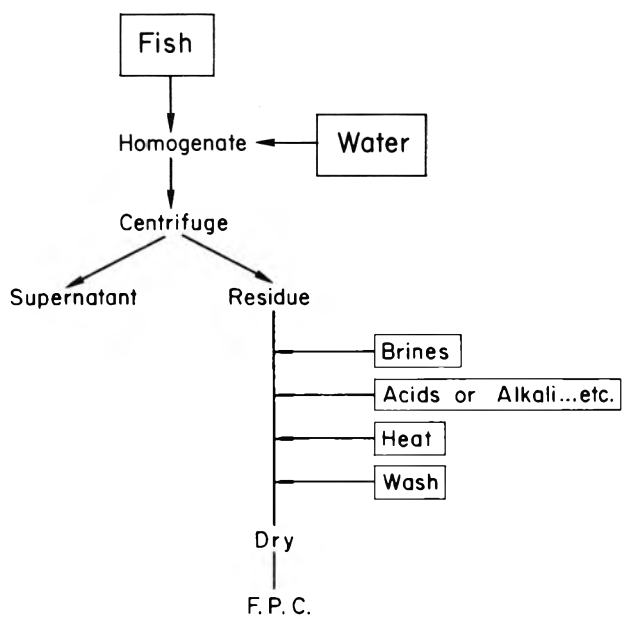

Fig. 2—General scheme for FPC production with aqueous methods.

dyl choline, lysophosphatidyl ethanolamine, sphingolipids, glycolipid and two other unidentified polar lipids.

This paper presents the results of actin-lipid interaction in aqueous medium, studied by sucrose gradient centrifugation and polyacrylamide gel electrophoresis techniques. Results of the effect of transforming G-actin into F-actin, effect of room temperature, cold, heating, pH, and ionic strength on actin-polar lipid and actin-neutral lipid complex formation are presented.

MATERIAL & METHODS

Actin

Actin was extracted and purified as previously described (Shenouda and Pigott, 1975). The lyophilized actin was dissolved in H_2O at 4°C, centrifuged at 40,000 rpm/3 hr, before use. Actin concentration was measured by absorbancy at 280 nm, using an extinction coefficient of 1.09 ml per mg-cm, as recommended by Rees and Young (1967).

Yellow perch (*Perca flavenscens*) were used throughout this work, owing to their availability in a fresh state all year. However, since it was difficult to keep yellow perch alive after catch, rockfish (*Sebastes auriculatus*) were used for preparing the 14-C fish lipids.

Labeling the fish lipids with carbon-14

The method described by Shenouda and Pigott (1974) was followed. The dose of isotopes, consisting of 50 μCi triolein, 50 μCi tripalmitin, 50 μCi glycerol and 250 μCi sodium acetate, was injected into a 233g rockfish. The fish was kept in a salt water tank at 10°C, and the lipid was extracted from the whole fish by the Bligh and Dyer method (1959) after 4 days. The lipid was further fractionated by partition between petroleum ether and 87% ethanol, as described by Galanos and Kapoulas (1962) to yield 13.92g neutral lipids (8.95×10^5 counts/min/g) and 1.96g polar lipid (9.06×10^6 counts/min/g). The purity of separation into polar and neutral lipids was checked by TLC.

Sucrose gradient

Preparing the gradient. The sucrose solution was purified to minimize the UV background absorbance by boiling the sucrose in a ratio of 10g activated charcoal to 100 ml solution for about 5 min and filtering through a charcoal pad on a Buchner funnel. A refractometer was used in preparing 5% and 20% sucrose stock solutions. 5 ml from each of the 20% and the 5% sucrose solutions were used in forming the gradient in polyallomer tubes (Beckman No. 331372 9/16″ × 3-1/2″) using the gradient forming apparatus.

Procedure

The 14-C lipids were dispersed by ultrasonication in distilled water, mixed with the actin solution, and the mixture was subjected to the desired treatment. The treated mixture was then layered over the freshly prepared sucrose gradient. Centrifugation was performed at 40,000 rpm for 48 hr in a Beckman centrifuge Model L2 with rotor No. SW 41 at 4°C. Fractionation of the gradient followed, using the density gradient fractionator Model 183 (ISCO, Neb.) at upward flow rate of 0.75 ml/min. The fractions were continuously monitored at 280 nm in a UV analyzer Model UA-2 (ISCO, Neb.) and 1 ml fractions were collected in scintillation vials for carbon-14 counting. For counting the actin pellets, NCS tissue solubilizer (Amersham-Searle, Ill.) was used for digestion. Benzoyl peroxide was added to decolorize the digest before counting.

Samples and treatments

Control. Control runs of sonicated lipids, as well as actin alone, were done to show the specific profile of each. The actin run showed three major peaks. These peaks were further analyzed electrophoretically on SDS polyacrylamide gels. Also, the spectrophotometric absorbancy of the top peak was scanned over the wavelength range 240–300 nm.

Actin + lipids at room temperature and at 4°C. 11.83 mg polar lipid or 13.86 mg neutral lipid were sonicated in 2 ml H_2O. 0.1 ml of the sonicated lipid was added to 1 ml of the actin preparation (10 mg actin/1 ml H_2O) and the mixture (mixture I) was either incubated at room temperature or at 4°C overnight before layering it over the sucrose gradient for fractionation.

Actin + lipids in the presence of $CaCl_2$ or $MgCl_2$. To the actin-lipid mixture (I) 0.1 ml of $CaCl_2$ or $MgCl_2$ (1.3×10^{-1} and 9×10^{-2} Mol respectively) was added. The final mixture was incubated overnight at 4°C before fractionation.

Effect of agitation and foam formation. A solution of actin-lipid mixture (I) was shaken vigorously on a test tube shaker for 3 min. The shaken mixture was placed on sucrose gradient for fractionation.

Actin + lipid; effect of heating. The actin-lipid mixture (I) was placed in a water bath at 75°C for 3 min. The heated mixture was layered over the sucrose gradient for fractionation. Fraction #5 from the polar lipid-actin and fraction #3 from the neutral lipid-actin were further checked by SDS-polyacrylamide gel electrophoresis for protein detection confirmation.

Actin + lipid; effect of ionic strength. 15.5 mg of polar lipid or 14.8 mg neutral lipid were sonicated in 2 ml H_2O. 1.1 ml of the actin preparation (12 mg actin/1 ml H_2O) was mixed with 0.12 ml of the sonicated lipid (mixture II). Solid KCl was added to the mixture to give the following ionic strength: 0.1, 0.3, 0.5, 1.0, 2.0 and 3.0. The mixture was left at room temperature for 3 hr. The last three treatments (ionic strength 1.0, 2.0 and 3.0) were heavier than the gradient and they were layered at the bottom of the gradient. Centrifugation and fractionation were carried out as usual.

Actin + lipid; effect of pH. To actin-lipid mixture II, HCl or NaOH was added to adjust the pH to 1.0. 3.0, 4.5, 6.0, 7.5 and 11.0. The mixture was incubated at room temperature for 3 hr before layering over the gradient and being fractionated by centrifugation.

Actin-lipid interaction on SDS electrophoresis

109 mg 14-C polar lipid or 162.5 mg 14-C neutral lipid were sonicated in 2 ml H_2O. 0.5 ml of the sonicated lipid were mixed with 1 ml actin preparation (30 mg actin/1 ml H_2O). The mixture was heated in a steam chamber at 100°C for 20 min. After cooling the mixture was treated as mentioned earlier for SDS-polyacrylamide gel electrophoresis runs. The stained bands or the corresponding parts on the gel were cut off and transferred into scintillation vials. These gel slices were solubilized as described by Kaplan and Criddle (1970) by heating them with 0.5 ml H_2O_2 (30%) until the gels were completely dissolved. After cooling, 1 ml NCS tissue solubilizing fluid was added, followed by 10 ml of the scintillation fluid and the samples were counted.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The method employed was as described before (Shenouda and Pigott, 1975).

RESULTS

Actin + fish lipid interaction

The sucrose gradient centrifugation profile for actin alone, C-14 fish lipids alone, and the mixture of fish lipid and actin previously incubated at either room temperature or at 4°C overnight are presented in Figures 3 and 4.

The actin preparation gave three peaks fractionated on the sucrose gradient (Fig. 3a and 4a). The top peak (A) was a protein-free fraction as tested by the Lowey method and by SDS-PAGE. Spectrophotometric scanning of this peak over the range 240–300 nm indicated that it consisted of nucleotides (ratio of O.D. 260/280 was 0.65/0.15). These nucleotides were derived from the ATP-Tris-Ascorbate buffer used in the preparation and purification of actin.

The SDS-PAGE analysis of the middle peak of the actin preparation, as well as the bottom peak (B and C, Fig. 3 and 4) indicated that both peaks were pure G-actin. Thus, the G-actin monomers (peak B) tend to form polymers of higher molecular weights which are easily dissociated into monomers on treatment with SDS and urea during the SDS-PAGE analysis.

Figure 3b shows that neutral lipids floated on the top of the gradient. Figures 3c and 3d indicate the formation of a neutral lipid-actin complex either with the monomer or polymer forms of actin. The polymerization of actin definitely increased the amount of binding to neutral lipids.

The amount of bound lipids in each fraction of actin on the sucrose gradient was calculated from the corresponding cpm under each peak. Table 1 presents this data as a percentage of bound lipid to total added lipids. The data show that the total bound neutral lipids amount to 33% of the added lipids (400 μg neutral lipids were added to 7 mg actin in these runs). The polymer form of actin binds 2 to 3 times more neutral lipids than the monomer form.

Raising the temperature from 4°C to room temperature enhanced the transformation of G-actin to the polymer form (the area of the monomer form, Peak B, scanned at 280 nm

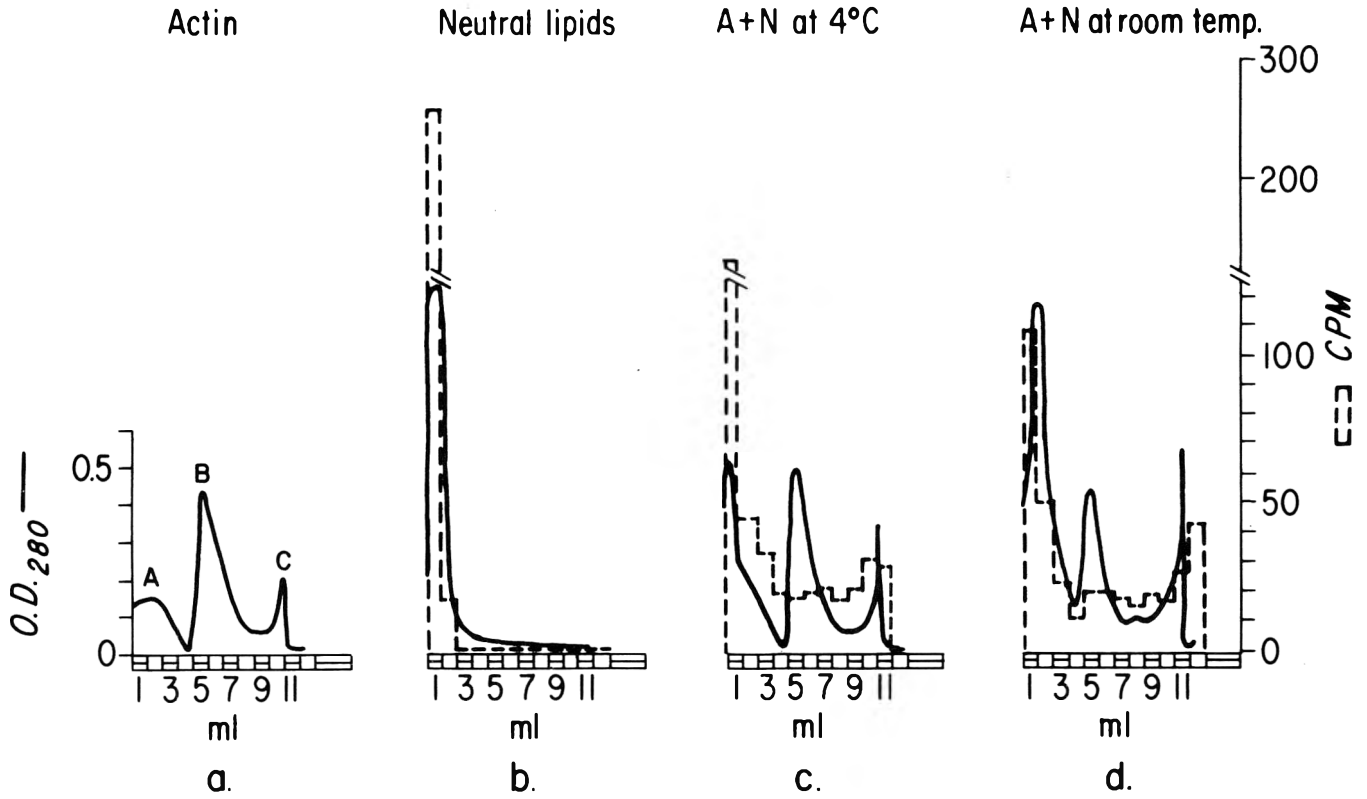

Fig. 3—Fractionation over sucrose gradient. Conditions are given under "Materials and Methods." Mobility from left to right. (a) Actin alone as monitored by absorbancy at 280 nm, showing three peaks. The top peak (A) is nucleotides derived from the buffer; the middle peak (B) is actin monomers; and the bottom peak (C) is soluble polymers of actin; (b) 14-C neutral lipids as monitored at 280 nm and the corresponding counting activity; (c) actin incubated with neutral lipids overnight at 4°C; (d) actin incubated with neutral lipids overnight at room temperature.

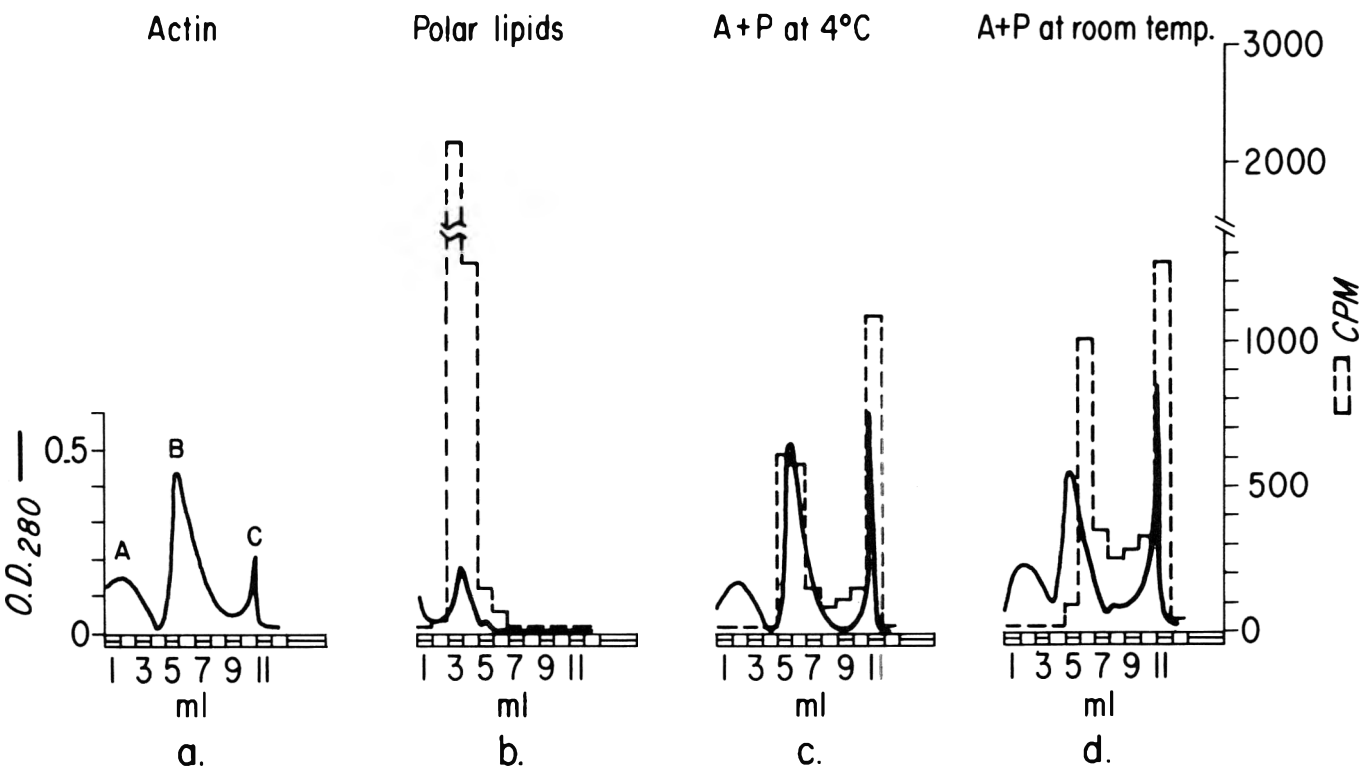

Fig. 4—Fractionation profile over sucrose gradient. The system is the same as mentioned in Fig. 3. (a) Actin alone; (b) 14-C polar lipids; (c) actin incubated with polar lipids overnight at 4°C; (d) actin incubated with polar lipids overnight at room temp.

decreased as temperature of incubation increased) and, consequently, increased the amount of bound lipids from 33% to 46% of the neutral lipids added.

Figure 4 (a–d) shows the fractionation profile in the case of polar lipids. Figure 4b shows that the polar lipids migrate deeper into the gradient after the centrifugation period. Pitlick and Nemerson (1970) recorded a similar observation with phospholipids. Various ranges of sucrose gradients and different centrifugation times were tried in an attempt to increase the resolution between the free polar lipids and the actin monomers on the gradient. The system used in this work (5 to 20% sucrose concentration, centrifugation at 40,000/48 hr) gave the most satisfactory results. In spite of the fact that there is unavoidable overlap, the binding between the monomer actin and polar lipids was revealed by raising the ionic strength (μ) to 0.3 (Fig. 11). The data show that about 10% of the added polar lipids were bound to the monomer form at 0.3 (μ) ionic strength. Moreover, the polar lipid-actin monomer interaction was confirmed by using electron spin resonance technique (under publication). Figure 4 shows the formation of a polar lipid-actin (polymer form) complex. Similarly, as in the case of neutral lipids, raising the incubation temperature of the polar lipid-actin mixture increased the bound polar lipids (from 48% to 57%) in conjunction with increasing the transformation of monomer actin to polymer form.

Effect of agitation and heating on actin

A solution of actin preparation (Actin I) [Actin I is actin preparation before the final purification on Sephadex column (Shenouda and Pigott, 1975).] was severely agitated on a test tube shaker for 3 min. The foamy preparation was centrifuged and the pellets were collected and subjected to SDS-PAGE. A similar solution of Actin I preparation was subjected to heating at 100°C for 3 min and the pellets, collected after centrifugation, were also tested on SDS-PAGE. The results in Figure 5 show that the coagulated pellets obtained by agitation are more easily dissociated into monomers on the gels than the heated pellets.

The presence of fish lipids has a noticeable increase on the heat denaturation of fish actin. Figure 6 shows that the polar lipids have a distinctive effect on the aggregation of actin during heating at higher temperatures (100°C for 3 min). Neutral lipids showed a lesser effect than polar lipids in this respect. The figure also shows that the contaminating tropomyosin is the least affected by heating.

Effect of agitation on lipid-actin interaction

The results of agitating the actin-lipid mixture on the lipoprotein formation are presented in Figure 7. Actin pellets were detected after the centrifugation period on the bottom of the gradient tubes. These pellets possess a high counting activity, as depicted in the figure. Table 1 shows that approximately one-third of the total bound lipids was detected in the pellets.

Fig. 5—Stained SDS disc gels prepared electrophoresed as described in the text. (a) Actin prep.-I, the top band is actin and the bottom band is tropomyosin; (b) actin prep.-I after heating at 100°C for 3 min; (c) actin prep.-I after vigorous agitation for 3 min.

Table 1—Effect of various treatments on the percentage of lipids bound to actin[a]

Treatment	% bound to monomer	% bound to soluble polymers	% bound to pellets	% total bound
I — Neutral lipids				
Incubated at 4°C	10	23	0	33
Incubated at room temp.	13	33	0	46
Presence of Ca^{++}	3	0	90	93
Presence of Mg^{++}	21	6	62	89
Effect of heating	16	25	8	49
Effect of agitation	12	31	20	63
II — Polar lipids				
Incubation at 4°C	–	48	0	48
Incubation at room temp.	–	57	0	57
Presence of Ca^{++}	–	8	31	39
Presence of Mg^{++}	–	12	21	33
Effect of heating	–	13	13	26
Effect of agitation	–	20	12	32

[a] 400 µg of lipids were added to 7 mg actin.

$$\text{Percent bound lipid} = \frac{\mu\text{g bound lipid}}{\mu\text{g total added lipids}} \times 100$$

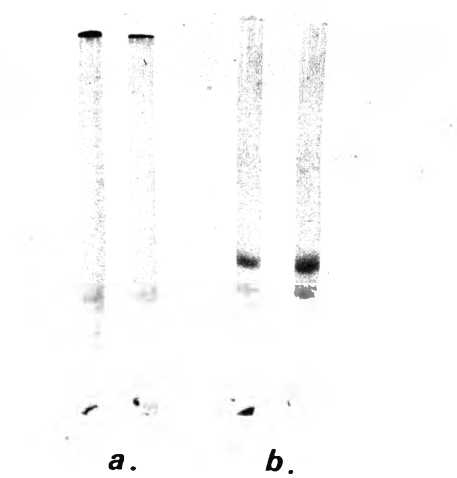

Fig. 6—The effect of heating in the presence of fish lipids on the degree of aggregation of fish actin. Conditions for preparing, running and staining the gels are described in the text. (a) Actin prep.-I heated at 100°C for 3 min in the presence of fish polar lipids; (b) actin prep.-I, same heat treatment, in the presence of fish neutral lipids.

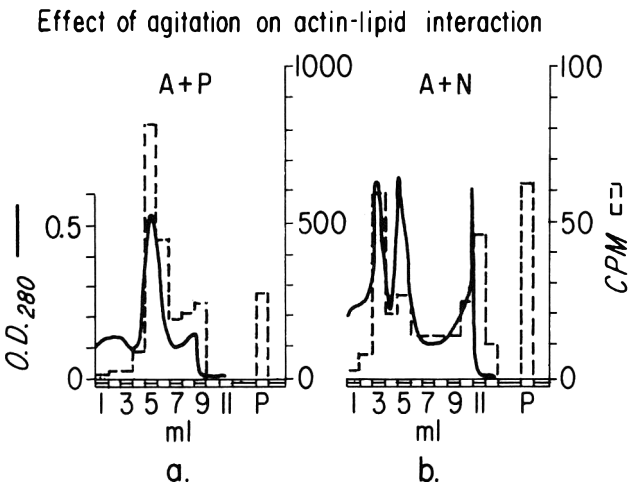

Fig. 7—Sucrose gradient fractionation profile showing the effect of agitation on actin-lipid mixtures. Conditions of fractionation as described in the text. (a) Actin agitated in the presence of polar lipids; (b) actin agitated in the presence of neutral lipids. P = collected pellets in the bottom of the gradient tube.

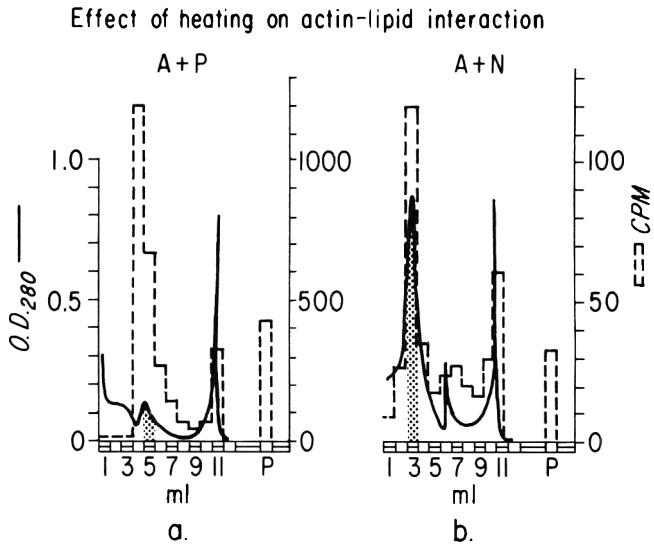

Fig. 8—Sucrose gradient fractionation profile, showing the effect of heating (70°C for 3 min) on actin-lipid mixtures. Conditions of fractionation as described in text. (a) Actin heated in the presence of polar lipids; (b) actin heated in the presence of neutral lipids. P = collected pellets in the bottom of the gradient tube.

Agitation of the actin-lipid mixture causes binding of 63% and 32% of the added neutral and polar lipids respectively. Agitation and foam formation probably cause exposure of hydrophobic regions on the actin molecules, which leads to an increase in the bound neutral lipids and a decrease in the bound polar lipids.

Effect of heating on actin-lipid complex formation

Heating the actin-lipid mixture at 75°C for 3 min produced a different pattern on the sucrose gradient (Fig. 8). The majority of the actin monomers disappeared and, instead, either soluble or insoluble polymers were formed. Fraction #5 from actin-polar (Fig. 8a) and fraction #3 from actin-neutral (Fig. 8b) were protein-free, as tested by SDS-PAGE. The actin pellets under such heat treatments were dissociated into monomers by SDS and urea. The data (Table 1) show that such heat treatment causes a small increase in the total amount of bound neutral lipids to actin (49% of the added lipids); however, a reverse effect was observed in the case of polar lipids, where about half of the bound polar lipids were released (from 50% to 25%).

Effect of calcium and magnesium cations

The effect of addition of divalent cations such as Ca^{++} or Mg^{++} on actin behavior is presented in Figure 9. The figure shows precipitation of the soluble actin polymers from the

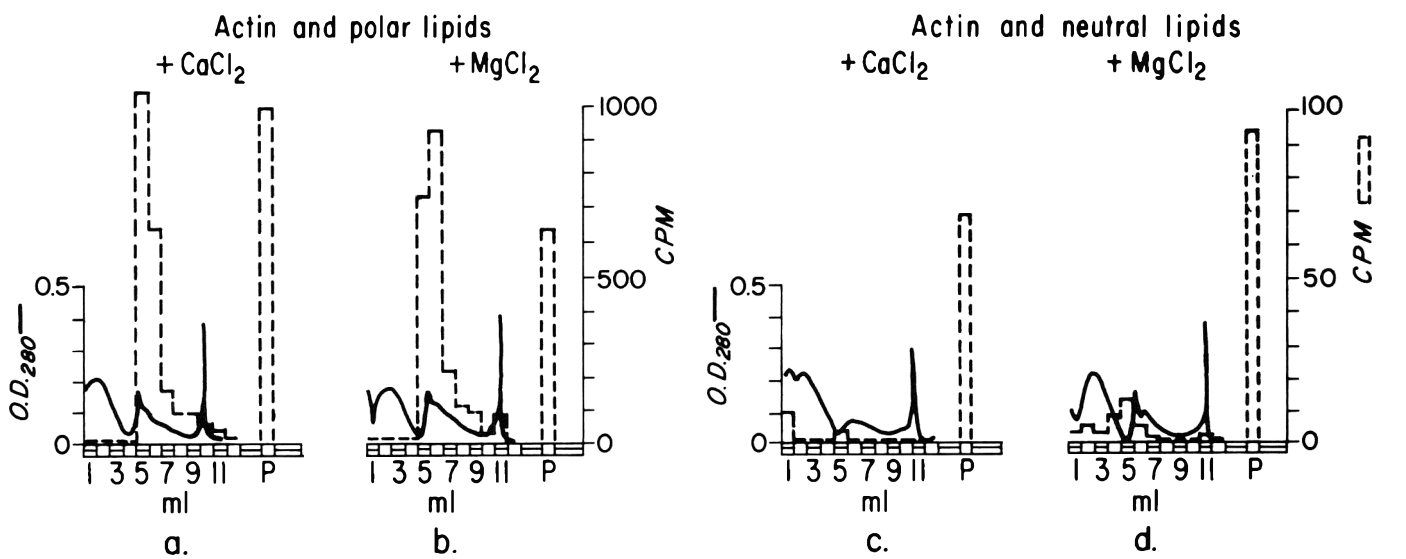

Fig. 9—Sucrose gradient fractionation profile of actin-lipid mixtures in the presence of divalent cations. Conditions of fractionation are described in the text. (a) Actin and polar lipid mixture containing 1×10^{-2} M $CaCl_2$; (b) actin-polar lipid mixture containing 7×10^{-3} M $MgCl_2$; (c) actin-neutral lipid mixture containing 1×10^{-2} M $CaCl_2$; (d) actin-neutral lipid mixture containing 7×10^{-3} M $MgCl_2$.

gradient, which indicates an increase in the molecular weight of the polymers. Briskey and Fukazawa (1971) cited that transformation of G-actin to F-actin appears to take place by the polymerization of globular molecules into linear aggregates, which may have a weight of many millions. They also reported that Mg^{++} cations in concentrations of 7×10^{-4}M and Ca^{++} cations at 1×10^{-3}M cause 50% polymerization.

The results (Fig. 9) indicate that the divalently polymerized actin shows specificity in binding to the lipids; i.e., it increases bound neutral lipids (up to 93% of the added lipids) and decreases bound polar lipids (33–39% of the added polar lipids). The results also show that calcium polymers bind more polar lipids than the magnesium polymers.

Effect of ionic strength (μ)

Briskey and Fukazawa (1971) reported that the maximum polymerization of actin was at 0.1–0.15μ KCl, and that further increases in salt concentration cause a decrease in the extent of polymerization and eventually abolished it. However, Figures 10 and 11 show that in the presence of lipids there is a gradual increase in the formation of higher molecular weight actin polymers with increasing ionic strength. The actin monomers disappeared completely from the gradient at ionic strengths higher than 1.0. It is also noticed that the actin behavior was the same in the presence of either polar or neutral lipids. Figure 10 shows that at ionic strength 0.3 and 0.5, a nonsoluble lipoprotein complex of actin and neutral lipids was detected near the top of the gradient tube (fractions 2 and 3).

Figure 12 shows a linear increase in the bound lipid, polar or neutral, with increasing ionic strength. Increasing the ionic strength over 1.0 gave a steeper increase in the bound neutral lipid and depressed, to some extent, the increase of bound polar lipids. This indicates the existence of electrostatic forces between the polar lipids and actin in the lipoprotein complexes.

Effect of pH

The actin patterns on the sucrose gradient as a function of pH are presented in Figures 13 and 14. Similar behavior of actin was observed in the presence of either polar or neutral lipids. At pH lower than 6.0, no monomers were detected and all the actin accumulated in the pellets. Increasing the pH increased the dissociation into the monomer form. At higher pH (pH 11.0), the monomers were less dense and floated near the top of the gradient tube.

Figure 15 presents the interaction pattern of lipid-actin as a function of pH. The neutral lipids exhibit a biphasic pattern, where the lowest binding of the lipids was at pH 3.0. Shifting the pH on either side increased the binding; however, there was another decline at neutral pH or higher.

All the polar lipids were tightly bound to actin at pH 4.5 or lower. Raising the pH towards the alkaline side was accompanied by a gradual decrease in the bound polar lipids.

The results of the SDS-PAGE experiments are presented in Figures 16 and 17. The actin used in these runs (actin Prep. I) was taken before the Sephadex purification step and, there-

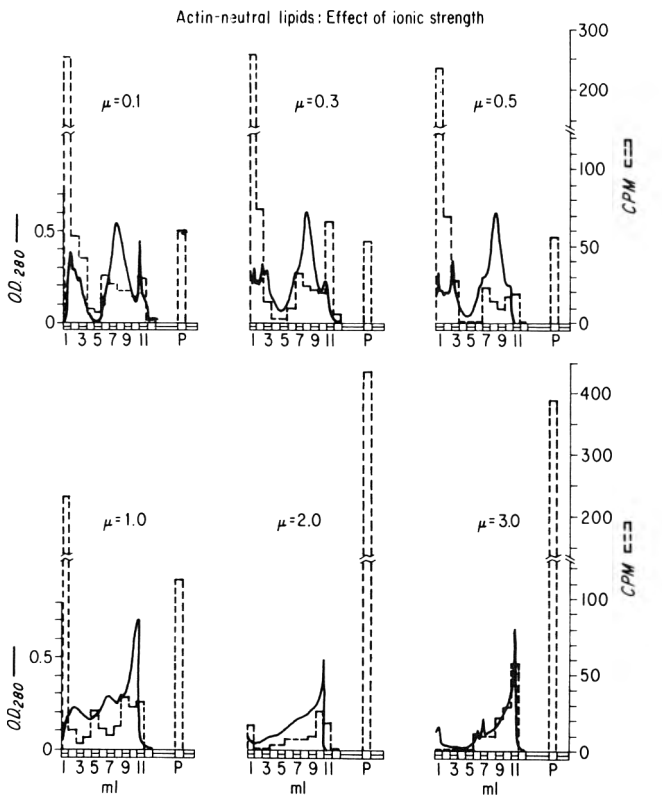

Fig. 10—Effect of ionic strength on the fractionation profile of actin-neutral lipid mixtures. Conditions as described before. The ionic strength of the mixtures was adjusted by KCl to the designated μ as shown in the figure. The mixtures were incubated at room temp for 3 hr before fractionation on the gradient.

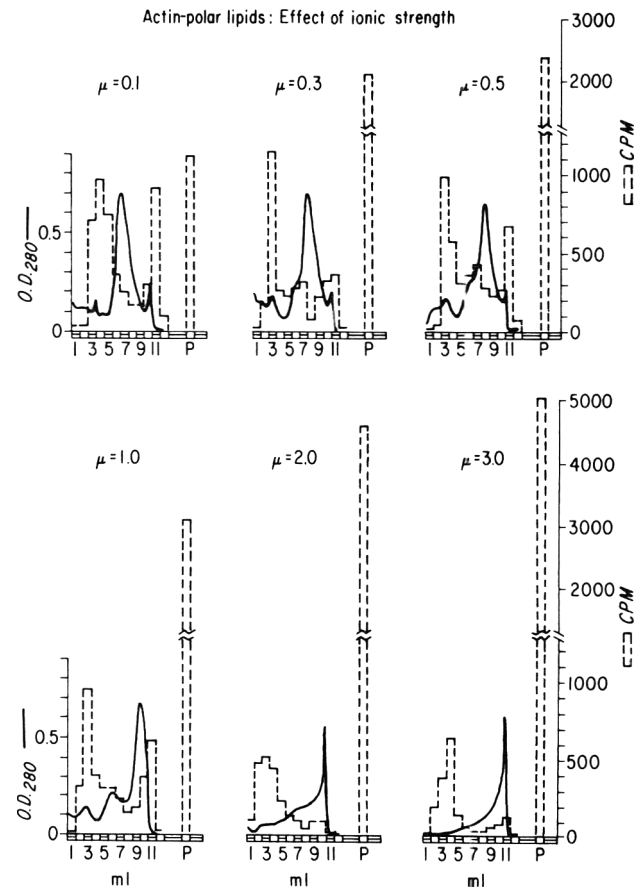

Fig. 11—Effect of ionic strength on the fractionation profile of actin-polar lipid mixtures. Conditions as described before. The ionic strength of the mixtures was adjusted by KCl to the designated μ as shown in the figure. The mixtures were incubated at room temp for 3 hr before fractionation on the gradient.

fore, the preparation contains about 12% tropomyosin as impurity. The control run with polar lipids alone or neutral lipids alone shows that all the polar lipids and the majority of the neutral lipids migrate through the gel and are located as a large band ahead of the actin band. The rest of the neutral lipids remained at the top of the gels.

Incubating actin and polar lipids at room temperature resulted in monomers and aggregates which bound to the lipids. The binding was strong enough to resist the effect of SDS and urea, and the actin-lipid complex migrated as a unit under the influence of the electrical current.

Heating the actin-polar lipid mixture at 100°C for 15 min resulted in the formation of a high molecular weight aggregate which did not dissociate with the detergent and stayed at the top of the gels. Heating also caused dissociation of part of the lipoprotein complex, as indicated by a decrease in the counts accompanying the actin bands.

Neutral lipids showed less effect on actin aggregation than polar lipids, and the lipoprotein complexes formed also showed resistance to dissociation when treated with SDS and urea. Similarly, heating released part of the bound lipids, as was the case with polar lipids.

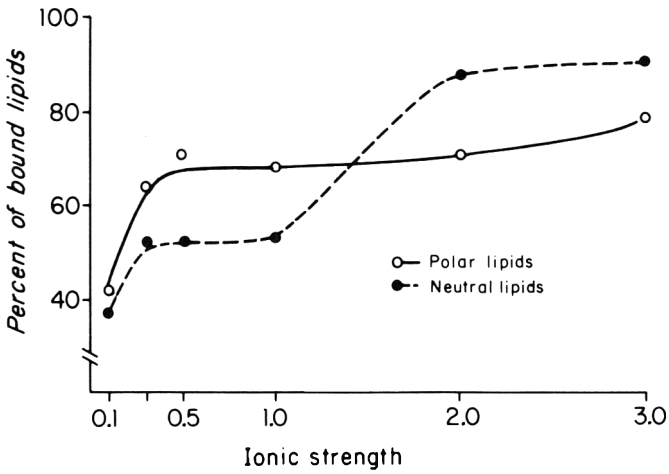

Fig. 12–Percent of bound lipids as a function of increasing ionic strength. 600 μg 14-C polar lipids or 14-C neutral lipids were incubated with 12 mg actin at different ionic strengths. Incubation was at room temp for 3 hr. The mixtures were fractionated by density gradient centrifugation (details in text), and the bound lipids were calculated. The percent of bound lipids = (mg bound lipids/mg added lipids) × 100.

DISCUSSION

THE INTERACTION between lipids and proteins is dependent on different types of forces, namely electrostatic forces be-

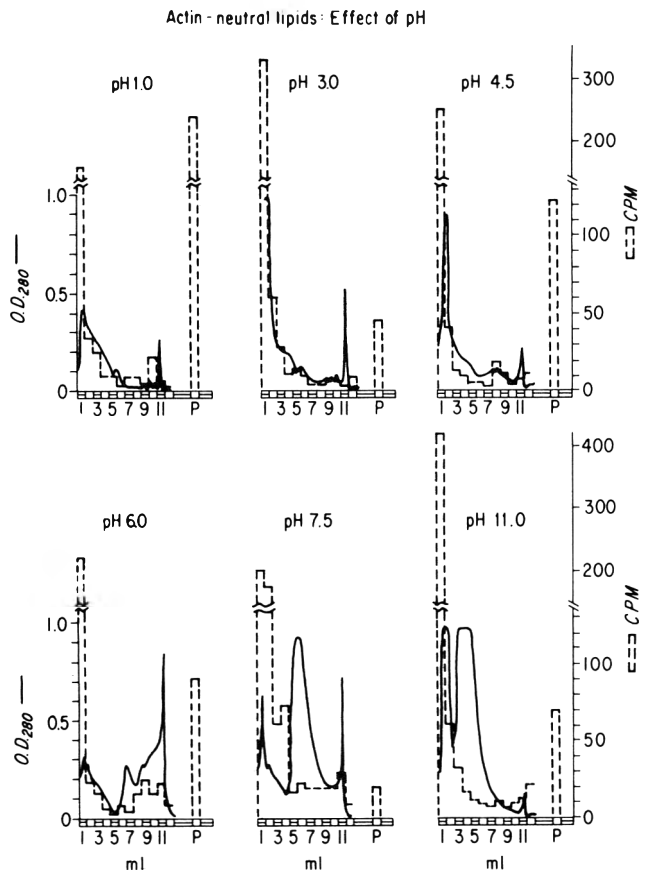

Fig. 13–Effect of pH on the fractionation profile of actin-neutral lipid mixtures. Condition of fractionation as described in text. The pH's of the mixtures were adjusted to the designated pH, as shown in the figure. The mixtures were incubated at room temp for 3 hr before fractionation on the gradient.

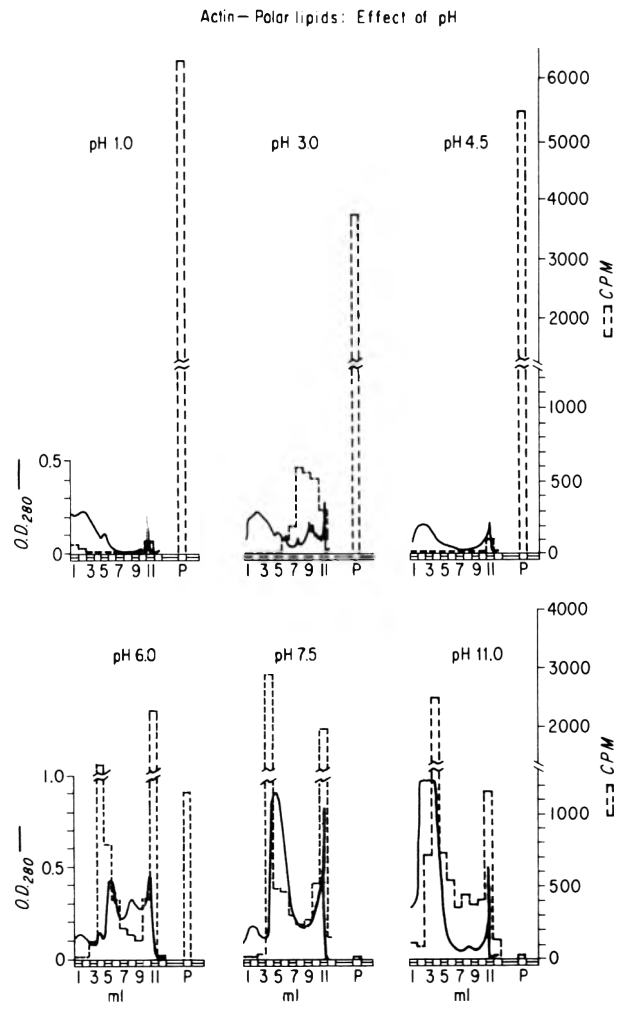

Fig. 14–Effect of pH on the fractionation profile of actin-polar lipid mixtures. Conditions of fractionation as described in text. The pH's of the mixtures were adjusted to the designated pH as shown in the figure. The mixtures were incubated at room temp for 3 hr before fractionation on the gradient.

tween charged groups, Van der Waals forces between the hydrocarbon chains, hydrogen bonding and hydrophobic forces. Covalent bonding between lipids and proteins has been reported to a lesser extent. The specificity of interaction may be mutually dependent on the overall size, shape and charge of both the protein and the complex lipid micella. However, each type of interaction is predicted to exist when the proteins and lipids are known to possess certain groups or residues. For example, it can be expected that the phosphate groups on the phospholipids play a role in the binding through electrostatic forces with charged lysine or arginine residues of the protein.

Besides the sophisticated methods used in detecting and measuring these different types of forces, simple techniques were used also for testing the participation of such forces. For

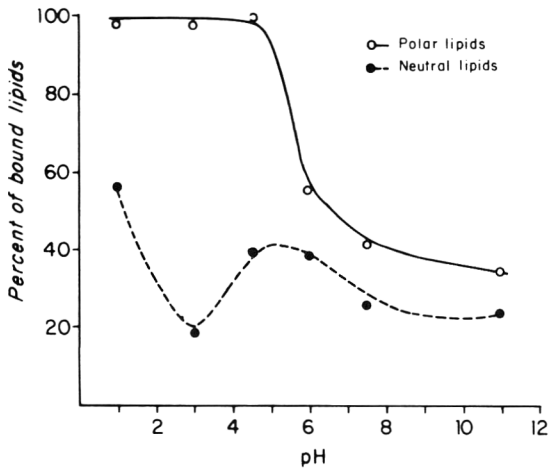

Fig. 15—Percent of bound lipids as a function of pH. 600 μgm 14-C polar lipids or 14-C neutral lipids were incubated with 12 mg actin at different pH's. Incubation was at room temp for 3 hr. The mixtures were fractionated by density gradient centrifugation (details in text) and the bound lipids were calculated. The percent of bound lipids = (mg bound lipids/mg added lipids) × 100.

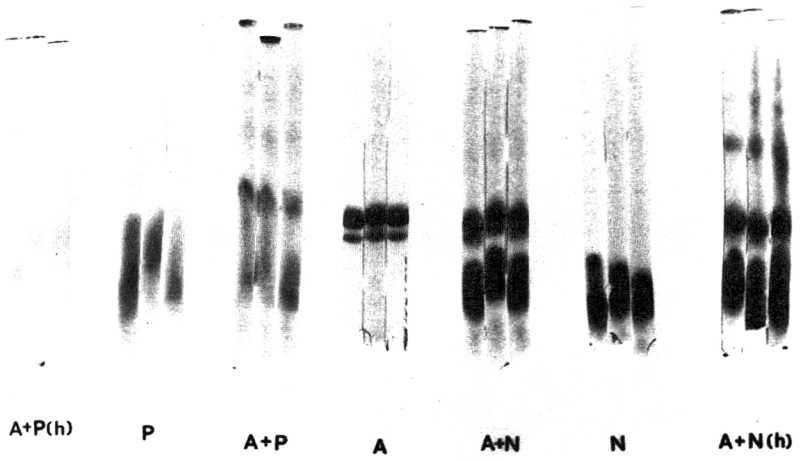

Fig. 16—Typical electrophorograms of fish actin (prep.-I) subjected to different treatments. Actin 1 mg/gel, 14-C neutral lipids 1.3 mg/gel and 14-C polar lipids 0.9 mg/gel. A = actin prep.-I as a control; P = polar lipids alone; N = neutral lipids alone; A + P = actin + 14-C polar lipids incubated at room temp for several hours; A + P(h) = A + P heated at 100°C for 15 min; A + N = actin + 14-C neutral lipids incubated at room temp for several hours; and A + N(h) = A + N heated at 100°C for 15 min.

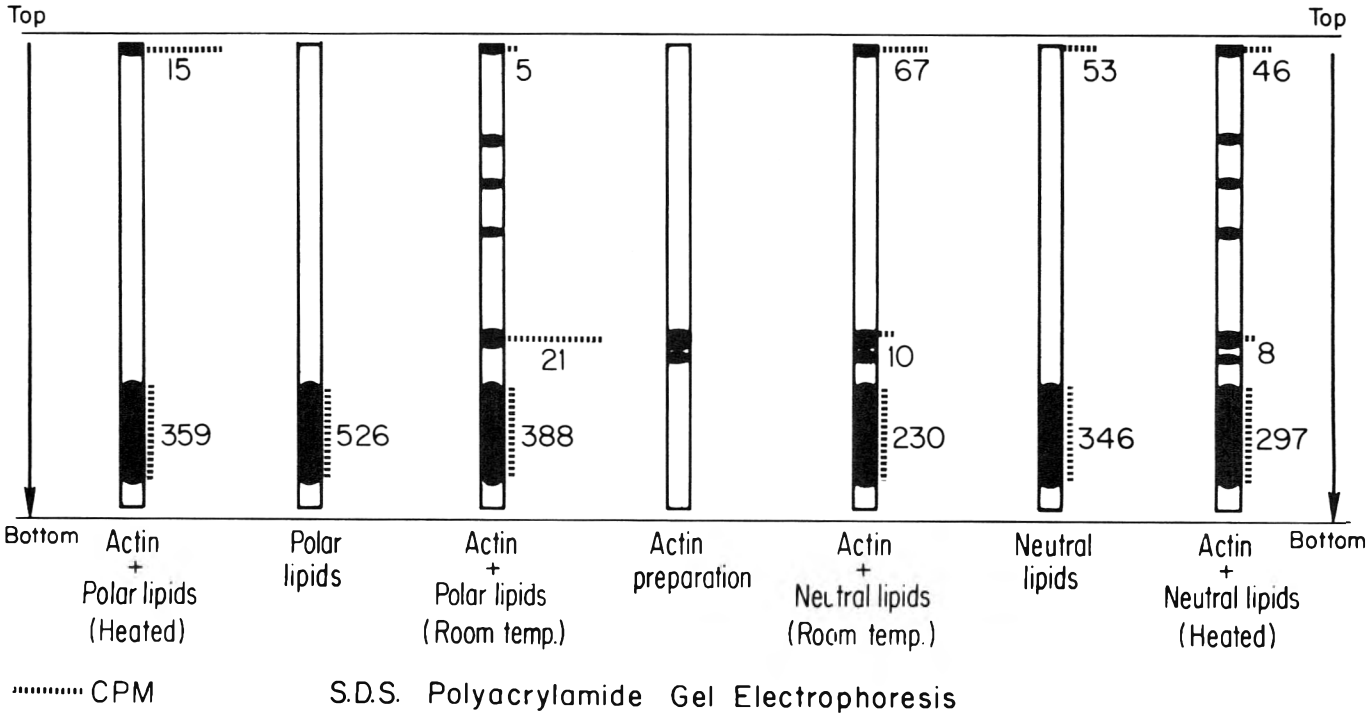

Fig. 17—Schematic presentation of the main bands seen on sodium dodecyl sulfate gel electrophoresis, as shown in Fig. 16, with the corresponding counts/min of carbon-14 activity. Method described in text.

example, the competitive effect of increasing the concentration of ions on the electrostatic interaction was used in detecting such binding. Also, measuring the change in the free SH or ϵ-NH_2 groups was used as indication of the covalent bonding and so on.

From the amino acid analysis of fish actin (Shenouda and Pigott, 1975) one may expect the participation of a variety of forces in actin-lipid lipoprotein complexes. Marinetti and Pettit (1968) reported that Lys., His., Tyr., Met. and Cys. are amino acids which play a role in the binding between cardiolipin and γ-globulin. The neutral lipid-actin complex formation on the sucrose gradient experiments suggests the presence of hydrophobic forces as well as H-bonding. The existence of lipid protein complexes after the urea and SDS treatment (Fig. 16 and 17) indicates that hydrogen bonding is not primarily responsible for the binding of lipids to actin in the lipoprotein. Evans et al. (1968a) stated that hydrophobic bonding appeared to be involved either in the binding of part of the lipid to the protein or in holding the protein molecules in shapes so that the lipids are enclosed. Chiu and Pomeranz (1966) noticed that much more polar wheat flour lipids than nonpolar components were bound during dough mixing. Actin binds more polar lipids than neutral lipids (Table 1). Participation of ionic interaction between the charged groups on the polar lipids and the charged residues on the actin is expected. Moreover, due to the fact that fish polar lipid is more highly unsaturated than the neutral lipids (Roubal, 1967), a stronger hydrophobic interaction may take place. Ionic interaction was reported to be the sole binding force in some lipid-protein systems (Palmer and Dawson, 1969). Camejo et al. (1968), using the monolayer technique, reported that the charged lipids vary in their degree of interaction with proteins, and Pitlick and Nemerson (1970) attributed these differences to the different micellar properties of each phospholipid.

The increase in bound lipids (polar or neutral) to the polymerized form of actin suggests that polymerization of actin is associated with conformational changes which cause a considerable increase in the interacting sites on the actin molecules. This conformational change is accelerated by raising the incubation temperature (Fig. 3 and 4). The results also show that polymerization of actin in the absence of divalent cations gives a different binding pattern than that given by lipids in the presence of such cations (Fig. 9 and Table 1). These differences in binding should be attributed to differences in the polymerization mechanism of the actin itself. Apparently polymerization of actin in the presence of Ca^{++} or Mg^{++} causes exposure of more hydrophobic regions on the actin molecules, which stimulates the binding to neutral lipids and depresses the ionic interaction forces between the lipids and actin. Hendrickson and Fullington (1965) attributed the stability of some lipid-metal-protein complexes to the fact that three of the six metal coordination bonds of metals such as Ca^{++}, Mg^{++}, or Ni^{++}, are free to accept additional donor groups and consequently stable mixed complexes could be formed. Their data showed that the equilibrium stability constant values are the same for calcium and magnesium. Braun and Radin (1969) suggested that the divalent cations stabilized the lipid-protein complexes by inter- or intra-chain crosslinking through protein carboxyl or lipid phosphate groups. They also reported that insoluble lipid-protein complexes were resistant to resolubilization when calcium was present. Fullington (1969) reported that interaction of phospholipid and wheat proteins can take the form of mixed chelation on divalent cations. Joos and Carr (1969) indicated that the binding of calcium to phospholipid-protein complexes is pH dependent. The results (Table 1) showed that the presence of Ca^{++} or Mg^{++} does not increase the binding of polar lipids and, on the contrary, a significant decrease was recorded. This indicates that the major role of the divalent cations in the lipid-actin interaction is not formation of metal bridges between lipids and proteins, but is the transformation of the monomer actin into the fibrillar high molecular weight actin polymers.

Heating and foam formation of actin have a similar effect on lipid binding (Fig. 7 and 8; Table 1). The increase of bound neutral lipids and the decrease in polar lipid binding could be due to exposure of more hydrophobic regions, presumably from the interior of the molecule. Therefore, foam formation could cause a random unfolding of actin molecules as the general effect of heating on proteins.

Proteins vary considerably in their stability to heating, a phenomenon which is related to their structure. For example, small-size proteins in which disulfide bridges stabilize their structure, are more resistant to heat than large polysubunit proteins in which their cysteine residues are in sulfhydryl form. Camejo et al. (1968) reported that the absence of disulfide bridges appears to be a feature of proteins associated with lipids, either in soluble lipoproteins or in cell membranes. It was reported recently that the presence of lipids stabilizes the structure of some proteins. Taguchi and Ikeda (1968) reported that lecithin plays an important role in activating the fish actomyosin ATP-ase. Lux et al. (1972) cited that delipidation of human plasma decreases the helical content about 20%, with a corresponding increase in disordered structure. Heating the actin in the presence and absence of lipids (Fig. 5 and 6) showed the positive role of lipids, especially the polar lipids, on the degree of actin aggregation and provides the effect of the lipids in disrupting the actin structure during heating.

The effect of pH on lipid-actin interaction is the result of the effect of pH on the charges on the actin and lipids and the effect of pH on the shape of the actin molecules. Colacicco (1969), using the monolayer technique, found that lipid protein interaction is affected by the degree of surface denaturation and protein conformation which is dependent, in most proteins, on pH. He also reported that some proteins expand at low pH and therefore facilitate lipid penetration. This is probably the case with actin at low pH, where the majority of the lipids were complexed at this pH range (Fig. 15). Raising the pH value over the isoelectric precipitation of actin should create a negative charge on the actin, which should cause repulsion with the phosphate moiety of the phospholipids and a decline in the polar lipid-actin complex formation, as was observed. On the other hand, the effect of pH on the neutral lipid-actin binding seems to be a composite of different effects. The neutral lipids showed two distinct subgroups on SDS-PAGE (Fig. 16 and 17). The major component exhibits some degree of polarity and migrates toward the anode, while the other subgroups did not move under the influence of the electrical current. The last group is expected to show minimum interaction with actin near its isoelectric point, where the actin will be covered with the maximum charge load, and the binding increases with increasing or decreasing pH. The other subgroup probably will show more attraction to the positive parts of the actin and a relative decrease in the binding will be observed at higher pH's. The sum of the behavior of these two subgroups could probably produce the biphasic curve observed with neutral lipids (Fig. 15).

The sudden shift of the actin peak at pH 11.0 in the sucrose gradient to a region of lower density (Fig. 13 and 14) indicates structural rearrangement of the actin molecules to less dense, less reactive monomers, with the binding sites for lipids either hidden or destroyed.

The characteristics of interaction between lipids and actin with increasing ionic strength demonstrates the direct effect of salt concentration on the actin. The logical deduction made from these experiments is that the actin molecules unfold greatly and vast numbers of residues are exposed and become available to bind with lipids, ionically and hydrophobically. Lenaz et al. (1970) found an opposite observation in mitochondrial membranes, where the binding for phospholipids decreased randomly at higher salt concentrations. Braun and

Radin (1969) reported that increasing the monovalent cations reduced the hydrophilicity of the protein-lipid complex. Pomeranz et al. (1968) demonstrated that sodium chloride affected the gluten and reduced the nonpolar-gluten interaction.

The relative inhibition (Fig. 12), by high ionic strengths of the media, on the interaction between polar lipids and actin clearly indicates the existence of electrostatic forces in these lipoproteins.

Evans et al. (1968b) in their studies on the effect of various detergents on lipoprotein dissociation stated that anionic detergents such as SDS increased the extraction of lipids from lipovitellin, primarily by breaking down the hydrophobic bonds which presumably hold the protein molecules in shape. Helenius and Simons (1972) stated that lipid removal by detergents from lipoprotein complexes could be an exchange of bound lipid for bound detergents. The treatment of lipid-actin complexes with urea and SDS for SDS-PAGE (Fig. 16 and 17) revealed that the lipids still remain firmly bound under these conditions, and this suggests that either covalent bonding or an unusually strong electrostatic bonding and/or hydrophobic bonding exists between lipids and actin.

SUMMARY & CONCLUSIONS

FISH ACTIN interacts with polar or neutral fish lipids at room temperature or cold temperature. Actin interacts either in monomer form (G-actin) or polymer forms (F-actin). F-actin interacts more strongly (2–3 times) than G-actin. Any treatment which induces the transformation of G-actin into F-actin, such as Mg^{++}, Ca^{++}, or raising the temperature, increases the lipid-actin complex formation. Hence, the use of sea water may not be recommended in producing FPC. Agitation and/or heating probably cause exposure of hydrophobic regions in the actin molecules, which consequently increases the neutral lipid binding and depresses, to some extent, the polar lipid binding. pH affects primarily the state of charges on actin molecules, as well as the conformations of the proteins, which in turn affects the degree and type of binding with lipids. Actin-lipid interaction is not inhibited completely by increasing the ionic strength (μ), which indicates the participation of the hydrophobic interaction. However, μ has a noticeable effect on actin-polar lipid interaction, which indicates the involvement of the electrostatic interaction and, finally, the SDS and urea treatments during the sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) suggest either the existence of covalent bonding or an unusually strong electrostatic and/or hydrophobic bonding between fish lipids and actin in the actin-lipid complexes.

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REPRESSION OF Vibrio parahaemolyticus BY Pseudomonas SPECIES ISOLATED FROM PROCESSED OYSTERS

INTRODUCTION

MICROORGANISMS are in constant interaction with one another in numerous environments including the food we eat. The quality and shelf life of a refrigerated food product can be greatly affected by the numbers and types of microorganisms present. Cultured foods have received a great deal of attention in this regard; many studies having been conducted on the repression of microorganisms due to the production of acids, peroxides, antibiotics and other substances by lactic acid bacteria (Hurst, 1972).

Little information is available on the interactions of microorganisms found in processed oysters. Oysters commercially distributed as freshly shucked shellfish must be refrigerated until consumed. Among the microbial species which predominate in Pacific and eastern oysters are *Pseudomonas, Flavobacterium, Achromobacter, Alcaligenes* and *Vibrio* (Colwell and Liston, 1960; Lovelace et al., 1968; Murchelano and Brown, 1968). In a recent report by Vanderzant et al. (1973), a range of 0–79.3% (average, 14.9%) was given for *Pseudomonas* of the total microbial flora of 31 processed oyster samples obtained from the Texas Gulf Coast area. Price and Lee (1970) isolated from Pacific oysters species of *Lactobacillus* which were capable of inhibiting *Pseudomonas, Bacillus* and *Proteus* through the production of hydrogen peroxide. Vanderzant and Custer (1968) in a study of interactive inhibitory activities among certain psychrotrophic bacteria isolated from refrigerated foods noted that certain *Pseudomonas* species demonstrated inhibitory activities against species of *Achromobacter* and other *Pseudomonas* species. Inhibition against *Achromobacter* was variable according to plating medium, temperature of incubation and ratio of effector to test species. In another report (Scannell et al., 1971), an unidentified *Pseudomonas* sp. was shown to produce an antimetabolite, thioguanine, during a fermentation process which was effective in the inhibition of a strain of *E. coli*. The purpose of the present investigation was to describe the ability of *Pseudomonas* sp. isolated from processed Maryland oysters to inhibit *V. parahaemolyticus*. In addition, the influence of selected environmental factors on this inhibitory activity was studied.

MATERIALS & METHODS

Cultures

Bacterial isolates were obtained from processed Maryland oysters (*Crassostrea virginica*) which has been stored at 5°C for 10 or 13 days from date of processing by direct plating of dilutions on Standard Methods Agar (SMA) (BBL, BioQuest), containing 1% NaCl. Isolates were maintained on slants of SMA 1% NaCl and stored at 5°C. For testing purposes, cultures were streaked on SMA 1% NaCl and incubated 24 hr at 25°C. Single colonies were then used to inoculate tubes of trypticase soy broth (TSB) (BBL, BioQuest) containing 1% NaCl and incubated at 25°C for 24 hr.

Five *V. parahaemolyticus* strains, obtained through the courtesy of Dr. M. Fishbein, Div. of Microbiology, Food & Drug Administration, Washington, D.C. were tested: strains 8700, 33C10, IDI (Kanagawa positive); strains 3525, 33C9 (Kanagawa negative). All stock cultures were maintained at 25°C on trypticase soy agar (TSA) (BBL, BioQuest) slants containing 2.5% NaCl and a long-term-preservation-medium (B.A.M., May 5, 1972). For testing purposes, cultures were streaked on thiosulfate-citrate-bile salts-sucrose (TCBS) (BBL, BioQuest) agar and incubated 24 hr at 32°C. Single colonies were used to inoculate tubes of TSB 1% NaCl and incubated at 32°C for 14–16 hr. Cell numbers of *V. parahaemolyticus* cultures in TSB 1% NaCl were standardized by adjusting the optical density to 0.100 (620 nm, Spectronic 20, Bausch & Lomb). Dilution blanks used contained 0.5% peptone, 1.0% NaCl and were tempered to room temperature before use.

Screening for inhibitory activity

A spot-plate method was used in which 0.1 ml of appropriate dilutions of *V. parahaemolyticus* were spread on SMA 1% NaCl plates (previously dried for 24 hr at 32°C) yielding approximately 10^5 and 10^4 cells per plate. After drying, six oyster isolates were spotted on duplicate plates of each dilution using an Accu-drop apparatus (The Sylvania Co., Milburn, N.J.). Following incubation at 25°C for 24–48 hr, inhibitory action was graded in the following manner: absent (–), weak or slight (+), moderate (++) and strong (+++). Isolates showing inhibitory activity were identified according to the criteria of Vanderzant and Nickelson (1969) and Vanderzant and Patel (1967).

Effect of various factors on inhibition

The spot-plate method was used and degree of inhibition expressed in mm by measuring the zone of clearing between the edge of the drop and growth of test species on the plate. Effect of incubation at 25°C versus 35°C was studied using SMA 0.5% NaCl plates (pH 7.5). Effect of media pH and NaCl content was studied at 25°C. Plating media consisted of several NaCl concentrations (0.5, 1.0, 1.5, 2.0, 2.5% w/v) at each pH value (6.2, 7.5, 8.8). Media were brought to desired pH with 1N HCl or 5N NaOH before autoclaving. Since inhibition was detected on SMA 0.5% NaCl plates but not on TSA plates the following combinations of ingredients were tested as plating media: SMA 0.5% NaCl (control), SMA 0.5% NaCl and 0.5% phytone, SMA 0.5% NaCl and 1.0% trypticase, TSA (control), TSA and 0.25% yeast extract (BBL, BioQuest), TSA 0.1% glucose.

Inhibitory activity of culture filtrate

Pseudomonas X3-7 was inoculated into 1 liter of broth consisting of 0.1% glucose, 0.25% yeast extract, 0.5% NaCl, and distilled water followed by incubation at 25°C for 5–7 days until strong pigment formation and fluorescence was evident. Fluorescence was viewed using a portable ultraviolet lamp (Mineralight UVS-11, Ultra-Violet Products, Inc., San Gabriel, Calif.). Cells were removed by centrifugation at 5,860g for 30 min and the supernatant filter-sterilized through a 0.20 μm pore size filter (Metricel, T.M.G. Gelmen). To determine whether the inhibitory substance was heat stable, the filtrate was autoclaved for 15 min at 121°C. In addition, a dialysate of the filtrate was prepared against water at 5°C for 1–3 days using cellophane dialysis tubing (Union Carbide Corp., Food Products Div., Chicago, Ill.). All samples were tested for the presence of inhibitory activity using a tube-assay method in which *V. parahaemolyticus* strain 8700 cultures were serially diluted in a constant amount of sample. Each assay tube (10 × 75 mm) contained 0.1 ml of sterile 10% NaCl and 0.1 ml of autoclaved 5X TSB plus 0.8 ml of sample or water (control). To the first tube of each sample series, 0.1 ml of an appropriate dilution of *V. parahaemolyticus* strain 8700 was added to yield approximately 10^5 cells/tube. This tube was serially diluted in subsequent sample tubes to a concentration of 10^2 cells/tube. Total volume of each tube was 1.0 ml. Uninoculated controls of each sample were included. Observations for turbidity were made after incubation at 25°C for 24 and 48 hr. Confirmation of positive tubes was accomplished by streaking on sections of TCBS plates followed by incubation at 32°C for 24 hr.

RESULTS

Isolation and identification of inhibiting colonies

45 cultures were isolated from processed Maryland oysters which had been stored for 10 and 13 days at 5°C. Nine oyster

Table 1—Detection of inhibition of V. parahaemolyticus by Pseudomonas isolates using a spot-plate method on Standard Methods Agar containing 1% NaCl

V. parahaemolyticus strain	X1-10	X1-13	X1-16A	X1-26	X3-2	X3-3	X3-4	X3-7	X3-8
Kanagawa negative									
3525	+[a]	+	+	+++	++	+	+	++	+
33C9	++	++	+	+++	++	+	+	+++	+
Kanagawa positive									
8700	++	+++	+	+++	++	+	+	++	+
33C10	–	–	+	++	+	–	–	+	–
IDI	–	–	+	++	+	–	–	+	–

[a] +++ = strong inhibition, ++ = moderate inhibition, + = slight inhibition, – = no inhibition.

isolates demonstrating inhibitory activity were identified as belonging to the genus *Pseudomonas*, Group I (Vanderzant and Nickelson, 1969; Vanderzant and Patel, 1967). These species demonstrated various degrees of inhibitory activity depending upon strain of *V. parahaemolyticus* tested (Table 1). *Pseudomonas* spp. X1-26 and X3-7 demonstrated the strongest inhibitory activity against most of the *V. parahaemolyticus* test strains. Consequently, these *Pseudomonas* spp. were chosen for further study on the effects of media pH, salt and temperature of incubation on the repression of *V. parahaemolyticus*. All *Pseudomonas* spp. tested showed some degree of repression of the Kanagawa negative strains 3525 and 33C9 and the Kanagawa positive strain 8700. In general, a lesser degree of inhibition of the Kanagawa positive strains 33C10 and IDI occurred, with some *Pseudomonas* spp. showing no or only weak inhibition of these strains (Table 1).

Table 2—Effect of temperature of incubation on inhibition of V. parahaemolyticus by Pseudomonas isolates on Standard Methods Agar (pH 7.5) containing 0.5% NaCl

V. parahaemolyticus strain	Inhibition Pseudomonas isolate X1-26 25°C	X1-26 35°C	X3-7 25°C	X3-7 35°C
8700	2.0[a]	1.6	2.1[b]	0
3525	4.1[b]	2.3	3.6[b]	0.9

[a] Average of two samples of two replicates expressed in mm
[b] Indicates significantly ($P \leq 0.05$) greater inhibition as compared to means at 35°C within each Pseudomonas species by variance analysis

Effect of temperature of incubation

In general, the degree of inhibition observed was greater at 25°C as compared to 35°C (Table 2). *Pseudomonas* sp. X3-7 demonstrated no inhibition of *V. parahaemolyticus* strain 8700 and only weak inhibition of strain 3525 at 35°C. Further studies of the influence of other factors on inhibition were therefore carried out at 25°C.

Effect of media pH and NaCl content

Optimal inhibition of *V. parahaemolyticus* strain 3525 by both *Pseudomonas* spp. appeared to occur at pH 7.5 and 0.5% NaCl (Table 3). Some inhibition by *Pseudomonas* sp. X1-26 was observed at the highest concentration of NaCl tested (2.5%); however, little or no inhibition by *Pseudomonas* sp. X3-7 was evident at either 2.0 or 2.5% NaCl. Although insufficient growth of *V. parahaemolyticus* strain 8700 was obtained on plating medium pH 6.2 and 0.5% NaCl, it was evident that inhibition by both *Pseudomonas* spp. was maximal at pH 8.8 and 0.5% NaCl (Table 4). *Pseudomonas* sp. X3-7 appeared to be less effective in general than *Pseudomonas* sp. X1-26 in the repression of strain 8700 at NaCl concentrations above 0.5% and had no inhibitory effect at 2.5% NaCl. Analysis of variance revealed a significant ($P \leq 0.01$) interaction between media pH and NaCl concentration indicating that changes in the level of one factor modified the effect of the other factor on the degree of inhibition produced. Concentration of *V. parahaemolyticus* strain 3525 cells had a significant ($P \leq 0.05$) effect on the amount of inhibition observed. A greater inhibitory effect was observed at lower populations irrespective of type of plating media.

Effect of peptone media ingredients

During preliminary trials in which TSA was used as a plating medium, little or no inhibition was observed (data not

Table 3—Effect of pH and NaCl content of Standard Methods Agar on inhibition of V. parahaemolyticus strain 3525 by Pseudomonas isolates X1-26 and X3-7

pH	CFU/plate[a]	Pseudomonas isolate X1-26 % NaCl 0.5	1.0	1.5	2.0	2.5	Pseudomonas isolate X3-7 % NaCl 0.5	1.0	1.5	2.0	2.5
6.2	6.2×10^5	4.8[b]	2.7	1.8	0.5	0.5[c]	4.4	1.0	0	0	0
	6.2×10^4	5.3	3.7	2.7	1.5	0.5	4.7	1.0	0.5	0	0
7.5	6.2×10^5	7.5	5.5	4.0	3.5	1.2	6.9	3.4	2.3	0.5	0.5
	6.2×10^4	8.3	6.4	4.9	4.6	3.7	8.3	4.7	2.8	0.5	0.5
8.8	6.2×10^5	6.6	4.2	3.0	2.4	1.0	1.8	1.7	2.2	0	0
	6.2×10^4	7.0	5.0	5.8	2.8	1.3	5.5	3.1	2.3	0	0

[a] Colony forming units of V. parahaemolyticus per plate
[b] Average of two samples of two replicates expressed in mm
[c] Measurements less than 1 mm but greater than zero were listed as 0.5 mm for purposes of statistical evaluation

Table 4—Effect of pH and NaCl content of Standard Methods Agar on inhibition of V. parahaemolyticus strain 8700 by Pseudomonas isolates X1-26 and X3-7

		Pseudomonas isolate X1-26 % NaCl					Pseudomonas isolate X3-7 % NaCl				
pH	CFU/plate[a]	0.5	1.0	1.5	2.0	2.5	0.5	1.0	1.5	2.0	2.5
6.2	3.1×10^5	–[b]	2.0[c]	1.5	0.5	0.5[d]	–	1.4	0.5	0	0
	3.1×10^4	–	2.9	2.3	0	0.5	–	1.3	0.5	0	0
7.5	3.1×10^5	4.0	2.5	2.0	1.3	0.5	3.9	1.0	0.5	0	0
	3.1×10^4	4.2	2.5	2.7	2.2	1.3	4.5	1.8	0.5	0	0
8.8	3.1×10^5	5.1	3.3	2.9	1.8	1.0	5.9	1.8	0.5	0	0
	3.1×10^4	5.8	4.5	3.8	2.8	1.0	7.3	3.4	1.0	0.5	0

[a] Colony-forming units of V. parahaemolyticus per plate
[b] No growth of V. parahaemolyticus Strain 8700 on these plates
[c] Average of two samples of two replicates, expressed in mm
[d] Measurements less than 1 mm but greater than zero were listed as 0.5 mm for purposes of statistical evaluation.

shown). Various plating media consisting of combinations of SMA and TSA ingredients were therefore tested. Little or no inhibition was evident on TSA (control) plates whereas strong inhibition was obtained on SMA 0.5% NaCl (control) plates (Table 5). Addition of either glucose (0.1%) or yeast extract (0.25%) to TSA did not increase inhibition. Addition of peptone to SMA 0.5% NaCl plates, however, resulted in a significant ($P \leq 0.05$) "quenching" of inhibition. Phytone appeared to be more effective than trypticase per unit weight. Where inhibition was strongest, production of a water soluble pigment by both *Pseudomonas* spp. was observed.

Inhibition of *V. parahaemolyticus* by *Pseudomonas* sp. X3-7 culture filtrates

Since the possibility existed that competition for an essential nutrient in a "low-peptone" medium could account for the repression of *V. parahaemolyticus*, a culture filtrate (pH 6.9) of *Pseudomonas* sp. X3-7 was tested to determine whether an "inhibitory factor" was being elaborated into the growth medium. In addition a dialysate of the filtrate and an autoclaved sample of the filtrate were tested. Following incubation at 25°C for 48 hr, growth of *V. parahaemolyticus* was observed at all dilutions in the control tubes (Table 6). In the test sample tubes, growth was observed at only the highest concentration of *V. parahaemolyticus*. Approximately 10^4 cells/ml were inhibited by the filtrate, autoclaved filtrate and dialysate. It thus appeared that an inhibitory substance which is dialyzable and stable to autoclaving at pH 6.9 is elaborated by *Pseudomonas* sp. X3-7 into the growth medium.

DISCUSSION

CERTAIN *Pseudomonas* spp. showed inhibition of some but not all of the *V. parahaemolyticus* strains tested. The degree of inhibition varied with effector species and test strain. The variation in growth rates of the *V. parahaemolyticus* strains under these environmental conditions could be an important influence on the degree of inhibition noted. It is also possible that more than one type of inhibitory substance is involved or that the *Pseudomonas* spp. differ in the rate of production of one or more inhibitory substances.

Environmental conditions such as pH, NaCl content and temperature of incubation were shown to influence the inhibition of *V. parahaemolyticus* strains 3525 and 8700 by *Pseudomonas* spp. X3-7 and X1-26. Optimal pH for inhibition by both *Pseudomonas* spp. was different for strain 8700 than for strain 3525. The effect of pH may be related to the effect of different inhibitory principles or to the rate of growth of *V. parahaemolyticus* strains as compared to rate of production of the inhibitory substance under these conditions. Growth of strain 8700 at pH 6.2 and 0.5% NaCl was scant; however, at pH 6.2 and 1.0% NaCl growth was adequate. Beuchat (1973) reported that minimum pH values for growth of strain 8700

Table 5—Effect of plating medium on inhibition of V. parahaemolyticus by Pseudomonas isolates

	Inhibition by Pseudomonas isolate			
	X3-7		X1-26	
	V. parahaemolyticus		V. parahaemolyticus	
Plating medium	Strain 8700	Strain 3525	Strain 8700	Strain 3525
Trypticase soy agar:				
control	0.5[a]	0	1.0	0.5
0.25% yeast extract	0.5	0	1.0	1.0
0.1% glucose	0.5	0	1.3	0.3
Standard methods agar 0.5% NaCl:				
control	3.3	7.4	4.2	6.7
0.5% phytone	0.8[b]	0.5[b]	1.2[b]	1.0[b]
1% trypticase	1.7[b]	2.6[b]	3.0[b]	2.6[b]

[a] Average of two samples of two replicates expressed in mm
[b] Indicates significant ($P \leq 0.05$) quenching of inhibition as compared to the standard methods agar 0.5% NaCl control by variance analysis and least significance difference test

Table 6—Inhibition of V. parahaemolyticus strain 8700 by culture filtrates of Pseudomonas isolate X3-7 replenished with trypticase soy broth nutrients

	V. parahaemolyticus CFU/ml				
Sample	2.3×10^5	2.3×10^4	2.3×10^3	2.3×10^2	uninoculated
Control	++[a]	++	++	++	–
0.20 μm filtrate	++	–	–	–	–
Dialysate of 0.20 μm filtrate	+	–	–	–	–
Autoclaved 0.20 μm filtrate	++	–	–	–	–

[a] ++ = heavy growth, + = light to moderate growth, – = no growth.

and other strains of *V. parahaemolyticus* were influenced by media salt content as well as temperature of incubation. Little or no inhibition of *V. parahaemolyticus* by either of the two *Pseudomonas* spp. occurred at NaCl concentrations of 2.0 or 2.5%. In a study of Staphylococci in competition, Peterson et al. (1964) noted that inhibition by saphrophytes at higher salt concentrations (above 3.5%) was lessened. Apparently the increase in salt concentration affected the saphrophytes by materially lengthening their lag phase.

The type of plating medium, in particular the concentration and type of peptone ingredients, influenced the inhibition of both *V. parahaemolyticus* strains 8700 and 3525 by *Pseudomonas* spp. X1-26 and X3-7. Pigment production was likewise affected and was maximal where the strongest inhibition was observed. *Pseudomonas* sp. X3-7 consistently produced a yellow-green fluorescent pigment while *Pseudomonas* sp. X1-26 produced lesser amounts of yellow-green pigment and varying amounts of a greenish-blue-gray pigment. The observation that both inhibitory activity and pigment production are variable suggests that under these different environmental conditions *Pseudomonas* spp. are being influenced to make use of alternative enzymes and biochemical pathways. In effect, this could mean that different types and amounts of inhibitory substances are being elaborated. King et al. (1954) studied the effect of five peptone ingredients on pigment production by weak pigment producing strains of *Pseudomonas*. The results showed that the kind of peptone ingredient used influenced not only the amount but also the type of pigment produced. Others have noted that the nitrogen source (organic and inorganic) in a growth medium will also influence pigment production (Burton et al., 1947; King et al., 1948). Martineau and Forget (1958) found that of the peptones (Difco) investigated, only "proteosepeptone" or "neopeptone" allowed pigment formation on Sabouraud maltose agar. In the same study the carbohydrate source was also observed to have an effect and almost any sugar except glucose, galactose and arabinose when added to a medium containing neopeptone permitted the occurrence of strong pigmentation. The presence or absence as well as the relative proportions of inorganic ions have been shown to exert a definite influence on type and amount of pigment formation (King et al., 1948; Burton et al., 1948; King et al., 1954). King et al. (1948) reported that the relative proportions of $MgCl_2$ and K_2SO_4, and the level of PO_4 and Na ions influenced type and amount of pigment formation. Sodium salts were found to be slightly inhibitory. Peptone sources commercially available differ considerably in composition in the aforementioned constituents (Difco Manual, 1953; BBl Manual, 1973). Since these constituents have been shown to affect pigment production it is perhaps not surprising to find that the production of inhibitory substances by *Pseudomonas* spp. is likewise affected.

Results indicate that an inhibitory substance is elaborated into the growth medium by *Pseudomonas* sp. X3-7 which is low molecular weight, not protein in nature (dialyzable) and stable to autoclaving at pH 6.9. In addition, it is apparent that environmental factors exert a significant influence on the degree of repression of *V. parahaemolyticus* by the *Pseudomonas* isolates. Further studies are desirable to determine whether production of inhibitory substances by *Pseudomonas* spp. is of consequence in oysters with respect to the inhibition of *V. parahaemolyticus*, particularly under proper conditions of storage (5°C). Salt content and the pH of oysters would appear to be important influences. During processing, oysters are subjected to a washing process ("blowing") which may reduce salinity of the surface area of the oysters (Vanderzant et al., 1973). Sensitivity of *V. parahaemolyticus* to effector species may be increased under adverse conditions, such as low salt content and low temperatures. Survival of *V. parahaemolyticus* in fish homogenate has been shown to be affected by salt content (Covert and Woodburn, 1972). Temperature also influences the viability of *V. parahaemolyticus* (Goatcher et al., 1974; Johnson and Liston, 1973). The production of inhibitory substances may be directly affected by temperature since *Pseudomonas* spp. are known to utilize different enzyme systems at different temperatures (Farrell and Rose, 1965). Lower levels of *V. parahaemolyticus* were shown to be more readily inhibited. The ratio of effector species to *V. parahaemolyticus* would therefore be a significant factor in the resultant repression of *V. parahaemolyticus* in processed oysters in the event of mishandling by exposure to temperatures above refrigeration.

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FOOD USE OF SOYBEAN 7S AND 11S PROTEINS
Heat Denaturation of Soybean Proteins at High Temperature

INTRODUCTION

HEAT TREATMENT at high temperature is one of the novel processing techniques of soybean as illustrated in Aburage (fried tofu) manufacture from ancient times and also during processing of textured protein products. In spite of these extensive applications and their increased acceptance in food industries, little has dealt with protein denaturation in these temperature ranges, probably because protein becomes extremely insoluble and difficult to submit to existing analytical methods. For instance, the study by Cummings et al. (1973) was limited to the fate of water-soluble soybean protein during thermoplastic extrusion. In a previous paper, high temperature expansion characteristics of soybean 7S and 11S proteins were studied and discussed (Saio et al., 1974). On the basis of the previous data, an investigation was undertaken in order to clarify the qualitative changes in soybean 7S and 11S proteins during heat treatment at 100–170°C.

In the present paper, qualitative changes in proteins of heat-induced gels solubilized with various solvents, are reported.

MATERIALS & METHODS

Sample

Partially purified soybean 11S and 7S globulins. From defatted soybean meal, cold insoluble fraction (CIF) was prepared by the method of Briggs and Wolf (1957) and crude 7S by the method of Koshiyama (1965). Each protein was spray dried at 150–160°C inlet and 80–90°C outlet temperature. The crude 7S obtained was again dissolved with water, repeatedly precipitated by acid at pH 4.8 to remove 2S components, and lyophilized after neutralization.

Experimental methods

The flow sheet of the experimental methods is shown in Figure 1. Each procedure is detailed below.

Measurement of solubility. 125 mg of protein sample were suspended into 25% paste with 0.375 ml of phosphate buffer (μ = 0.1, pH 7.6), phosphate buffer (μ = 0.5, pH 7.6) or HCl-NH_4OH buffer (μ = 0.1, pH 8.5). The paste was placed in a conical flask and autoclaved at 100–170°C under appropriate pressure for 5 min. After cooling to room temperature, 10 ml of the solution containing different concentrations of sodium dodecyl sulfate (SDS), 2-mercaptoethanol (ME) and urea were added to the heat-induced gel and the gel was ground with a spatula to make it easy to dissolve. The mixture was kept in a shaking bath (6 cm shaking width and 60 rpm) at 40°C for 15 hr and centrifuged at 3,000 rpm (1,207 × G) for 15 min. Absorbance of the protein in the supernatant from the centrifugation was measured at 280 nm or by the method of Lowry et al. (1951). Solubility was expressed as percentage of dissolved protein to total protein in the original sample.

Ultracentrifugal analysis. Heat-induced gel, which was prepared from protein paste suspended with phosphate buffer (μ = 0.1, pH 7.6), was solubilized with 10 ml solution containing 0.075M SDS and 0.025 ME following the procedure described above. The solubilized solution was submitted to ultracentrifugal analysis (Hitachi UCA-1), immediately after dialysis at 25°C for 46 hr against water containing a drop of toluene. The centrifugation was carried out at 51,200 rpm (190,500 × G) at 20°C.

Disc polyacrylamide gel electrophoresis (DPE) and SDS-disc polyacrylamide gel electrophoresis (SDS-DPE). Heat induced gel, which was prepared from 500 mg of protein paste suspended with phosphate buffer (μ = 0.1, pH 7.6) was completely solubilized with 6 ml of solution containing 0.075M SDS and 0.025M ME, was submitted to DPE analysis after dialysis for 24 hr against water. Disc polyacrylamide gel electrophoresis was carried out according to the method of Davis (1964), and 0.05 ml of 0.5% protein solution was applied to each gel after dilution with the same volume of 0.8% sample gel. Electrophoresis was performed in 7% polyacrylamide gel and carried out at 2.5 mA per gel.

The method of Shapiro et al. (1967) was used for SDS-DPE. To the mixture of 0.5% protein sample, 2% SDS (in 50% glycerin) and ME (1:1:0.02 in column), 1/5 volume of 0.05% Bromo Phenol Blue was added as a tracker dye. On each gel, 0.05 ml of the solution was applied after incubation at 25°C overnight. Electrophoresis was performed in 10% polyacrylamide gel and carried out at 8 mA per gel. Gel after electrophoresis was stained with Amido Black 10B and decolored with 7% acetic acid solution with stirring.

RESULTS & DISCUSSION

FIGURE 2 shows the solubility changes of heat-induced gels from CIF and crude 7S in phosphate buffer containing 0.05M SDS and 0.025M ME. The solubility of CIF-gels was higher than that of crude 7S-gels at all temperature ranges. In both gels the increase of ionic strength with NaCl increased the solubilities and the solubility at pH 8.5 was higher than that at pH 7.6, although the difference between both gels is fairly distinct.

The results described above were in good agreement with the previous results (Saio et al., 1974) using calcium gels pre-

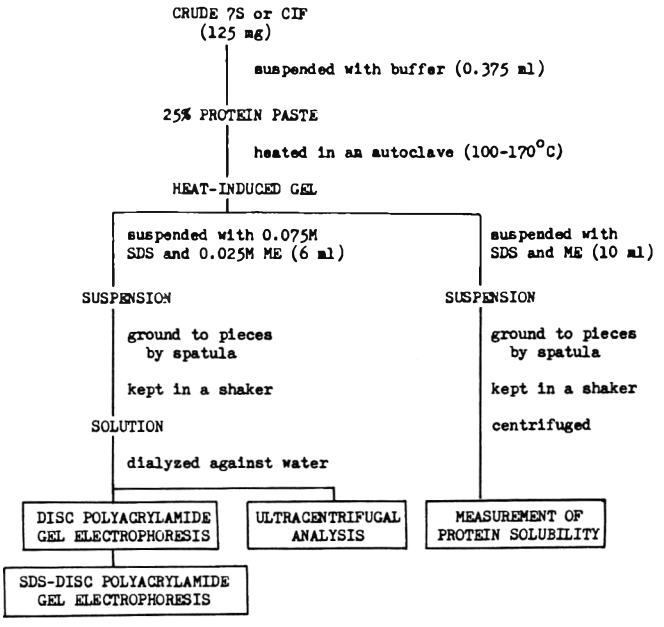

Fig. 1—Flow sheet of experimental methods.

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pared from CIF and crude 7S, which showed that gels from CIF heated at 132°C were more soluble in SDS containing ME than those from crude 7S.

It was also noted that crude 7S-gels showed the lowest solubility at 105–130°C and that the solubility increased at temperatures higher than 140°C, while the solubility of CIF-gels increased markedly at temperatures above 110°C, as shown in Figure 2.

Figure 3 shows the solubility changes of CIF-gels and crude 7S-gels in phosphate buffer containing urea and ME. The difference between both gels was not marked, when compared with the difference of solubility changes in SDS and ME, but with an increase of ME containing urea, solubility of CIF-gels increased more distinctly, while in crude 7S-gels the temperature showing the lowest solubility transferred to a higher range. On the basis of results with SDS or urea, it may be summarized that the solubility of both gels decreased distinctly after heating at 110°C; however, the solubility of CIF-gel markedly increased at temperatures above 110°C, while that of crude 7S-gel did not increase even at 110–130°C, but at temperatures higher than 150°C both gels showed higher solubility.

The qualitative changes in heat-induced gels dissolved with SDS and ME, were examined by ultracentrifugal analysis. Figure 4 shows the ultracentrifugal pattern of heat-induced gels of both proteins, after solubilizing with SDS and ME. When the protein sample dissolved with SDS and ME was submitted directly to ultracentrifugal analysis, every pattern showed only one peak which sedimented slowly. The ultracentrifugation was undertaken immediately after dialysis for 46 hr. After dialysis and storage in a refrigerator for several hours, samples heated at 110–130°C showed a fast sedimented component, because of the formation of aggregates. As shown in Figure 4, the aggregated peak which sedimented rapidly, was shown at 130°C in crude 7S and at 100°C in CIF and in both proteins at above 150°C only one peak migrated slowly was shown. The difference of ultracentrifugal patterns between both samples seems to be similar to that of their solubilities. The results from measurements of solubility and ultracentrifugal analysis may suggest that in heat-induced gel of 7S protein, cross-linkages are formed at 105–130°C by the binding forces such as hydrophobic bond, hydrogen bond and electrostatic bond, while in CIF protein-gel the binding forces are weaker than those of 7S protein-gel.

Qualitative changes in gels solubilized with SDS and ME were analyzed by disc polyacrylamide gel electrophoresis (DPE) and a SDS-disc polyacrylamide gel electrophoresis (SDS-DPE). Some of them are somewhat difficult to interpret for their diffusion pattern caused by a large number of minor components. Figures 5 and 6 show electrophoretical patterns of DPE and SDS-DPE of CIF-gels, respectively. The uppermost photo shows the pattern of unheated CIF and the lower photo shows the pattern of unheated CIF which was solubilized with the same concentration of SDS and ME as samples heated at 100–170°C.

In Figure 5 the patterns were almost similar from "unheated" to 110°C, each band gradually became obscure at 120–130°C and at temperatures higher than 140°C, the two

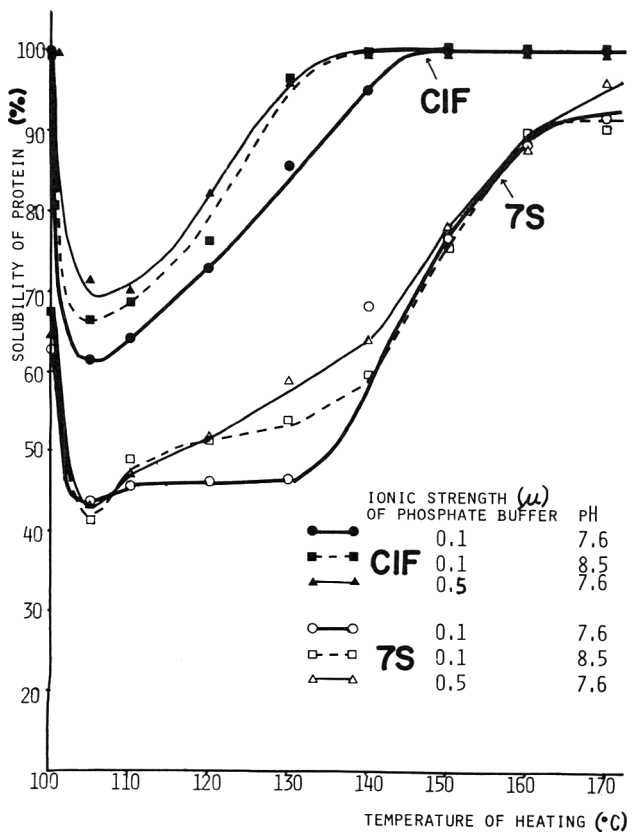

Fig. 2—Solubility of gels induced at different temperatures in buffer containing SDS (0.05M) and ME (0.025M).

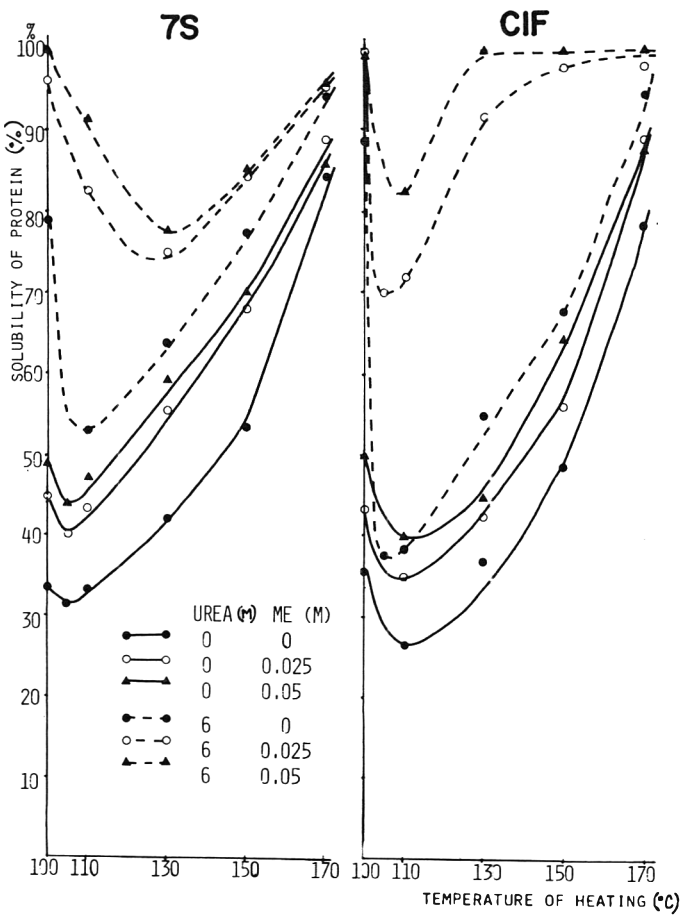

Fig. 3—Solubility of gels induced at different temperatures in buffer containing urea (0 and 6M) and ME (0.025M and 0.05M).

original main bands could not be recognized clearly and an increase in the prominence of the front was observed. The patterns of DPE up to 110°C were in good agreement with previous results of protein solutions heated at 100°C (Saio et al., 1971).

SDS-DPE is an excellent analytical method to measure molecular weight. Catsimpoolas et al. (1971) reported that 11S globulin is composed of subunits having molecular weights of about 22,300 and 37,200, the larger value corresponding to acidic subunits and the smaller to basic one. Yanagi-Ochiai et al. (1973) discussed the exact molecular weights of both subunits and a minor subunit by use of SDS-DPE and other analytical methods. The pattern of 11S globulin reported by Cummings et al. (1973) showed a variety of bands but the multiple appearance of the pattern seems to be attributed to insufficient dissociation of protein to subunits.

In Figure 6 unheated CIF showed two principal bands and a minor one, which were derived from 11S globulin and a few minor bands, derived from 7S globulin. Similar clear patterns as unheated were obtained up to 130°C, but they became gradually obscure at 130–140°C and finally no bands were observed on gel at heating above 150°C. Figures 7 and 8 show electrophoretic patterns of DPE and SDS-DPE of crude 7S-gel, respectively. In Figure 7 the main band on polyacrylamide gel was almost unchanged up to 120°C, became obscure at 130–150°C and broader at above 150°C with remarkable increase of a band on the front line as in the case of CIF.

In Figure 8 unheated crude 7S showed one main band and two minor bands derived from 7S globulin and two minor bands, from 11S globulin. The bands derived from 7S globulin migrated slower than those from 11S globulin. The patterns were not so changed up to a temperature of 140°C but changed above 150°C with disappearance of bands. The effects of heating temperature on changes of electrophoretical patterns were roughly similar in both crude 7S and CIF proteins. And it may suggest that subunits of both proteins in heat-induced gels were unchanged up to 120–130°C and degraded to lower molecular substances at 150°C. Partially dissolved protein gel, which was used for the measurement of solubility, was also submitted to DPE and SDS-DPE. The gel was dissolved with SDS and ME and centrifuged. The supernatant was applied on acrylamide gels. The pattern was identical to those of total protein sample with an exception, namely, that the

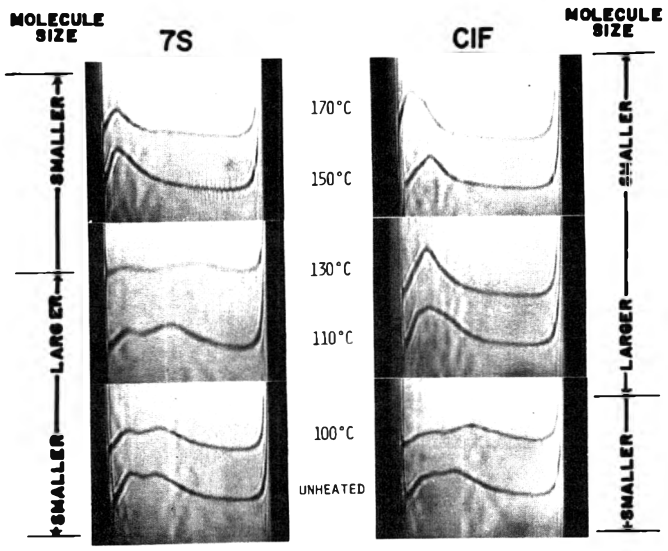

Fig. 4—Ultracentrifugal pattern of heat-induced gels solubilized with SDS and ME. (Photographs were taken at 36 min after reaching maximum speed, at 20°C. Protein concentration is 1.25% and bar angle was 60°.)

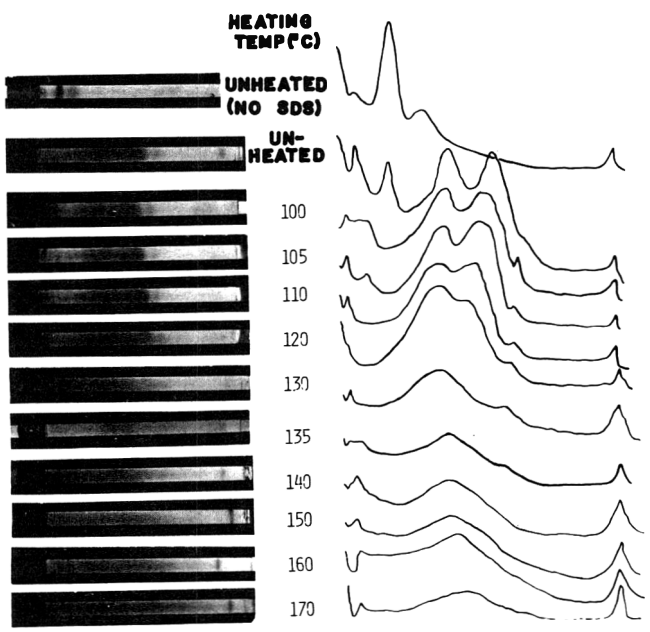

Fig. 5—Disc polyacrylamide gel electrophoresis of CIF-gel solubilized with SDS and ME.

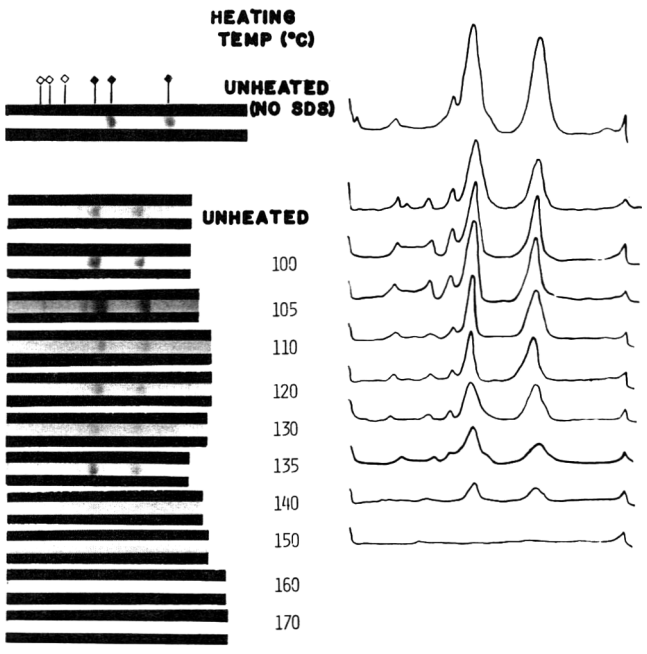

Fig. 6—SDS-disc polyacrylamide gel electrophoresis of CIF-gel solubilized with SDS and ME. (♦ Bands derived from 11S globulin; ★ Bands derived from 7S globulin.)

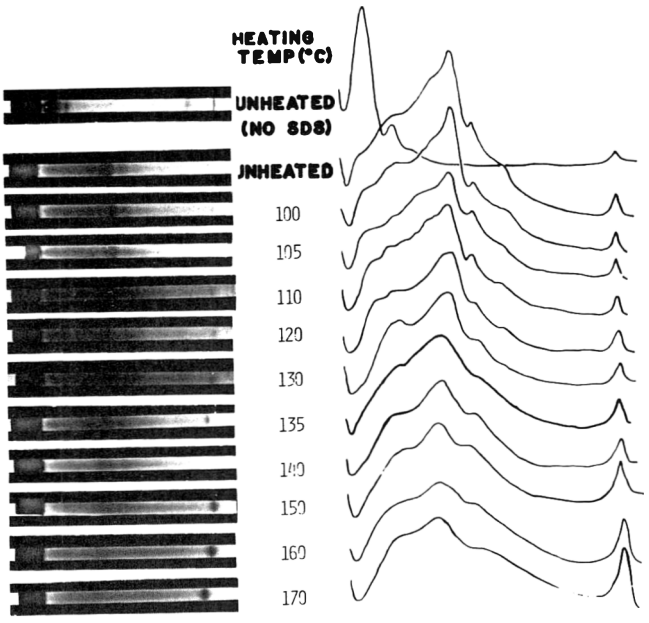

Fig. 7—Disc polyacrylamide gel electrophoresis of crude 7S-gel solubilized with SDS and ME.

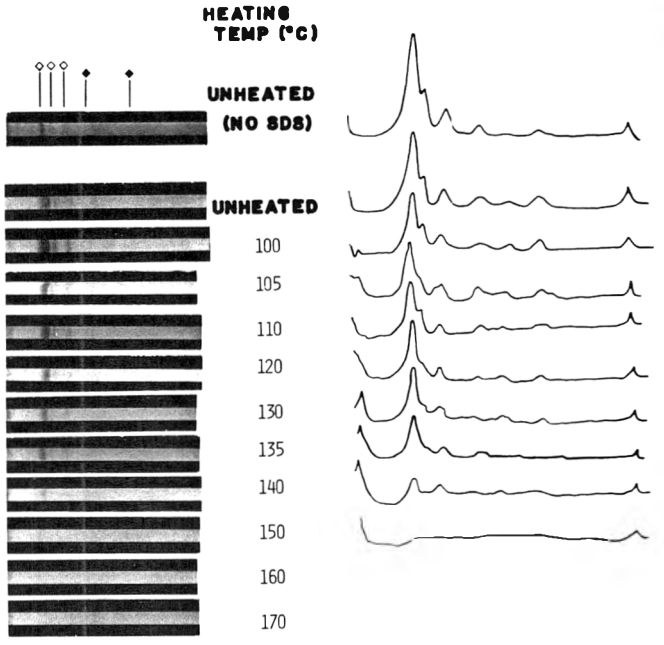

Fig. 8—SDS-disc polyacrylamide gel electrophoresis of crude 7S-gel solubilized with SDS and ME. (◇ Band derived from 7S globulin; ◆ Bands derived from 11S globulin.)

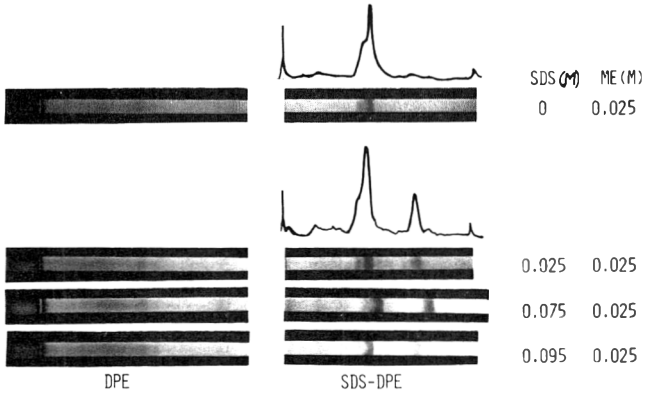

Fig. 9—Disc acrylamide gel electrophoresis of supernatant from heated CIF-gel, solubilized with different concentrations of SDS.

supernatant of CIF-gel dissolved with ME and without SDS contained only a high molecular subunit. The pattern is shown in Figure 9. Hashizume et al. (1974) reported that smaller subunits were more easily precipitated than larger subunits by heating at 60–100°C. This agrees with the above result of heating at above 100°C and appears to be of interest to investigate further the differences between the two cases.

In a previous paper (Saio et al., 1974), soybean protein gel coagulated with calcium showed the maximum expansion property by heating at around 130°C and this property was more dominant in CIF-gel than in 7S-gel. The authors presume, on the basis of available data, that soybean protein may show expansion properties as porous materials, when cross-linkage in insoluble gel becomes weaker by heating above 110°C, but the gross structure of its subunits are unchanged by heating below 140°C. Discussion of the changes of the protein molecule during heating will be done in a successive report.

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FOOD USE OF SOYBEAN 7S AND 11S PROTEINS
Changes in Basic Groups of Soybean Proteins by High Temperature Heating

INTRODUCTION

IN PREVIOUS papers, high temperature expansion characteristics of soybean 7S and 11S proteins (Saio et al., 1974) and the qualitative changes in proteins during heating at 100–170°C were investigated (Saio et al., 1975). In the course of the investigation using SDS-disc polyacrylamide gel electrophoresis, no bands were found on electrophoretical gel when heated over 150°C, in spite of application of a large amount of protein sample on gel.

The results by ultracentrifugal analysis and measurement of solubility suggested the degradation of protein molecules to lower molecular weight substances. The authors then did not discuss the reason why protein heated over 150°C was not stained with Amido Black 10B. Amido Black 10B reacts with basic groups of protein by the sulfonic radical which is highly negatively charged. It was reported that soybean protein lost the ability to form gel by heating in excess (meta-sol) and that the formation of cross-linkage which may be accompanied by gel formation was significantly prevented by deamidation during excess heating (Catsimpoolas et al., 1971). With reference to this information, the present investigation was initiated to investigate the disappearance of disc electrophoretic bands of soybean protein by excess heating. It was undertaken to clarify the changes in basic groups of soybean 7S and 11S proteins during heating at 100–170°C by measurement of amount of Amido Black 10B bound to proteins in the presence of SDS, analysis of basic amino acids and determination of amide groups and also to follow the changes of disc electrophoretic bands of protein during heating by staining with o-phthalaldehyde instead of Amido Black 10B.

MATERIALS & METHODS

Samples

Partially purified soybean globulins. Cold insoluble fraction (CIF) and crude 7S were used. The method of preparation was described in a previous paper (Saio et al., 1975).

Experimental methods

The method of preparation of heat-induced gels was the same as described previously (Saio et al., 1975). Namely, the protein paste (25%) was autoclaved at 100–170°C for 5 min and then cooled to room temperature. The resultant heat-induced gel was solubilized with 0.075M SDS (sodium dodecylsulfate) and 0.025M ME(2-mercaptoethanol) by grinding to fine pieces with a spatula and keeping in a shaking bath at 40°C overnight. The protein solution was submitted to the following analyses.

Measurement of Amido Black 10B bound to proteins. To 2 ml of 2% protein solution derived from heat-induced gel, 25 ml of the reagent of Amido Black 10B were added. To prepare the reagent, 0.616g of Amido Black 10B was dissolved with 1 liter of HCl-sodium citrate buffer, pH 2.8. The mixture was shaken by hand for 15 sec, kept for 5 min and centrifuged at 2,000 rpm (536 × G) for 5 min. The absorbance of the supernatant at 610 nm was measured after dilution to 50 times volume. The blank, using water for the protein sample, was measured as described above. The amount of Amido Black 10B bound to proteins was shown as the difference between absorbances of blank and sample. Measurement was made following the method of Shiga et al. (1959).

Determination of amide groups. To 120 mg of heat-induced gel in a 50-ml flask, 4 ml of 2N HCl were added with a few drops of octyl alcohol. The mixture was refluxed for 3 hr. After cooling in an ice bath, the mixture was neutralized with NaOH up to the point just before changing color of Methyl Red. The neutralized solution was transferred completely into a flask and 5 ml of borate (pH 8.6) buffer added. To prepare the buffer, 12.4g of boric acid and 14.9g of potassium chloride were dissolved in 1 liter of water and 240 ml of 0.2N NaOH was added to this solution. Similarly to the nitrogen determination by Kjeldahl method, the liberated NH_3 was absorbed in H_2SO_4 solution by steam-distillation for 3.5 min and back-titrated with 1/20N NaOH. The amount of free NH_3 in the heat-induced gel before HCl hydrolysis was determined after adding 5 ml of water to the gel, neutralizing with HCl just before changing color of Methyl Red and holding at room temperature for 1 hr. The method of Bailey (1937) was modified for soybean gel.

Analysis of basic amino acids. 1 ml of protein solution derived from heat-induced gel was hydrolyzed with 6N HCl for 24 hr at 110°C. The hydrolyzed protein solution was submitted to an Amino Acid Analyzer (Hitachi KCA-1) after elimination of HCl. The analysis was conducted using a short column.

OPT system and SDS-disc polyacrylamide gel electrophoresis. SDS-disc polyacrylamide gel electrophoresis (SDS-DPE) was carried out according to the method of Shapiro et al. (1967) and the OPT (o-phthalaldehyde) system followed the method of Weidekamm et al. (1973). After overnight incubation at 25°C of the mixture of 0.5% protein solution, 2% SDS (in 50% glycerin solution) and ME (1:1:0.02 in volume), 5μg o-phthalaldehyde dissolved in methanol in 1% was added. The solution was kept for 2 hr in the dark at room temperature. Electrophoresis was performed in 10% polyacrylamide gel and carried out at 8 mA per gel. Weidekamm monitored the migration of OPT-marked protein by taking X-ray photographs of fluorescence from the excited OPT-protein with a hydrogen lamp at 360 nm. However, as the amount of fluorescence was too low to measure by densitometer, purple color of OPT was photometrically measured by means of a densitometer (Atago 801) with No. 57 filter (at 570 nm). After this measurement, to compare patterns, the gel was successively stained with Amido Black 10B, decolored with 7% acetic acid and then measured with No. 61 filter (at 610 nm) as described in another report (Saio et al., 1975).

RESULTS & DISCUSSION

IN A PREVIOUS PAPER (Saio et al., 1975), it was reported that protein heated over 150°C was not entirely stained with Amido Black 10B on electrophoretical gels for SDS-DPE.

The major factors responsible for the amounts of SDS bound to proteins are presumed to be molecular weight, electro-charge and strength of intra-molecular bonding. In the SDS-DPE system, the proteins or their subunits migrate to the cathode as highly negatively charged species, protein-SDS complex, It was reported that the amount of SDS bound to protein at saturation is 1–1.4 (Pitt-Rivers and Impiombato, 1968) or 1.2–2.2 (Nelson, 1971), as far as proteins exist at least as subunits.

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Table 1—Changes in basic amino acids of heat-induced gels during heating at 100—170°C

Sample	Amino acid	Unheated	Temperature of heating gels (°C) 100	105	110	120	130	140	150	160	170
7S-gel	Lys	7.10	6.80	6.66	7.18	7.09	7.21	7.26	6.63	6.61	6.92
	His	2.52	2.48	2.91	2.36	2.38	2.38	2.09	2.92	3.08	2.31
	Arg	7.35	7.70	7.42	7.45	7.52	7.39	7.63	7.42	7.29	7.75
	Total	16.97	16.98	16.92	16.99	16.99	16.98	16.98	16.97	16.98	16.98
11S-gel	Lys	5.25	5.39	5.56	5.60	5.22	5.47	5.29	5.41	4.99	5.01
	His	2.65	2.99	2.35	2.65	2.02	2.61	2.45	2.70	2.85	2.75
	Arg	8.10	7.60	8.06	7.75	8.78	7.91	8.25	7.87	8.16	8.22
	Total	16.00	15.98	15.97	16.00	16.02	15.99	15.99	15.98	16.00	15.98

% of each amino acid to total protein

As Amido Black 10B binds with basic groups of protein by its negatively charged sulfonic radical, it is reasonable that the negatively charged SDS-protein complex inhibits the binding of Amido Black 10B.

Figure 1 shows the changes in the amount of Amido Black 10B bound to proteins derived from gels unheated and heated at 170°C, in the presence of different concentration of SDS. Both gels were selected for this experiment because they may be almost soluble with an SDS solution of lower concentration containing 0.025M ME. The amount of binding rapidly decreased in the range of 0—0.03M SDS, and in the range over 0.06M little was bound to proteins. The rate of decrease of binding was slightly higher in gels heated at 170°C than in unheated gels and in CIF-gel than crude 7S-gel.

The difference in amount of Amido Black 10B bound between CIF-gel and crude 7S-gel at different temperatures of heating is shown in Figure 2. Gels shown in Figure 2 were solubilized with 0.025M SDS containing 0.025M ME. The concentrations of SDS in the SDS-DPE system were 0.039M in the incubated solution and 0.007M in electrophoretical buffer, respectively. Considering the SDS concentrations described above, the difference of amounts bound between unheated and heated at 170°C may not be enough to understand the difference between SDS-DPE gels unheated and heated.

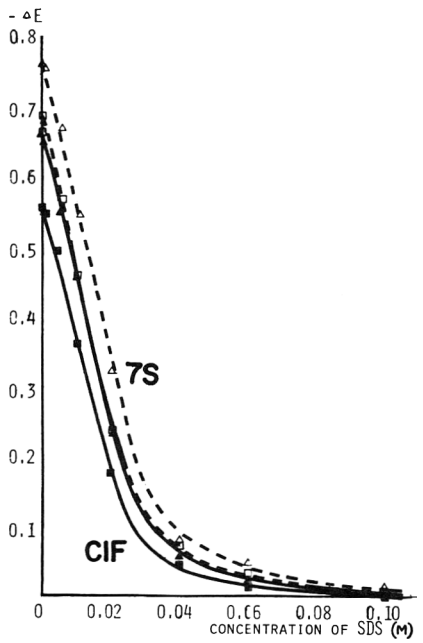

Fig. 1—Binding amount of Amido Black 10B with protein of heat-induced gels in different concentrations of SDS. [■——■ CIF-gel heated at 170°C; □-------□ 7S-gel heated at 170°C; ▲——▲ CIF-gel unheated; △-------△ 7S-gel unheated; −ΔE: Absorbance of blank − Absorbance of sample (610 nm)]

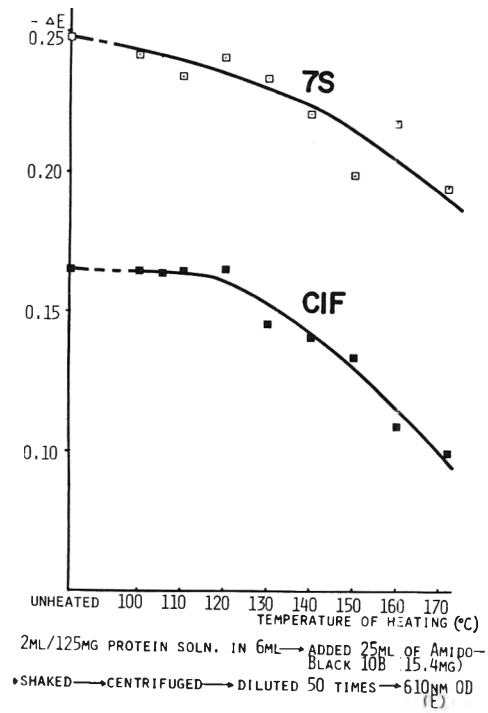

Fig. 2—Binding amount of Amido Black 10B with protein of heat-induced gels solubilized with SDS and ME. [−ΔE: Absorbance of blank − Absorbance of sample (610 nm)]

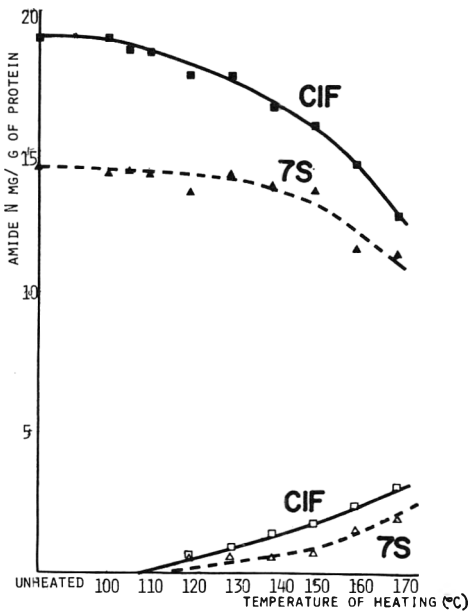

Fig. 3—Decrease of amide in heat-induced gels during heating. [■——■ amide in CIF-gel; ▲-------▲ Amide in 7S gel; □——□ Free ammonium in CIF-gel; △-------△ Free ammonium in 7S-gel]

Table 2–Changes in characteristics of soybean protein at high temperature heating

Temperature of heating (°C)	100	105	110	120	130	140	150	160	170
gross-structure of subunits		intact			little degraded			degraded	
solubility	rapid decrease		slow increase					rapid increase	
binding force (degree of aggregate)	rapid increase		slow decrease					rapid decrease	
expansion property		increase				rapid decrease			
texture	hard fragile				soft elastic		like sol		

From Figure 2, the amount of binding by Amido Black 10B per one protein molecule was calculated as 184 in crude 7S and 109 in CIF, respectively, on the basis of molecular efficiency of Amido Black 10B (43,250) and molecular weights of proteins 7S (180,000) and 11S (350,000). After heating at 170°C, the amount of Amido Black 10B bound decreased to as low as 7–8 in crude 7S-gel and 22–23 in CIF-gel.

In order to see the changes in basic amino acids of both proteins (paste) by heating at 100–170°C, lysine, histidine and arginine were analyzed. The results were shown in Table 1. As far as these experimental conditions were concerned, no significant changes were recognized. But as in a preliminary experiment in 2% protein solution, a decrease of arginine was recognized by heating at 170°C; the high concentration of protein and ionic strength with phosphate buffer in this experiment may prevent the degradation of basic amino acids.

Next, changes in amide groups by heating were investigated. The weakly positively charged amide groups may bind with Amido Black 10B by their resonance structure, especially at pH 2.8. The changes in amide groups by heating are shown in Figure 3. There was good agreement with the changes in the amount of Amido Black 10B bound as shown in Figure 2, namely, the amide groups of CIF-gel began to decrease from 105°C and decreased rapidly as the temperature of heating increased, while those of crude 7S-gel began to decrease from around 140°C. Liberated NH_3 increased from 105–110°C and the amount liberated was higher in CIF-gel than in crude 7S-gel. The sum of amide groups and NH_3 was approximately equivalent in the range 140–150°C but decreased at above 150°C, probably because NH_3 liberated escaped from the gel as the amount of liberation increased.

The amounts of amide groups per one protein molecule were 186 in crude 7S and 475 in CIF before heating and decreased after heating at 170°C to 49 in crude 7S and 155 in CIF (26% in crude 7S and 33% in CIF). The figures were larger when compared to the amount of Amido Black 10B bound, but may be reasonable because amide groups form hydrogen-bonds with other amide groups or with carboxyl groups in the protein molecule and they do not always bind with Amido Black 10B by their resonance structure. On the basis of data described above, the authors understand that the decrease of amide groups is related to the decrease of Amido Black 10B bound to proteins.

Now, in order to discuss more in detail the disappearance of electrophoretic bands of protein gels heated at above 150°C, o-phthalaldehyde (OPT), which was reported to bind with protein by formation of a covalent bond, was used as a staining dye instead of Amido Black 10B. Figure 4 shows the comparative results of staining by OPT and successive staining by Amido Black 10B. OPT appeared as a big band near the front which seemed to interfere with the appearance of clear bands of fast-migrating subunits. But electrophoretic patterns of the OPT system showed clearly the gradual diminishing of subunit bands at 130–140°C and their disappearance at above 150°C, similar to the case by Amido Black 10B. The OPT band near the front increased in amount with an increase in temperature of heating, especially above 150°C and also seemed to migrate slightly faster with an increase of temperature.

From the results it may be concluded that the gross-structure of subunits degraded to form lower molecular weight substances by heating at above 150°C and that a sharp increase of

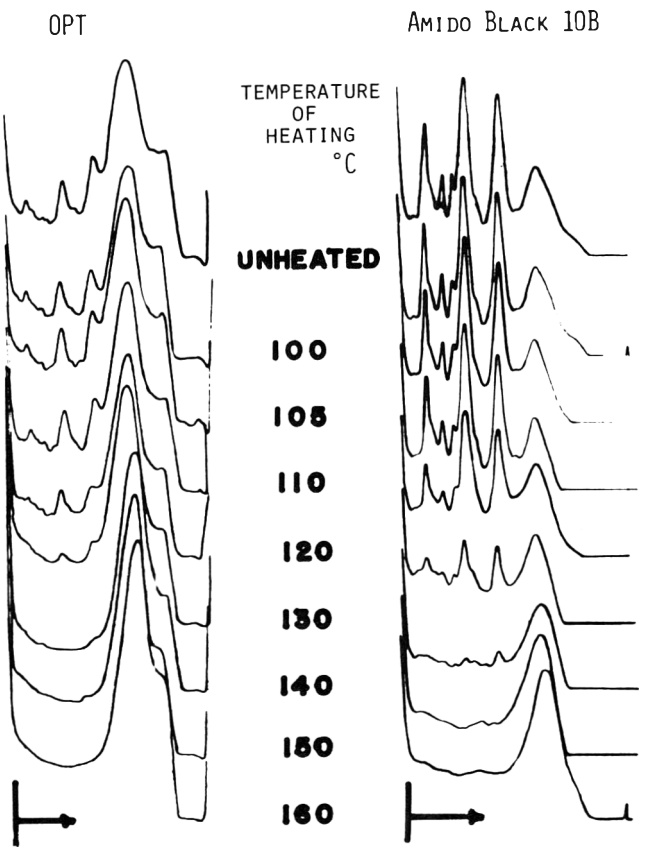

Fig. 4–SDS-disc polyacrylamide gel electrophoresis of CIF-gels stained with OPT or Amido Black 10B.

bound SDS resulted from the increase of surface area of the protein molecule by dissociation definitely preventing the binding of Amido Black 10B.

On the basis of available data, including previous papers (Saio et al., 1974; 1975), the conclusive scheme of chemical and physical changes of soybean protein gels during heating is shown in Table 2.

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EFFECTS OF FEEDING OXIDIZED OR HEATED SOYBEAN OIL ON TISSUE COMPOSITION AND HEMATOLOGICAL STATUS OF RATS

INTRODUCTION

VARIOUS metabolic effects of fats subjected to prolonged heating or oxidation have been reported [see reviews by Quackenbush (1945) and Artman (1969) for example]. Crampton et al. (1953) observed that portions of damaged oils that will not form adducts with urea are toxic if isolated and fed to animals. More recently, there has been widespread interest in ascertaining the wholesomeness for human consumption of oils that may be subjected to heat and aeration damage by prolonged use to fry various foods. For example, Kaunitz et al. (1965; 1966) studied the effects of mildly oxidized oils and observed differences in the longevity of rats depending upon the source of the oil. The reduced rate of growth of rats (Nolen et al., 1967) and dogs (Nolen, 1973) fed heated hydrogenated soybean oil was attributed to a decrease in absorbability of the damaged fat. Used hydrogenated soybean oil had no effect on reproduction or teratology in rats (Nolen, 1972) although an isolated distillable nonurea-adducting fraction of the oil elicited manifestations of acute toxicity (Nolen et al., 1967). Nakamura et al. (1973) found that the liver of rats fed autoxidized safflower oil was increased in size but that the triglyceride content was markedly decreased. Enlarged liver is often reported as one of the physiological effects of damaged lipids in the diet. Sometimes this is associated with an increase in hepatic lipid, but the opposite may occur also. In an earlier study by the authors, reduced liver lipid content was observed in rats that were fed heated soybean oil (unpublished data). The damaged oil also appeared to have an effect on the hematological status of these animals. No explanation has been noted in the literature for the enlarged livers and only passing reference has been made to the occurrence of anemia in animals fed heat-damaged fats. In order to evaluate the effects of damaged oils on tissue composition and hematological status, rats were fed, ad libitum, diets containing fresh, aerated and heated oil and several blood parameters were measured. The inanition caused by the addition of damaged fats to these diets made it difficult to ascribe the effects to either the damaged fats per se or the reduced intake of food or protein. Therefore a second experiment was conducted in which feed intake of some animals given the fresh oil diet was limited to that of the animals given the heated oil diet ad libitum. In addition a group of rats was fed a diet containing the heated oil with an increased protein concentration so that the protein intake of this lot of animals was equal to that of the rats fed the fresh oil diet ad libitum.

This is a report of the effects of these diets on the hematological profile of the animals and the relationship of these to alterations in the composition of the liver, spleen, and carcass associated with dietary treatment.

EXPERIMENTAL

Diets and feeding protocol

The oil used in the first experiment was refined, deodorized soybean oil. No antioxidants had been added and the oil was not hydrogenated. The linolenic acid content of this oil was determined to be 7.1% using the procedure of Worthington et al. (1972) and it had a peroxide value of 2.0 determined by the official method of the AOCS (1970). One batch of this oil was aerated by a stream of compressed air for several months. This treatment resulted in an oil having a linolenic acid content of 6.7% and a peroxide value of 217. The oil had some rancid odor and off-flavor; however, no change in color of the oil was observed. A second portion of the oil was flushed intermittently with carbon dioxide for several days while being heated at 195°C. The linolenic acid content of this lot of oil was reduced to 5.4% and it had a peroxide value of 70. This oil was darkened somewhat and had a strong rancid odor and flavor.

For the second experiment table grade soybean oil was purchased from a local super-market. This was refined and partially hydrogenated oil with BHA and BHT added as antioxidants. It has a linolenic acid content of 3.1% and a peroxide value of 1.2. A portion of this oil was heated at 195°C while compressed air was bubbled through it for about 100 hr. This lot of oil had a linolenic acid content of 2.1%, a peroxide value of 5.4 and a strong rancid odor and flavor with some darkening in color.

Each diet contained 40% of fresh or treated oil, 0.0175% d,l-α-tocopherol, 10% cellulose, 4% mineral mixture, 2.2% vitamin mixture (P-H salt and vitamin diet fortification mixture, Nutritional Biochemical Corp. Cleveland, Ohio). All diets but one contained 20% casein. The casein content of one of the diets used in the second experiment was adjusted so that the average protein intake of the animals consuming it would equal that of the group fed the fresh oil diet ad libitum. This diet was mixed daily. The amount of casein to be added was based on feed and protein intake of the two groups of animals for the previous 24 hr. Equal quantities of sucrose and corn dextrin were added to the diets to make up 100%.

The salt mixture used in the first experiment provided about 170 mg of iron per kg of diet, which is four to five times the requirement of the rat. In order to avoid the excessive dietary iron content in the second study, a salt mixture without ferric citrate was obtained and iron was added to the diets at the rate of 35 mg per kg.

Male Sprague-Dawley rats were divided by weight into groups of 15 animals per diet in each of the two experiments. The rats were 4 wk old at the beginning of each study and were fed the test diets for 8 wk. The animals were housed individually in stainless steel cages and had free access to deionized water. In the first experiment, all diets were fed ad libitum and food intake was measured every second day. In the second study, animals were fed daily. One group of rats was given the fresh oil diet and a second group was fed the heated oil diet ad libitum. Each animal of a third group was given an amount of the fresh oil diet equal to the average amount of the heated oil diet consumed by the animals of the second group for the previous 24 hr. The fourth group of rats was given ad libitum a diet prepared each day with the heated oil and added casein as described above. These last two dietary groups are listed in the tables as fresh oil, rst'd (restricted) and heated oil, hipro (high protein), respectively.

Tissue preparation and analysis

The animals were exsanguinated under ether anesthesia. Total hemoglobin was determined by the method of Evelyn and Malloy (1938). Hematocrits were measured in heparinized capillary tubes and red blood cells were counted in a hemacytometer. Transferrin was saturated with iron as described by Goodwin et al. (1966) and appropriate aliquants of serum or iron-saturated serum were wet ashed with sulfuric, nitric and perchloric acids for mineral analysis. Serum iron and total iron binding capacity (Stookey, 1970) and copper (Carter, 1972) were determined colorimetrically. Ceruloplasmin was measured by its p-phenylenediamine oxidase activity as described by Sunderman and Nomoto (1970).

The liver, spleen and epididymal fat pads were removed and weighed. In the second experiment the remainder of the carcass was prepared for mineral analysis by excising the remaining viscera, washing them free of adhering blood and removing the contents of the gastrointestinal tract. The carcass and remaining viscera (including the epididymal fat pads) were disintegrated in nitric acid and made to volume after removing the separated fat. The spleens, liver samples and aliquants of the carcass digest were wet ashed in sulfuric, nitric and perchloric acids and iron content was determined with ortho-phenanthroline (Saywell and Cunningham, 1937). Copper was measured in ashed samples of liver and carcass as noted above for serum.

Liver protein content was measured by the method of Lowry et al. (1951). Nucleic acids were extracted as described by Munro and Fleck (1966); ribonucleic acid (RNA) was measured by ultraviolet spectroscopy and deoxyribonucleic acid (DNA) was determined by the method of Ceriotti (1952). Liver lipids were determined as described previously (Miller, 1974).

During the eighth week of the second study, feces excreted during a 24-hr period were collected from each animal and oven-dried. The dried feces were ground in a mixture of water, ethanol and hexane and lipids were extracted with hexane and measured gravimetrically.

The data were subjected to analysis of variance and differences associated with dietary treatment were determined according to the multiple range tests of Duncan (1955).

RESULTS & DISCUSSION

Growth and feed efficiency

Feed intake and growth of the animals were depressed by the treatments imposed on the dietary oils and the extent of the depression increased with the severity of the oil treatment (Table 1).

In the second experiment, the animals given the fresh oil diet in an amount limited to that of rats fed the heated oil diet consumed their entire allotment of food within a short period of time. The increase in feed efficiency observed for these animals is in accord with other observations (Muiruri and Leveille, 1972) on the effects of "meal eating" versus "nibbling" on growth of rats. The increased rate of growth in the animals fed a limited quantity of the fresh oil diet in comparison to those that consumed an equal quantity of the heated oil diet is evidence that poor performance of animals fed the damaged oil cannot be attributed solely to decreased palatability of the diet. When the protein content of the heated oil diet was increased so that animals consumed the same amount of protein as those fed the fresh oil diet ad libitum, feed efficiency was increased to that of the fresh oil diet. Feed intake and growth were also improved but did not reach levels obtained with the diet containing fresh oil. The additional protein in the diet either improved its palatability, or the increased ratio of protein to calories caused an increase in growth rate which triggered an enhancement in appetite.

The average lipid content of the 24-hr fecal collection was 100 mg for the animals fed the fresh oil ad libitum and 93 mg for those with restricted intake of this diet. Of the two groups of animals given the heated oil the lipid loss per day was 253 mg in those fed 20% casein and 279 in the rats given the extra protein. This difference in fecal lipid loss is not sufficient to account for the reduced feed efficiency of animals fed the heated oil.

Tissue composition

The hepatomegaly observed in animals fed heated oils was further defined in the second experiment by examining the nucleic acid and protein content of the liver tissue (Table 2). The reduced number of liver cells, as indicated by DNA content, associated with restricted intake of the fresh oil diet is in

Table 1—Effects of aerated and heated soybean oil on feed intake and growth of rats[a]

Dietary treatment	Protein intake (g)	Feed intake (g)	Feed[b] eff. (g)	Final wt (g)	Epididymal fat wt (g)
Experiment 1					
Fresh, ad lib	160.0	800a	0.401a	391a	6.83a
Aerated, ad lib	133.0	665b	0.365b	314b	4.69b
Heated, ad lib	111.6	558c	0.320c	251c	2.75c
Experiment 2					
Fresh, ad lib	158.0	790a	0.436b	418a	6.15a
Fresh, rst'd[c]	103.2	516c	0.466a	313b	3.72b
Heated, ad lib	104.6	523c	0.327c	249c	2.48b
Heated, hipro[d]	161.9	632b	0.416b	336b	3.56b

[a] In each experiment, values within a column not having the same letter are significantly different at $P < 0.01$.
[b] Feed efficiency, grams of weight gained per gram of feed consumed
[c] Feed intake of each animal was restricted to the average intake for the previous 24 hr of the animals fed the heated oil diet with 20% casein.
[d] Casein concentration of the diet was increased so that protein intake of these animals matched that of animals fed the fresh oil diet, ad lib.

Table 2—Effects of aerated and heated soybean oil on liver composition of rats[a]

Dietary treatment	wt (g)	% of body wt	H_2O (%)	Lipid (%)[b] Total	TG	CE	Protein (%)	No. of cells[c] (millions)	RNA/cell (pg)	Prot/cell (pg)
Experiment 1										
Fresh, ad lib	14.1a	3.6b	69.0b	7.75a	3.25a	0.41a				
Aerated, ad lib	11.5b	3.7b	71.3a	5.62b	1.24b	0.30b				
Heated, ad lib	12.6ab	5.0a	71.6a	4.64c	0.35c	0.11c				
Experiment 2										
Fresh, ad lib	14.9b	3.6c	68.8c	7.83a	3.44a	0.36a	16.4b	3400a	39.8ab	765bc
Fresh, rst'd[d]	11.1c	3.5c	69.8b	6.42ab	2.12b	0.23b	16.4b	2800b	34.8b	662c
Heated, ad lib	13.7b	5.6a	71.1a	4.87b	0.62c	0.10c	18.2a	2800b	38.9ab	895b
Heated, hipro[e]	17.1a	5.1b	70.4ab	5.12b	0.87c	0.11c	18.8a	3080ab	43.7a	1074a

[a] In each experiment, values within a column not having the same letter are significantly different at $P < 0.01$.
[b] Abbreviations: TG, triglycerides, CE, cholesterol esters
[c] Number of cells based on DNA content
[d] Feed intake of each animal was restricted to the average intake for the previous 24 hr of the animals fed the heated oil diet with 20% casein.
[e] Casein concentration of the diet was increased so that protein intake of these animals matched that of animals fed the fresh oil diet, ad lib.

agreement with results obtained in animals subjected to protein-calorie malnutrition (Dallman and Manies, 1973). The dietary restriction in the study reported here was less severe than in the work cited and caused no significant reduction of RNA or protein content of the liver cells.

Livers of rats fed the heated oil had the same number of cells as their pair-fed controls but the protein content per cell was significantly increased. Thus at equal levels of protein intake, less protein was used for somatic growth and more was retained in the liver of animals fed the heated oil in comparison to rats given the fresh oil. When the protein concentration of the diets containing heated oil was increased, bodily growth increased but there was also an even further increase in protein content of the liver cells. The amount of lipid stored in the liver as triglycerides was greatly reduced in rats fed diets containing the heated oil.

Table 3—Effects of aerated and heated soybean oil on total iron and copper content of carcass, liver and spleen of rats[a]

	Iron				Copper	
Dietary treatment	Carcass (mg)	Liver (mg)	Spleen (mg)	Total (mg)	Carcass (μg)	Liver (μg)
Experiment 1						
Fresh, ad lib		1.95a	0.24a			78.4a
Aerated, ad lib		1.30b	0.18b			74.1ab
Heated, ad lib		1.41b	0.16b			67.0b
Experiment 2						
Fresh, ad lib	4.63a	0.91ab	0.14	5.68a	389a	72.2a
Fresh, rst'd[b]	3.70b	0.83b	0.13	4.66bc	314b	52.4c
Heated, ad lib	3.01c	1.07a	0.12	4.22c	216c	61.4b
Heated, hipro[c]	3.95b	0.96ab	0.11	5.02b	360ab	72.9a

[a] In each experiment, values within a column not having the same letter are significantly different at $P < 0.01$.
[b] Feed intake of each animal restricted to the average intake for the previous 24 hr of the animals fed the heated oil diet with 20% casein.
[c] Casein concentration of the diet was increased so that protein intake of these animals matched that of animals fed the fresh oil diet, ad lib.

In the first experiment, in which the diets contained a plethora of iron, the content of this mineral in the liver was decreased by aerating or heating the oil used in the diets. The iron content of the diets used in the second study was adequate for the animal's need but considerably lower than that of the diets in the previous experiment. Of the two groups of animals fed equal quantities of the diets containing fresh or heated oil in the second study, those given the heated oil had more iron in the liver and less in the carcass than those fed the fresh oil. Thus in the animals fed the damaged oil, more of the iron that was absorbed was stored in the liver rather than being used for hemoglobin synthesis. Since hemoglobin synthesis was decreased and there was no increase in total iron content of the solid tissues, the total amount of iron absorbed must have been reduced in animals fed the heated oil. The effects of the dietary treatments on copper content of liver and carcass were similar to those on iron content of these tissues (Table 3).

Hematolgoical parameters

Both the number of red blood cells (RBCs) produced and the mean cell hemoglobin concentration (MCHC) were depressed in animals given diets containing the heated fats (Table 4). Consequently the hemoglobin content and volume of cells in the whole blood were significantly reduced in these animals in comparison to the animals fed the fresh oil diets. In the second experiment, restriction of food intake of the fresh oil diet led to a reduction in number and volume of cells in the blood with relatively little decrease in hemoglobin concentration in comparison to that of animals provided with unlimited quantities of the same diet. Use of heated oil in the diet caused a significant decrease in hemoglobin and hematocrit with no change in number of cells in comparison to the animals pair-fed the fresh oil diet. Addition of extra protein to the heated oil diet brought about very little improvement of the hematological status of the rats. Ito and Reissmann (1966) have shown that dietary protein restriction causes a depression of cell division in the bone marrow and Reissmann (1964a, b) presented evidence that the effect is due to diminished erythropoietin formation rather than to any rate-limiting effect on red blood cell formation per se.

In the second experiment, serum levels of iron and copper and of their respective binding proteins, transferrin (TIBC) and ceruloplasmin (Cp) were reduced by use of heated fats in the diets. Total iron-binding capacity was also decreased in animals

Table 4—Effects of aerated and heated soybean oil on hematogoical status of rats[a]

							Serum			
Dietary treatment	RBC[b] (X 10^{-6})	Hb[b] (%)	Ht[b] (%)	MCV[b] (μ^3)	MCH[b] (pg)	MCHC[b] (%)	Iron	TIBC[b] (μg/100ml)	Copper	Cp[b] (mg/100 ml)
Experiment 1										
Fresh, ad lib	8.72a	15.8a	53.0a	61.1b	17.5	29.7ab	313	776a	124	53
Aerated, ad lib	8.31a	15.2b	50.2b	60.5b	18.3	30.1a	302	700a	129	62
Heated, ad lib	7.45b	14.0c	49.2b	66.4a	18.9	28.5b	299	531b	140	59
Experiment 2										
Fresh, ad lib	9.02a	15.7a	52.1a	57.9b	17.5ab	30.2a	361a	762a	136a	60a
Fresh, rst'd[c]	8.34b	15.1a	50.0b	60.1ab	18.2a	30.2a	327ab	698a	131ab	56a
Heated, ad lib	8.24bc	13.7b	47.6c	58.0b	16.7b	28.7b	280b	575b	113c	47b
Heated, hipro[d]	7.78c	14.2b	48.8bc	62.8a	18.3a	29.2b	270b	579b	119bc	48b

[a] In each experiment, values within a column not having the same letter are significantly different at $P < 0.01$.
[b] Abbreviations used: RBC, red blood cell count; Hb, hemoglobin; Ht, hematocrit; MCV, mean cell volume; MCH, mean cellular hemoglobin; MCHC, mean cellular hemoglobin concentration; TIBC, total iron binding capacity; Cp, ceruloplasmin.
[c] Feed intake of each animal was restricted to the average intake for the previous 24 hr of the animals fed the heated oil diet with 20% casein.
[d] Casein concentration of the diet was increased so that protein intake of these animals matched that of animals fed the fresh oil diet, ad lib.

fed the heated oil in the first study but serum levels of iron and copper were not altered by dietary treatments in this study.

SUMMARY

IN THESE EXPERIMENTS, the extra-hepatic tissues of the animals responded to the use of heated fats in the diets as though there had been a restriction of dietary protein-calories. The reduced feed efficiency associated with use of the heated fats supports the concept of decreased availability of calories. The reduced production of hemoglobin and the serum proteins, transferrin and ceruloplasmin, and the increase of cellular protein in the liver of rats fed the heated oil diet suggest that an abnormal portion of the dietary protein was being retained in the liver to cope with the metabolic effects of the damaged fat. Possibly the protein is used to synthesize hepatic enzymes which metabolize the nonphysiological portion of the dietary lipid to a form, or forms, which can be expelled either through the lungs or the kidneys.

The addition of extra protein to the diet containing heated oil partially alleviated the effects seen when the diet was fed with 20% protein. This was accomplished with an increase in quantity of protein per hepatic cell indicating that, at the level of the hepatic cell, protein was one of the factors limiting metabolic functions.

The aerated and heated fats used in these studies were considered to be unacceptable for human consumption and the level of fat in the diets was considerably higher than is recommended for human diets. However, as Nolen et al. (1967) have pointed out "some factor of exaggeration is necessary and appropriate in feeding studies for detecting mild toxicity..." It is not anticipated that fats consumed in commercially fried foods would ever create the metabolic disturbances observed in this study, in diets that are nutritionally adequate. However, if the quantity or quality of dietary protein was marginal, the metabolic balance might already be precarious. If so, inclusion of damaged fats in the regime might tip the balance of one or more of the above mentioned functions, e.g., absorption of iron, to an inauspicious level and thus have an unwholesome effect.

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ULTRASONIC EXTRACTION OF PROTEINS FROM AUTOCLAVED SOYBEAN FLAKES

INTRODUCTION

WITH MORE and more soybean protein going into human food, efficient extraction from soybean flakes assumes more and more importance. Commercial extraction yields 30% or less of the weight of flakes, and ideal laboratory extraction yields 42% of defatted flakes (Smith, 1958). For maximum yield of proteins, the flakes are unheated; but for maximum nutritive quality, the flakes require a moist heat treatment (Circle and Smith, 1972). Toasting causes protein denaturation which means low yields, diminished functionalities and limited applications.

Factors that affect the dispersion of soybean proteins in water include temperature and time of extraction, meal age, particle size, solvent-to-meal ratio, pH, salt type and concentration and rate of stirring (Circle, 1950). High-speed stirring has been reported to grind the sample during extraction (Paulsen et al., 1960). Other factors affecting protein solubility have been reviewed (Circle, 1950; Wolf, 1972).

Although sonication has been applied in the past to disintegrate and solubilize animal and plant tissue components (El'Piner, 1964; Ensminger, 1973), a search of literature indicates that it has not been applied to improve the extraction of proteins in soybean flakes. This paper reports the comparison between ultrasonic extraction on a laboratory scale with conventional-stir extraction upon heated and unheated soybean flakes.

MATERIALS & METHODS

Preparation of flakes

Whole soybeans from a 1971 crop of Kanrich variety were cracked, dehulled and flaked. The flakes were defatted by extraction with a hexane-pentane mixture on a laboratory scale. The defatted flakes, which contained 46.4% protein based on 7.41% total nitrogen (air-dry basis, moisture content 10.7%) were stored in a refrigerator at 4°C before use. A portion of flakes was autoclaved at 120°C for 20 min and stored at room temperature. The respective nitrogen solubility indexes (NSI) were 25 and 91 for autoclaved and unautoclaved flakes (AOCS, 1970).

Extraction of proteins

Sonication. To 5g of flakes in a 2-oz glass jar were added 50 ml distilled water. The jar was chilled in an ice slurry, and the mixture was sonicated usually for 8 min (except in one experiment for a varied length of time) with a Sonifier Model S125 manufactured by Heat Systems-Ultrasonics, Inc., Plainview, N.Y. The sonifier contains a standard mechanical transformer with a step horn and a power unit with a meter measuring input power. The step horn was dipped about 1 in. deep into solution, and the sonication was tuned to a maximum input power at which point the instrument operates at a frequency of 20 kHz and delivers an output power of 125 watts. After sonication, the mixture was centrifuged at 10,000 × G for 15 min, and the supernatant was poured through a thin layer of glass wool into a cylinder. The volume of the supernatant was recorded, and a portion was taken to measure the amount of proteins by the biuret method (Layne, 1957). The amount of soluble protein was calculated on the basis of 50 ml solvent used. Averages of duplicated protein determinations are reported.

Conventional stir. In 50 ml of distilled water, 5g of sample were stirred mechanically at 1200 rpm (three 1/2-in. bladed type) at room temperature for 1 hr. The sample was treated the same way as in the sonication-extraction regarding centrifugation, filtration and measurement of protein. This treatment was used because of its convenience. In one experiment the amount of protein extracted in three consecutive stirrings was compared with sonication-extractions.

Protein fractions

Cold-insoluble fraction (CIF). A portion of water extract from 5g of flakes was chilled overnight in a refrigerator (4°C) and then centrifuged at 10,000 × G at 4°C to collect the proteins (Wolf and Sly, 1967). The proteins were dissolved in 0.03M phosphate buffer –0.04M NaCl (pH 7.6, μ = 0.5) for further analyses.

Acid-precipitated fraction (APF). A portion of water extract was titrated with 1N HCl to pH 4.2 and then centrifuged at 34,000 × G for 10 min to collect the proteins. The proteins were treated the same way as described for CIF.

Ultracentrifugation

Proteins in CIF, APF and water extracts were dialyzed with two successive 3-liter batches of distilled water at 4°C for 48 hr. After dialysis the proteins were equilibrated with the same phosphate buffer as described above for 4 hr before adjusting the protein concentration to 7 mg per ml. The protein samples were centrifuged at room temperature with a Spinco Model E centrifuge at 47,600 rpm and their schlieren patterns were examined qualitatively for their protein components.

RESULTS & DISCUSSION

Extraction with sonication and conventional-stir

Experiments to compare sonication and the conventional-

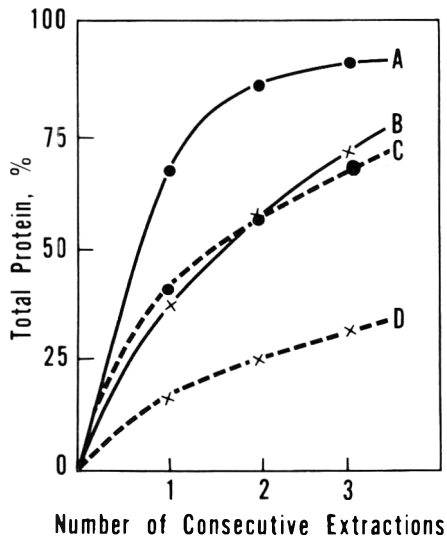

Fig. 1—Percentages of total soybean proteins in consecutive extractions with 50 ml water from Kanrich defatted flakes (5g). Treatments of the flakes include (A) unautoclaved, sonicated-extraction; (B) unautoclaved, conventional-stir extraction; (C) autoclaved, sonicated-extraction; and (D) autoclaved, conventional-stir extraction.

stir method included those with (a) three consecutive extractions of proteins with water and (b) single extraction with water or 1N NaOH as solvents.

(a) In three consecutive extractions 70% total protein in unautoclaved flakes was solubilized in water by the conventional-stir method, and 90% by sonication-extraction (Fig. 1).

When autoclaved flakes were used, on the one hand, conventional-stir extracted only 32% protein. Sonication, on the other hand, solubilized 69% of the total protein. Proteins in the autoclaved flakes were denatured, and obviously conventional-stir extraction was ineffective in dispersing the proteins in water. By comparison, sonication effectively dispersed more of the denatured proteins in autoclaved flakes in water.

(b) In a single extraction, results with water were compared to that with 1N NaOH (Table 1). When water was used in a single extraction, sonication dispersed 58% of the proteins in autoclaved flakes, whereas conventional stirring extracted only 16% of the proteins.

The NaOH solvent extracted 73% and 91% proteins, respectively, from autoclaved and unautoclaved flakes with conventional-stir, whereas sonication with NaOH solvent recovered 99% and 95%, respectively. Although water was less efficient in protein extraction than the NaOH solvent, sonication extracted more proteins in either solvent whether a single extraction or three consecutive extractions.

Table 1—Comparison of soy protein extracted by sonication and conventional-stir with water and with 1N NaOH as solvents

Defatted flakes (5g)	Soybean protein (mg) extracted by			
	Sonication		Conventional-stir	
	Water	1N NaOH	Water	1N NaOH
Autoclaved	1350 (58%)	2290 (99%)	375 (16%)	1700 (73%)
Unautoclaved	2050 (88%)	2200 (95%)	1400 (60%)	2100 (91%)

Table 2—Yield of soybean protein in cold-insoluble fractions, acid-precipitated fractions and water extracts

Defatted flakes (5g)	Yield of protein (mg)		
	Cold insoluble	Acid precipitated	Water extract
Unautoclaved, conventional-stir	155	592	862
Autoclaved, conventional-stir	92	245	547
Unautoclaved, sonication	259	632	1296
Autoclaved, sonication	179	610	1172

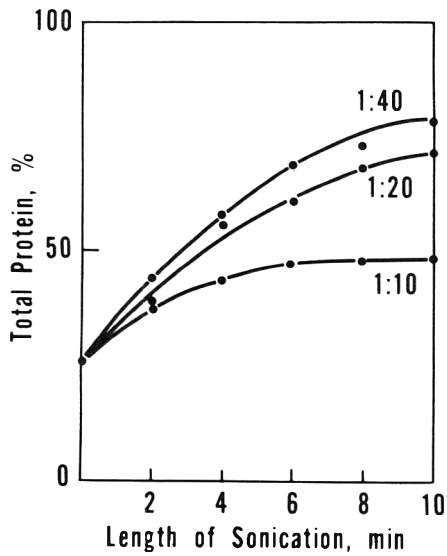

Fig. 2—Percentages of total soybean proteins from 5g autoclaved Kanrich defatted flakes in a single extraction with water at varied time and meal-to-solvent ratios.

Dispersion of denatured proteins

The amounts of protein in autoclaved flakes dispersed in water by sonication are affected by extraction time and meal-to-solvent ratios (Fig. 2). With a meal-to-solvent ratio of 1:10 the amount of protein extracted with sonication reached a maximum of 48% at 6 min and then leveled off. When the ratio was varied to 1:40, a new maximum of 78% was reached at 10 min. Further increase of the solvent or length of time did not improve the percentage significantly. Other factors, such as pH of solvent, salt type and concentration and extraction temperature, were not tested extensively; nevertheless, data indicate that an irreversible loss of protein in autoclaved flakes is about 20% of the total and that sonication extracted nearly 80% of the total proteins in autoclaved flakes.

Yield of protein fractions

To compare the qualitative differences in proteins dispersed by sonication and conventional-stir, the yields of protein fractions in CIF, APF and water extract were measured (Table 2). Sonication improved the yields of protein in CIF, APF and water extract from autoclaved or unautoclaved flakes, compared to the respective categories by conventional-stir. With sonication the yield (179 mg) of CIF from autoclaved flakes was significantly lower than that from unautoclaved (259 mg). The differences in APF and water extract were not so great as in CIF. By conventional-stir extraction, all three categories were lower from autoclaved flakes. Regardless of which method of extraction was used, the loss of CIF in autoclaved flakes was more than 60% (61% by conventional-stir, 69% by sonication).

The similarities of components in these three categories of proteins (CIF, APF and water extract) are seen in Figure 3. There are no qualitative differences in their components between the methods used. The quantitative yields of individual components in these patterns were not determined.

Sonication represents a new way to isolate soybean proteins. It not only extracts more proteins from flakes, but more significantly, it does so from autoclaved flakes analogous to ones produced commercially (Fig. 1). Up to now, extraction of soybean proteins has been mostly done by conventional-stir (Circle and Smith, 1972). Results from that method are good on unautoclaved flakes, but poor on autoclaved flakes. If sonication is applied on autoclaved flakes, the efficiency of protein extraction can be improved.

Soybeans used in this study are of Kanrich "vegetable" variety which are larger in size than field varieties. Soybeans for industrial use are of field varieties. Although the varietal difference has not been studied, the efficiency of protein extraction by sonication as affected by other factors—variety of beans, size of flakes, extraction time, temperature, salt, pH, etc.—will be investigated later.

Sonication may also provide a partial solution to the flavor

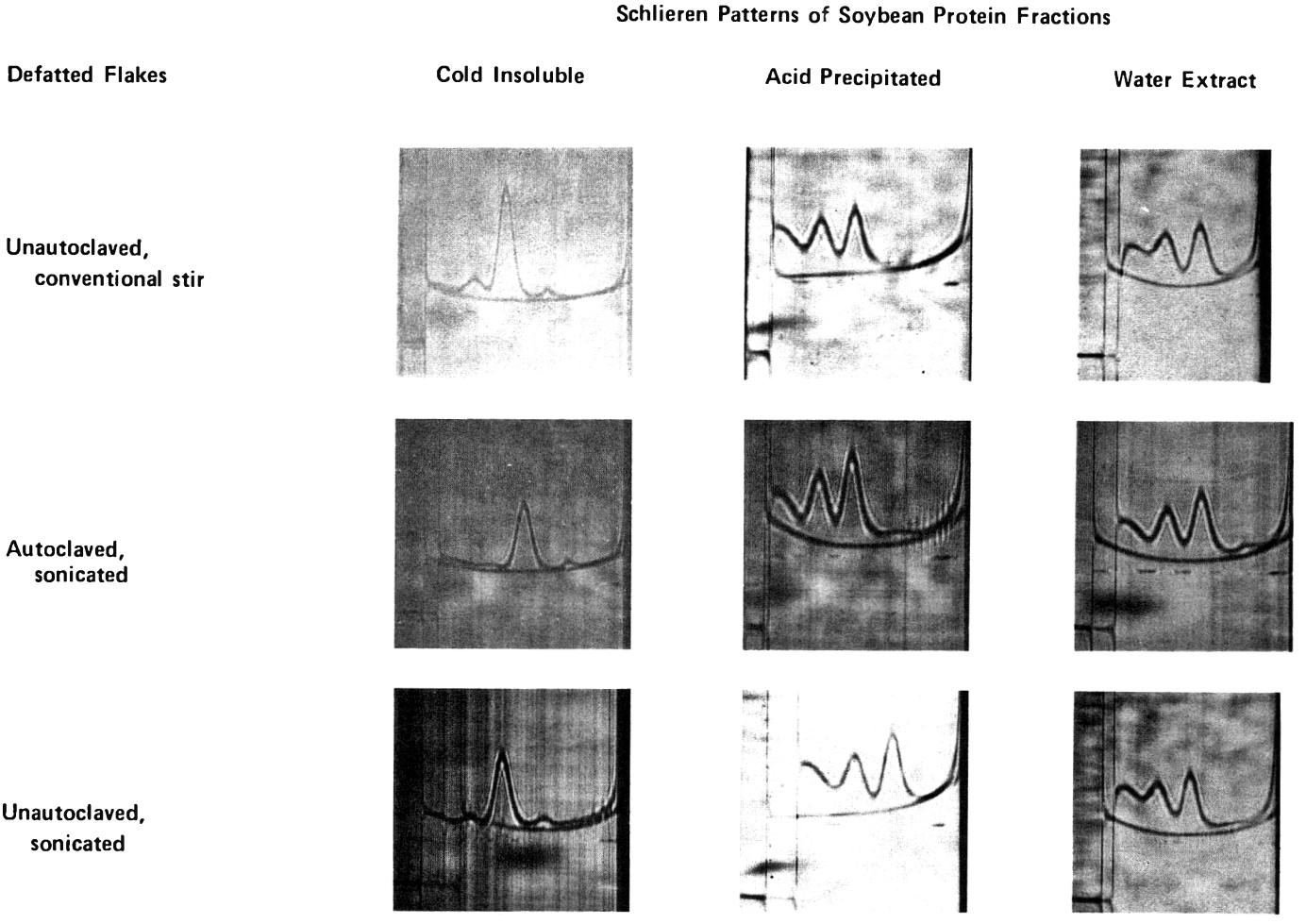

Fig. 3—Ultracentrifuge patterns (48 min after starting centrifugation) of soybean protein in cold-insoluble fractions, acid-precipitated fractions and water extracts from autoclaved and unautoclaved Kanrich defatted flakes extracted by sonication and conventional-stir.

problem of soybean proteins. Since autoclaving increases the nutritional value of proteins and also removes undesirable flavors in soybeans, proteins solubilized by sonication should have improved flavor and good functionality. These proteins are similar to those extracted by conventional-stir in some of their chemical and physical properties.

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SPROUTING OF SEEDS AND NUTRIENT COMPOSITION OF SEEDS AND SPROUTS

INTRODUCTION

THE SPROUTING of soybean (*Glycine max*) and mung bean (*Phaseolus mungo*) was developed by the Chinese centuries ago. However, limited data have been published relating to sprouting of seeds and their nutritive composition.

Young, in 1782, first observed that germinating seeds acquired antiscorbutic properties (Chayen, 1953). Several studies have shown that dry seeds have measurable amounts of ascorbic acid (Ahlberg, 1935; Bhagvat and Rao, 1942a, b; Cravioto et al., 1945), but the amount is increased by germination (Ahlberg, 1935). Varieties of beans and peas can be grown extensively in different regions. Dried shelled bean or pea seeds can be stored for an extended period. The sprouting of bean or pea seeds does not require sunshine or soil and is not limited to seasonal growth. The time of sprouting, as found in this laboratory was short (average of 4–5 days), and the yield of sprouting is high.

One purpose of this study was to develop a rapid and simple method that could be used in homes, or developed for commercial production of sprouts. With increased emphasis on the nutritive value of foods and especially the so-called natural foods, this study was also designed to determine the nutritive value of several varieties of bean and pea seeds before and after sprouting. Sprouts could be of value as a fresh, low-cost vegetable, grown indoors in any season, and could contribute vitamin C and other nutrients to the diet.

MATERIALS & METHODS

Sprouting of seeds

Untreated cellulose sponge was used for the sprouting of seeds. Six varieties of pea seeds and 12 varieties of bean seeds were obtained locally. Mature, unbroken seeds were washed and soaked in five times volume of water. For those seeds which developed mold growth during sprouting, chlorinated lime (Merck & Co., 1960) solution was used instead of water to inhibit the growth of molds. A 0.05% aqueous solution was used unless sprouting was inhibited, in which case 0.02% solution was used. Seeds were soaked overnight or for 6 hr if soft or broken testa developed.

Plain cellulose sponges, boiled in water for 10 min, were placed in sterilized, opaque plastic pans and allowed to cool to room temperature. The soaked seeds were washed thoroughly and then placed in a layer on the sponges. Fresh soaking medium (water or chlorinated lime solution) was poured to one-half height of the sponge to provide moisture during sprouting. The pan was then covered with aluminum foil to exclude light and held at room temperature (23–25°C).

The germinating seeds were washed once a day with the soaking medium if mold appeared; otherwise, the sponge was kept moist only with the soaking medium. Sprouts of 1–2 inches were collected before the development of rootlets. Sprouting time was 4–6 days.

Analytical methods

Proximate analysis, determination of five vitamins (vitamin C, thiamin, riboflavin, niacin and tocopherol), carotenes and six minerals (iron, calcium, magnesium, manganese, potassium and phosphorus) were carried out with each variety of seed before and after sprouting in duplicate. Only the sprout (hypocotyl) was analyzed since in practice the remainder of the sprouted bean is usually discarded.

Proximate analysis

Water content of seeds and sprouts was determined by drying the ground sample in a convection oven at 105°C for 5 hr (to constant weight). For the determination of ash, samples were heated at 600°C overnight. Total protein content was determined by the rapid micro-Kjeldahl method of Concon and Soltess (1973). Total lipid was extracted by a modified method of Bleigh and Dyer (1959), using a chloroform and absolute methanol mixture (2:1).

Determination of vitamins

All samples were analyzed for ascorbic acid by a modification of the 2,4-dinitrophenylhydrazine procedure of Roe et al. (1948).

Roe (1961) showed this procedure to be specific for ascorbic acid with no interference from sugars or other chromagens when the coupling reaction is run at 37°C. In confirmation of this, both Roe (1961) and our laboratory observed no increase in optical density when amounts of glucose up to 5 mg/ml were added to a 10 mcg ascorbic acid standard.

The method was modified by using an aliquot of the oxalic acid extract directly for determination of oxidized ascorbic acid. Another aliquot was used for determination of total ascorbic acid after oxidation with bromine. Reduced ascorbic acid was obtained by difference.

Total tocopherol was determined by the Emmerie-Engel reaction on the nonsaponifiable fraction of diethyl ether extract (Strohecker and Henning, 1965).

Riboflavin and thiamin were both extracted from the same sample by using a combination of Pearson's (György and Pearson, 1967) acid and enzymic extraction procedure to ensure complete extraction. The validity of the results was tested by recovery of the vitamins added as internal or external controls. The entire analysis was carried out under controlled illumination.

10g of sprouts (or 5g of pulverized seeds) were blended with 75 ml of water and transferred into a 500 ml amber pyrex flask in which all subsequent heating and incubation steps were carried out. The volume was adjusted to 150 ml and the pH to 2–3 with 0.1N HCl. 1/2g of pepsin was added and the sample was incubated at 37–40°C for 4 hr. The sample was then heated just below the boiling point on a hotplate for 1/2 hr before and after addition of 25 mg of cystine hydrochloride. After cooling to room temperature, the pH was adjusted to 4–4.5 with sodium acetate. After addition of 0.5g of diastase and 0.5g of Klerzyme (Baxter Laboratories, Staten Island, N.Y.) the sample was incubated overnight at 37–40°C.

The sample was filtered and the volume adjusted quantitatively to 500 ml. Aliquots were used for riboflavin and thiamin analysis.

Riboflavin was determined by using an irradiation blank. In the procedure, the riboflavin was extracted into a butanol-pyridine mixture after the permanganate oxidation, and the blank was prepared by destruction of the riboflavin by irradiation with ultraviolet light (Blak-Ray, Ultra-violet Products Inc., San Gabriel, Calif.).

Thiamin was determined by the chemical assay method involving alkaline oxidation of thiamin to thiochrome. The sample extract was purified by using a synthetic Zeolite Decalso column, and the eluate containing thiamin was treated with an alkaline ferricyanide solution for the production of thiochrome. The thiochrome was extracted with isobutanol for fluorometric measurements. A blank and thiamin standard was run for each series of determinations.

The carotenoids were determined by an adaption (Tichenor et al., 1965) of the chromatographic method described by the Association of Vitamin Chemists (1951). An aliquot of the sample was blended with a 12% ethanolic potassium hydroxide solution. The carotenoids were extracted with petroleum ether and read at 440 nm for an index of total carotenoids.

For niacin determination, 30g of ground seeds or fresh sprouts was mixed with 200 ml of 1N sulfuric acid solution in a blender. This mixture was then analyzed for niacin by the AOAC method (1970).

Determination of minerals

Iron, magnesium, calcium, phosphorus, potassium and manganese were analyzed by atomic absorption spectrophotometry using a Perkin-Elmer Model 303 instrument. Each sample was prepared by dissolving a weighed portion of seed ash (approximately 50 mg) or sprout ash (approximately 25 mg) in 1 or 2 ml of 2N hydrochloric acid solution. The sample was made up to a final volume of 5 or 10 ml with distilled water.

RESULTS & DISCUSSION

Sprouting of seeds

Methods reported in the literature for sprouting of seeds have used chiefly paper towels as a medium (Courter, 1972), and controlled conditions for growth of microorganisms were not stated. A piece of cheesecloth as a medium was used by Chen (1970). However, with these methods, the seeds needed to be washed every 4 hr to prevent the growth of mold. With the use of sponges the moisture content could be regulated, less washing was required, and no washing during the entire process was required for Thomas Laxton peas, Wando peas and mung beans. Table 1 gives the sprouting conditions of the seeds. Grades were given to each variety of seed to show the relative suitability for sprouting.

Proximate analyses of seeds and sprouts

The results of the proximate analyses are summarized in Table 2. With the exception of soybeans, the protein composition of all seeds analyzed fell within a relatively narrow range (18–26%). Patwardhan (1962) reported a range of 22–28% for 11 varieties of legumes. Soybeans contained 42.7% protein as opposed to the value of 34.1% for mature, dry, raw seeds reported by USDA Handbook No. 8 (Watt and Merrill, 1963).

The protein content of the sprouts (removed from the seed coat) was relatively low (2.7–5.0%). This compares with USDA Handbook No. 8 data of 3.8% for raw sprouted mung beans and 6.2% for sprouted Soybeans (Watt and Merrill, 1963). The lowered values for protein in the sprouts are, of course, primarily a result of the large increase in water content upon sprouting. Handbook No. 8 lists a value of 35 kcal of energy for 100g of sprouted mung beans containing 3.8g of protein. Assuming a value of 4 kcal/g protein and a digestibility of 80% (FAO, 1970), this would mean that approximately 35% of the available energy in the sprout comes from protein.

The lipid content of the seeds was relatively low (2.1–4.0%), with the exception of the soybeans (21%). The sprouts ranged from 0.1–0.8% in lipid. Values in Handbook No. 8 are for sprouted seeds rather than the separate sprouts and are listed as 1.4% for sprouted soybeans and 0.2% for sprouted mung beans (Watt and Merrill, 1963). Values for total ash are also shown in Table 2.

Vitamin concentrations of seeds and sprouts

As shown in Table 3, dry seeds contained from 2.2–9.0 mg/100g of total ascorbic acid. Cravioto et al. (1945) and Cravioto (1951) reported comparable values in *Phaseolus vulgaris* (up to 3.4 mg/100g), *Cicer arietinum* (1.3 mg/100g), *Vicia faba* (5.4 mg/100g) and *Lens esculata* (7.5 mg/100g). Ahlberg (1935) also showed minimal amounts of total ascorbic acid in dry seeds.

The total ascorbic acid content of the pea seed sprouts ranged from 18.8–50.0 mg/100g. The bean sprouts ranged from 12.6–42.2 mg/100g. A 100g serving of several varieties of these sprouts could exceed or come close to supplying 100% of the 1973 Recommended Dietary Allowance (National Academy of Sciences, 1973) for ascorbic acid (45 mg). Wells et al. (1963) showed varieties of green beans to differ in ascorbic acid content. They found Tenderlong 15 variety to contain the highest amount of ascorbic acid (19.8 mg/100g) in whole, fresh, uncooked beans. This is less than half the amount found in the Bunch, Executive bean sprouts (42.2 mg/100g).

Reduced ascorbic content was relatively low in some varieties (Table 3). In others it comprised the major part of the total ascorbic acid.

Table 3 also shows the amount of tocopherol and carotene in various varieties of seeds and sprouts. The content of these

Table 1–Sprouting conditions of seeds

Varieties	Soaking time	Soaking medium	Sprouting time (days)	Washing frequency	Yield (%)	Grade[a]
Peas						
Dwarf Gray	overnight	water	4–5	once/2 days	60	A
Early Alaska	overnight	water	5–6	once/2 days	60	A
Laxton Progress	overnight	0.05% c[b]	5	none	65	B
Mammoth Melting	overnight	0.05% c	5	daily	53	B
Thomas Laxton	6 hr	0.05% c	5	none	20	C
Wando	overnight	0.05% c	4	none	80	A
Beans						
Bunch, Executive	6 hr	0.02% c	5–6	daily	32	D
Bunch, Top Crop	6 hr	0.02% c	5–6	daily	65	D
Burpee, Stringless	6 hr	0.02% c	4–5	daily	48	C
Great Northern	overnight	0.05% c	4–5	daily	41	D
Tenn. Green Pod	6 hr	0.02% c	4–5	daily	60	C
Pinto	overnight	0.05% c	4–5	daily	50	B
Red Kidney	6 hr	0.05% c	5	daily	43	D
Red Valentine	6 hr	0.02% c	4–5	daily	55	C
Sulfur	6 hr	0.02% c	4–5	daily	40	C
White Navy	6 hr	0.02% c	4–5	daily	40	B
Soybean	6 hr	0.02% c	4–5	daily	60	B
Mung bean	6 hr	water	3–4	none	80	A

[a] A, excellent for sprouting; B, good for sprouting; C, poor for sprouting; D, not suitable for sprouting
[b] Chlorinated lime solution

two nutrients is low in both seeds and sprouts; however, there is considerable variation among varieties. The amount of tocopherol in some varieties of dry seeds is significant in view of the 1973 Recommended Daily Dietary Allowance of 12–15 mg for adults (National Academy of Sciences, 1973).

The thiamin, riboflavin and niacin content of seeds and sprouts is shown in Table 4. A 100g serving of some varieties of pea and bean sprouts is capable of supplying close to 1/3 of the 1973 RDA of thiamin and riboflavin (1.0 and 1.2 mg/day, respectively) for the adult female (National Academy of Sci-

Table 2—Proximate analysis of seeds and sprouts[a]

Varieties	Water (%)		Ash (%)		Protein (%)		Lipids (%)	
	Seeds	Sprouts	Seeds	Sprouts	Seeds	Sprouts	Seeds	Sprouts
Peas								
Dwarf Gray	6.5	92.9	2.6	0.4	18.7	3.9	3.2	0.5
Early Alaska	6.2	93.1	2.6	0.4	21.9	3.9	2.6	0.5
Laxton Progress	5.3	93.9	3.2	0.3	26.9	4.2	4.0	0.4
Mammoth Melting	6.6	93.0	2.9	0.5	22.7	4.0	2.7	0.6
Thomas Laxton	5.5	92.6	2.7	0.4	23.9	3.1	3.5	0.6
Wando	10.9	90.4	3.9	0.6	26.3	4.5	4.0	0.5
Beans								
Bunch, Executive	7.3	92.7	2.9	0.7	22.0	4.2	2.4	0.3
Bunch, Top Crop	9.0	93.0	3.6	0.7	19.2	3.4	2.2	0.4
Burpee, Stringless	7.9	92.8	3.4	0.3	24.4	4.3	2.5	0.4
Great Northern	10.8	93.7	3.7	0.4	21.7	3.2	2.0	0.3
Tenn. Green Pod	8.8	93.4	3.8	0.5	26.3	3.4	2.5	0.4
Pinto	10.8	92.8	4.1	0.5	21.0	3.3	2.1	0.3
Red Kidney	9.3	90.7	3.8	0.5	25.5	4.2	2.4	0.5
Red Valentine	9.1	89.1	3.5	0.8	18.6	5.0	2.6	0.6
Sulfur	11.1	92.3	3.6	0.7	22.0	4.2	2.6	0.8
White Navy	16.3	92.6	3.5	0.6	22.4	3.6	2.2	0.4
Soybean	5.7	92.3	4.4	0.4	42.7	3.8	21.0	0.5
Mung bean	10.6	91.5	3.1	0.8	24.1	2.7	2.4	0.1

[a] Analyses were made in duplicate

Table 3—Ascorbic acid, tocopherol and carotene contents of seeds and sprouts[a]

Varieties	Ascorbic acid (mg/100g)				Tocopherol (mg/100g)		Carotene (mcg/100g)	
	Seed[b]	Sprout[b]	Seed[c]	Sprout[c]	Seed	Sprout	Seed	Sprout
Peas								
Dwarf Gray	2.9	32.3	6.4	50.0	0.314	0.055	10.13	0.29
Early Alaska	4.9	24.8	9.0	44.5	0.715	0.148	37.35	0.30
Laxton Progress	1.8	26.4	3.9	46.3	0.889	0.123	16.59	2.51
Mammoth Melting	3.3	4.2	4.9	18.8	1.240	0.212	3.16	0.17
Thomas Laxton	4.7	5.3	8.9	26.9	0.024	0.198	13.00	3.63
Wando	4.9	12.1	8.1	41.2	0.037	0.124	22.50	1.55
Beans								
Bunch, Executive	1.3	24.2	2.4	42.2	0.805	0.050	2.15	0.23
Bunch, Top Crop	1.0	20.0	2.2	33.4	0.208	0.019	1.87	2.30
Burpee, Stringless	3.7	13.0	5.6	26.0	1.830	0.130	0.23	1.04
Great Northern	4.3	6.1	5.3	21.4	2.300	0.063	1.91	1.37
Tenn. Green Pod	5.1	6.0	6.4	18.6	1.610	0.195	3.10	0.24
Pinto	4.3	15.5	7.3	28.6	1.400	0.210	3.22	0.59
Red Kidney	3.8	28.8	4.5	38.7	0.718	0.048	3.04	1.79
Red Valentine	5.2	6.4	8.3	12.6	1.210	0.019	1.79	0.59
Sulfur	4.3	17.1	6.3	39.8	0.782	0.069	3.34	0.30
White Navy	2.0	10.9	3.0	22.3	1.510	0.064	2.68	4.05
Soybean	5.5	3.6	7.5	21.1	1.87	0.085	16.34	3.28
Mung bean	3.0	7.3	5.4	38.3	1.97	0.200	4.05	0.84

[a] Analyses were made in duplicate
[b] Reduced form
[c] Total

ences, 1973). The nutrient/energy ratio of these vitamins in sprouts is quite high considering that 100g of raw bean sprouts contains only 35 kcal of energy as opposed to 340 kcal in 100g of dry beans (Watt and Merrill, 1963). The niacin content of the sprouts was relatively low (Table 4). Banerjee et al. (1955) reported that the concentration of niacin, pyridoxine, pantothenic acid, inositol, biotin, tocopherol and vitamin K increases in certain legumes after germination.

Mineral concentration in seeds and sprouts

The concentration of Fe, Mg, Ca, P, K and Mn in both seeds and sprouts is shown in Table 5. The dry seeds generally were a good source of iron, and appreciable amounts were also

Table 4—Thiamin, riboflavin and niacin content of seeds and sprouts[a]

Varieties	Thiamin (mg/100g)		Riboflavin (mg/100g)		Niacin (mg/100g)	
	Seed	Sprout	Seed	Sprout	Seed	Sprout
Peas						
Dwarf Gray	0.80	0.19	0.16	0.11	3.97	0.90
Early Alaska	0.89	0.30	0.24	0.24	2.18	1.22
Laxton Progress	0.68	0.26	0.35	0.38	1.99	0.63
Mammoth Melting	1.07	0.29	0.23	0.30	2.71	0.96
Thomas Laxton	0.89	0.25	0.19	0.25	2.12	1.56
Wando	1.27	0.25	0.36	0.24	3.56	3.48
Beans						
Bunch, Executive	0.49	0.33	0.28	0.28	1.74	2.31
Bunch, Top Crop	0.37	0.17	0.15	0.19	2.71	2.64
Burpee, Stringless	0.44	0.22	0.32	0.19	2.00	2.01
Great Northern	0.94	0.20	0.21	0.11	1.05	2.51
Mung bean	0.43	0.12	0.22	0.21	2.30	4.12
Pinto	0.88	0.24	0.14	0.18	2.20	2.30
Red Kidney	1.39	0.37	0.17	0.25	3.14	2.92
Red Valentine	0.73	0.16	0.24	0.10	1.27	1.96
Soybean	1.03	0.18	0.32	0.15	2.20	2.72
Sulfur	0.69	0.16	0.33	0.29	2.43	2.04
Tenn. Green Pod	0.86	0.26	0.21	0.27	3.26	1.77
White Navy	1.08	0.46	0.25	0.20	2.01	0.62

[a] Analyses were made in duplicate; values are expressed on a wet weight basis.

Table 5—Mineral composition of seed and sprouts[a]

Varieties	Fe (mg/100g)		Mg (mg/100g)		Ca (mg/100g)		P (mg/100g)		K (mg/100g)		Mn (mg/100g)	
	Seed	Sprout	Seed	Sprout	Seed	Sprout	Seed	Sprout	Seed	Sprout	Seed	Sprout
Peas												
Dwarf Gray	6.52	0.55	140	17	94	13	307	20	736	94	16.1	0.9
Early Alaska	5.65	1.24	141	22	94	20	311	36	708	145	19.5	12.9
Laxton Progress	4.39	0.53	118	13	68	29	371	44	923	112	7.0	1.2
Mammoth Melting	5.54	0.84	135	15	97	13	354	40	763	157	20.8	6.8
Thomas Laxton	5.88	0.82	146	15	89	13	383	75	737	133	11.2	1.0
Wando	8.32	1.14	171	17	118	19	471	52	1299	215	18.7	4.2
Beans												
Bunch, Executive	5.18	0.70	154	26	88	15	353	70	849	187	18.3	2.1
Bunch, Top Crop	6.94	0.59	164	23	107	15	450	29	1068	100	22.9	9.1
Burpee, Stringless	6.92	0.47	222	21	135	18	357	57	1198	253	15.5	10.2
Great Northern	1.29	0.48	222	24	157	18	379	46	1234	153	17.3	13.0
Tenn. Green Pod	7.31	0.49	224	21	175	13	441	53	1270	173	12.5	10.7
Pinto	7.51	0.54	162	20	103	16	509	50	1526	187	18.9	11.0
Red Kidney	2.20	0.81	194	21	125	17	428	37	1274	187	21.9	9.0
Red Valentine	7.13	0.59	171	29	148	22	314	85	1237	285	20.6	13.8
Sulfur	7.01	0.90	222	22	166	42	488	76	1042	256	34.3	1.8
White Navy	8.30	1.06	183	19	166	15	365	62	1085	205	23.2	9.1
Soybean	9.53	0.60	200	15	318	32	427	12	1362	157	18.2	10.2
Mung bean	6.18	1.89	221	36	307	43	327	28	961	276	29.8	3.8

[a] Values are expressed on a wet weight basis.

Table 6—Effect of sprouting medium on calcium composition of sprouted seeds and sprouts (mg/100g)[a]

	Sprouting medium (% chlorinated lime)					
	0%		0.02%		0.05%	
Varieties	Sprouts	Sprouted seeds	Sprouts	Sprouted seeds	Sprouts	Sprouted seeds
Soybean	27.9	45.6	31.6	49.4	34.1	56.2
Wando pea	38.0	16.1	47.1	21.0	51.6	27.9

[a] Values are expressed on a wet weight basis.

found in the sprouts considering their lower energy content. The mung bean sprouts were the highest in both magnesium and calcium. Patwardhan (1962) also reported a relatively high concentration of calcium in mung beans as opposed to most other legume seeds.

The greatest variability was found in the manganese concentration of the sprouts. Whereas, there was a fivefold variability among the manganese concentration of the dry seeds there was a 14-fold variability among the sprouts. This is significant in view of the recent report of Leveille (1974), that manganese concentration of some varieties of vegetables can vary as much as 14-fold depending on the location in which they are grown. Variety and seasonal differences were also shown to affect the manganese concentration (Leveille, 1974).

Availability of the mineral nutrients in seed sprouts has not been studied to any extent. Singh and Banerjee (1953) reported a greater availability of iron after germination. Belavady and Banerjee (1953) reported a decrease in phytate phosphorus upon germination of legumes.

Effect of sprouting medium on calcium composition

The concentration of calcium in the sprouts and sprouted seeds of two varieties is shown to be directly related to the concentration of chlorinated lime in the sprouting medium (Table 6). Chlorinated lime consists of varying proportions of $Ca(OCl)_2$, $CaCl_2$, $Ca(OH)_2$ and H_2O (Merck and Co., 1960).

This study shows that selected varieties of sprouted seeds may be a significant source of nutrients. The level of ascorbic acid is dramatically increased during germination of the seeds. In addition, the concentration of protein, some B-vitamins and minerals may make a considerable contribution to human dietaries when considered in light of the high nutrient/energy ratio in sprouted seeds.

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SEVERAL FACTORS AFFECTING COLOR, TEXTURE AND DRAINED WEIGHT OF CANNED DRY LIMA BEANS

INTRODUCTION

THE LEGUMES are an important source of vegetable proteins in many parts of the world. White (1970) made a survey on consumer use of dry beans, peas and lentils. She concluded that bean consumption might be increased through development of spicy, quick-cooking or ready-prepared bean dishes with built-in appeal.

Occasionally, canned lima beans show a gray discoloration which influences the marketability of the product.

In the present investigation, White Ventura 65 and B-51-114 dry lima beans were compared for their properties after canning. The effects of soaking methods, chelating agents and calcium ions on quality of the canned product are reported.

MATERIALS & METHODS

Dry lima beans

100 lb each of White Ventura 65 and B-51-114 dry large lima beans (*Phaseolus lunatus* L.) grown at Westley, King City and Santa Maria areas of California were supplied by Professor Carl L. Tucker of the Dept. of Agronomy, University of California, Davis. The dry beans were kept at room temperature in tightly covered 5-gal cans for 1 month prior to canning. The moisture content of the dry beans was between 11–12%. White Ventura 65 dry large limas grown on the University Farm at Davis were also used for some of the experiments.

Canning procedures

Rehydration. In the rehydration experiment, 2.2-kg batches of dry beans were soaked in solutions containing 0, 0.25 and 0.50% citric acid in distilled water. One part of dry beans was immersed in four parts of soaking medium at room temperature (20–22°C) for 12 hr.

In the EDTA experiments, 2.2 kg of dry beans were soaked in four parts water containing 0.5% EDTA · Na_2 (ethylenediaminetetraacetic acid disodium salt) for 12 hr.

In the calcium chloride study, 2.2-kg batches of dry beans were soaked at room temperature for 12 hr in 8.8 liters of distilled water containing 0, 0.10 and 0.30% $CaCl_2 \cdot 2H_2O$.

Blanching. The soaked beans were drained on a stainless-steel screen, rinsed with tap water and then blanched in water or steam at 100°C for 10 min or a specified time, allowing 5 min for preheating. The blanched beans were drained, rinsed with tap water, poured into a stainless-steel tub containing 3 volumes of ice water, kept there for 5–15 min, and then drained.

Canning. The blanched beans were sorted to remove defective ones. 250g of sorted beans and 230 ml of brine were added to each 303 × 406 enameled cans (National Can Company, #130). The standard brine contained 1.5% salt, 3% sugar and 0.1% monosodium glutamate in distilled water (Raab et al., 1973).

Effect of calcium chloride on textures. $CaCl_2 \cdot 2H_2O$ (C.P. grade) was added to the standard brine in the amounts of 0, 0.1, 0.2, and 0.3% (w/v).

Effect of EDTA · Na_2. Ethylenediaminetetraacetic acid disodium salt was added to the standard brine in the amounts of 0, 100, 300, and 500 ppm.

The cans were sealed at room temperature under a vacuum of 22 in. Hg in a Rooney semi-automatic vacuum sealer and heat processed in a retort at 118.3°C for 30 min, with a come-up time of 5 min. After retorting, the canned beans were cooled in tap water for 10 min, using compressed air to replace steam during the cooling periods. The canned products were stored at 7.2°C prior to chemical analysis and quality evaluation.

Analytical procedures

Drained weight. The contents of each can were drained for 5 min on an 8-in. screen to remove the brine. The weight of the drained beans was determined. The average of four determinations for each sample was recorded.

pH. The pH of brine was determined with a Beckman Zeromatic SS-3 pH meter.

Titratable acidity. 100g of drained beans were macerated with 100 ml of distilled water in a Waring Blendor for 2 min. 20g of the resulting puree was diluted with 100 ml of distilled water and titrated to pH 8.0 with 0.1N sodium hydroxide. The average of four determinations was reported as percent citric acid in the drained beans.

Total phenols. Total phenols were determined by the Folin-Denis phosphomolybdate colorimetric method (Syn and Luh, 1965).

Total solids. 2g of diatomaceous earth were placed into each aluminum dish and dried at 100–110°C for 1 hr. The dishes were cooled in a desiccator for 30 min and weighed with a Mettler analytical balance. Approximately 10g of sample were placed into the aluminum dish and weighed. The sample was then mixed thoroughly with the diatomaceous earth, and placed on a steam bath until reaching apparent dryness. The dish was dried in a vacuum oven for 3 hr at 70°C under a vacuum of 29 in. Hg. After drying, the dishes were cooled in a desiccator for 30 min and weighed again. The percent total solids in the sample was then calculated.

Color. A Gardner model AC-1 automatic color difference meter was used to measure the color of the drained beans. A light yellow porcelain plate with $Rd = 60.7, a = -2.1, b = +22.3$ was used as a reference plate. The results are reported as Rd (brightness), a and b values.

Texture. A Lee-Kramer Shear Press equipped with an electronic recording attachment was used for texture evaluation (Mohammadzadeh-Khayat and Luh, 1968). The average of three determinations was reported. 55g of canned beans were placed in a cylindrical cell (5.25 cm i.d. × 4.3 cm inside ht) in each determination. The plunger descended at a speed of 50 ± 2 sec/stroke. A 500-lb gauge ring was used. The area under the curve was measured with a planimeter. The results are expressed as the area under the curve in sq in. (Binder and Rockland, 1964).

Metal ions. Copper, iron and calcium ions were determined with a Perkin-Elmer model 303 atomic absorption spectrophotometer (AOAC, 1970).

5g of drained beans were accurately weighed into a 800-ml Kjeldahl flask and mixed with 10 ml of 7:1 (v/v) perchloric-sulfuric acid mixture. The solution was kept at room temperature overnight. It was then treated with 45 ml of conc nitric acid, and digested on an electric heater until most of the nitric oxide fumes were expelled. After cooling, 15 ml of distilled water was added. The solution was mixed thoroughly. The Kjeldahl flask was washed three times with 5-ml portions of water. The filtrate and washings were combined and diluted to 50 ml in a volumetric flask. A 10-ml aliquot of digested sample solution was pipetted into a 25-ml volumetric flask and diluted to volume for analysis of calcium and copper ions.

Calcium. Calcium standard in lanthanum and sulfuric acid was prepared from dried C.P. grade $CaCO_3$ in 25-ml quantities of 2, 5, 10, 20, 40 and 60 μg/ml calcium. Enough sulfuric acid was added to provide 1% in diluted solution. Total volume was increased to about 12 ml with distilled water. 5 ml of the stock lanthanum solution was added to avoid interference caused by phosphorous. The flask was filled to 25 ml with distilled water. The samples were analyzed for calcium at a wavelength of 422.7 nm.

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Table 1—Effect of soaking in acidified water on color of canned large lima beans, White Ventura 65 variety, 6 months after canning

Code	Soaking medium	pH of brine	Total acidity in beans %	Gardner colorimeter readings Rd	a	b	Avg visual color score[a]
A	Distillec water	6.16	0.19	25.1	+0.4	+15.5	5.2
B	Distillec water + 0.25% citric acid	5.85	0.19	27.8	+0.3	+16.2	6.5
C	Distillec water + 0.50% citric acid	5.65	0.22	27.8	+0.5	+17.3	6.8
LSD	(P = 0.05)	0.09					1.2

[a] Based on a 1–10 scale: Excellent, 9–10; good, 7–8; fair, 5–6; poor, 3–4; very poor, 1–2.

Copper. For copper analyses, the wavelength was set at 324.7 nm.

Iron. For iron analyses, the wavelength was set at 248.3 nm.

Total nitrogen and crude protein. The Kjeldahl method described in AOAC (1970) for determination of total nitrogen and crude protein was used.

Pectin. For total pectin the versene-pectinase carbazole method described by McCready and McComb (1952) was utilized.

Sensory evaluation. A panel of 15 judges evaluated the color, texture and flavor of the canned samples. Two cans of each sample were used for each evaluation. Separate sets of samples were placed at random in small paper cups under dim light in separate booths. The judges were asked to evaluate texture and flavor on a 1–10 scale: excellent, 9–10; good, 7–8; fair, 5–6; poor, 3–4; and very poor, 1–2.

For color evaluation, the judges were asked to proceed to another booth under a standard light source approaching daylight. The method of score was the same as that for texture and flavor on a 1–10 scale.

The results of sensory evaluations were subjected to analysis of variance. The least significant difference (LSD) at the 95% probability level (P = 0.05) was calculated.

RESULTS & DISCUSSION

Rehydration in acidified water

Experiments were carried out to study the effect of rehydration in acidified water containing 0.25 and 0.50% citric acid on the color of the canned product. The results are presented in Table 1.

It was observed that the color of the canned beans was improved by soaking the dry beans in acidified water for 12 hr prior to processing. This was evidenced by the increase in Gardner *Rd* value from 25.1 in the control to 27.8 in the acid-soaked samples. The panel gave a higher visual color score to the acid-soaked samples than to the control. The results are significant at the 95% probability level. There was no significant difference in color score between the acidified samples.

The improvement in color of the canned beans through soaking in acidified water may be related to the lowering in pH value of the canned beans from 6.16 to 5.85 and 5.65, respectively, in the acid-soaked samples.

It is likely that citrate ion can form complex ions with the trace elements such as copper or iron, making them unavailable for reactions with the phenolic compounds and sulfides which tend to cause discoloration in the canned beans.

Effect of acidified brine

Color. The effect of acidified brine on the color of the canned beans is presented in Table 2. Acidification of the brine with citric acid at 0.25% and 0.50% levels improved the color of the canned product. It is postulated that the beans contain sulfur-containing amino acids and proteins which can yield hydrogen sulfides during the heat sterilization process. The sulfides thus formed will react with copper ions resulting in a grayish color. When citric acid was added, the formation of gray colored compounds decreased which may be due to: (a) lowering of the pH of the medium causing dissociation of metal copper sulfide complexes; and (b) formation of complex ions between citrate and copper, making it unavailable to the sulfide.

Texture Addition of citric acid to the brine increases the firmness of the canned beans (Table 3). The phenomenon may be explained by the denaturation of some proteins and suppression of hydration of the proteins and starch in the beans by the acid.

pH and drained weight. The pH and drained weight of the canned beans (B-51-114 variety) as influenced by the acidified brine are presented in Table 4. The pH of the brine decreased as the level of citric acid in the brine increased. Acidification of the brine with citric acid results in a lower drained weight.

The decrease in drained weight may be explained by the decrease in hydration of proteins and starch resulting in some shrinkage in volume of the canned beans.

Table 2—Effect of acidified brine on visual color of canned large lima beans, 4 months after canning

Treatment	Brine	Average visual color score[a] B-51-114 Westley	B-51-114 King City	B-51-114 Santa Maria	White Ventura 35 Westley	White Ventura 35 King City	White Ventura 35 Santa Maria
A	Control[b]	4.7	4.5	4.6	5.7	4.5	5.0
B	Control + 0.25% citric acid	6.7	7.8	7.6	6.7	8.0	6.8
C	Control + 0.50% citric acid	8.0	8.7	7.8	8.2	8.8	7.2
LSD	(P = 0.05)	0.7	0.9	0.7	1.1	1.1	0.9

[a] Panel of 15 judges scored the color of each variety from the three growing areas on a hedonic scale of 1–10: Excellent, 9–10; good, 7–8; fair, 5–6; poor, 3–4; very poor, 1–2.

[b] The control brine contained 1.5% salt, 3% cane sugar and 0.1% monosodium glutamate in water.

Table 3–Texture of canned large lima beans as affected by adding citric acid to the brine

Treatments	Brine	Lee-Kramer shear press readings, (sq in. X 6.25)											
		B-51-114 variety						White Ventura 65 variety					
		Westley (inland)		King City		Santa Maria (coast)		Westley (inland)		King City		Santa Maria (coast)	
		2 mo	4 mo	2 mo	4 mo	2 mo	4 mo	2 mo	4 mo	2 mo	4 mo	2 mo	4 mo
A	Control[a]	6.38	7.81	6.63	7.81	7.63	7.88	7.38	8.00	7.37	8.00	10.13	7.81
B	Control + 0.25% citric acid	8.38	8.63	8.50	11.44	7.88	8.31	10.00	11.75	11.50	12.50	17.25	13.88
C	Control + 0.50% citric acid	8.75	9.63	10.75	13.13	7.55	11.25	12.38	12.94	13.50	13.50	18.00	17.06

[a] The control contains 1.5% salt, 3% cane sugar and 0.1% monosodium glutamate in water.

Polyphenols and metal ions. Polyphenolic compounds are known to be capable of forming colored complexes with copper ions. The tannin-like substances in the canned beans were determined as total phenols by the Folin-Denis reagent. The results are presented in Table 5. There was no significant difference in level of total phenols between the B-51-114 and White Ventura 65 varieties. The Folin-Denis reagent reacts with the aromatic hydroxyl groups of phenolic compounds to form a deep blue color due to the reduction of the phosphomolybdate reagent. Under the conditions of this investigation, the levels of total phenols in the beans from three growing areas were approximately equal.

Copper and iron. The copper and iron contents of the drained beans are shown in Table 5. The level of copper in the

Table 4–Effect of acidified brine on pH and drained weight of canned dry large lima beans, 4 months after canning

Treatments	Brine store	Westley (inland)		King City		Santa Maria (coast)	
		pH of brine	Drained wt (g)	pH of brine	Drained wt (g)	pH of brine	Drained wt (g)
B-51-114 Variety							
A	Control[a]	6.14	317.3	6.15	307.2	6.15	319.3
B	Control + 0.25% citric acid	5.58	298.6	5.58	277.7	5.52	301.8
C	Control + 0.50% citric acid	5.21	292.4	5.20	274.6	5.20	297.0
White Ventura 65 Variety							
A	Control[a]	6.09	314.9	6.10	313.5	6.12	312.8
B	Control + 0.25% citric acid	5.54	308.3	5.53	299.4	5.55	311.6
C	Control + 0.50% citric acid	5.15	302.9	5.13	291.5	5.17	304.8
LSD	(P = 0.05)	0.10	4.5	0.09	3.8	0.11	4.3

[a] The control brine contained 1.5% salt, 3% sugar and 0.1% monosodium glutamate.

Table 5–Total phenols and metal ions in canned large lima beans[a]

Variety	Growing area	Total phenols (mg/100g)	Copper (mg/100g)	Iron (mg/100g)	Calcium (mg/100g)	Total solids
B-51-114	Westley	31.6	0.223	2.52	11.68	29.41
White Ventura 65	Westley	30.3	0.178	2.11	5.94	29.39
B-51-114	King City	30.1	0.231	1.88	9.38	29.01
White Ventura 65	King City	30.1	0.156	1.73	7.99	28.21
B-51-114	Santa Maria	31.5	0.163	2.77	8.30	29.45
White Ventura 65	Santa Maria	30.6	0.126	2.36	6.85	29.36
LSD	(P = 0.05)	1.5	0.028	0.16	0.70	1.52

[a] The beans were drained prior to analysis.

Table 6—Effect of adding calcium chloride to the brine or the soaking water on color and texture of canned large lima beans, White Ventura 65 variety, Davis, Calif.

Code	Soaking medium	$CaCl_2 \cdot 2H_2O$ added to brine %	pH of brine	Gardner color readings Rd 2 mo	Rd 6 mo	a 2 mo	a 6 mo	b 2 mo	b 6 mo	Texture[a] sq in. X 6.25 2 mo	Texture[a] sq in. X 6.25 6 mo
A0	Distilled water	none	6.10	25.5	23.7	1.4	1.2	9.5	9.2	7.1	8.4
A1	Distilled water	0.1	6.00	26.3	27.4	1.2	0.4	9.6	9.2	7.0	9.0
A2	Distilled water	0.2	6.00	26.6	28.1	0.9	0.5	9.7	9.1	11.0	12.5
A3	Distilled water	0.3	6.00	29.7	28.9	0.7	0.7	9.3	9.3	13.1	15.9
B	$CaCl_2 \cdot 2H_2O$ (0.1%)	none	6.10	24.6	23.1	1.0	1.1	9.3	10.5	7.6	8.1
C	$CaCl_2 \cdot 2H_2O$ (0.3%)	none	6.10	25.6	23.9	1.4	1.0	9.1	8.7	9.8	11.1
LSD	(P = 0.05)		0.09							0.7	0.6

[a] Texture was measured with a Lee-Kramer shear press.

Table 7—Effect of calcium chloride treatments on the sensory quality and calcium content of canned large lima beans, White Ventura 65 variety, 6 months after canning

Code	Soaking medium	$CaCl_2 \cdot 2H_2O$ added to brine (%)	Average sensory score Color	Texture	Flavor	Calcium content drained beans (mg/100g dry basis)
A0	Distilled water	none	5.6	6.0	6.7	20.3
A1	Distilled water	0.1	6.3	6.4	6.5	50.9
A2	Distilled water	0.2	6.9	7.2	6.4	88.6
A3	Distilled water	0.3	7.9	7.3	6.9	132.4
B	Distilled water + 0.1% $CaCl_2 \cdot 2H_2O$	none	7.2	7.2	7.6	47.4
C	Distilled water + 0.3% $CaCl_2 \cdot 2H_2O$	none	7.8	8.9	7.3	78.4
LSD	(P = 0.05)		0.8	1.2	1.0	1.2

product may have some bearing on the discoloration of the canned products. It is important to avoid contamination of the beans with copper during harvesting, handling and canning. The difference in soil properties may also contribute to the difference in levels of copper ions in the beans.

Calcium. The calcium content of the canned beans are presented in Table 5. It appears that the B-51-114 variety contained more calcium than the White Ventura variety. Similar trends were found in the beans from the three growing areas.

Effect of calcium chloride

It is generally known that addition of small amounts of calcium ions can improve the texture of certain canned foods (Mohammadzadeh-Khayat and Luh, 1968).

Calcium chloride was added either to the brine during canning, or to the soaking medium prior to blanching. The color and texture of the canned beans after storage for 2 and 6 months were evaluated. The drained beans contained 20.86% protein on the dry basis (N X 6.25), and 29.6% total solids.

Color of the canned beans was improved by adding calcium chloride to the brine during canning. The *Rd* values of the treated samples were higher than those of the control (Table 6). Visual color scores made by the panel also indicate an improvement in color of the canned product resulting from $CaCl_2$ treatment (Table 7).

The effect of adding calcium chloride to the brine on texture of the canned products was measured with a Lee-Kramer shear press recorder. The results are presented in Table 6. It is apparent that addition of $CaCl_2$ to the brine resulted in a firmer texture in the canned product. Soaking the dry beans in water containing 0.1 and 0.3% $CaCl_2$ (Samples B and C) also increased the firmness of the beans. The texture of the beans was firmer in the samples stored for 6 months after canning than in those stored for 2 months only. This phenomenon may be explained by the diffusion of calcium ions into the beans after canning. The calcium ions can combine with the pectin in the cell walls to strengthen the binding between the cells (Mohammadzadeh and Luh, 1968).

Organoleptic data of the canned beans 6 months after canning are presented in Table 7. The panel detected differences in firmness between the samples with and without 0.2% $CaCl_2 \cdot 2H_2O$ added to the brine. Soaking the dry beans in water

Table 8—Pectin in canned lima beans with calcium chloride treatment

Code	Soaking medium	$CaCl_2 \cdot 2H_2O$ added to brine (%)	Total pectin in drained bean on dry basis (%)
A0	Distilled water	none	6.6
A1	Distilled water	0.1	7.4
A2	Distilled water	0.2	7.5
A3	Distilled water	0.3	8.4
B	Distilled water + 0.1% $CaCl_2 \cdot 2H_2O$	none	7.2
C	Distilled water + 0.3% $CaCl_2 \cdot 2H_2O$	none	7.5

Table 9—Effect of EDTA · Na_2 on color and texture of canned large lima beans, White Ventura 65 variety grown in Davis, Calif., 6 months after canning

Code	Soaking medium	EDTA · Na_2 added to brine ppm	Gardner colorimeter readings Rd	a	b	Average visual color score	Texture Lee-Kramer shear press sq in. X 6.25	Textural score
A1	Distilled water	none	23.6	1.8	9.7	4.1	7.8	6.7
A2	Distilled water	100	23.1	1.7	10.7	5.2	9.4	6.6
A3	Distilled water	300	28.9	1.3	15.1	6.4	8.9	6.9
A4	Distilled water	500	28.7	1.1	15.2	7.1	7.9	7.3
B	0.5% EDTA · Na_2	none	29.8	1.7	16.0	8.8	10.1	7.3
LSD	(P = 0.05)					0.6	0.3	1.0

[a] On a 1–10 scale: Excellent, 9–10; good, 7–8; fair, 5–6; poor, 3–4; very poor, 1–2.

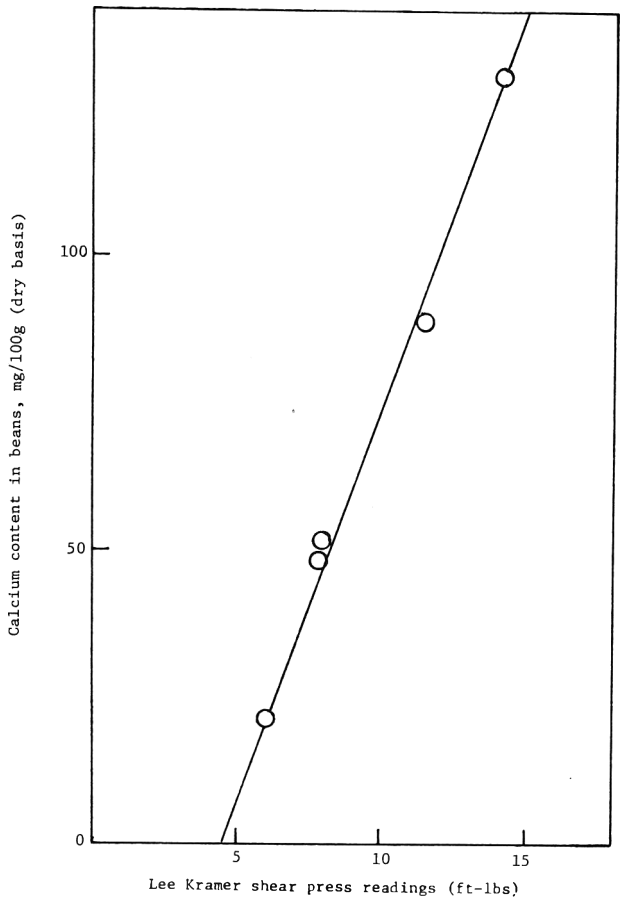

Fig. 1—Relationship between calcium content and Lee-Kramer shear press readings of canned dry large lima beans.

containing 0.1 and 0.3% $CaCl_2 \cdot 2H_2O$ also resulted in a firmer texture. There was no significant difference in flavor score of the beans at the 95% probability level.

Calcium content. The distribution of calcium ion between the drained beans and the brine are presented in Table 7. About 80% of the calcium was in the beans, and the remainder in the brine. Addition of calcium chloride to the brine resulted in an increase in calcium content both in the beans and in the brine.

The relationship between calcium content of the beans and the texture (Lee-Kramer Shear Press readings) is shown in Figure 1. In the concentration range of this experiment, a linear relationship exists between calcium contents and the shear press readings. Beans of firmer texture were higher in calcium content.

Pectin. The total pectin contents of the canned beans as related to $CaCl_2$ in the brine are presented in Table 8. When calcium chloride content was increased in the brine, the total pectin content in the drained beans was increased. The calcium ions can combine with the pectin in the cell walls to form a bridge, causing a firmer texture in the canned product.

Effect of treatment with EDTA disodium salt

The compound EDTA disodium salt can prevent discoloration in foods because of its ability to immobilize metal ions through chelation (Martell et al., 1952) incorporating it into a ring-like texture. Metal ions, particularly iron and copper, can catalyze the oxidation of ortho-dihydric phenols to form quinones which then polymerize to form brown or grayish colored polymers (Swain, 1962). Polyphenolic compounds such as catechin and chlorogenic acid, aside from being prone to oxidation, also possess the ability to bind iron or copper ions to form colored complexes.

Color. The Gardner difference meter readings of the canned beans are presented in Table 9. The color of beans was improved when the EDTA · Na_2 concentration in the brine was increased. Either soaking the dry beans in EDTA · Na_2 solution or adding EDTA · Na_2 to the brine was effective in improving the color of the canned products. The improvement in color of the canned beans may be attributed to the binding of iron or copper ions with EDTA · Na_2.

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FOLIC ACID CONTENT OF CANNED GARBANZO BEANS

INTRODUCTION

ONE OF THE important factors to be considered in food processing is retention of nutrients. Information concerning the nutritional effects during cooking, processing and storage of food stuffs has been published by Bender (1966), Schweigert (1966), Hurdle et al. (1968), Sukewer et al. (1970), Mitsuda (1971) and Schroder (1971).

The principal biochemical reactions in which folate takes part in man are: (a) protein synthesis—interconversion of serine and glycine; interconversion of histidine and glutamic acid; and methionine biosynthesis; and (b) nucleic acid synthesis—synthesis and catabolism of purines and pyrimidines; and DNA synthesis (Rothman, 1970). The biochemistry of folic acid and related pteridines has been reviewed by Blakely (1969) and Robinson (1966).

Folic acid is a vitamin required for growth, reproduction and prevention of anemia in animals and for treatment of several types of anemia in human beings. Beans are considered a good source of folic acid. Although there are a number of studies concerning folic acid content of beans (Ives et al., 1946; Toepfer et al., 1951; Chung et al., 1961), information concerning that of garbanzo beans (*Cicer arietimum*) is lacking.

In the present study, the folic acid content of canned garbanzo beans was determined by the microbiological assay method, using *Streptococcus faecalis* (ATCC 8043) as test organism. Chicken pancrease enzyme treatment of the samples was applied to obtain extracts containing all the folic acid in forms utilized by the microorganism, measurable as total folic acid content. The effects of soaking, blanching and thermal processing on folic acid retention in canned garbanzo beans were also studied.

MATERIALS & METHODS

Garbanzo beans

100 lb of dry garbanzo beans (*Cicer arietinum*) were supplied by the California Dry Bean Advisory Board, Dinuba, Calif.

Canning

Unless otherwise stated, one part of dried beans was soaked in three parts of water for 12 hr at room temperature (22–25°C). The soaked beans were drained, rinsed twice, covered with water in an enamelled pan, and blanched in a steam chamber for 10 min at 100°C, allowing 5 min of come-up time. The blanched beans were drained on a stainless steel screen, cooled in an ice water bath for 3 min, drained again and sorted to remove moldy and defective ones. 250g of blanched beans were added to each 303 × 406 enamelled can (National Can Co., #130) together with 230 ml of brine containing 3.0% sucrose, 1.5% NaCl and 0.1% MSG (monosodium glutamate). The cans were sealed under a vacuum of 22 in. Hg, and retorted in a stationary retort at 118.3°C for 30 min with 5 min come-up time (Raab et al., 1973). The heat processed cans were cooled in water, dried, kept at room temperature for 1 wk, and then stored at 7.2°C.

Effect of soaking, blanching and heat processing

Soaking. 5 kg of dry garbanzo beans were soaked in 15 liters of distilled water at room temperature for 12 hr. The excess water was discarded. A 200-g sample of soaked beans was frozen in covered glass jars at −18°C for folic acid determination. The remainder was used for the blanching study.

Water blanching. 200g portions of soaked beans were blanched in 200 ml of distilled water at 100°C for 0, 5, 10 and 20 min.

Steam blanching. 200g portions of soaked beans were blanched with steam at 100°C for 0, 5, 10 and 20 min.

Cooling. After both steam and water blanching, the beans were immediately dipped into ice water. The beans were drained and sorted to remove defective ones. Representative samples were frozen in tightly covered bottles and kept at −18°C in the dark until needed for folic acid analysis. The remaining soaked beans were blanched with either steam or water at 100°C for 10 min. The blanched beans were used for heat processing studies.

Heat processing. Each No. 303 enamelled bean can was filled with 250g of blanched beans and 230 ml of brine. Two different brine compositions were used:

Standard brine. The standard brine contained 3.0% sugar, 1.5% NaCl and 0.1% monosodium glutamate in distilled water.

Brine with 0.2% ascorbic acid. The standard brine was treated with 0.2% ascorbic acid to prevent photo oxidation (Toennies et al., 1956).

The filled cans were sealed under a vacuum of 22 in. Hg in a Rooney semi-automatic double seamer. The beans were heat processed in a vertical stationary retort at 118.3°C for 29.8, 38.5, 46.5 and 53.4 min corresponding to lethal values (F_0) of 8, 13, 16 and 20 respectively. The time necessary to achieve the F_0 values was calculated on the basis of an initial temperature of 27°C. The come-up time was 5 min to preheat the cans in the retort before timing. The cans were cooled immediately after heat processing in the retort with cold water while compressed air was used to replace steam pressure.

Preparation of bean samples for folic acid assay

The dry beans were first ground in an Enterprise meat grinder, using a plate with 5 mm holes. A portion of these partially ground samples was reground in a Wiley grinder with a 20-mesh screen. The ground samples were stored in the dark in closed glass bottles at −18°C.

Soaked beans and blanched beans

200g of soaked or blanched beans were homogenized with 200 ml of distilled water for 30 sec in a Waring Blendor. Portions of this homogenate were frozen in closed glass bottles at −18°C.

Canned beans

Four cans from each processing treatment were taken randomly as a representative sample.

To determine the free and total folic acid in the whole can the beans and brine were homogenized for 60 sec in a Waring Blendor. Portions of the sample were frozen in sealed jars at −18°C.

Extraction of folic acid

The extraction procedure described by Toepfer et al., (1951) and Hoppner (1971) was adapted. 1g of ground dry beans or 10g of homogenized beans was transferred to an Erlenmeyer flask containing 40 ml of 0.2M phosphate-ascorbate buffer, pH 7.2 (2.723g KH_2PO_4, 0.560g NaOH and 0.2g ascorbic acid diluted to 100 ml with distilled water) and 50 ml water. Three drops of 1-octanol were added to prevent foaming. The mixture was heated in an autoclave for 15 min at 121°C. When the mixture was cool, 20 mg of Bacto-chicken pancreas enzyme preparation, first wet with a drop of glycerol and suspended in 5 ml of water, was added. The enzyme was omitted at this point if free folic acid was being assayed. After adding 2–3 ml toluene, the mixture was incubated at 37°C for 24 hr. The flasks and contents were autoclaved for 3 min at 121°C. After cooling, the mixture was filtered through a Whatman No. 1 filter paper. Aliquots of the filtrate were diluted with ascorbate buffer (pH 6.1) to the desired concentration of 0.5–2.0 ng of folic acid per ml. To prepare the ascorbic buffer, 27.8g $NaH_2PO_4 \cdot H_2O$ was dissolved in 1000 ml distilled water (solution A) and 71.7g $Na_2HPO_4 \cdot 12H_2O$ in 1000 ml (solution B). Then 212.5 ml

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of solution A and 37.5 ml of solution B were diluted to 1000 ml with distilled water. The pH should be 6.1. Before using, the buffer was treated with ascorbic acid (2 mg/ml) as described by Baker and Frank (1967).

Microbiological assay procedure

Folic acid in the samples was assayed microbiologically with *Streptococcus faecalis* (ATCC 8043) by the method recommended by Herbert and Bertino (1967), Baker and Frank (1967) and Hoppner (1971), but with certain modifications. *S. faecalis* was maintained in a medium containing 0.3% beef extract, 0.5% tryptone, 0.1% dextrose, 0.2% KH_2PO_4, 0.1% L-cysteine HCl, 1.5% agar (w/v). The pH was adjusted to 6.8–7.0 with KOH. 10 ml of the medium was dispensed into screw capped test tubes and autoclaved for 30 min. The stab culture was incubated at 37°C for 24 hr and stored at 4°C. It was subcultured once every 2 wk.

Inocula preparation

The organism was inoculated from the stock cultures into working-strength sterilized assay medium to which 0.5 ng/ml (for *S. faecalis*) of pure folic acid (crystalline pteroyl-glutamic acid) were added. After incubation at 37°C for 20 hr, the well-grown culture was transferred into new working-strength sterilized assay medium containing 0.1 mg folic acid per ml.

Assay medium

A single turbidimetric assay medium (Flynn et al., 1951; Toepfer et al., 1951; Baker et al., 1959; Eigen and Shockman, 1963; Baker and Frank, 1967) was modified. It is presented in Table 1. The double-strength liquid basal medium was frozen in an amber-colored bottle with a glass stopper. The medium was thawed and filtered through a medium porosity sintered glass filter just before use.

Table 1—Composition of assay medium (double strength)[a]

Component	mg per 100 ml of assay medium
Acid-hydrolyzed casein (Darco treated)[b]	1000
Na acetate (anhydrous)	1000
Na citrate · $2H_2O$	1000
KH_2PO	500
K_2HPO_4	500
Ascorbate	200
L-tryptophan	20
Adenine sulfate	1.0
DL-α-alanine	4.0
Guanine HCl	1.0
Uracil	1.0
Xanthine	2.0
L-asparagine H_2O	60
L-cysteine (free base)	50
Riboflavin	0.1
para-Aminobenzoic acid	0.2
Pyridoxine HCl	0.4
Thiamine HCl	0.04
Ca pantothenate	0.08
Nicotinic acid	0.08
Biotin	0.002
Glucose	5000
Tween 80[c]	0.01 ml
Glutathione (reduced)	0.5
Salt mix[d]	1.0 ml
$MnSO_4$ · H_2O[e]	20

[a] Boiled to dissolve the constituents and drive off CO_2. After cooling, adjust pH to 6.6–6.8 with H_2SO_4 or KOH and bring to final volume.
[b] Vitamin free casein hydrolysate (acid), salt free (Nutritional Biochemicals Co., Cleveland, Ohio) had been acid hydrolyzed by the methods of Toepfer et al. (1951) and Kavanagh (1963).
[c] Atlas Powder Co., Wilmington, Del. The commercial preparation is diluted with ethanol to appropriate concentrations.
[d] 1.0 ml contains: 40 mg $MgSO_4$ · $7H_2O$, 2 mg NaCl, 2 mg $MnSO_4$ · $4H_2O$, 2 mg $FeSO_4$ · $7H_2O$.
[e] Added after pH adjustment.

Folic acid standards

A stock solution of folic acid (1 mg/ml) was prepared by dissolving crystalline pteroyl-glutamic acid (PGA) in 20% ethanol in water (v/v). The pH was adjusted with 0.1N NaOH to 10–11 to dissolve the PGA. Then 0.06N H_2SO_4 was used to readjust the pH to 7.0. The solutions were kept deep frozen at −18°C, and used when needed. Tenfold dilutions of this stock solution were made serially in phosphate-ascorbate buffer to obtain working standards of 2, 1 and 0.2 ng/ml. The standard curve was prepared by making various additions to 2.5 ml assay medium in individual 16 × 150 mm culture tubes covered with Kap-uts caps. Cotton wool should not be used for plugging the tubes as it may contain folic acid which is leached out in the sterilizing process.

A control tube consisting of the assay medium and water, without addition of folic acid, was included in the standard curve to check the carryover from the inoculum.

Sterilization, inoculation and incubation

The addition of the assay medium to individual culture tubes was completed first. The capped tubes were autoclaved at 121°C for 15 min and then placed into a cold water bath for rapid cooling. One drop of inoculum was added aseptically to each tube. The details of aseptic technique have been discussed by Hunter et al. (1958). The assay tubes were incubated in the dark at 37°C for 24 hr. All the assay procedures should be carried out away from direct light, because folic acid is rapidly inactivated by light with formation of p-aminobenzoylglutamic acid and 2-amino-4-hydroxy-6-formylpteridine (Lowry et al., 1949). The latter was converted first into the corresponding acid and then into 2-amino-4-hydroxypteridine.

Turbidimetry

After incubation, the assay tubes were heated at 100°C for 2 min to stop the growth and then cooled. Microbial growth was measured at 650 nm in a Bausch-Lomb spectrophotometer. It was necessary to shake the tubes well to suspend the organisms uniformly. If air bubbles were present in the solution the tubes were allowed to stand for approximately 30 min (Freed, 1966). After that, the contents were again suspended uniformly. If the optical density (OD) was greater than 1.0, the cultures were diluted with water to read below OD 1.0. The reading was then multiplied by the appropriate dilution factor. The amount of folic acid in the sample was calculated from readings obtained from the standard curve.

Recovery studies

Recoveries from samples with known amounts of added folic acid were determined to show the reproducibility of the assay procedures. 0, 5, 10, 15 and 20 ng of folic acid were added to 1g samples of ground beans before extraction. The percent recovery was determined. The average value of four determinations is reported.

Proximate analysis

Total solids. 2g of dry, ground sample or 5g of homogenized sample were weighed in tared aluminum dishes. 3–4g dried diatomaceous earth was added to the aluminum dishes of homogenized samples to facilitate drying. The dishes were put on a steam bath for 30 min for preliminary drying; they were then placed in a Heraeus vacuum oven for 6 hr at 70°C at 100 mm Hg. After removal from the oven, they were covered, cooled in a desiccator and weighed.

RESULTS & DISCUSSION

Standard curve

The standard curve for microbiological assay of folic acid by *S. faecalis* was obtained by plotting concentration of folic acid versus optical density (OD) at 650 nm on a linear scale. A linear relationship existed between the concentration of folic acid and the optical density. The coefficient of correlation between folic acid concentration and growth (OD at 650 nm) was 0.980.

The term folic acid generically refers to all the pteroylglutamic acids. Through long usage it has come also to be a synonym for pteroylmonoglutamic acid. One may use folate as the generic term and pteroylmonoglutamic acid for the pure substance. The uses of folate assays are to define the pteroylmonoglutamic acid or folinic acid (N^5-formyltetrahydrofolic

Table 2—Effects of washing, soaking and blanching on folic acid retention in garbanzo beans, assayed with S. faecalis (ATCC 8043)

Garbanzo beans	Free folic acid[a] Mean[a] (μg/g dry wt)	Retention (%)	Total folic acid[a] Mean (μg/g dry wt)	Retention (%)
Raw and dry	0.328 ± 0.010	100	3.47 ± 0.11	100
Washed, soaked	0.313 ± 0.015	95	3.26 ± 0.21	94
Steam blanched				
5 min	0.252 ± 0.011	77	3.06 ± 0.07	88
10 min	0.219 ± 0.008	67	2.71 ± 0.09	78
20 min	0.208 ± 0.006	63	2.64 ± 0.09	76
Water blanched				
5 min	0.239 ± 0.007	73	2.69 ± 0.06	78
10 min	0.225 ± 0.016	69	2.60 ± 0.05	75
20 min	0.177 ± 0.023	54	1.88 ± 0.14	54

[a] Average of four determinations

acid) content of pharmaceutical preparations, to define the folate content of foods and to determine the state of folate nutrition of man (Freed, 1966; Herbert and Bertino, 1967).

The naturally occurring folates are the various coenzymatically active forms of the vitamin, which are tetrahydrofolates and usually exist as conjugates with more than one molecule of glutamic acid incorporated into the structure. Most of the naturally occurring folates carry a one-carbon unit attached to the N^5 or N^{10} position, or attached to both nitrogens. Of these various one-carbon adducts of tetrahydrofolate, the N^5-formyl adduct and perhaps N^5-methyltetra-hydrofolate appear to be stable enough to resist most isolation procedures (Herbert and Bertino, 1967).

The finding that polyglutamate conjugates of folate compounds occur naturally further complicates attempts to identify the folates in foods. These polyglutamate conjugates containing more than three glutamate residues do not serve as growth factors for the usual assay organisms. For total folate assay, the extracts were hydrolyzed enzymatically to the monoglutamate level with Bacto-chicken pancreas. The concentration of folic acid, established in each assay, was read from the standard curve which was prepared in each experiment.

Recovery studies

To check the reproducibility of the assay procedures, recovery studies were performed. Various amounts of standard folic acid (0–20 ng) were added directly to 1g samples of dry garbanzo beans before extraction. The results represent the effect of experimental procedure on the recovery of folic acid by the organisms. The average recovery was 96% (range = 93–98) for *S. faecalis*.

The nutritive balance of the assay medium is important in microbiological assays. The modified assay medium used in the present investigation supports excellent growth of *S. faecalis*. The growth response can be measured by the turbidimetrical method in preference to the acidimetry method.

Presence of heavy metal contaminants in water may inhibit the growth of the test organisms. Therefore, distilled and deionized water should be used in the investigation.

The prepared assay medium and folic acid standard solutions should be stored in amber-glass containers under deep freezing to make sure that microbial action or chemical degradation will not occur.

All buffer solutions used for dilution and extraction should contain 0.2% ascorbic acid to protect against photooxidation. The protective effect of ascorbic acid has been reported by Toennies et al. (1956). In addition, the pH values of the medium and buffer should be carefully controlled before inoculation with the organisms.

Some commercial brands of "vitamin-free" casein hydrolysate contain some folic acid to cause appreciable growth in the blanks. Therefore, it was necessary to remove residual folic acid and other interfering elements from casein in the laboratory by treatment with 5N HCl and decolorization with activated charcoal (Kavanaugh, 1963).

Effects of soaking and blanching

In the washing and soaking processes the retention of free folic acid was 95.4% and the corresponding retention in total folic acid was 93.9% (Table 2).

To compute percent retention, the folic acid content of the dry garbanzo beans was considered as 100. In the water blanched beans, free folic acid retention decreased from 72.9% to 45.0% as the blanching time lengthened from 5 min to 20 min; total folic acid decreased from 77.5 to 54.2% under the same conditions. Steam blanching somewhat improved folic acid retention. Blanching losses may occur as a result of leaching of the water-soluble vitamin when the cells are killed by

Table 3—Effect of heat processing on free and total folic acid retention in canned garbanzo beans (beans and brine combined), using S. faecalis (ATCC 8043) as test organism

Brine type	Retorting time at 118.3°C (min)	Free folic acid Mean (μg/g dry wt)	Retention (%) Based on original dry beans[a]	Retention (%) Based on steam blanched beans[b]	Total folic acid Mean (μg/g dry wt.)	Retention (%) Based on original dry beans[a]	Retention (%) Based on steam blanched beans[b]
Standard brine	29.8	0.199 ± 0.003	60.7	90.9	2.43 ± 0.06	70.0	89.7
	38.5	0.213 ± 0.006	64.9	97.3	2.52 ± 0.10	72.6	93.0
	46.5	0.192 ± 0.009	58.5	87.7	2.49 ± 0.16	71.8	91.9
	53.4	0.291 ± 0.007	61.3	91.8	2.42 ± 0.05	69.7	89.3
Standard brine with 0.2% ascorbic acid	29.8	0.209 ± 0.006	63.7	95.4	2.55 ± 0.10	73.5	94.1
	38.5	0.212 ± 0.002	64.6	96.8	2.50 ± 0.10	72.0	92.3
	46.5	0.210 ± 0.005	64.0	95.9	2.67 ± 0.10	76.9	98.5
	53.4	0.202 ± 0.003	61.6	92.2	2.42 ± 0.09	69.7	89.3

[a] The raw dry garbanzo beans contained 0.33 μg free folic acid/g dry wt and 3.47 μg total folic acid/g dry wt.
[b] The steam blanched beans contained 0.22 μg free folic acid/g dry wt and 2.71 μg total folic acid/g dry wt.

the blanching process. A reduction in blanching time seems to be desirable for better folic acid retention. Total folic acid retention in garbanzo beans was 78.1% when steam blanched for 10 min; 74.9% when water blanched for 10 min.

Folic acid was retained more in the steam blanched beans than in the water blanched ones. This is logical since leaching will be greater in water than in steam. After water-blanching at 100°C for 20 min, free and total folic acid retentions were approximately 54%. When steam blanching was applied, free folate retention was 63.4%.

Effect of heat processing

The effect of heat processing on folic acid retention in canned garbanzo beans is presented in Table 3. There was no significant decrease in either free or total folic acid content even when the processing time at 118.3°C was lengthened from 29.8 (F_0 = 8) to 53.4 min (F_0 = 20). The phenomenon may be explained by the fact that the pH of canned garbanzo beans is between 5.8 and 6.2, a range at which folic acid is quite stable toward heat under the conditions of the experiment.

Folic acid in garbanzo beans was quite stable toward heat processing under the conditions of this investigation. The retention of total and free folic acid in the canned beans was 70.0% and 73.5%, respectively, of that present in the original dry bean.

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RED LIGHT INTENSITY AND CAROTENOID BIOSYNTHESIS IN RIPENING TOMATOES

INTRODUCTION

LIGHT in the visible region causes an increased synthesis of carotenoids in ripening tomatoes. McCollum (1954) found higher carotenoid levels in tomatoes ripened in the light when compared to tomatoes ripened in the dark. Shewfelt and Halpin (1967) found that Standard Gro-Lux fluorescent lamps were more effective than cool white or wide spectrum fluorescent lamps for color development in detached green tomatoes. Worthington et al. (1969) showed that Gro-Lux lamps increased the temperature inside the tomatoes more than did the incandescent or cool white fluorescent lamps, and thus attributed the increase in color development of ripening tomatoes to a temperature effect. Shewfelt (1970) measured the radiant output of Gro-Lux lamps and found predominantly red light. Jen (1974b) observed that red monochromatic light enhanced the color development of ripening green tomatoes more than did green or white light. Also, Jen (1974a) demonstrated that blue light was most effective in increasing carotenoid synthesis as the absorption maxima of the carotenoids was in the blue region. Khudairi and Aboleda (1971) showed that red light stimulated carotenoid biosynthesis in tomatoes and that the level of carotenoid synthesis was regulated by plant hormones.

In this report the effect of light intensity as well as light quality on the biosynthesis of carotenoids in ripening tomatoes was examined. The possible involvement of phytochrome in carotenoid biosynthesis was also investigated.

EXPERIMENTAL

Tomatoes

Mature green tomatoes of the Homestead cultivar were obtained from the local market and sorted for uniform size, maturity and specific gravity in aqueous ethanol (Jen, 1974a). Light treatments of tomatoes were started at the breaker stage of maturation.

Light treatments

For each light treatment, 30 tomatoes were placed, styler end up, in one of four identical bench-style environators as described previously by Jen (1974a). The tomatoes were placed 20 cm from the light source, and lighting periods were set for 14 hr per day. Thermal barriers were used to minimize temperature variations. Thermocouples were placed just underneath the skin of one tomato in each environator, and the temperatures were monitored with a 24-point potentiometer to maintain a temperature of 26 ± 0.5°C in all tomatoes by adjusting environator temperatures. This was to eliminate the possibility of a temperature effect which Worthington et al. (1969) suggested as the real cause of increased color development in illuminated tomatoes. Humidity was kept at a high level by placing pans of water at the bottom of each environator. The following three light treatment experiments were conducted:

Experiment 1

Light Treatment	Intensity
Dark control	0 mW/cm²
4 Gro-Lux lamps	159 mW/cm²
8 cool white lamps	536 mW/cm²

Experiment 2

Dark control	0.0 mW/cm²
1 Gro-Lux lamp	1.2 mW/cm²
2 Gro-Lux lamps	84.0 mW/cm²
6 Gro-Lux lamps	243.0 mW/cm²

Also, during Experiment 2, 30 tomatoes were exposed to far-red light for 30 min and then kept in darkness for the remainder of the ripening period. The light intensity of the far-red light in the region of 700–800 nm was 488 mW/cm². After 7 days, samples from these tomatoes were removed and analyzed for carotenoids, along with similar samples from dark control and the 6 Gro-Lux lamps (red light) treatment.

Experiment 3

Light Treatment	Intensity
2 Gro-Lux lamps	84 mW/cm²
4 Gro-Lux lamps	159 mW/cm²
6 Gro-Lux lamps	243 mW/cm²
8 Gro-Lux lamps	416 mW/cm²

Radiant energy output was measured with an ISCO model SR spectroradiometer with a remote probe at a photodistance of 20 cm. The spectral intensity curves were recorded on an ISCO model SSR recorder. Radiant energies were calculated from the area under the spectral intensity curve as described previously (Jen, 1974a).

Pigment analysis

Tomatoes were held from 7–9 days since the initial maturity varied with each lot. Three fruits were removed at random at 2-day intervals at the beginning and then at 1-day intervals near the end of the ripening period. Carotenoids were extracted and quantitated as described previously (Jen, 1974a).

RESULTS & DISCUSSION

In Experiment 1, total carotenoid production after 8 days of light treatment was highest in tomatoes illuminated with four Gro-Lux fluorescent lamps (Fig. 1). It is important to note that tomatoes treated with four Gro-Lux lamps received 159 mW/cm² of radiant energy, and that tomatoes treated with eight cool white fluorescent lamps received 536 mW/cm² of radiant energy. Both light treatments resulted in greater carotenoid production than the dark control. The results of Experiment 1 indicate that light quality is a factor in carotenoid production. Light emitted by Gro-Lux lamps was predominately in the red region and was more effective in stimulating carotenoid production than that emitted by the cool white lamps, which have very little light in the red region (Fig. 2). Shewfelt and Halpin (1967) reached the same conclusion in color development of tomatoes using the same number of Gro-Lux and cool white fluorescent lamps.

Experiments 2 and 3 were designed to determine the effect of various intensities of red light on carotenoid production. Figure 3 shows that carotenoid production increased as red light intensity increased. (Gro-Lux lamps were used as source of red light.) It was interesting to note that carotenoid content continued to increase in tomatoes treated with red light up to 13 days of light treatment whereas carotenoid production in

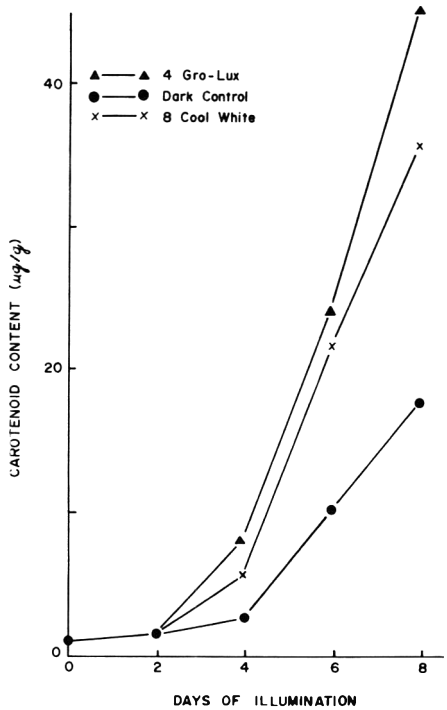

Fig. 1—Carotenoid biosynthesis in tomatoes illuminated with Gro-Lux and Cool-White fluorescent lamps.

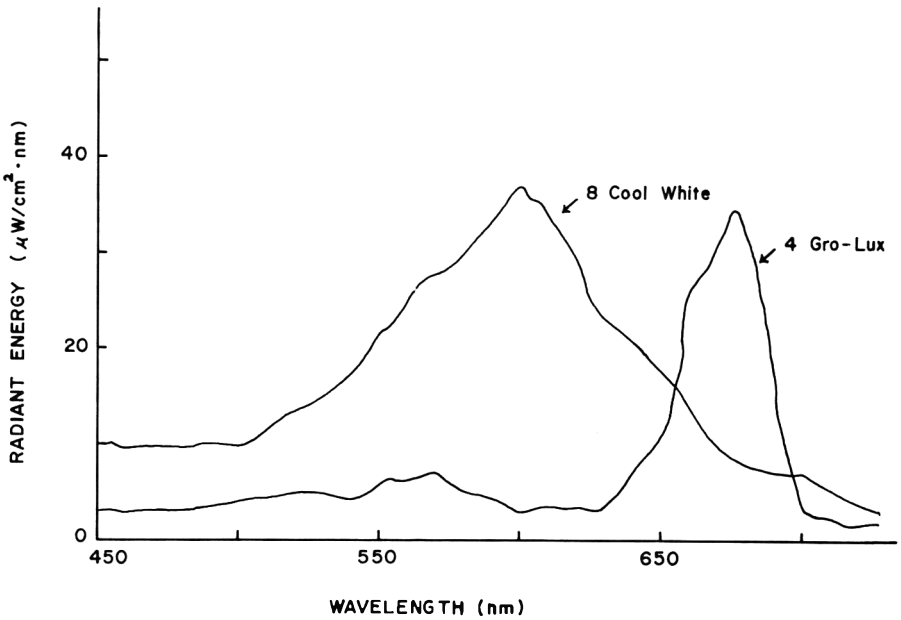

Fig. 2—Radiant energy distribution curves of Gro-Lux and Cool-White fluorescent lamps for Figure 1.

tomatoes held in darkness leveled off as the fruits continued into senescence, as shown by the dotted lines in Figure 3. This is of particular interest to the food industry interested in color development of detached green tomatoes. If the overall effect, represented by the slope as determined by the linear least square method, is plotted versus the log of the incident energy, a linear progression is observed as shown in the lower half of Figure 4. Results from Experiment 3 (Fig. 5) again showed that carotenoid production increased as red light intensity increased up to six Gro-Lux lamps. The six Gro-Lux lamps and eight Gro-Lux lamps were equally effective in enhancing carotenoid biosynthesis, indicating a saturation of light intensity below 243 mW/cm^2. The upper part of Figure 4 shows that the red light effect, when plotted versus the log of the energy, was linear up to six Gro-Lux lamps. These results establish that the intensity of red light does have an effect on carotenoid synthesis, and that this effect is not a temperature effect since the temperature inside the tomatoes under all lighting conditions was held at 26°C. According to Krogman (1973), below saturation but above a critical minimum, the phytochrome-mediated response to red light depends linearly on the log of the incident energy. Tomatoes exhibit this phe-

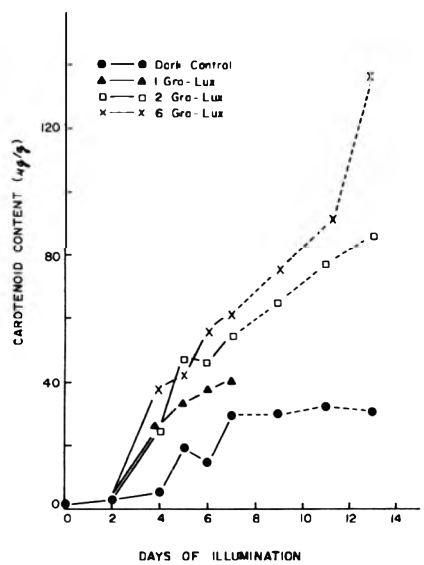

Fig. 3 (left)—Carotenoid biosynthesis in tomatoes illuminated with one, two or six Gro-Lux fluorescent lamps and dark control.

Fig. 4—Relationship between carotenoid biosynthesis and the energy received in ripening tomatoes. Lower curve represents data from Figure 3; upper curve represents data from Figure 5. The rate of carotenoid biosynthesis was calculated from the slope of data in Figures 3 and 5 by linear least square method.

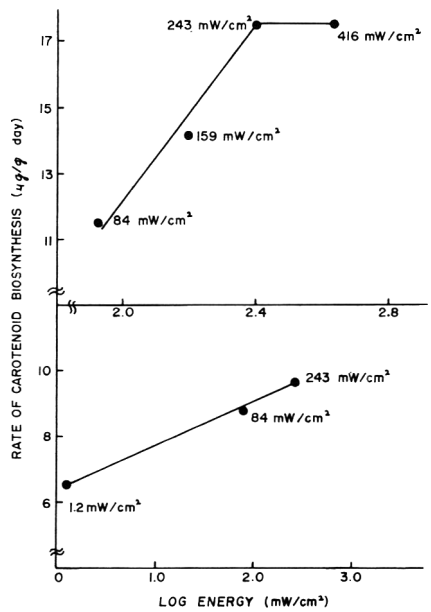

Table 1—The effects of red and far-red light on carotenoid biosynthesis in ripening tomatoes

Light treatment	Carotenoid levels (μg/g) after 7 days
Red (14 hr per day)[a]	60.04
Dark control	30.44
Far-Red (30 min + dark)[b]	14.24

[a] Gro-Lux fluorescent lamps were used as source of red light.
[b] Only one application of far-red light was used since P_r is stable in the dark and does not revert back to P_{fr} (Butler et al., 1963).

nomena (Fig. 4), suggesting the involvement of phytochrome in carotenoid biosynthesis.

The results in Table 1 showed that red light stimulated and far-red light suppressed carotenoid production, as compared to the dark control. Cohen and Goodwin (1962) subjected etiolated maize seedlings to red and far-red light treatments and found that red light enhanced carotenoid synthesis. They also observed that the effect of the red light can be reversed by far-red light treatment. Schnarrenberger and Mohr (1970) demonstrated a similar response in mustard seedlings. This type of effect with red and far-red light is usually accepted as proof of a phytochrome-mediated response. Table 1 shows that the phytochrome response can be demonstrated in the tomato fruit. Khudairi and Aboleda (1971) reached the same conclusion except that in their experiment, the far-red light did not suppress carotenoid biosynthesis in comparison to the dark control.

It is concluded from these data that light quality and light intensity both are important in determining the amount of carotenoids synthesized by the tomato. Evidence also indicates that carotenoid biosynthesis in tomatoes is under the control of phytochrome.

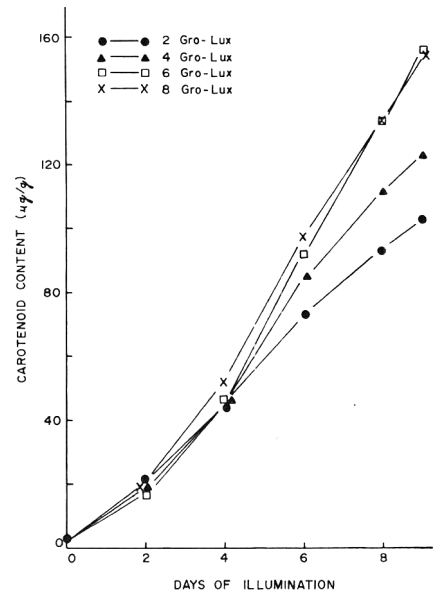

Fig. 5—Carotenoid biosynthesis in tomatoes illuminated with two, four, six and eight Gro-Lux fluorescent lamps.

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FACTORS INFLUENCING BLOATER FORMATION IN BRINED CUCUMBERS DURING CONTROLLED FERMENTATION

INTRODUCTION

BLOATER DAMAGE in commercially brined cucumbers, particularly the larger sizes, is a source of serious economic loss to the pickle industry. The recent advent of mechanical harvesting, which favors harvest of larger sizes, combined with the increased demand for larger-sized brine-stock for making hamburger dill chips, dill spears, relishes, etc., has increased the need for reduction of bloater development.

The successful pure culture fermentation of cucumbers, which had been heat-shocked (77°C, 5 min) and brined in containers ranging from 1 qt to 5 gal, by certain lactic acid bacteria (Etchells et al., 1964, 1968a), encouraged us to consider adapting the process for commercial brining in bulk containers. Heating to rid green-stock of contaminating microbes was considered impractical for bulk-brining in commercial tanks. Alternatively, a procedure not requiring use of heat was used which afforded a means of obtaining a desired fermentation, predominated by *Lactobacillus plantarum*. This procedure, developed over several years, included thorough washing of the green-stock; chlorination of the cover brine; acidification; buffering; and inoculation with *L. plantarum*. The equilibrated brine strength and temperature were carefully controlled according to Etchells and Hontz (1972).

Serious bloater damage resulted, however, when the above procedure was used, due to the small amounts of CO_2 produced by *L. plantarum* and the respiring cucumbers (Fleming et al., 1973a, b). This illustrated that bloater formation can occur under certain conditions even with a homofermentative lactic acid bacterium; gas-forming microbes such as yeasts, coliforms and heterofermentative lactics do not necessarily have to be present for bloater damage to occur as was previously thought. Purging of dissolved CO_2 from the brine with nitrogen resulted in essentially bloater-free brine-stock cucumbers (Fleming et al., 1973a).

Based on these findings, a "controlled fermentation" process was outlined for use by commercial briners (Etchells et al., 1973). A fermentation predominated by *L. plantarum*, and purging of CO_2 from the brine with nitrogen are two primary features in the process.

The present study preceded the outlined process of Etchells et al. (1973), and served as a basis on which the process was founded. CO_2 was not purged from the brines in the present work. Rather, our objective was to study environmental factors which influence bloater formation in nonpurged fermentations predominated by *L. plantarum*, including brining depths, pack-out ratio and temperature. Also, comparisons of chemical and microbiological changes in natural and controlled (i.e., predominated by *L. plantarum*) fermentations illustrate basic problems encountered in the development of the controlled fermentation process, particularly in relation to directing the fermentation.

MATERIALS & METHODS

SIZE NO. 3, pickling cucumbers (1-1/2–2 in. diam), hand-harvested, were brined in epoxy-coated, 55-gal, steel drums using essentially the same basic brining procedure described earlier for controlled fermentation of cucumbers brined in bulk (Etchells and Hontz, 1972; Etchells et al., 1973). Briefly, this procedure, with some modifications necessary for present objectives, was as follows: cucumbers were thoroughly washed, preshrunk in 25° salometer brine containing 80 ppm available chlorine for 2–3 hr, or until the cucumbers were sufficiently flaccid to obtain a pack-out ratio of cucumbers:brine on a percentage by wt basis of 65:35, or 270 lb cucumbers and 17.5 gal brine for a "full" drum (50 gal). This brine was drained off and replaced with fresh 25° salometer, chlorinated brine, to which was added 6 ml of glacial acetic acid per gallon of brined cucumbers. The cucumbers were kept immersed in the brine by means of a perforated, 1/4-in. thick, flat, plastic "false head" mounted inside the top of the drums, at the 50 gal mark, and about 1–2 in. below the brine surface. Chlorinated nylon cloth or a large, circular piece of filter paper was placed on the "false head;" then, the desired amount of dry salt was carefully added to maintain the brine strength at 25° salometer. The salt was added on the head at the rate of 6 lb per 100 lb cucumbers; 1/2–2/3 was added immediately after heading (and brining) and the remainder about 24 hr later. Sodium acetate ($3\ H_2O$), sufficient to equilibrate at 0.5%, was added to the head about 18 hr after brining.

The initial pH of the acidified cover brine was about 2.8. The pH rose during the 24-hr equilibration as acetic acid diffused from the cover brine into the cucumbers. With the addition of sodium acetate, several hours prior to inoculation, the brine was buffered at pH 4.7 (± 0.2), which is suitable for growth of *L. plantarum*. The brine has consistently shown no visual turbidity after 1–2 days' equilibration with the cucumbers prior to inoculation.

The brine-cucumber-mass was next inoculated with a frozen (liquid nitrogen) culture concentrate of *L. plantarum* (Chr. Hansen's Lab., Inc., Milwaukee, Wisc.) at a concentration of about 4 billion cells/gal of brined material about 24 hr after brining. In some instances, we have used inocula prepared by growing the cultures in cucumber juice broth (CJB; Fleming and Etchells, 1967).

The times for salt and acetate additions, and inoculation were extended up to 12 hr longer in some instances, depending on characteristics of the cucumbers, so that the brine strength would be below 28° (7.4%/wt) salometer before addition of the second salt and before inoculation. In all instances, however, the sequence of additions was the same.

The 55-gal drums were incubated in "walk-in," controlled-temperature rooms, located at a cooperating pickling plant, or in the Food Science building at North Carolina State University. Low-ozone ultraviolet (2537 Angstroms) germicidal lamps (Atlantic Ultraviolet Corp., Long Island City, N.Y.) were placed 20 in. directly above the uncovered brine surface to prevent growth of film yeasts. Alternatively, in some drums, film yeasts were inhibited by placing about 1-in. diam, plastic bubbles (cut from packing material), containing mustard oil, on the brine surface. Vapor from the mustard oil escaped gradually through the needle puncture made when the oil was injected by syringe into the bubbles. Plastic sheeting was loosely draped over each drum to retard loss of mustard oil vapor, but allow the more volatile CO_2 to escape and thereby prevent a build-up of pressure.

Evaluation of the brine-stock for bloater damage, determined 2–3 wk after brining, and methods for titratable acidity (calculated as lactic acid), pH, NaCl and reducing sugars were those described or referred to

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Table 1—Populations and isolation of lactic acid bacteria in controlled and natural fermentations of brined cucumbers[a]

	Controlled			Natural		
		Isolates			Isolates	
Days after brining	Lactic acid bacteria (Millions/ml)	No. picked	No. gas formers[b]	Lactic acid bacteria (Millions/ml)	No. picked	No. gas formers[b]
1	0.03[c]	15[c]	14[c]	1.08	—	—
2	8.5	12	0	12.5	—	—
3	265.0	12	0	13.5	12	4
4	185.0	9	0	40.5	9	0
5	93.0	9	0	12.0	11	1
7	17.6	9	0	1.6	12	0
9	2.2	9	0	0.5	12	1
12	0.4	9	0	0.06	12	1
14	5.8	9	0	4.4	12	2
20	0.03	9	0	2.4	9	1

[a] See Materials & Methods for brining procedures. Lactic counts for controlled and natural fermentations were from single, 55-gal drums of each. The controlled fermentation was inoculated 1 day after brining. Incubation, 27°C.
[b] Number of gas-formers out of the total number of isolates picked from LBS agar plates
[c] This sample was taken immediately prior to inoculation.

previously (Fleming et al., 1973a). Dissolved CO_2 was determined by the method of Fleming et al. (1974). Samples were taken with sterile 12-ml disposable syringes through rubber serum stoppers, positioned at appropriate locations in the side of the drum. All values reported are averages of duplicate fermentations unless otherwise indicated.

Media used for plate counts of the various microbial groups were: LBS medium (BBL), for lactic acid bacteria (Rogosa et al., 1951, as modified by Costilow et al., 1964); dextrose agar (BBL), acidified with 5 ml of 5% tartaric acid/100 ml medium immediately before pouring, for yeasts; and violet red bile agar (BBL), for coliform bacteria. Incubation was at 30°C for lactics and yeasts, and 37°C for coliforms.

For isolation of lactic acid bacteria, colonies were picked from LBS plates, transferred to CJB tubes and incubated at 30°C. Cell morphology was determined microscopically at 1350X magnification under an oil immersion objective. Final pH, acidity and residual reducing sugars were determined after 2 wk incubation of the isolates. These data were used as supporting evidence in classifying the isolates.

Heterofermentative (gas-forming) lactic acid bacteria were determined by a modification of the general procedure of Gibson and Abdel-Malek (1945). A drop of an active broth culture of each isolate was transferred to 5 ml of CJB with 2% NaCl in 19 × 100 mm tubes. 2 ml of sterile petrolatum were added to each tube, which was then loosely capped with a Bacti-capall (Preiser Scientific). Heterofermentative lactic acid bacteria, *Lactobacillus brevis* and *Leuconostoc mesenteroides*, produced sufficient CO_2 after 3 days at 30°C to force the petrolatum upwards, leaving a gas pocket. Homofermentative bacteria, *L. plantarum* and *Pediococcus cerevisiae*, did not produce enough CO_2 to form a gas pocket. This test for gas formation was verified by using pure cultures of the above-named species.

RESULTS

Comparison of controlled and natural fermentations

In the controlled fermentation process (Etchells et al., 1973) applied in the present experiments, except without purging, fermentation by the added starter culture, *L. plantarum*, predominated. Brines from cucumbers which had been chlorinated and acidified according to this process contained only 350 coliform bacteria per ml 1 day after brining and just before inoculation with *L. plantarum;* none was detected 1 day after inoculation and thereafter. In contrast, a natural fermentation contained 15,000 coliform bacteria per ml 1 day after brining, but the count dropped to about 100/ml after 3 days, and none was detected thereafter. Yeast counts were 50–100/ml in both fermentations during the first 3 days, and were less than 100/ml thereafter until 7 days when a slight growth of film yeast began in a small area of the brine surface shaded from UV light. Coliform and yeast counts in brines of natural fermentations are variable, and should be expected to deviate widely from the examples above.

One day after brining, populations of lactic acid bacteria in the brines of unwashed cucumbers (natural fermentation) were 36X those of brines from washed-chlorinated-acidified (controlled fermentation) cucumbers before inoculation (Table 1). The lactic count reached a maximum of 265 × 10^6 cells/ml 2 days after inoculation of the controlled fermentation and then declined. Maximum lactic count in the natural fermentation, 40.5 × 10^6 cells/ml, was reached 4 days after brining.

Prior to inoculation, lactic isolates from the brines of controlled fermentations were practically all gas-formers, cocci or short rods, single and in chains, and similar to species in the genus *Leuconostoc*. After inoculation, isolates were all nongas-formers (Table 2) and short rods typical of the *L. plantarum* starter culture. Isolates from the natural fermentation over a 20-day period were about 86% nongas-forming lactobacilli, 9% gas-forming lactobacilli, 2% leuconostocs and 2% pediococci

Table 2—Isolation of lactic acid bacteria from controlled and natural fermentations of brined cucumbers[a]

	Controlled	Natural	
Isolate type	No. of isolates[b]	No. of isolates	Time of isolation[c]
Total	87	89	3–20 days
Leuconostoc sp.	0	2	3rd day
Lactobacillus sp.			
Non-gas-formers	87	77	3–20 days
Gas-formers	0	8	3–20 days
Pediococcus sp.	0	2	3rd & 5th days

[a] Isolates were picked from colonies of LBS agar platings of brine samples taken from the controlled and natural fermentations represented in Table 1.
[b] Refers to number of isolates taken after inoculation and 2–20 days after brining.
[c] Refers to time after brining the cucumbers

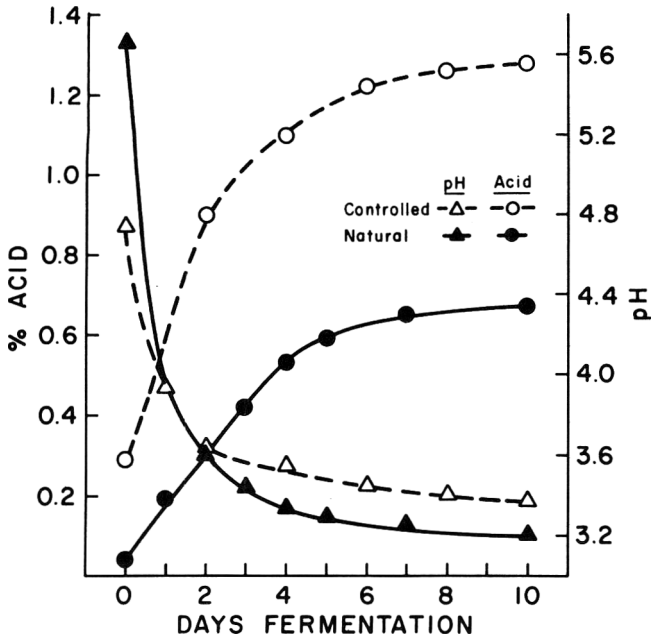

Fig. 1—Acid production in controlled and natural fermentations of brined cucumbers. Initial values obtained 1 day after brining and just prior to inoculation.

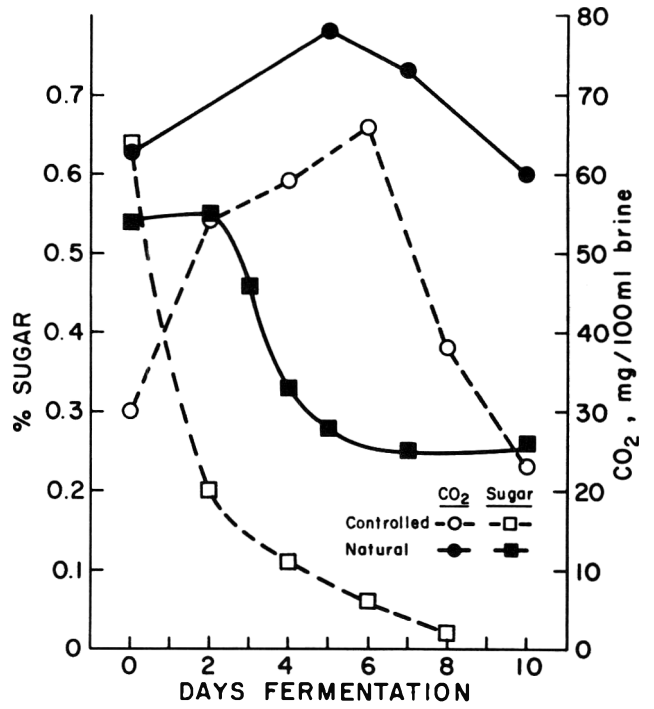

Fig. 2—Sugar utilization and CO_2 production in controlled and natural fermentations of brined cucumbers. Initial values obtained 1 day after brining and just prior to inoculation.

(Table 2); gas-forming lactobacilli were isolated throughout the 20-day period.

In controlled fermentations of brined cucumbers incubated at 32°C and 25° salometer, fermentable sugars were rapidly and completely converted to acid, usually within 7–10 days (Fig. 1 and 2). The amount of sodium acetate added provided sufficient buffering action to permit *L. plantarum* to ferment all of the brine sugars which diffused from the cucumbers. After completion of fermentation, the pH was 3.3–3.4, which is above the range that inhibits the *L. plantarum* culture used. Criteria used to determine completion of fermentation were: absence of reducing sugars and of changes in titratable acidity and pH over a 2-day interval between analyses.

In natural fermentations, times required for conversion of sugars to acid were longer and unpredictable in comparison with controlled fermentations. About 0.25% sugar remained after the production of lactic acid had ceased (Fig. 1 and 2). In this particular case, the pH had dropped to 3.2 and further activity by the natural lactic acid bacteria was inhibited. Residual sugars in such instances usually are fermented by subsurface yeasts with resulting bloater formation (Etchells and Bell, 1950; Etchells et al., 1952, 1953). We have observed some natural fermentations in which all sugars were metabolized as rapidly as in controlled fermentations, and others in which fermentation ceased when up to 0.5% sugar, still remained. We attribute such variation in natural fermentations to the variability in populations of natural microflora that convert sugar to carbon dioxide, acetic acid, various alcohols, etc., in addition to lactic acid. Coliform bacteria, heterofermentative lactic acid bacteria and yeasts contribute to this

Table 3—Bloater formation and brine analyses of controlled and natural fermentations of brined cucumbers

	Bloater damage[a]							Brine analyses[b]			
	Balloon		Lens		Honeycomb		Total		Acid	Sugar	Maximum CO_2
Treatment	%	D	%	D	%	D	%	pH	%	%	mg/100 ml
Controlled											
32°C	22.0	(A-M)	9.5	(M)	16.5	(M-S)	48.0	3.34	1.32	0.02	68.5
27°C	10.0	(S)	8.0	(S)	6.5	(S)	24.5	3.38	1.30	0.00	66.5
Natural											
32°C	59.0	(A)	24.0	(A)	10.5	(M)	93.5	3.26	0.65	0.26	79.5
27°C	50.0	(A)	27.5	(A)	12.5	(M-S)	90.0	3.28	0.62	0.08	91.5

[a] Capital letters in parentheses under "D" in Table refer to severity of bloating: S = Slight, M = Moderate and A = Advanced condition. When two letters appear, the first indicates the category in which most of the damage was placed. Values are averages of duplicate fermentations.

[b] Analyses after 10 days' fermentation, except for maximum CO_2 values which usually occurred 4–6 days after inoculation. Values are averages of duplicate fermentations.

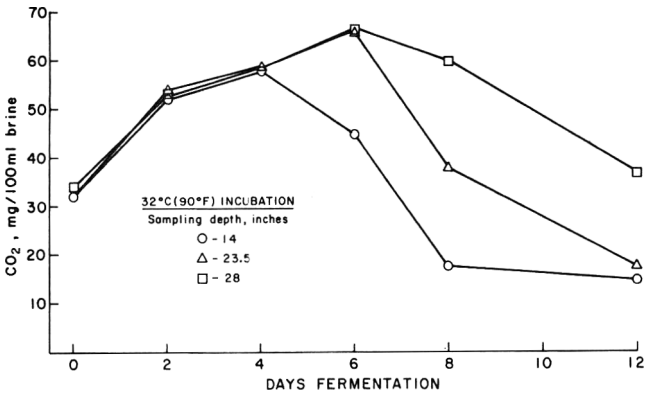

Fig. 3—Concentrations of dissolved CO_2 at various depths in full drums of brined cucumbers undergoing controlled fermentation at 32° C. Initial values obtained 1 day after brining and just prior to inoculation.

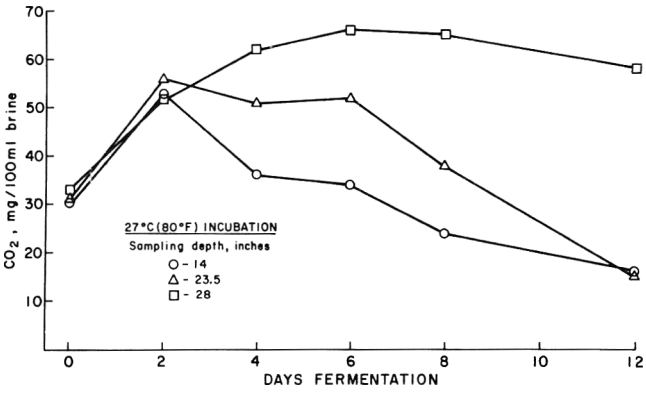

Fig. 4—Concentrations of dissolved CO_2 at various depths in full drums of brined cucumbers undergoing controlled fermentation at 27° C. Initial values obtained 1 day after brining and just prior to inoculation.

variability. Also, cucumbers vary in their contents of fermentable sugars; those with high amounts (particularly large sizes), usually are not fully fermented by the lactic acid bacteria in an unbuffered brine (Etchells and Moore, 1971).

Bloater damage occurred in the controlled fermentations (Table 3), although it was much less than in natural fermentations (Table 3). Furthermore, bloater damage was less at 27°C than at 32°C for both controlled and natural fermentations. Carbon dioxide content of the brine reached and maintained higher levels in natural than in controlled fermentations (Fig. 2). This probably accounts for the higher incidence and severity of bloater damage in the natural fermentations.

Brining to various depths

Cucumbers were brined in 55-gal drums at three levels of fill, with the pack-out ratio (i.e., cucumbers:brine) remaining constant at 65:35% by wt. Thus, only the depth of the cucumber-brine-mass varied.

Bloater damage when cucumbers were brined to a depth of 27 in. (full drum; 50 gal at head level), 18 in. (2/3 full), and 9 in. (1/3 full) is given in Table 4. Statistical evaluations of these data are summarized in Table 5. Examination of Table 5 shows that differences in percent bloaters due to "type" are significant, but none of the interactions with "type" are. The means in Table 4 show that "type" differences arise from the much higher incidence of balloon than either lens or honeycomb type. The lack of interactions with "type" suggests that the effects of temperature and depth of brining are essentially consistent for all bloater types. Hence, effects of temperature and depth of brining may be inferred from the means of percent total bloaters.

Bloater damage, whether expressed as percent affected or severity of those affected, was greater when cucumbers were brined at 32°C than at 27°C; and there was more damage in cucumbers brined at greater depths within each temperature (Table 4). The significant interaction of the linear effect of depth with temperature (Table 5) arises from the fact that the increase in bloaters at 32°C is much sharper than at 27°C. At 27°C the average increase in percent total bloaters is 5% for each 9-in. increase in depth; at 32°C this increase averages 18% (Table 4).

Table 4—Influence of brine depth and temperature on bloater formation in controlled fermentations of brined cucumbers[a]

Brine-cucumber-mass depth	Bloaters[b]							Bloaters induced by mechanical damage[c]					Brine analyses[d]	
	Balloon		Lens		Honeycomb		Total	Balloon		Lens		Total	Acid	
Inches	%	D	%	D	%	D	%	%	D	%	D	%	%	pH
32° C Incubation														
27	22.0	(A-M)	9.5	(M)	16.5	(M-S)	48.0	3.5	(S)	0		3.5	1.32	3.34
18	10.5	(M-A)	7.0	(M)	3.5	(M)	21.0	3.0	(S)	3.0	(S)	6.0	1.25	3.35
9	7.0	(M-S)	3.5	(S)	1.5	(S)	12.0	3.0	(S)	1.5	(S)	4.5	1.18	3.36
Average	13.2		6.7		7.1		27.0	3.2		1.5		4.7	1.25	3.35
27° C Incubation														
27	10.0	(S)	8.0	(S)	6.5	(S)	24.5	5.0	(S)	4.0	(S)	9.0	1.30	3.38
18	7.5	(M-S)	4.5	(S)	5.0	(M)	17.0	3.0	(M)	2.0	(M)	5.0	1.22	3.35
9	8.5	(S)	3.5	(S)	2.5	(S)	14.5	5.5	(S)	0		5.5	1.14	3.35
Average	8.7		5.3		4.7		18.7	4.5		2.0		6.5	1.22	3.36

[a] The pack-out ratio was constant for all treatments; 65% cucumbers, 35% brine, by weight. All values are averages of duplicate fermentations, except for the 9-in. depth which are averages of triplicates.
[b] Letters in parentheses indicate the severity of bloating; see footnote a, Table 3 for description.
[c] Bloaters induced by mechanical damage are also included with the normal bloaters which are given in the preceding columns.
[d] Values after 12 days' fermentation. Reducing sugars were less than 0.1% in all cases.

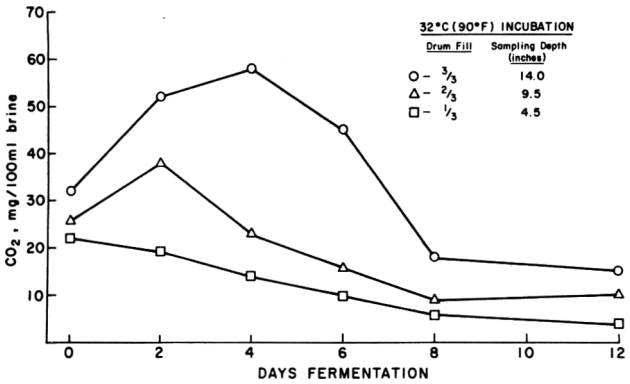

Fig. 5—Concentrations of dissolved CO_2 in brines of cucumbers undergoing controlled fermentation at various levels of container fill at 32° C. Samples were taken from mid-depth for each level of fill. Initial values obtained 1 day after brining and just prior to inoculation.

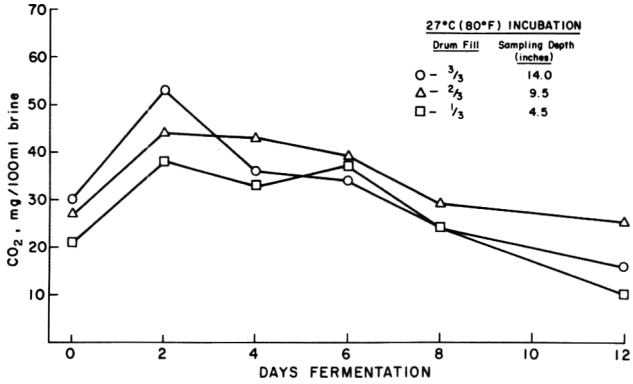

Fig. 6—Concentrations of dissolved CO_2 in brines of cucumbers undergoing controlled fermentation at various levels of container fill at 27° C. Samples were taken from mid-depth for each level of fill. Initial values obtained 1 day after brining and just prior to inoculation.

Concentrations of dissolved CO_2 at 3 depths in the full drums were monitored (Fig. 3 and 4). At both 32 and 27°C, concentration and retention of CO_2 were directly related to the depths from which samples were taken. At both temperatures, concentration of CO_2 reached a maximum at the greatest brine depths after about 6 days, and the values were similar, about 66 mg/100 ml brine. Over the next 6 days, however, the CO_2 concentration remained higher at 27°C than at 32°C.

CO_2 was also monitored at mid-depth of the three basic levels of container fill (Fig. 5 and 6). Concentrations of CO_2 were correspondingly lower in the brines of cucumbers packed at correspondingly more shallow depths.

Mechanical injury

For several years we have observed that brine-stock pickles of all sizes, showing clear-cut evidence of bruises from handling of the green-stock, are practically always bloated in and around the bruises. Bruises usually can be detected by cutting the raw cucumbers or partially-cured brine-stock at an injured area of the flesh. Here, the usual white, opaque, uncured stock is translucent around the bruise. Heretofore, we have not differentiated between bloaters that were associated with mechanical damage and those that were not. Table 4 indicates that bloaters associated with mechanical damage occurred with about the same relative frequency at all brining depths, at 32 and 27°C, whereas, the total percentage of cucumbers bloated was decidedly higher at 32°C. Mechanical harvesting causes more damage to cucumbers than hand harvesting (Marshall et al., 1972), and therefore may result in a higher incidence of bloater formation.

Brine circulation

Since CO_2 concentrations were lower near the brine surface exposed to the atmosphere (Fig. 3 to 6), we thought that circulation might help reduce the CO_2 concentration throughout the brine. Brine circulation did reduce CO_2 accumulation

Table 5—Analysis of variance for the effects of brine depth and temperature on bloater formation in cucumbers[a]

Source of variation	d.f.	Mean square
Bloater type	2	98.36**
Temperature	1	69.44**
Type X temperature	2	7.69
Depth (linear)	1	352.67**
Depth (deviation from linear)	1	29.39
Type X depth (linear)	2	9.54
Type X depth (deviation from linear)	2	4.85
Temp X depth (linear)	1	112.67**
Temp X depth (deviation from linear)	1	9.39
Type X temp X depth (linear)	2	20.04
Type X temp X depth (deviation from linear)	2	10.02
Pooled error	18	12.33

[a] Combined analysis made from orthogonal comparisons of replicate totals. See Table 4 for details of the experiment.
** Significant at the 0.01 level of probability.

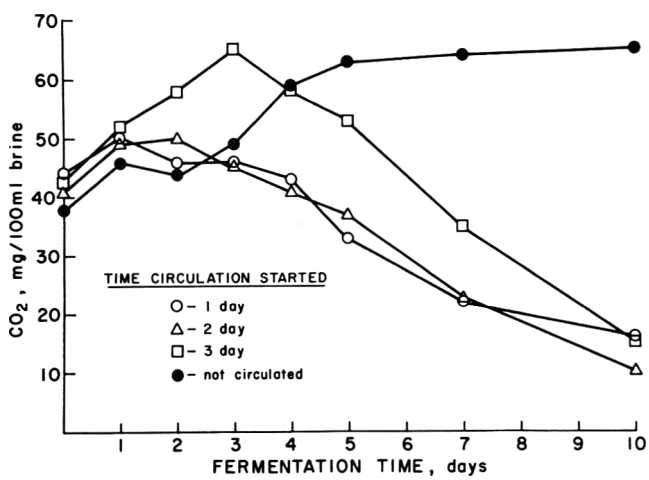

Fig. 7—Effect of brine circulation on CO_2 retention in brines covering cucumbers undergoing controlled fermentation at 27° C. (See footnote a, Table 6, for circulation treatment.) Values represent single drums. Initial values obtained 1 day after brining and just prior to inoculation.

Table 6—Influence of brine circulation on bloater formation

Time circulation started[a]	Bloater damage[b] Balloon %	D	Lens %	D	Honeycomb %	D	Total %	Maximum CO_2 mg/100 ml
32° C Incubation								
Uncirculated	36	(A)	27	(M)	15	(M)	78	56
1 Day	17	(A)	41	(M)	9	(M-S)	67	54
2 Day	20	(S-M)	24	(S-M)	12	(S)	56	59
3 Day	29	(A)	36	(M)	13	(S)	78	60
27° C Incubation								
Uncirculated	7	(S)	20	(S)	0	—	27	65
1 Day	7	(S)	20	(S)	0	—	27	50
2 Day	7	(S)	10	(S)	0	—	17	50
3 Day	17	(S)	11	(S)	1	(S)	29	65

[a] The brine was circulated, beginning on the day indicated, after inoculation, for 30 min twice a day at two, 12-hr intervals. A submerged pump circulated the brine up through a rubber tubing located inside a 3-in. diam, plastic pipe nto a perforated, 12-in. diam, plastic bowl located above the brine surface, from which the brine trickled back into the drum. Circulation was at the rate of 3 gal/min.
[b] Values are from single drums from each treatment. Letters in parentheses indicate the severity of bloating; see footnote a, Table 3.

(Fig. 7), but not bloater damage (Table 6). A more efficient circulation system, particularly if started sooner, might reduce CO_2 in the brine enough to reduce bloater development.

Effect of pack-out

Cucumbers were brined at pack-out ratios (cucumbers: brine) of 65:35, 55:45 and 45:55% by wt. Bloater damage decreased at lower pack-out ratios (Table 7). Percents of total bloaters and balloon bloaters ($P < 0.01$) and honeycomb bloaters ($P < 0.05$) were significantly higher at 65:35 than at 45:55. Although CO_2 concentrations were not determined, the smaller proportion of cucumbers would be expected to give off less CO_2, which would be dissolved in greater amounts of brine. Furthermore, less brine sugars would be available for CO_2 production by microbial metabolism.

Table 7—Influence of pack-out ratio on bloater formation in controlled fermentation of brined cucumbers[a]

Pack-out ratio, by wt Cucumbers %	Brine %	Bloater damage[b] Balloon %	D	Lens %	D	Honeycomb %	D	Total %
65	35	20	(M)	2	(S)	32	(S-M)	54
55	45	17	(M-A)	0		18	(S-M)	35
45	55	10	(S-M)	0		9	(S)	19

[a] Incubation temperature, 30° C
[b] Letters in parentheses indicate the severity of bloating; see footnote a, Table 3, for description.

DISCUSSION

A BRINE FERMENTATION of cucumbers predominated by *L. plantarum* will not necessarily insure bloater-free brine-stock. Bloater damage is influenced by factors which favor retention in the brine-mass of the relatively small amounts of CO_2 produced in such fermentations.

The uncovered containers we used simulated commercial brining conditions. The maximal amount of CO_2 accumulated at any given time during fermentation in such containers depends on two opposing factors, namely, the rate at which CO_2 is formed, and the rate at which it diffuses into the atmosphere from the brine surface.

In a rapid fermentation during the first few days after the initial brining, microbial activity causes a rapid accumulation of CO_2, which, combined with CO_2 from the respiring cucumbers, may readily favor bloater formation. Thus, inoculation with a vigorous strain of a lactic acid species, even a homofermentative such as *L. plantarum*, may actually promote bloater development unless CO_2 is removed during this period by some means such as purging with nitrogen (Fleming et al., 1973a). Optimum temperature for growth of the culture might contribute to bloater formation. Also, higher temperatures reduce the solubility of CO_2 (Quinn and Jones, 1936); and, according to Boyle's gas law, gaseous CO_2 would either occupy more space and/or create a greater pressure at higher temperatures. Thus, CO_2 present inside the cucumber, might be forced from solution, increase gas pressure and cause a bloated area.

The depth of the cucumber-brine-mass influences the retention of dissolved CO_2, in uncovered containers. An increase in the ratio of exposed brine surface area to total volume of brine-cucumber-mass would be expected to decrease accumulation of CO_2. In cylindrical, uncovered brining tanks, depth is the primary geometrical dimension which influences CO_2 retention in the brine. Commercial cucumber brining tanks are essentially cylindrical, about 8–16 ft in diameter and 7–8 ft deep. Deep tanks would be expected to retain high concentrations of CO_2 for longer times than shallow tanks, especially near the bottom, and bloater damage may be greatest at the bottom. The manner in which head boards are placed on the top of the cucumbers could also be important. Tightly fitting head boards would restrict diffusion of CO_2 from the brine into the atmosphere. A more desirable procedure would probably be to allow spacing between or, better, to perforate the head boards (Etchells et al., 1973).

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FRACTIONATION AND CHARACTERIZATION OF PEROXIDASE FROM RIPE BANANA FRUIT

INTRODUCTION

THE APPARENT ubiquity of peroxidase in plant tissues has prompted research into its function in key processes of development and senescence of plant tissues. Peroxidase has been implicated in ethylene biogenesis (Yang, 1967), which is believed to be an important regulator of ripening and senescence of fruit tissue. Peroxidase has also been shown to catalyze the oxidation of indole-3-acetic acid (IAA) (Hinman and Lang, 1965). It has been proposed that peroxidase functions in the degradation of chlorophyll (Walker, 1964), a characteristic reaction during ripening of most fruit.

A multiplicity of isoenzyme forms has been demonstrated for peroxidase. Shannon et al. (1966) identified seven isoenzymes in peroxidase isolated from horseradish root (HRP). These isoenzymes were fractionated and classified as anionic or cationic on the basis of ion exchange chromatography. Kay et al. (1967) demonstrated that the properties of cationic and anionic isoenzymes differed in pH optima, substrate specificities, specific activities, apparent Km's and affinities for inhibitors.

Novacky and Hampton (1968) showed that the spectrum of isoperoxidases revealed on starch gel and acrylamide gel electrophoresis was constant for a given species or tissue under specific conditions and is apparently related to age, species or variety, growth regulating substances and disease.

We have examined the electrophoretic patterns and the ionic and catalytic characteristics of peroxidase extracted from ripe banana fruit. The electrophoretic and catalytic properties of the anionic and cationic forms of peroxidase were observed to differ.

MATERIALS & METHODS

Materials

The bananas were generously donated by the United Fruit Company and were of the Valery variety grown in Honduras and Costa Rica. The fruit was received green within 8–10 days of harvest. During transport the fruit was maintained at 13–15°C in ventilated chambers with 85–95% relative humidity. The fruit was ripened at 18°C and 80–95% relative humidity after gassing with 100–150 ppm ethylene for 24 hr. Tissue was extracted from fruits which had reached full yellow peel color (#6 as described by Loesecke, 1950).

Preparation of cell free extracts

Fruit was peeled and slices from the middle two-thirds of the fruit were frozen in liquid nitrogen. The frozen slices were ground in a Sorvall omnimixer for 3–5 min to a fine powder. The powders were combined: 1:1 (w/v) with an extraction medium containing 0.1M Tris, 17% sucrose, 0.1% cysteine-HCl, 1% ascorbic acid and 0.8M $CaCl_2$, pH 8.5. The slurry was centrifuged at 75,000 × G for 30 min at 4°C. The resulting supernatant was treated with 1% Macerozyme (Calbiochem Co.) for 4 hr at 20°C. The resulting digest had markedly reduced viscosity and was used for fractionation.

Fractionation

The digested extracts were chromatographed on a Sephadex G-25C column equilibrated with 0.005M trishydroxymethylamino methane-hydrochloride (Tris-HCl), pH 8.5. Fractions exhibiting peroxidase activity with Peroxtesmo KO test paper (Galliard-Schlesinger) were collected and pooled. The pooled eluent (S) was incubated for 1 hr at 4°C and centrifuged for 10 min at 75,000 × G. This step resulted in the coagulation of mucilaginous materials which would otherwise clog the ion exchange columns in succeeding steps. The resulting supernatant (CS) was applied to a diethylaminoethyl (DEAE) cellulose column equilibrated with 0.005M Tris-HCl, pH 8.5. The protein fraction not adsorbed to the column was collected and pooled (cationic fraction, C). The column was eluted either by a linear gradient or batchwise elution using 0.025M Tris-HCl, pH 8.5 containing 0.25M NaCl as the final buffer. The resulting eluent from the DEAE column was designated as anionic (A). The CS, C and A fractions were used for pH optimum, determinations for peroxidase and IAAoxidase activities, polyacrylamide gel electrophoresis, and isoelectric focusing in both columns and gels.

Peroxidase assay

The assay medium consisted of 0.01M sodium phosphate buffer (pH 6.0), 0.008% o-dianisidine and 0.003% H_2O_2. It was demonstrated that these substrate concentrations saturated the enzyme preparation from banana fruit. Enzyme activity was determined using 2 ml of the above medium, 0.9 ml distilled water, and 0.1 ml sample. $A_{(460)}$ was determined with a recording spectrophotometer.

The pH optima of CS, C and A fractions were determined using Tris-citrate buffer 0.01M instead of phosphate at pH values ranging from 3.0–9.0 (0.5 pH unit intervals) and assayed as above.

IAAoxidase assay

Fractions were assayed by the method of Meudt and Gaines (1967) using 1.0 mM-IAA, 0.5 mM $MnCl_2$, 0.5 mM dichlorophenol in 0.01M sodium phosphate buffer, pH 6.1. pH optima were determined using the above incubation mixture by adjusting to pH values between 4.0–7.0 (0.5 pH unit intervals) with NaOH or HCl.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was performed according to Ioannou et al. (1973) using gels containing 0.2% amylopectin. Samples of 100 µl were applied to the origin of the gel. Peroxidase bands were stained by incubation in the following solution: 100 ml distilled water, 0.5g benzidine-HCl, 10 ml glacial acetic acid. After 5 min, 3 ml H_2O_2 was added and zones of activity stained blue. A similar, although more diffuse pattern of staining was observed when o-dianisidine was employed as substrate.

Isoelectric focusing in gels

Samples were focused in polyacrylamide gels with a pH gradient according to the method of Haglund (1971). 100 µl samples were incorporated into the gels before polymerization. Visualization of zones of activity was accomplished using the peroxidase assay described above.

Column isoelectric focusing

Column isoelectric focusing was performed by the techniques described in the LKB 8100 Ampholine Instruction Manual. Samples were dialyzed for 18–24 hr at 4°C against distilled H_2O with four changes of water to achieve a salt level of less than 5 µmoles in the total sample. The sample (100 ml) was used in the preparation of the sucrose density gradient.

RESULTS & DISCUSSION

Fractionation

The peroxidase extract was fractionated into anionic and cationic forms upon ion exchange chromatography. Elution of the DEAE cellulose column using a linear gradient resulted in

one broad peak with several shoulders as shown in Figure 1. Electrophoresis of samples taken from various fractions throughout the peak revealed some differences in isoenzyme distribution (Fig. 2). This lack of more complete separation by DEAE chromatography may be the result of very small differences in net charge of the isoenzymes. This property would result in only very small differences in binding capacity of the isoenzymes for the ion exchange resin.

Batchwise elution resulted in one sharp peak with peroxidase activity, and this method was used to obtain anionic peroxidase for further study. This fraction contained 22.4% of the peroxidase activity at assay pH 4.9 and 19.7% of the activity at assay pH 6.0 that was found in the CS fraction. The cationic fraction obtained showed a very strong positive reaction with Peroxtesmo KO test paper; however, when assayed with o-dianisidine the activity recovered was only 14.0 and 7.8% for assay pH 4.9 and 6.0, respectively. This is a recovery of only 36.4% (pH 4.9) and 25.7% (pH 6.0) of the activity found in the CS fraction; however, protein recovery was 100.8%. Thus, there is an indication that some form of denaturation of the enzyme occurred during fractionation. One explanation is suggested by the fact that electrophoresis of the CS fraction shows a fast-moving doublet (bands 8 and 9) which was not found in the A fraction (Fig. 3). There is a possibility of polymerization of the enzyme with the resulting polymer exhibiting lower activity.

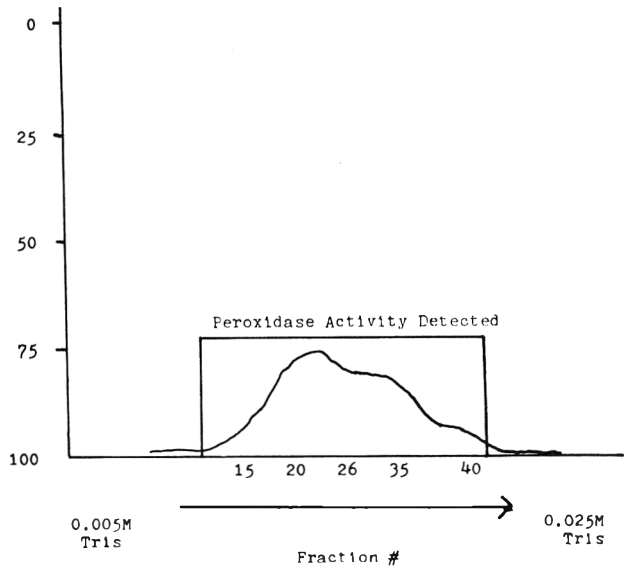


Fig. 1—Elution profile of peroxidase obtained with a linear gradient elution of DEAE cellulose. Results are for one experiment and representative of two other trials.

pH optima

As seen in Figure 4 the activity of peroxidase in the CS fraction was significant from pH 4.0–8.0 with a plateau of maximal activity between pH 5.0–6.0. The anionic fraction exhibited a plateau of maximal activity between pH 4.5–5.0, while the cationic fraction showed an optimum at pH 4.5. However, it is evident from the figure that all three fractions of peroxidase exhibit activity over a wide range of pH. The slight difference in optima observed between the anionic and cationic fractions is similar to results obtained by Kay et al. (1967) with HRP fractions.

It can also be seen that the activity in the high pH range appears to be lost during ion exchange chromatography. It is a possibility that it was retained on the ion exchange column due to exceptionally strong binding of specific isoenzymes.

IAA pH optima

The pH optimum for IAAoxidase activity of each fraction was determined. The CS fraction showed a pH optimum of pH 6.5, the anionic at 4.5–5.0 and the cationic at pH 5.5 as seen in Figure 5. The shoulder seen in the CS fraction at pH 4.5 appears to be entirely due to the anionic fraction. It also appears that there is a loss of the high pH activity of the more purified fractions. This could be due to selective denaturation of isoenzymes with alkaline pH optimum.

Gel electrophoresis

Polyacrylamide gel electrophoresis of the S fraction resulted in six bands with similar mobility, a fast moving doublet and one zone near the origin. A densitometric scan of this gel is presented in Figure 3. The staining near the origin (band #1) was possibly due to enzyme bound to the precipitate which forms upon standing of this fraction.

The anionic fraction exhibits the same pattern with the exception of the loss of the fast moving bands as seen in Figure 6. No discernable bands were found with the cationic fraction on the basic gel system; however, upon acid electrophoresis one zone was detected in the cationic fraction.

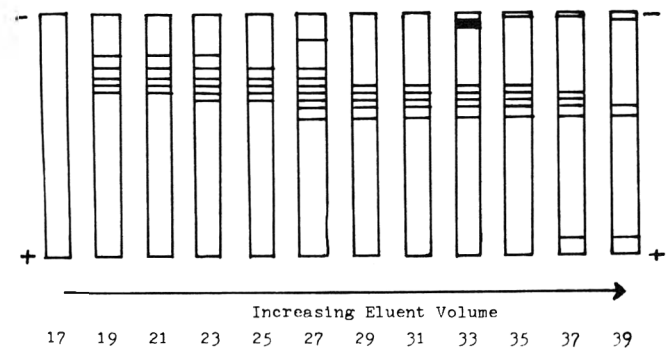


Fig. 2—Patterns observed upon polyacrylamide gel electrophoresis of samples from differing elution volumes of a linear gradient eluted DEAE column. Results are for one experiment and representative of two other trials.

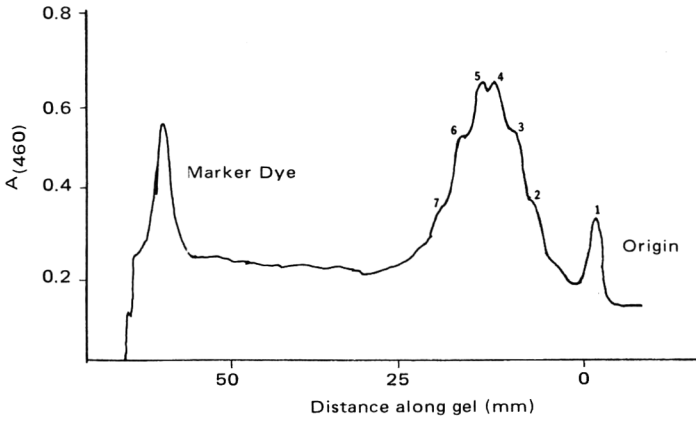


Fig. 3—Densitometric scan (A_{460}) at 50 mm/min of polyacrylamide gel electrophoretic separation of the S fraction.

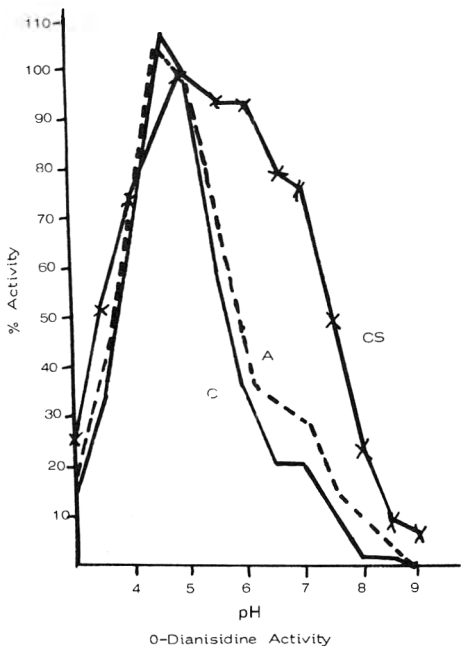


Fig. 4—The pH optima of the CS, C and A fractions of peroxidase activity toward O-dianisidine. All activities are expressed as % activity found at pH 5.0. Results are of one experiment and are representative of one other trial.

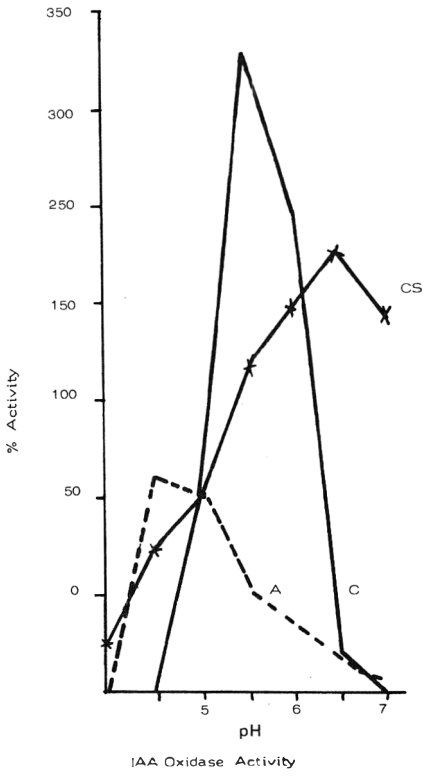


Fig. 5—The pH optima of the CS, C and A fractions of peroxidase activity toward IAA. All activities are expressed as % activity found at pH 5.0. Results are of one experiment and are representative of one other trial.

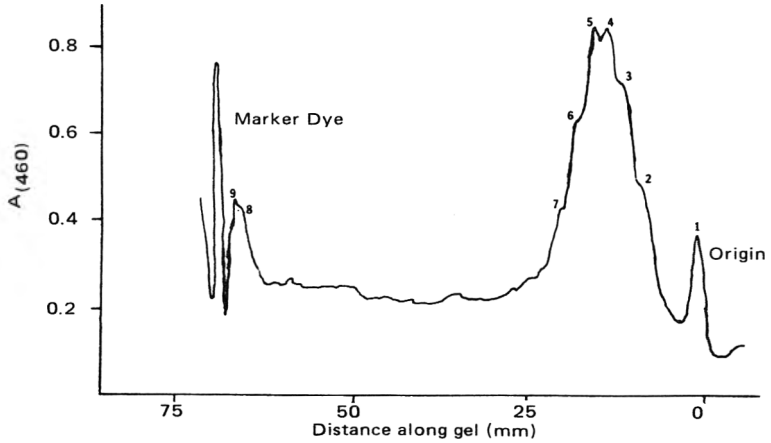
Isoelectric focusing in gels

Samples of the S, C and A fractions were focused in a pH 3.0–10.0 gradient in polyacrylamide gels and the resulting patterns are shown in Figure 7. The S sample exhibited staining throughout the gel indicating some form of interference with focusing. The two anionic fractions showed differences in pattern as expected from the results of electrophoresis of these samples. The cationic fraction showed no activity in the low pH zone as was predicted.

Column isoelectric focusing

An anionic fraction obtained from batchwise elution of a DEAE cellulose column was focused in a pH 3.0–6.0 gradient. As seen in Figure 8 the gradient was linear from pH 3.5–5.0. For each 2-ml fraction collected from the 110 ml column the pH was measured and peroxidase and IAAoxidase activities were determined. There were eight zones of peroxidase activity and nine zones exhibiting IAAoxidase activity, the latter being low in all samples. All of the isoelectric points were

Fig. 6—Densitometric scan (A_{460}) at 50 mm/min of polyacrylamide gel electrophoretic separation of the A fraction.



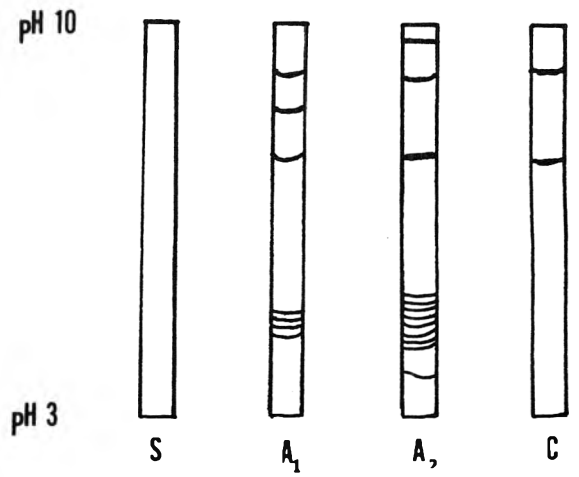


Fig. 7—Results of isoelectric focusing in polyacrylamide gels on a pH 3.0–10 gradient of the S, A and C fractions. The two A fractions were from different portions of the elution profile in Figure 1.

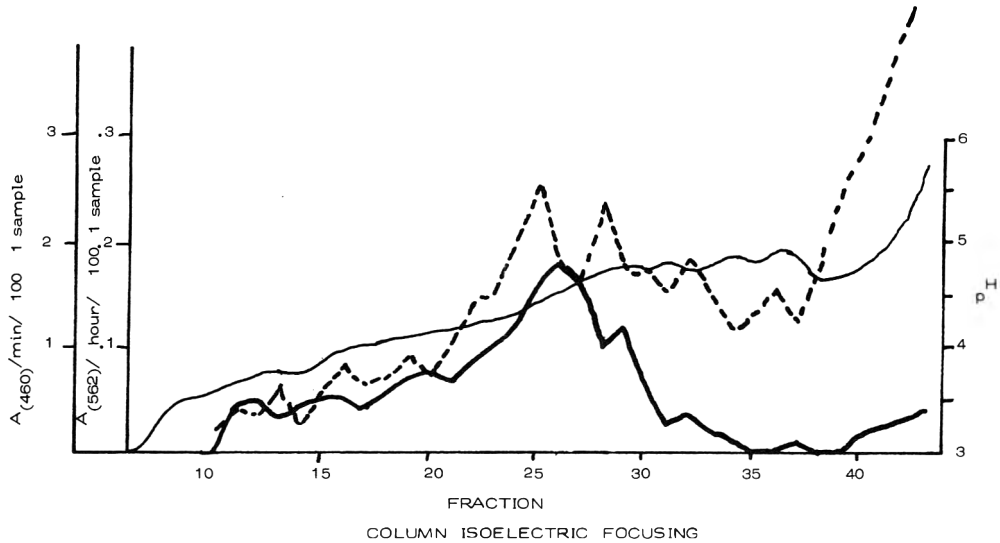


Fig. 8—Results of column isoelectric focusing in a pH 3.0–6.0 gradient on an anionic fraction obtained from a batchwise elution of a DEAE cellulose column. Legend: pH (——), peroxidase activity (——), IAAoxidase activity (-------).

between pH 3.72–4.92. The isoelectric points were very close, accounting for the difficulty in separation obtained by other methods.

Further studies will concentrate upon the IAAoxidase function of peroxidase as a possible regulator of ripening.

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JFS APPLIED SCIENCE and ENGINEERING

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BREAD BAKING PROPERTIES OF AQUEOUS PROCESSED PEANUT PROTEIN CONCENTRATES

INTRODUCTION

BREAD available commercially carries only 8% protein. Food scientists are investigating means to increase bread protein by utilizing oilseed protein products. Commercially available soy protein products have been successfully incorporated in bread. Alternative sources of high protein products are being investigated for bread fortification. Rooney et al. (1972) compared the baking properties of several oilseed flours and concluded that defatted peanut flour had excellent baking properties and produced bread with good taste, texture, crumb grain and loaf volume. Rhee et al. (1973) reported the use of aqueous processed peanut protein concentrates (PPC) in bread baking. A 10% level of substitution of wheat flour with PPC, increases the cost of bread $0.01–0.02, compared to the commercial bread. However, the extra protein supplied by PPC fortified bread, compensates for the high cost.

Objectives of the work reported here were:

(1) To compare the baking properties of PPC with an experimental defatted peanut flour, commercial defatted peanut, and full fat soy flours.
(2) To evaluate the performance of three different forms of PPC in bread baking.

MATERIALS & METHODS

Production of experimental peanut protein concentrates and defatted peanut flour

Experimental peanut protein concentrates were produced according to the pilot plant scale procedure described by Rhee et al. (1973). Prior to spray drying, the wet peanut protein concentrate (PPC) was divided into three sub-lots. Each sub-lot was adjusted to a different pH prior to spray drying. The first sub-lot was adjusted to pH 4.0 with 1N HCl, the second sub-lot was left "as is" (pH 5.5) and the third sub-lot was adjusted to pH 7.0 with 1N NaOH. These three sub-lots will be referred to as PPC 4.0, PPC 5.5 and PPC 7.0. The PPC 4.0 and PPC 5.5 represent isoelectric forms of peanut proteins and the PPC 7.0 is the sodium salt form. Production of the experimental defatted peanut flour was described by Rooney et al. (1972).

Analytical data

The oilseed protein products and the bread baked with them were analyzed for protein, ash, fat, moisture and crude fiber with standard AOCS (1971) procedures. Amino acid analysis (except tryptophan and cystine) was carried out by the procedure of Spackman et al. (1958). Tryptophan was determined by the method of Kohler and Palter (1967). Color readings of the protein sources were made with the Hunter Colormeter. "L" values were used as an index of the color of the protein sources.

Physical dough and baking properties

Preparation of flour blends. Each of the oilseed protein products was substituted for 10, 15 and 20% of the wheat flour in the baking formula. A total of 22 blends were studied in a randomized complete block design.

Physical dough properties. Strength and stability of the dough mixed with 50g of the flour blend at optimum water absorption were studied with a Brabender Farinograph according to the standard AACC (1970) procedure.

Baking properties. Pound loaves were baked using each flour blend with a short-time dough system with the following formula and procedure. The formula based on 100g flour blend was: sugar 5g; yeast 3g; salt 2g; shortening 3g; and sodium stearoyl-2-lactylate (SSL) 0.5g. Each flour blend was mixed in a Hobart–120 mixer equipped with McDuffy bowl under optimum absorption, oxidation (ppm of potassium bromate) and mixing time which were pre-determined by running a series of preliminary experiments. Dough was scaled to 540g. The residual dough was frozen for analysis later. Dough was fermented for 40 min at 32°C and 95% R.H. It was sheeted through a National Sheeter at 9/32 in. and 7/32 in. settings. It was moulded using a "Roller Up" Moulder from National Manufacturing Company (Lincoln, Nebr.). The dough was proofed to constant height at 32°C and 95% R.H. and baked at 218°C for 25 min.

Loaf volume was measured immediately after baking by rapeseed displacement. The bread was weighed and stored overnight. The crumb grain score, crumb color, pH of the crumb and dough were measured 18 hr later. pH was measured according to an AACC procedure (1970).

All the flour blends were baked on the same day and the whole bake was replicated on each of three different days.

Organoleptic studies

A six-member taste panel scored texture, crumb color, taste and flavor of bread baked with 100% wheat flour and that prepared by substituting 20% of the oilseed products for the wheat flour except experimental defatted peanut flour. The taste and flavor detections were made before and after toasting. A hedonic scale of 1 to 5 was used; 1 = poor and 5 = excellent.

Softness measurements

A control containing 100% wheat flour and blends with 15% each of PPC, commercial defatted peanut and full fat soy flours were baked, on each of the two different days. The baking procedure and formulation were the same except 0.1% calcium propionate was added as the preservative. Crumb softness was measured over a 5-day period using a Precision Penetrometer. The combined weight of the cone and the rod of the penetrometer was 150g (Precision Scientific Co., 1967). Crumb softness was measured on three 1-in. slices, from each loaf. Three penetrometer readings were taken on each slice. Crumb softness was measured after 24, 72 and 120 hr storage at 25°C.

Statistical analysis

Analyses of variance were calculated for loaf volume, crumb grain score, crumb color and softness data.

RESULTS & DISCUSSION

Composition and physical properties of protein sources

Protein content of the PPC used in these experiments ranged from 54–56% and fat content was 17–19% (Table 1). Experimental and commercial defatted peanut and full fat soy flours contained 63.5, 61.5 and 44.5% protein, respectively. The full fat soy flour was chosen for this study because of its high fat content which is comparable to that of PPC. The ash content of the experimental and commercial defatted peanut flours was two to three times higher than that of PPC. The dark color of the commercial peanut flour was attributed to the use of unblanched peanuts as starting materials and partly to the heat generated during the pre-press operation.

Essential amino acid profiles of the different protein sources are compared in Table 2. Lysine content of PPC and commercial peanut flour was lower than the full fat soy flour. The profile of PPC and commercial defatted peanut flour was almost similar except PPC contained slightly larger amounts of cystine, valine, phenylalanine and tyrosine.

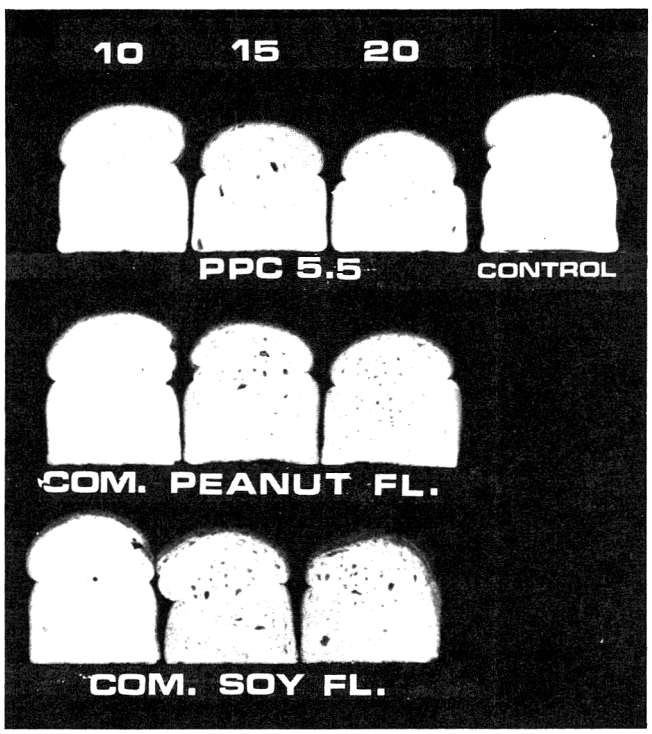

Fig. 1—Pound loaves of bread baked by short-time dough procedure with peanut protein concentrate spray dried at pH 5.5, commercial defatted peanut flour, and commercial full fat soy flour substituting 10, 15 and 20% wheat flour. Control is 100% wheat flour.

Table 1—Proximate analysis of the oilseed protein sources and wheat flour

Protein Source	Protein[a] (%)[b]	Fat (%)[b]	Ash (%)[b]	Color[c]
PPC 4.0	55.5	19.3	1.4	81
PPC 5.5	56.1	17.0	1.7	74
PPC 7.0	54.3	17.9	2.1	75
Experimental defatted peanut flour	63.5	1.0	4.9	84
Commercial defatted peanut flour	61.3	0.7	5.0	77
Commercial full fat soy flour	44.8	22.9	–	80
Wheat flour	15.8	–	–	91

[a] Protein = N X 6.25 for oilseed products; N X 5.7 for wheat flour
[b] % dry weight basis
[c] "L" Value of Hunter colormeter

Table 2—Essential amino acid composition of PPC, commercial defatted peanut and full fat soy flours

Amino acid	Grams of amino acid/100g protein		
	PPC[a]	Commercial defatted Peanut flour[b]	Full fat soy flour[c]
Lysine	2.9	3.0	6.7
Cystine	1.4	1.0	1.0
Methionine	1.1	0.9	1.4
Valine	4.7	3.8	5.3
Leucine	6.8	6.4	7.9
Isoleucine	3.5	3.2	5.3
Tryptophan	0.9	1.0	1.5
Threonine	2.5	2.6	3.9
Phenylalanine	5.5	4.7	5.1
Tyrosine	4.1	3.7	4.3

[a] Spray dried at pH of 5.5; average of four observations
[b] Data for the sample supplied by Gold Kist Inc.
[c] Data for the sample supplied by ADM Co.

Table 3—Farinograph and baking properties of PPC, experimental defatted peanut, commercial defatted peanut and full fat soy flours

Protein Source	Substitution level (%)	Farinograph properties		Baking properties		
		Absorption (%)[a]	Peak time (Min)	Loaf vol (cc)	Crumb grain score (%)	Crumb color[b]
Control	–	66.0	10.5	3258	80	80
PPC[c]	10	65.6	7.5	2817	71	73
	15	67.1	8.0	2675	65	72
	20	68.7	9.0	2285	55	68
Experimental	10	68.5	6.5	2938	80	71
defatted	15	70.6	6.5	2875	70	70
peanut flour	20	72.4	6.5	2613	40	65
Commercial	10	65.9	8.0	3050	70	69
defatted	15	67.3	7.0	2825	60	67
peanut flour	20	69.2	8.0	2550	40	62
Commercial	10	63.1	7.0	3075	76	76
full fat	15	66.0	7.5	2917	73	71
soy flour	20	63.7	7.0	2567	53	69
LSD 0.05				136	9	2

[a] Expressed on 14% moisture basis
[b] "L" value of Hunter colormeter
[c] Spray dried at pH of 5.5

Farinograph properties. Farinograph absorption of the dough increased with the addition of the oilseed proteins (Table 3). The high fat content of PPC did not affect farinograph absorption. There was no difference in mixing properties.

Baking properties

Loaf volume, crumb grain and color scores of the bread baked with the different protein sources are presented in Table 3; photographs of the bread are presented in Figure 1.

Loaf volume of the bread baked with 15% and 20% PPC was significantly reduced compared to that baked with the same concentrations of experimental defatted peanut, commercial defatted peanut, and full fat soy flours. This may be due to the high fat content of PPC; but, preliminary experiments in our laboratory indicate that bread baked with PPC containg 11% fat had the same loaf volume as that baked with PPC containing 18% fat.

Different vegetable proteins such as soy and peanut behave differently in the bread system. Tsen and Hoover (1973) reported that full fat soy flour gave a better baking performance than defatted soy flour. Differences in performance of PPC and defatted peanut flour could be attributed to the selective extraction of certain proteins of PPC. The crumb color of the breads baked with both PPC and the experimental defatted peanut flour were lighter than that baked with commercial peanut flour. Bread baked with full fat soy flour had an unacceptable yellow crumb color.

The crumb grain score deteriorated with increasing levels of the various oilseed protein products. Crumb grain score of bread containing 15% PPC and experimental defatted peanut flour was superior to that baked with the same concentration of commercial defatted peanut flour. Bread baked with 15% full fat soy flour had superior grain to the one that had 15% PPC.

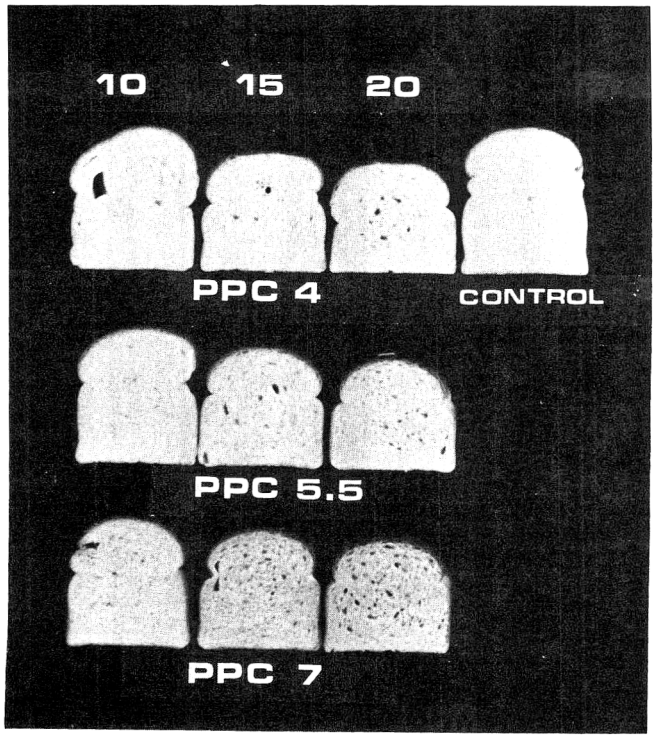

Fig. 2—Pound loaves of bread baked by short-time dough procedure with peanut protein concentrate spray dried at pH of 4.0, 5.5 and 7.0 substituting 10, 15 and 20% wheat flour. Control is 100% wheat flour.

On an average, bread protein due to PPC, experimental defatted peanut flour, commercial defatted peanut, and full fat soy flours increased by 32, 36, 38 and 21%, respectively (Table 4). Fat content of the bread containing full fat soy flour and PPC was higher than the bread that had 100% wheat flour or various concentrations of experimental and commercial defatted peanut flours.

Over a 5-day period, bread with 15% full fat soy and commercial defatted peanut flours was softer than that baked with 15% PPC (Table 5). This may be due to higher specific loaf volume of bread with full fat soy and commercial defatted peanut flours, than the one baked with 15% PPC.

Table 4—Average proximate analysis of bread baked with PPC,[a] experimental defatted peanut, commercial defatted peanut, and full fat soy flours

Protein source	Moisture (%)	Protein (%)[b] N X 6.25	% over control	Fat (%)[b]	Ash (%)[b]	Crude fiber (%)[b]
Control	35.4	16.5	—	1.8	2.7	0.2
PPC[a]	39.3	21.8	32	3.2	2.8	0.9
Experimental defatted peanut flour	37.2	22.4	36	1.4	—	0.8
Commercial defatted peanut flour	37.9	22.7	38	1.6	3.2	0.7
Commercia full fat soy flour	38.4	19.9	21	5.2	3.2	0.4

[a] Spray dried at pH of 5.5
[b] Dry weight basis

Table 5—Penetrometer readings of bread crumb with 100% wheat flour, 15% each of PPC,[a] commercial defatted peanut, and full fat soy flours

Protein Source	Hours after baking		
	24	72	120
Control	158	160	139
PPC[a]	149	133	120
Commercial defatted peanut flour	162	142	129
Commercial full fat soy flour	165	149	132
LSD 0.05	9		

[a] Spray dried at pH 5.5

Table 6—Taste panel data of the bread baked with 20% level of PPC, commercial defatted peanut, and full fat soy flours[a]

Protein source	Crumb color	Taste	Flavor	Texture
Control	4.7	4.1	4.2	4.7
PPC[b]	3.8	3.8	3.8	3.4
Commercial peanut flour	2.8	3.2	3.1	3.3
Full fat soy flour	3.1	3.3	2.8	3.1

[a] Scale: 1 = poor; 5 = excellent
[b] Spray dried at pH 5.5

Table 7—Effect of pH level prior to spray drying on the physical dough and baking properties of PPC substituted for part of the wheat flour

pH level prior to spray drying	PPC Conc (%)	Farinograph properties		Baking properties			
		Absorption[a] (%)	Peak time (Min)	Loaf vol (cc)	Crumb grain score (%)	Crumb color[b]	Crumb pH
Control		66.0	10.5	3258	80	80	5.5
4.0	10	65.5	7.0	2900	72	77	4.9
	15	67.1	6.5	2583	66	74	4.9
	20	68.6	7.5	2220	50	72	4.8
5.5	10	65.6	7.5	2817	71	73	5.2
	15	67.1	8.0	2675	65	72	5.2
	20	68.7	9.0	2285	55	68	5.2
7.0	10	65.4	8.5	2791	70	75	5.4
	15	67.1	10.0	2583	63	72	5.5
	20	71.6	10.0	2283	50	67	5.5
LSD 0.05				111	9	2	

[a] Expressed on 14% moisture basis
[b] "L" value of Hunter colormeter

The taste panel testing indicated that bread baked with PPC had better organoleptic qualities than that of commercial defatted peanut and full fat soy flours (Table 6). The flavor of the bread containing full fat soy flour was unacceptable to all the panel members. A strong peanut odor was detected during the dough mixing stage with commercial defatted peanut flour, but, none of the panel members detected a peanut flavor in either the raw or toasted bread baked with the peanut protein products.

Effect of pH prior to spray drying of PPC on the bread baking properties

Farinograph properties of the dough containing 10, 15 and 20% levels of PPC 4.0 was almost the same compared to the dough that had the same concentrations of PPC 5.5 and PPC 7.0 (Table 7). Loaf volume and crumb grain score of the bread baked with 10, 15 and 20% levels of PPC 4.0 was not significantly different from the ones containing the same levels of PPC 5.5 and 7.0 (Figure 2). These results indicated that there was no significant influence of pH level prior to spray drying on farinograph properties and baking properties of PPC; except that crumb color became slightly darker with an increase in pH level when compared at the same concentration. This is in contrast to the adverse effect of acidic pH prior to spray drying of the water-extracted cottonseed protein concentrates on loaf volume and crumb grain scores observed by Lawhon et al. (1972).

The findings reported above in this paper indicate that differences in protein systems of various oilseed materials and conditions used in processing them significantly affect the compatability of oilseed protein with wheat gluten during bread baking.

SUMMARY

BAKING PROPERTIES of three experimental peanut protein concentrates (PPC) produced by an aqueous extraction process were compared with a commercial and an experimental defatted peanut flour, and a commercial full fat soy flour. PPC concentrations higher than 10% reduced loaf volume significantly compared to that of bread baked with the other protein sources. However, bread with PPC was superior in flavor, taste and crumb color to the bread baked with the commercial defatted peanut and full fat soy flours.

All three forms into which PPC were spray dried did not show any significant difference in bread baking properties.

Peanut protein products have excellent potential for use in baking. PPC has many attributes (i.e., color, bland flavor) that are desirable for baking applications. It is possible that by further process modification a PPC can be obtained that has even better properties for use in bread baking.

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PROTEIN CONTENT AND AMINO ACID COMPOSITION OF DEVELOPING PEAS

INTRODUCTION

THE MAJOR legume crops, other than soybeans and peanuts, belong to five groups: chick-peas, peas, broad beans, lentils and common beans (Bressani and Elias, 1968). Legume seeds are characterized by a relatively high content (20–30%) of protein that is, in general, superior in quality to that of cereal grains. However, the digestibility of legume proteins may vary widely (from 51–92%). Cereal and legume proteins exert considerable supplementary effect; the protein value of mixtures exceeds those of the components fed separately. The S-containing amino acids are usually the first limiting amino acids in legume proteins; cystine has a sparing effect on methionine. Legumes are good sources of lysine and may be primary sources in cereal-based diets.

The main reserve proteins of pea seeds are albumins and globulins in the ratio of 1:1.4 (Beevers and Poulson, 1972). Storage globulins contribute 80–90% of the seed proteins in many legumes and in peas are principally vicilin and legumin (Boulter et al., 1973). Beevers and Poulson (1972), who followed changes in protein content of pea cotyledons from 9–33 days after flowering, reported that albumins were synthesized early in cotyledon development whereas globulin synthesis predominated with increasing maturity. Protein increased gradually with rapid deposition between 21 and 27 days after flowering, and then the rate of accumulation declined as seed dehydrated and matured. Danielsson and Lis (1952) showed that amino acid composition differs between albumin and globulin and also between the vicilin and legumin fractions of globulin. Albumin was high in tryptophan and lysine, and low in arginine.

Differences in protein contents among pea cultivars have been noted by several authors, including Esh et al. (1959), Ali-Khan and Youngs (1973) and Furedi (1970). Bajaj et al. (1971) found no correlation between protein level and protein quality in different pea lines, but noted differences in protein quality among lines. Furedi (1970) reported that the ratio of crude protein to total dry matter decreased in the developing pea seed, and attributed this to the accumulation of starch in the seed. According to Smith (1973), RNA and starch syntheses in *Pisum arvense* cease before maturation but protein synthesis continued until the seeds are ripe. Chitre et al. (1950) found that protein content of peas increased with maturity, but percent protein (dry weight basis) decreased. Weckel et al. (1963) found that protein on a fresh weight basis increased with size and maturity, but decreased on a dry weight basis. Protein content was greater in Alaska (smooth seeded) than in Perfection (wrinkle seeded) peas, either on the basis of size or stage of maturity. Changes in protein content and amino acid composition of maturing cereals have been extensively investigated (Pomeranz, 1973).

The Protein Advisory Group (PAG) of FAO/WHO/UNICEF (Anon., 1973) stated that the food legumes, major sources of protein and other important nutrients in many developing countries, have been neglected with regard to research necessary to improve their low productivity and consumer acceptance and to correct nutritional limitations. The PAG statement emphasized that supportive investigations on growth and development processes in food legumes are essential to legume improvement.

This investigation was stimulated by the observation in another study that the relationship between Kjeldahl nitrogen and dye-binding capacity varied according to maturity of pea seeds at harvest (Unpublished data, Shivaji Pandey and E.T. Gritton). Since Kjeldahl gives a measure of protein through estimation of nitrogen, and dye-binding is associated primarily with basic amino acids, it was suggested that amino acid balance might change with maturity of the peas. We have investigated the changes during maturation in amino acid composition of the three seed types of commercially grown canning pea cultivars, and compared these changes with those taking place in maturing cereal grains.

MATERIALS & METHODS

MANY PREVIOUS investigations either have not specified the pea cultivar used, or have used only one. Since it is important to know if differences exist among cultivars, the three seed types of the following canning pea cultivars were selected: 'Alaska' (smooth seeded), 'Alsweet' (wrinkle seeded due to r_b gene), and 'New Line Early Perfection' (wrinkle seeded due to the gene r_a). These plants were grown in the field during the summer of 1973. Enough flowers of each cultivar were tagged on the day of pollen shedding (June 13 for Alaska and Alsweet and June 19 for NLEP) to permit successive harvests. Thirty pods with developing seeds were harvested from each cultivar at 12 days after tagging, 20 at 18 days, and 10 each at 24, 36 and 48 days. Pods were placed in small glass jars or plastic bags and immediately taken to the laboratory where the ovules were removed, counted, weighed and then placed in a freeze dryer where moisture was reduced to about 0.5% (determined by oven-drying a portion of the sample at 100°C for 24 hr). Freeze-dried samples were ground in a micro-Wiley mill to pass a 20-mesh sieve. Nitrogen was determined by the Kjeldahl method on 0.5-g samples; crude protein was estimated by multiplying the nitrogen concentration by 6.25 and is reported on an as-is basis (freeze-dried samples). Amino acids were determined on hydrolysates with a Beckman 121 automatic amino acid analyzer by the method of Robbins et al., 1971. Values are expressed in grams of amino acid per 100g of amino acid recovered. Recoveries averaged 74.8%. The relatively low recovery can be explained by the fact that a fair proportion of the total N in legumes is nonprotein N (Bressani and Elias, 1968).

Samples of NLEP for preliminary analyses were grown in 1972 at the Arlington Agronomy Research Farm during the summer and in the greenhouse at Madison, Wisconsin, in the fall.

RESULTS & DISCUSSION

RESULTS of the preliminary analyses were fully confirmed by the more extensive studies in 1973, so only data from the 1973 samples are presented here. Because differences among cultivars were small, and to simplify presentation of amino acid analyses, results for the three cultivars combined are given.

Moisture contents and ovule weights (dry matter basis) of the developing peas are given in Figure 1. For canning, peas would normally be harvested at about 18–21 days after pollination. Physiological maturity (maximum dry weight) was attained at about 25 days after pollination when moisture content ranged from 57.1–66.4% (average 62.7%). This moisture content is substantially higher than the moisture content of physiologically mature barley (41.1–44.2%) (Pomeranz et al., 1971).

Protein content and amino acid composition of proteins in developing peas are given in Table 1. In all three cultivars, percent protein (dry weight basis) was highest at the earliest stage of maturity (12 days after pollination), decreased somewhat during the next 6 days, then increased but did not equal levels at 12 days. The decrease in protein up to about the 18th day is in agreement with the observations of Furedi (1970), and Weckel et al. (1963) who found protein, on a dry weight basis, decreased as seed matured. Protein concentration of the smooth seeded cultivar, Alaska, was slightly lower except at 36 days, than that of either of the other cultivars (data not shown). Weckel et al. (1963) reported the Alaska peas they analyzed were higher in protein than the Perfection type. Because dry weight of the ovule increased 6.2 to 11.6-fold during development, the relatively uniform percent protein indicated that additional protein was deposited at a rate roughly equivalent to the rate of increase in ovule dry weight.

Cultivar differences were small in the pattern of amino acid composition in the developing peas. Average increases during development in concentrations of amino acids were: lysine, 1.8-fold; histidine, 1.5-fold; aspartic acid, 1.7-fold; serine, 1.3-fold; proline, 1.8-fold; glycine, 1.9-fold; isoleucine, 1.4-fold; leucine, 1.9-fold; tyrosine, 1.8-fold; and phenylalanine, 2.1-fold. Average decreases were: ammonia, 1.8-fold; threonine, 2.2-fold; glutamic acid, 1.6-fold; cystine, 1.9-fold; alanine, 2.2-fold; and methionine, 1.1-fold. Concentration of valine was practically unchanged. Between the 12th and 18th day after pollination, concentration of arginine increased 1.6-fold, decreased thereafter, and at maturity was about 1.1 times the concentration in proteins in the ovules harvested 12 days after pollination. Arginine followed the same pattern in the preliminary analyses of the NLEP samples in 1972.

Comparison of amino acid concentrations in proteins of developing seeds of cereals (Pomeranz and Robbins, 1972) and peas shows few similarities and many major dissimilarities. In both, glutamic acid is the main amino acid; changes in amino acid composition were generally greatest between the first two sampling periods and smallest after physiological maturity; concentrations of theronine and alanine decreased and of

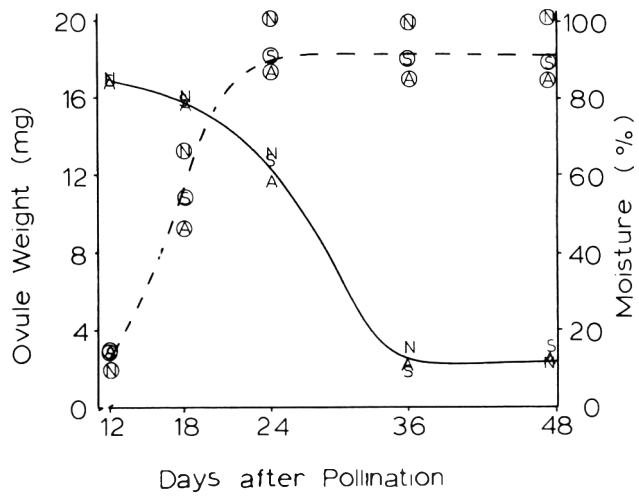

Fig. 1—Ovule weight (mg) and moisture content (%) of three pea cultivars harvested at various stages of development; S = Alsweet, A = Alaska, N = NLEP. Circled values and dashed line—ovule weight; others—moisture content.

Table 1—Protein content[a] and amino acid composition[b] of three pea cultivars harvested at various stages of development in 1973

Protein or amino acid	Days after pollination				
	12	18	24	36	48
Percent					
Protein	29.2–31.9	24.7–27.4	26.0–29.2	27.7–30.4	26.2–29.9
Lysine	4.4–4.9	6.4–7.0	7.4–7.8	7.4–8.5	7.8–8.2
Histidine	1.6–1.7	2.0–2.1	2.3–2.5	2.3–2.6	2.4–2.4
Ammonia	2.2–3.4	1.5–1.7	1.4–1.6	1.4–1.7	1.5–1.6
Arginine	6.1–9.6	10.4–15.2	7.4–10.6	6.7–10.5	6.9–8.8
Aspartic acid	7.4–7.8	11.0–11.6	12.2–13.2	12.4–12.6	12.7–13.0
Threonine	7.9–8.6	4.7–6.9	3.5–4.1	3.5–3.9	3.6–3.9
Serine	3.0–3.6	3.5–4.0	3.9–4.5	4.0–4.3	4.1–4.2
Glutamic acid	25.0–28.7	16.2–18.5	16.6–17.4	16.9–18.2	17.2–18.9
Proline	1.4–4.8	3.2–4.2	3.4–5.2	4.1–5.4	4.2–4.4
Cystine	1.1–1.6	0.7–0.7	0.6–1.1	0.6–0.9	0.6–1.0
Glycine	2.5–2.8	3.5–3.9	4.4–4.7	4.6–4.9	4.8–4.9
Alanine	9.9–11.9	5.2–5.8	4.6–5.0	4.5–5.0	4.7–5.0
Valine	4.9–5.5	5.2–5.5	5.2–5.6	5.2–5.5	5.3–5.6
Methionine	2.1–2.5	2.3–2.5	2.0–2.3	2.0–2.2	1.9–2.3
Isoleucine	3.1–3.3	4.0–4.4	4.4–4.8	4.4–4.8	4.5–4.8
Leucine	4.0–4.4	6.4–7.4	7.4–8.0	7.5–7.8	7.7–7.9
Tyrosine	1.2–1.7	2.1–2.3	2.7–3.3	2.3–2.5	1.6–2.5
Phenylalanine	2.2–2.4	3.6–4.2	4.6–5.2	4.6–5.1	4.7–5.0

[a] Kjeldahl N X 6.25, %
[b] Grams amino acid per 100g amino acid recovered

phenylalanine increased. The important differences were that concentrations of glutamic acid, the main amino acid of storage proteins in cereals, increased in cereals and decreased in peas; concentrations of lysine and aspartic acid increased in maturing peas and decreased in maturing cereals; concentrations of S-containing amino acids increased in cereals and decreased in peas.

The large increase in concentration of aspartic acid in pea proteins is of interest because aspartic acid is a key intermediate in the biosynthesis of lysine in bacteria, algae and higher plants (Vogel, 1965). The decrease of S-containing amino acids in pea proteins is significant because they are the first limiting amino acids in legumes. The unique pattern of arginine concentration in pea proteins suggests that arginine might be important in the biosynthesis of proteins, and may help to explain the disparity, mentioned earlier, between protein as estimated by Kjeldahl nitrogen and dye-binding. Whereas the concentrations of lysine and histidine increased with maturity, which would give a higher estimate of protein through dye-binding, the arginine concentration reached a peak somewhere between 12 and 24 days after pollination, and then decreased. Depending upon just when this peak is reached, dye-binding capacity could increase or decrease as peas pass through the normal range of canning maturity.

As mentioned before, cultivar differences were small in patterns of amino acids in the three cultivars studied. While those cultivars represent a relatively wide range of germ plasm in cultivated peas, apparently the range does not affect amino acid composition. In guidelines for plant breeders who want to improve the nutritional value of their legumes, the PAG of the United Nations (Anon., 1973) emphasized the need to broaden the genetic base by wide crossing at the interspecific level, induction of mutations, and chromosomal engineering. The potential of those approaches to increase content and improve quality of protein in peas was reported (Amirshahi and Tavakoli, 1970; Gottschalk and Muller, 1970; Micke, 1970; Zokhrabian and Sidorova, 1970).

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INTERRELATIONSHIPS BETWEEN STORAGE, SOAKING TIME, COOKING TIME, NUTRITIVE VALUE AND OTHER CHARACTERISTICS OF THE BLACK BEAN (Phaseolus vulgaris)

INTRODUCTION

BEANS as well as other legume foods, constitute traditional foods in the diet of populations of subtropical and tropical areas. They provide significant amounts of protein and calories for both rural and urban populations (INCAP, 1969).

Previous studies have shown that when black beans (*Phaseolus vulgaris*) are cooked under pressure, their protein quality is compromised with cooking times of less than 10 or more than 30 min (Bressani et al., 1963). The same authors (Bressani et al., 1963) demonstrated that a loss of available lysine occurred when the cooking periods used exceeded 30 min. Such observation is of importance since beans have been accepted as a natural protein complement of cereal grains that provide to this group of staple foods the lysine in which they are deficient (Bressani et al., 1962). Therefore, any lowering in the lysine availability in beans would compromise their quality as a protein complement of cereal grains.

It has been shown that for beans a soaking operation prior to cooking is necessary to eliminate completely the toxic factors contained in the raw seed (Kakade and Evans, 1966; Liener, 1962). Molina et al. (1972, 1974) found that in order to obtain the maximum protein quality of black beans, a much shorter cooking time was needed for the soaked samples than for the unsoaked ones. Furthermore, the same authors (Molina et al., 1972, 1974) point out that storage appears to have a strong influence on the optimum processing conditions to be chosen based on protein quality.

The present work was undertaken to study the effect that storage could have on the protein quality of black beans when subjected to different soaking and cooking times.

MATERIALS & METHODS

THE BLACK BEAN (*P. vulgaris*), variety S-19-N, used in this study was grown at INCAP's experimental farm "San Antonio Pachalí," Guatemala, at an altitude of 5,151 ft above sea level, and corresponded to the 1972 crop.

For the storage trials the beans were separated into 100-lb lots which were placed in cloth bags and kept under ambient conditions in the storage rooms of the experimental farm. The temperature and relative humidity of the rooms were recorded during this period; the average temperature and relative humidity, according to records, were 21 ± 2°C and 77 ± 4%, respectively.

The storage times evaluated were 0, 3 and 6 months. At each time, four samples (3 kg each) were subjected in triplicate to a soaking treatment at room temperature (25°C) in tap water (approximately 6 liters of water/sample) for 0, 8, 16 and 24 hr, respectively. Each of the three samples from each of the soaking times studied was then cooked in its soaking water in an autoclave (15 psi, 121°C) for 10, 20 and 30 min. The cooked beans were separated from the broth by filtration, dried in an air oven (70°C for 24 hr) and milled in a hammer mill equipped with a 40-mesh screen, to obtain the precooked bean flour.

The nitrogen solubility of the precooked bean flours was evaluated in H_2O, 1N NaCl and 0.05N NaOH using a 1:6 meal:solvent ratio at 40°C for 2 hr with constant agitation. The suspension was then centrifuged at 4,000 rpm for 20 min and nitrogen determinations carried out in the supernatant. Nitrogen was determined in duplicate according to AOAC (1970). Protein was calculated using the customary conversion factor 6.25.

Available lysine was determined according to the method of Conkerton and Framptor. (1959). Methionine and cystine were determined microbiologically as per the method described by Elías et al. (1964).

The protein efficiency ratio (PER) was evaluated essentially by the AOAC method (1970). Weanling rats of the Wistar strain from the INCAP animal colony were distributed in groups of four males and four females each. All diets were supplemented with a 4% salt mixture (Hegsted et al., 1941), 5% cottonseed oil, 1% cod liver oil and enough corn starch to adjust to 100g, to which 5 ml of a vitamin B solution (Manna and Hauge, 1953) were added.

True protein digestibility was determined applying the method described by Bressani et al. (1963). The rats and bean diets were the same used for the PER estimation. The experimental period comprised 4 days, starting 4 days after the 28 days required for the PER estimation. The rats on the nitrogen-free diet were those from the casein standard protein group used for the PER estimation, which were fed the nitrogen-free diet for an adaptation period of 4 days prior to the 4-day experimental period.

RESULTS & DISCUSSION

THE METHIONINE and available lysine content of the raw

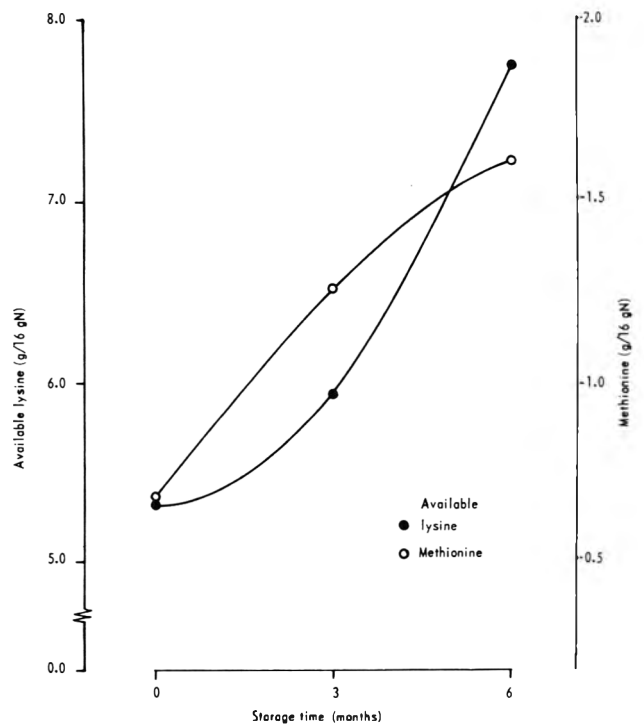

Fig. 1—Effect of storage on the methionine and available lysine content of raw beans.

beans at the different storage times studied, are shown in Figure 1. It is of interest to note that there did occur an increment in the content of both amino acids during the storage period. An increase in available lysine in raw black beans has also been reported by other authors (Ruiloba, 1973). However, in the latter study methionine was not determined. Because of the increment in methionine content, an improvement in the protein quality of the beans during storage could be expected, since this amino acid has been reported as the most limiting essential amino acid in bean protein (Bressani et al., 1961).

The PER values obtained for the beans at the different storage periods studied, after being subjected to the processing conditions evaluated, are shown in Figure 2. All PER values, including those for the control casein group, were adjusted to a standard hypothetical value of 2.5 for casein. This correction was made to eliminate any variation due to the different rat groups used to test the storage times studied. As may be appreciated, storage had a detrimental effect on the protein quality of the beans subjected to any of the processes studied; the only exception was the unsoaked beans processed after 6 months of storage. These results are of interest since they are at variance with what would be expected from the increment in methionine content of the raw bean during storage (Fig. 1).

The nutritional significance of cooking water to the protein quality of the product as a whole has not been assessed, even though it has been recognized that it may contribute to increased nutritional value in view of previous analytical values obtained for this fraction (Bressani, 1973).

Analysis of the biological data in Figure 2, indicates that cooking time had a statistically significant ($P < 0.05$) effect in lowering the protein quality of the beans processed either immediately after harvesting or after 3 months of storage. The same is true for beans stored during 6 months when subjected to a 16- or 24-hr soaking period prior to cooking. These results indicate that bean protein tends to become more susceptible to heat damage after 6 months of storage under the conditions evaluated, and that such susceptibility is decreased by a soaking treatment (16 or 24 hr) prior to cooking. This negative effect of soaking on the protein susceptibility to heat damage could probably be attributed to a hydration of the protein itself through the soaking operation, thus making it more susceptible to heat damage. The effect of soaking on the protein quality of the beans was found to be statistically significant ($P < 0.05$) only in the case of the beans stored for 6 months.

The effect of storage on bean protein quality was found to

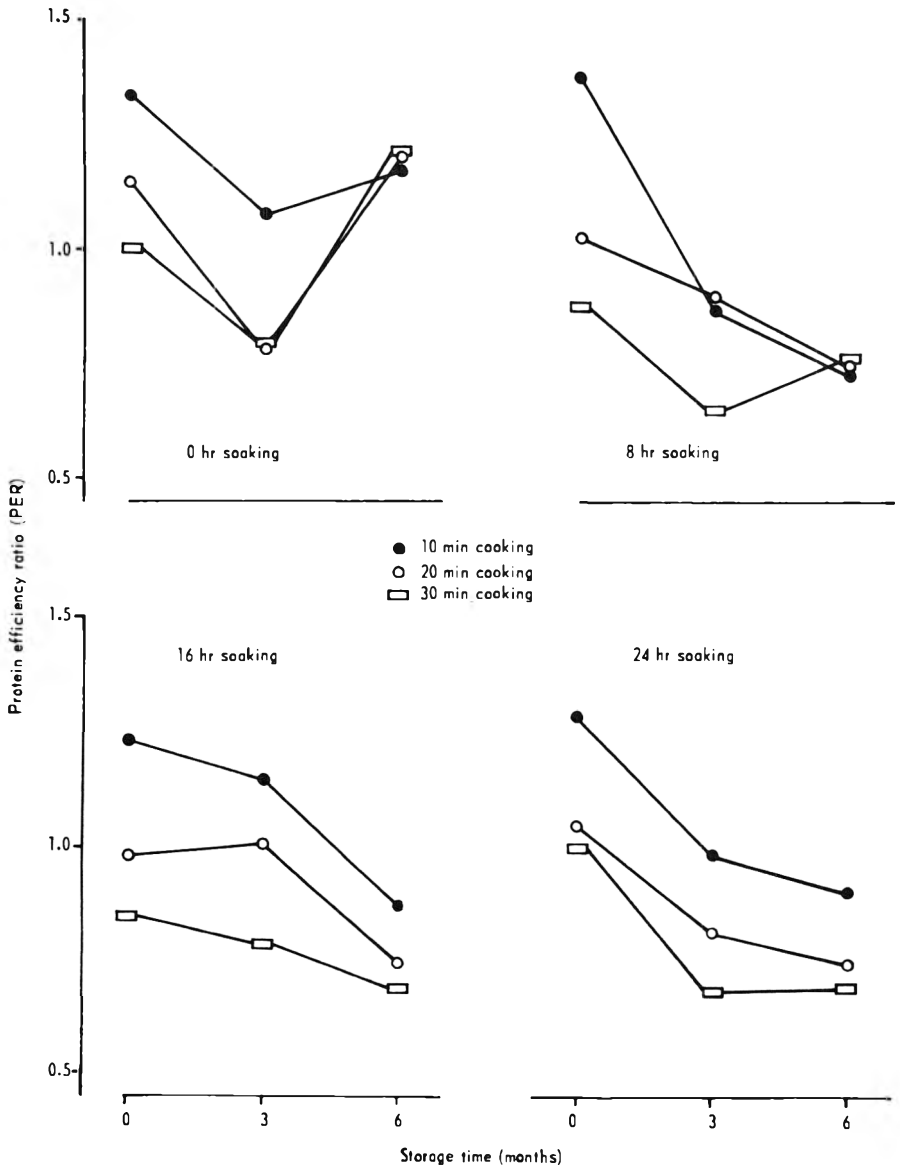

Fig. 2—Effect of storage on the protein efficiency ratio of beans subjected to different soaking and cooking times.

be statistically significant ($P < 0.05$), as shown in Figure 3, where the effect due to cooking time has been eliminated. The significant effect of soaking on the protein quality of beans stored for 6 months is also clearly appreciated.

In general, the biological behavior of the bean samples subjected to the different processes studied, followed the pattern that was to be expected according to preliminary observations of storage influence on the protein quality of beans reported by Molina et al. (1972, 1974). However, since such changes in protein quality did not correlate with those observed in the methionine content of raw beans (Fig. 1), it was thought of interest to examine other parameters which could explain, partly at least, the biological behavior of the processed samples.

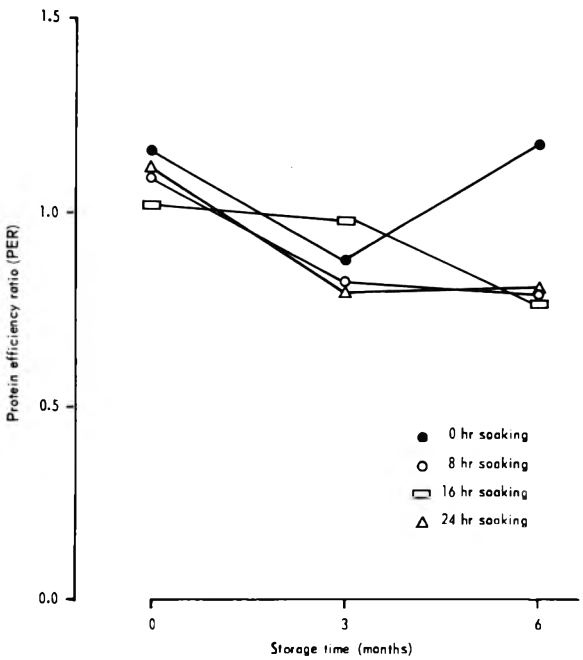

Fig. 3—Effect of storage on the protein efficiency ratio of beans subjected to different processes (eliminating the cooking effect).

The cystine, methionine and available lysine contents of the samples evaluated in this study are shown in Table 1. As the data reveal, the methionine and available lysine content of the processed samples tended to increase with storage, independent of the process to which they were subjected. These results reflect the increase in the content of both amino acids observed in the case of raw beans (Fig. 1). We cannot offer an explanation for these findings, but we can state that they could not be attributed to analytical errors, since all samples increased their available lysine and methionine content. On the other hand, the cystine content did not show a definitive pattern of change during storage. In this respect, it is of interest to note that the availability of bean methionine and cystine for the rat has been shown to be very poor when compared to that found for the same amino acids in the case of soybeans (Evans et al., 1974). Furthermore, the same authors (Evans et al., 1974) indicate that the degree of availability of these amino acids correlated with the PER values obtained for either beans or soybeans. Since in the present case the PER values (Fig. 2) did not correlate with the changes in the total methionine and/or cystine content of the processed samples (Table 1), the possibility exists that storage and processing may strongly affect the availability of sulfur-containing amino acids. This possibility is now being investigated in our laboratories.

The protein content and true protein digestibility of all samples studied are presented in Table 2. As the data show, the total protein content was unaffected either by storage or

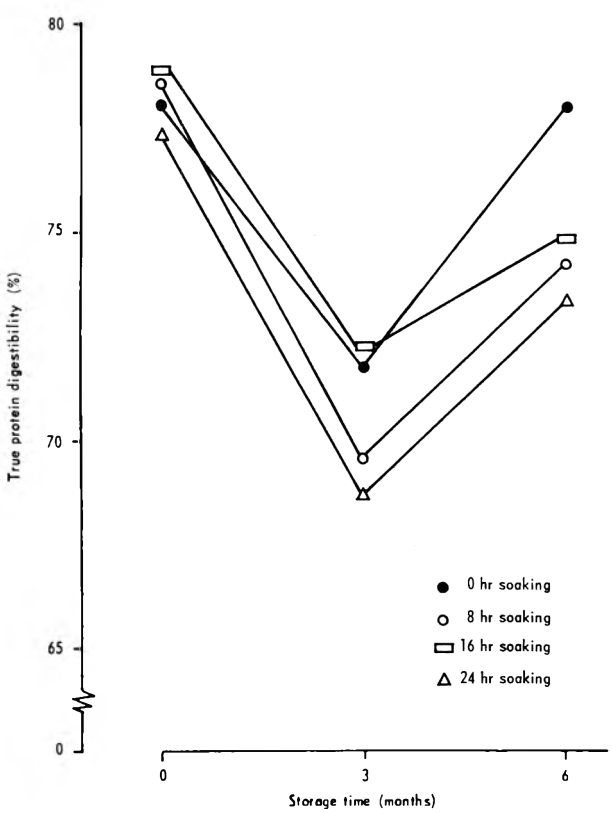

Fig. 4—Effect of storage on the protein digestibility of beans subjected to different processes (eliminating the cooking effect).

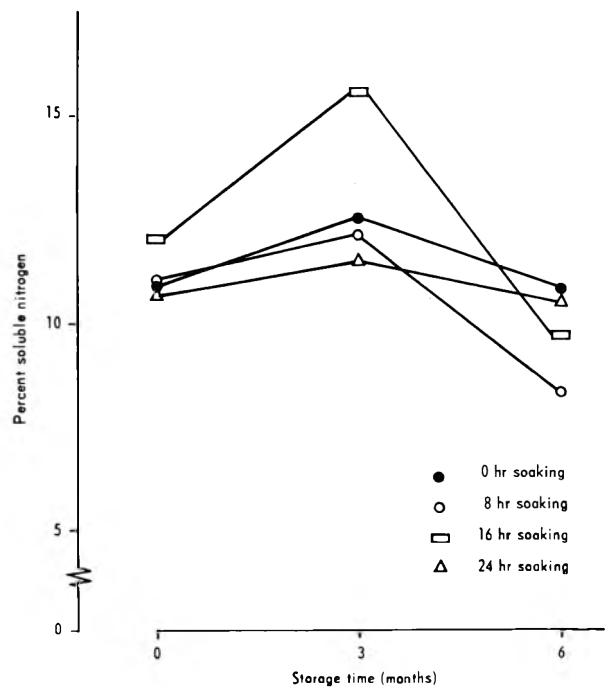

Fig. 5—Effect of storage on the nitrogen solubility in 1N NaCl of beans subjected to different processes (eliminating the cooking effect).

Table 1—Methionine, cystine and available lysine content of beans stored for different periods of time and subjected to different processes (g/16g N)

	Storage time (months)								
	0			3			6		
Process[a]	Available lysine	Methionine	Cystine	Available lysine	Methionine	Cystine	Available lysine	Methionine	Cystine
0–10	4.97	0.60	0.61	5.25	0.81	0.50	5.75	1.58	0.55
0–20	4.38	0.59	0.76	5.05	0.75	0.67	5.73	1.36	0.68
0–30	4.72	0.68	0.67	4.95	0.92	0.70	5.20	1.18	0.62
8–10	5.23	0.70	0.64	5.04	1.12	0.59	5.59	1.29	0.60
8–20	5.08	0.85	0.66	5.09	1.06	0.65	5.69	1.14	0.63
8–30	4.31	0.78	0.74	5.10	1.11	0.67	5.39	1.26	0.70
16–10	5.00	0.77	0.51	5.34	1.01	0.69	5.67	1.11	0.69
16–20	4.51	0.71	0.71	5.07	1.04	0.65	5.77	1.11	0.68
16–30	4.81	0.67	0.69	5.12	0.98	0.77	6.48	1.44	0.59
24–10	4.72	0.62	0.57	5.08	1.10	0.61	6.67	1.04	0.63
24–20	4.98	0.67	0.67	5.31	1.24	0.69	5.78	1.22	0.67
24–30	4.36	0.74	0.68	4.99	1.12	0.58	6.10	1.20	0.71

[a] Soaking hours—minutes cooking

by any of the processes evaluated. The protein digestibility of the samples subjected to any of the soaking periods studied, proved to be affected by cooking time, as happened in the case of the PER (Fig. 2). The effect of cooking time on the protein digestibility of the beans was not significant. Analysis of the data indicated that storage had a statistically significant ($P < 0.05$) effect on the digestibility of the bean protein. Soaking, however, had no significant effect on this parameter at any storage period evaluated.

The effect of storage on the digestibility of the bean protein is presented in Figure 4, where the effect of cooking time has been eliminated.

The nitrogen solubility of the processed samples in H_2O, 1N NaCl and 0.05N NaOH can be appreciated in Table 3. It is of interest to note that in the case of the samples subjected to any given soaking period, cooking time had an opposite effect on the nitrogen solubility in 1N NaCl and on the protein digestibility (Table 2). Also it is worthwhile noting that the samples stored for 3 months showed the highest nitrogen solubility in any of the solvents tested as well as the lowest protein digestibility when subjected to any particular process (Table 2, Fig. 4). Storage was found to have a statistically significant ($P < 0.05$) effect on the nitrogen solubility of the samples in any of the solvents evaluated.

The significant effect of storage on nitrogen solubility in 1N NaCl is shown in Figure 5, where the effect of cooking time has been eliminated. In this case one can observe that the tendency is opposite to that found in regard to protein digestibility.

Preliminary results show that the nitrogen fraction soluble

Table 2—Protein content and true digestibility of beans stored for different periods of time and subjected to different processes

	Storage time (months)					
	0		3		6	
Process[a]	Protein (N X 6.25)[b] (g/100g)	True protein digestibility (%)	Protein (N X 6.25)[b] (g/100g)	True protein digestibility (%)	Protein (N X 6.25)[b] (g/100g)	True protein digestibility (%)
0–10	25.5	80.2	23.9	73.5	24.9	77.1
0–20	25.7	78.5	24.7	71.9	24.4	77.1
0–30	25.6	75.5	24.6	70.2	24.6	80.2
8–10	25.6	79.9	25.1	72.9	24.4	77.2
8–20	25.0	78.9	25.7	68.2	24.6	75.1
8–30	26.0	77.1	26.2	67.5	24.3	70.5
16–10	26.2	80.8	25.2	74.7	24.7	77.5
16–20	24.4	79.1	26.0	71.4	25.4	73.8
16–30	26.0	76.6	25.8	70.7	25.1	73.0
24–10	25.8	77.8	24.9	69.7	25.2	75.3
24–20	24.7	77.6	25.7	69.4	24.9	72.8
24–30	25.7	76.6	25.3	67.3	25.4	72.1

[a] Soaking hours—minutes cooking
[b] On a 9% moisture basis

Table 3—Nitrogen solubility in 1N NaCl, 0.05N NaOH and H_2O of beans stored for different periods of time and subjected to different processes

	Storage time (months)								
	0			3			6		
	Nitrogen solubility (%)			Nitrogen solubility (%)			Nitrogen solubility (%)		
Process[a]	1N NaCl	0.05N NaOH	H_2O	1N NaCl	0.05N NaOH	H_2O	1N NaCl	0.05N NaOH	H_2O
0–10	9.4	14.1	11.4	11.0	14.1	11.1	12.0	17.6	9.3
0–20	11.3	13.2	12.1	13.0	14.9	12.2	10.6	17.9	8.8
0–30	12.1	14.8	13.7	13.6	14.8	14.3	9.8	14.0	8.9
8–10	9.8	14.1	11.2	11.2	18.2	17.2	7.0	16.8	8.2
8–20	11.2	14.8	10.8	12.1	19.0	16.6	8.5	14.2	8.5
8–30	11.9	14.2	12.7	12.4	18.2	17.7	9.4	14.0	10.8
16–10	10.7	14.5	13.0	11.8	18.2	17.0	9.3	12.4	9.7
16–20	12.7	14.8	12.7	13.7	22.5	21.3	9.8	15.0	10.4
16–30	12.7	15.4	12.5	20.6	28.3	23.5	10.5	13.0	8.5
24–10	10.1	15.2	10.5	10.2	17.2	14.0	9.5	15.1	8.7
24–20	10.5	14.2	11.7	12.4	17.9	16.3	10.4	12.9	10.5
24–30	12.1	14.8	11.7	12.9	16.8	12.4	11.9	16.1	11.3

[a] Soaking hours—minutes cooking

in 1N NaCl from beans subjected to any of the storage times or processes evaluated, is capable of lowering the protein in vitro digestibility of either beans or standard proteins (like zein) by 15–30% when using pepsin as the digestive enzyme. Furthermore, one of the authors (Bressani, unpublished data) has found that, in dogs, the black bean water-soluble nitrogen fraction shows a much lower digestibility (around 40%) than the water-insoluble nitrogen fraction (between 70–80%). These findings are not surprising if we consider that beans have been reported to have residual hemagglutinating activity after "proper" cooking (Korte, 1972) and to have specific globulin fractions which exhibit proteinase inhibitory action even after denaturation by heat or urea (Seidl et al., 1969).

At present, we are trying to establish whether the changes in nitrogen solubility in NaCl solutions—or other solvent—observed during storage or processing, represent also a change in the degree of inhibition of protein digestibility in vitro, and whether these nitrogen fractions affect in some way the availability of the most limiting essential amino acids in the bean protein. Likewise, the chemical and physical characteristics of such fractions and the changes occurring in the whole amino acid pattern of the bean protein during storage are being investigated.

Evidently, more research is necessary to explain the changes in biological behavior due to storage and processing herein presented.

Since legume foods in general are very susceptible to hardening during storage, thus causing large economic losses, more research is desirable to determine if there exists a correlation between the physical and biological changes in beans during storage. We believe that such a research would be of great value for most Latin American countries where beans supply large amounts of protein to the habitual diet and are becoming increasingly important during the period of protein shortage we are now facing.

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FROZEN STORAGE KEEPING QUALITY OF MINCED BLACK ROCKFISH (Sebastes spp.) IMPROVED BY COLD-WATER WASHING AND USE OF FISH BINDER

INTRODUCTION

MACHINES FOR efficiently separating the edible flesh from skin and bones of headed-and-gutted fish have been used in Japan for several decades to produce minced fish muscle. This machine-separated minced muscle has been used in food products that range from the traditional kamaboko fish cake to the newer fish sausages and hams. In 1970, over 1 million metric tons of kamaboko and fish sausages were produced in Japan (Okada et al., 1973). On the other hand, it is only recently that major fish processors in other countries have shown interest in developing new food products from mechanically-separated fish muscle. The scarcity of frozen cod and haddock fillet blocks and the increasing demand for this commodity to make breaded fish sticks and portions have resulted in the production of minced fish blocks by a number of countries for marketing in the United States (Teeny and Miyauchi, 1972; Whitaker, 1972).

During 1969, investigations were started at the Pacific Utilization Research Center to test the use of mechanically-separated minced muscle from under-utilized species in frozen fish blocks. Minced fish muscle offers great flexibility of processing to improve flavor, texture and storage stability and to enable the production of blocks from species not suitable for the production of conventional fish fillet blocks (Steinberg, 1972). Fish sorted commercially as black rockfish (*Sebastes melanops, S. brevispinis* and *S. flavidus*), whose dark muscle becomes rancid quickly during frozen storage (Teeny and Miyauchi, 1972; Stansby and Dassow, 1949), were selected for study because the successful stabilization of minced muscle of this species against oxidative rancidity would assure that many other species with similarly unstable lipids could also be used to produce minced fish blocks.

In the first phase of our study to develop a marketable minced black rockfish block, the best results were obtained by using sodium chloride and sodium tripolyphosphate to partially solubilize the muscle protein to bind the particles of coarse-minced muscle into a cohesive block. We called this a *modified* minced fish block (Teeny and Miyauchi, 1972). The use of butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) at levels ranging up to 0.006% increased the storage life at −18°C of the modified blocks to 9–12 months from only about 3 months for those containing no antioxidants.

In this, the second phase of the study, additional processing procedures were tried to improve the quality and the storage life of the modified black rockfish blocks. Meat-bone separating machines of the type in which the pressure applied to the fish material can be regulated as it passes through the machine permit the removal of primarily the light meat only. Stansby and Dassow (1949) demonstrated that removal of a small portion of the dark fatty flesh just beneath the skin of the fillet increased the cold-storage life of the frozen fillet from two to four times. Similarly, using predominantly the light meat in the minced blocks offers the possibility of increasing its cold storage life. Furthermore, in developing the process for making surimi, a semi-processed intermediate raw material for making kamaboko and fish sausages (Miyauchi et al., 1973), the Japanese found that washing the minced fish muscle well with chilled water to remove blood, flesh pigments, fat and other water-extractable constituents improved the color and odor and helped stabilize the functional properties of the wet fish protein during frozen storage. Accordingly, the purpose of this study was to determine whether the cold-storage characteristics of minced black rockfish blocks could be improved by (1) selectively separating and utilizing the light minced muscle of black rockfish; (2) washing the minced muscle to remove the water-extractable constituents; (3) the use of low-level antioxidants; and (4) the use of a fish binder.

EXPERIMENTAL

Sample preparation

In two replicate experiments in November and March, we used black rockfish in the round. They had been held in ice aboard commercial trawlers for about 5 days. On the basis of appearance and odor, the fish were judged to be average to better-than-average quality commercial fish. At our laboratory, some of the fish were used to prepare fillets and the remainder were used to prepare minced fish blocks. The fish to be mechanically deboned were headed, gutted and thoroughly washed to remove the various organs and extraneous soft materials from the gut cavity. The headed-and-gutted fish were next passed through a meat-bone separator (Bibun Model 15 with a drum perforated with 7-mm diameter holes) to separate the fish muscle from skin and bones. The belt tension on the meat-bone separator was set either at normal pressure to separate as much muscle as possible or at light pressure to separate primarily the light meat. The six types of minced muscle blocks listed in Table 1 were prepared as described below.

Preparation of unmodified minced muscle blocks. 500g quantities of the mixture of light and dark minced muscle were packed into waxed frozen food cartons (1-1/8 in. × 8-1/2 in. × 3 in.) and frozen under pressure in a vertical plate freezer (about 2 hr at −40°C) to form our mini-block control samples (Table 1, type 1). The mini-blocks were overwrapped with 2-mil polyethylene, sealed and stored at −18°C. Similarly, mini-blocks were prepared using the light minced muscle (Table 1, type 2).

Preparation of modified, unwashed minced muscle blocks. The modified minced muscle blocks were prepared as follows:

1. To prepare the fish binder to be used with each 100 lb of minced muscle, the ingredients listed below were homogenized in a

Table 1—Types of minced black rockfish blocks prepared

Type of blocks	Description of minced muscle	Processing variables		
		Washing	Antioxicants	Modified
1	Mixture of light & dark	Not washed	None	No binder
2	Mostly light	Not washed	None	No binder
3	Mostly light	Not washed	None	Binder
4	Mostly light	Not washed	Antioxicants	Binder
5	Mostly light	Washed	None	Binder
6	Mostly light	Washed	Antioxicants	Binder

1-gal Waring Blendor for about 2 min until the mixture became "sticky" or "tacky." The temperature of the binder was kept below 4.4°C by using ice water.

Ingredients used	Wt lb/each 100 lb of minced muscle	Function
Fish muscle	2.5	Improves textural properties
Sodium chloride	1.0	Solubilizes protein for textural properties; flavor
Sodium tripoly-phosphate	0.15	Solubilizes protein; improves water-holding capacity
Sugar	1.0	Taste
Monosodium glutamate	0.3	Flavor intensifier
Corn oil	1.0	Carrier for oil-soluble antioxidants
Ice water	5.0	Carrier for water-soluble ingredients

2. One-fourth of the binder-homogenate was added to 25-lb batches of light minced fish muscle and mixed for 2 min in a Hobart A-200 DT Food Mixer equipped with a dough arm agitator (Part E, S-62886) at low speed (about 50 rpm). 500g quantities of this mixture were packed (Table 1, type 3), frozen and stored as described in the preceding subsection.

3. The modified, unwashed minced muscle blocks containing antioxidants were prepared similarly except 0.001% by weight of BHA and 0.001% BHT were dissolved in the corn oil and used in the fish binder (Table 1, type 4).

Preparation of modified, washed minced muscle blocks. A portion of the light minced muscle taken from the meat-bone separator was weighed in a close-knit nylon mesh bag and gently agitated for 10 min in ice water (ratio of 5 parts by weight of water to 1 part by weight of minced muscle). The bag containing the fish-water slurry was suspended so as to permit the excess water to drain through the bag. Draining was continued until the weight of the washed muscle was approximately 3% less than that of the muscle before washing. This draining required several hours.

The washed minced muscle was used to prepare one lot of modified blocks containing no antioxidant (Table 1, type 5) and one lot containing antioxidant (type 6) as described in the preceding subsection.

Preparation of fillet blocks. Some of the whole rockfish were cut into skinless fillets. These fillets were washed, drained, packed into 5-lb frozen-food cartons, and plate frozen. These cartons of fillet blocks were overwrapped with 2-mil polyethylene, heat-sealed and stored at −18°C.

Preparation of reference samples. At each 4-month examination, modified, unwashed minced rockfish blocks were prepared from commercially caught, iced rockfish for use as freshly prepared reference samples.

Sensory evaluation

The various fillet and minced rockfish blocks were evaluated initially and after 4, 8 and 12 months of storage at −18°C. The various blocks were judged for odor and appearance (change in color). The blocks were then cut into 5/8-in. slices and examined organoleptically for development of rancidity and for changes in texture. Slices were prepared for the panel by steaming in covered aluminum containers for 15 min. They were presented as coded samples to a panel of 6 to 12 experienced judges. Samples were rated on a 5-point scale in comparison to the identified, freshly prepared reference sample, which was assigned the score of 5. The differences in the mean flavor and texture scores among the various samples were analyzed by analysis of variance (Snedecor and Cochran, 1956).

Moisture content determination

The moisture content was determined as follows: (1) Weigh 10–15g of fish sample into aluminum dishes containing about 20g of dried sand. (2) Using a spatula, mix the flesh thoroughly with the sand being careful to avoid loss of sand granules. (3) Heat overnight (12 hr) in air oven at 41°C. (4) Cool in desiccator for about 15 min. (5) Weigh. (6) Heat for another 2–4 hr, cool and weigh; repeat until constant weight is obtained. (7) Calculate moisture content.

RESULTS & DISCUSSION

Minced muscle yield

The yields of minced muscle, based on the weight of the round fish, obtained in the two experiments are given in Table 2. When the belt tension on the machines was set at normal pressure to recover the maximum amount of minced muscle, yields of 47.4% and 46.4% were obtained. When the belt tension was set at light pressure to recover primarily the light muscle, yields of 32.5% and 34.1% were obtained.

Table 2—Yields of minced muscle and skin-and-bone waste from headed-and-gutted black rockfish passed through the Bibun[a] meat-bone separator

Components		Yields[b] of various components Exp 1 (%)	Exp 2 (%)
(1)	Normal belt pressure:		
	Minced muscle	47.4	46.4
	Skin-and-bone waste	8.8	9.2
	Losses (by difference)	1.7	1.9
(2)	Light belt pressure:		
	Minced muscle	32.5	34.1

[a] Bibun Model 15 with drum perforated with 7-mm diameter holes
[b] Yields based on weight of whole fish used: 440 lb in Exp 1 and 395 lb in Exp 2

Fillet blocks

The black rockfish fillet blocks were moderately discolored and rancid in flavor when examined after 4 months of storage. This is in agreement with the 14–22-wk shelf life reported by Stansby and Dassow (1949) for fillets of the black rockfish, *Sebastes flavidus.*

Unmodified minced light-and-dark muscle blocks

The unmodified block made of unwashed, minced light-and-dark muscle was moderately rancid and judged unmarketable within 3 months of storage at −18°C.

Unmodified and modified minced light muscle blocks

The *unmodified* blocks made of unwashed, minced light muscle were moderately rancid and judged unmarketable after 4 months at −18°C (Table 3). Thus, the minced fish blocks made from primarily the light muscle had a shelf life about 1 month longer than those made from the mixture of light and dark muscle.

The modified blocks made of unwashed, minced light muscle, which was mixed with the fish binder, showed only slight-to-trace rancidity during 8 months of storage at −18°C. The texture of the modified blocks changed only slightly during the 8 months of storage. These results showed that the use of fish binder to prepare the modified minced fish block im-

Table 3—Changes in flavor (rancidity) and texture of unmodified and modified minced black rockfish blocks during storage at −18°C

Storage time (Months)	Mean flavor score (5-pt scale)[a] Unmodified	Modified	Mean texture score (5-pt scale)[b] Unmodified	Modified
0	4.0	3.9	3.9	4.0
4	2.9	3.5	3.4	3.7
8	2.6	3.4	2.9	3.8

[a] Five-point rating scale for rancidity, with mean score below 3 considered as "unmarketable": 5—None; 4—Trace; 3—Slight; 2—Moderate; 1—Excessive
[b] Five-point rating scale for texture: 5—Very good; 4—Good; 3—Fair; 2—Borderline; 1—Poor

proved its cohesiveness, water-holding capacity and succulence, and thereby significantly improved the cold-storage characteristics of minced black rockfish muscle.

Effect of washing

After washing the minced muscle with chilled water, the fish-water slurry was dewatered until the weight of the washed minced muscle reached a weight about 3% less than the starting weight of the minced muscle before washing. Although the optimum degree of dewatering was not determined, we did establish experimentally that this degree of dewatering enabled us to prepare modified blocks with good cold-storage and processing characteristics. The moisture contents of the modified blocks prepared from washed minced muscle were up to 4% higher than those made from unwashed minced muscle (Table 4).

In experiments to determine the effect of washing the minced muscle to remove some of the water-extractable constituents on flavor stability during frozen storage, the flavor scores (Table 5) of the modified, washed blocks (without antioxidants) were significantly higher at the 5% level than those of the modified, unwashed blocks (without antioxidants) at each examination during the 1-yr storage test at −18°C, except the 4-month examination in experiment 2. In addition, the modified, washed blocks were appreciably "whiter" in color than the modified, unwashed blocks. The storage life of the modified, unwashed blocks was between 8–12 months, whereas that of the modified, washed blocks was greater than 12 months. Thus, washing the minced muscle with chilled water to reduce the amount of blood, flesh pigments, fat and water-soluble proteins did improve the color, flavor and odor, and increased the storage life of the minced fish blocks.

Table 4—Moisture content of modified minced black rockfish blocks made from unwashed and washed minced muscle

Type of modified block	Moisture content Exp 1 (%)	Moisture content Exp 2 (%)
Unwashed, minced	78.2	77.4
Washed, minced	80.9	81.3

Table 5—Effect of washing minced flesh on flavor (rancidity) of modified black rockfish blocks with and without antioxidants during 12 months of storage at −18°C

Replicate experiment (No.)	Storage time (Months)	Mean flavor score[a] Unwashed flesh Without antioxidant	Unwashed flesh Antioxidant	Washed flesh Without antioxidant	Washed flesh Antioxidant
1	0	4.7	4.7	4.8	4.7
	4	3.5	3.5	4.3	4.3
	8	3.3	3.5	4.0	4.0
	12	2.9	3.2	3.7	3.8
2	0	4.7	4.6	4.7	4.5
	4	4.7	4.2	4.5	3.7
	8	3.0	2.8	4.1	4.0
	12	2.8	3.0	4.1	4.0

[a] Five-point rating scale for rancidity, with mean score below 3 considered as "unmarketable": 5—None; 4—Trace; 3—Slight; 2—Moderate; 1—Excessive

Effect of adding antioxidants

The antioxidant mixture (0.001% by weight of BHA and 0.001% BHT) in the fish binder had no beneficial effect on the cold-storage characteristics of either the modified, unwashed or modified, washed minced rockfish blocks (Table 5). Blocks made with washed, minced muscle had good flavor in the presence and absence of antioxidants throughout the 12-month storage period. Blocks made with unwashed, minced muscle, however, showed product deterioration of flavor both with and without antioxidants and were slightly rancid by around 8 months; at any storage period, differences in flavor between samples with and without antioxidants were small and inconsistent.

SUMMARY

BOTH THE fillet and minced muscle blocks of black rockfish (*Sebastes* spp.) had storage lives at −18°C of less than 4 months owing to the development of rancid flavors and discoloration. Modified fish blocks prepared by mixing the minced muscle with a fish binder had a longer storage life of 8–12 months. Modified blocks made with washed minced muscle were significantly better in color and flavor than those made with unwashed minced muscle during the 12-month storage test.

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FUNCTIONAL PROPERTIES OF ADDED PROTEINS CORRELATED WITH PROPERTIES OF MEAT SYSTEMS. Effect of Concentration and Temperature on Water-Binding Properties of Model Meat Systems

INTRODUCTION

FUNCTIONAL PROPERTIES of proteins for foods can be defined as physico-chemical properties, providing a certain amount of information about how a protein will act in a food system. However, few systematic investigations have been made on how these properties can be correlated with properties of complex food systems (Yasumatsu et al., 1972a, b). Of great interest are properties depending upon such water-protein relationships as solubility, viscosity, swelling and gel properties. The importance of solubility for beverages is obvious. The effects of these properties on semi-solid products such as meat systems are of a more complicated nature.

Water-binding properties of pure meat systems were carefully reviewed by Hamm (1972), who discussed the influence of various factors such as pH, presence of salts, temperature, time after slaughter, etc. Lack of water-holding capacity (WHC) will result not only in weight losses, but will also influence the texture (Hamm, 1972; Galloway et al., 1973). Recent publications have shown that the protein network responsible for the WHC can also enclose fat cells and is important for the fat-binding properties of the so-called meat emulsions as well (Hamm, 1973; van den Oord, 1973). The effect of various proteins added to meat on water binding properties have hitherto been very little studied.

The aim of this study was to investigate the effects of some functional properties of added proteins on the water-binding properties of meat systems. In order to focus all attention on the water-protein relationships, model systems were used, with all visible fat removed from the meat and nothing but protein and water added. Meat qualities common in minced meat products and the commercially available proteins soy protein isolate, sodium caseinate and whey protein concentrate were investigated.

Due to the complex nature of the meat systems, where interactions between the meat proteins and added proteins, and negative as well as positive effects of functional properties might have to be considered, proper analysis and interpretation of data in most cases necessitate the use of statistical methods of inference. In practical applications, the conventional procedures for analyzing structures of multivariate systems may be characterized either as (1) regression methods or as (2) factor analysis methods. From a statistical point of view, the fundamental problem of the present study consisted in mapping several independent variables (functional properties) into a set of statistically well-defined dependent variables (water-binding properties). Hence, the approach of trying some appropriate multiple regression procedure seemed natural.

MATERIALS

Soy protein isolate

Promine-D (Central Soya), a commercially available sodium soybean proteinate. Analysis (dry wt): protein (N × 5.69) 86.8%, ash (550°C) 4.7%, pH in 1% protein dispersion 7.4.

Caseinate

Sodinol V (A/S Lidano), a commercially available sodium caseinate. Analysis (dry wt): protein (N × 6.38) 91.5%, ash (550°C) 4.6%, fat (Röse-Gottlieb) 1.1% and pH in 1% dispersion 7.4.

Whey protein concentrate (WPC)

WPC concentrated by gel filtration was used. Analysis (dry wt): protein (N × 6.38) 76.8%, ash (550°C) 5.4%, fat (Röse-Gottlieb) 5.2%, lactose (polarimetric method) 8.3% and pH in 1% dispersion 7.2.

Meats employed

Parallel runs were made with meat from beef brisket and meat from pork shoulder, materials frequently used in minced meat. Two meat systems were used in order not to draw conclusions from one special case. After removal of all visible fat, the meat was ground twice through a 5 mm grinding plate and mixed thoroughly. Portions of ca 100g were packed in sausage casings of PVDC film, quickly frozen and stored at −30°C. Thawing was carried out in a water-bath at 20°C. Analysis of beef brisket: protein (N × 6.25) 20.1%, water (drying at 130°C) 74.7% and fat (NMR) 3.3%. Analysis of pork shoulder: protein (N × 6.25) 19.5%, water (drying at 130°C) 76.6% and fat (NMR) 2.8%. During the experiments the water-binding properties of the frozen stored meat samples were continuously checked, and no significant changes were found.

METHODS

Functional properties

Solubility tests were made on 1% dispersions at 25°C. If heat treated the dispersions were maintained at 70, 80, 90 and 100°C for 30 min and then cooled to 25°C. The method used has previously been described (Hermansson, 1973). For swelling and Brookfield measurements, see Hermansson (1972). Viscosity measurements were made in a Haake Rotovisco RVI, thermostated to 25°C at 42 s^{-1}, 1142 s^{-1} of untreated dispersions and at 15 s^{-1} of heat-treated dispersions. When the effect of heat treatment was studied, the dispersion was heat treated in the measuring system for 30 min and rapidly cooled to 25°C. Readings were always taken after 5 min.

Penetrometer. A SUR-penetrometer equipped with a 25g plunger, diameter 20 mm, was used. Readings were taken after 5 sec.

Properties of meat systems

Calculations on the composition. A constant protein/water quotient of 0.2 was preferred to calculations on added protein and added water. The advantages of a constant quotient have previously been discussed for model calculations on sausages by Hennig (1971) and Lindner and Stadelmann (1961). When proteins were added the calculation was based on percent added protein out of the total protein content i.e., as if part of the meat proteins had been exchanged. Table 1 shows the relationship of percentage exchanged meat protein and other ways of representing the amount of incorporated protein.

Mixing technique

A basic Sorvall Omnimixer with baffled stainless steel chambers was used at 2800 rpm. Mixing time was in 3–30 sec intervals. After each 30 sec interval the system was gently stirred with a glass rod. When protein was to be exchanged, meat, protein and water were mixed for 2–30 sec intervals. The protein was allowed to swell for 20 min and the system was then remixed for 30 sec. The sample size was about 200g.

Determinations of moisture loss (ML)

Fresh meat systems. Various techniques reported in the literature were tried. The pressure technique recommended by Grau and Hamm (1957) for raw meat was found unsuitable when caseinate or WPC was

incorporated. Instead, a centrifugation technique was developed, in which a 20g sample was centrifuged at 18,000 rpm (40,000 × G) for 30 min. The volume of released juice was measured, and moisture loss calculated as (ml of juice released/20) × 100. Three replicate runs were made, and the analysis had a standard deviation of 0.3. The effects of experimental conditions are shown in Table 2. When one parameter was varied, the other two were kept constant at the values referred to above. The method will be referred to as ML_c (c = centrifugated).

Heated meat systems. A modified version of the heated tube technique described by Wierbicki et al. (1957), and a drip method similar to that of Miller et al. (1968) were used. The former was found to be the better of the two, and ML_{ht} (ht = heated tube) will be reported in this and following publications. 2g of meat mixture were filled in open-end glass tubes (6 cm × 1 cm) and closed with rubber stoppers. The upper rubber stopper had a small hole to equalize pressure. The tubes were heated at 80°C for 15 min. After cooling, the stoppers were removed and the meat plug carefully transferred to a net of stainless steel fastened in a steel ring, and weighed. The net assembly was then placed on a hollow PVC cylinder ca 1.5 cm above the bottom of a centrifuge tube and centrifuged at 2,000 rpm for 5 min. The steel ring had grips and could easily be lifted out of the tubes. ML_{ht} was calculated as (weight difference/weight before centrifugation) × 100. Four replicate runs were made and the method showed a standard deviation of 0.5. At high levels of protein incorporation, differences in flow properties might have an effect on the dependence of the centrifugation speed, and thus influence the result. ML_{ht} as a function of centrifugation speed for systems with 50% exchanged protien is shown in Figure 1. The curves do not intersect in the range studied.

Statistical models

Regression models. The observed changes in moisture loss properties were related to corresponding changes in functional properties by means of certain regression procedures. Among others, the following types of regression models were used:

Linear additive model

$$ML_d = \beta_0 + \beta_1 U + \beta_2 U_2 + \ldots + \beta_p U_p + \epsilon;\ \epsilon \sim N(0,\sigma^2) \qquad (1)$$

ML_d denotes the observed changes in moisture loss; $\beta_0, \beta_1, \ldots, \beta_p$ are the unknown regression parameters to be estimated; ϵ is a normally distributed random error with the mean value 0 and the variance σ^2. For each i, i = 1, ..., p, U_i denotes some product of the observed changes in the k different functional properties. In the simplest possible case, each U_i involves only one variable. Model (1) may then be written as:

$$ML_d = \beta_0 + \beta_1 X_{1d} + \beta_2 X_{2d} + \ldots + \beta_k X_{kd} + \epsilon \qquad (1.1)$$

where $X_{1d}, \ldots, X_{kd}$ are the observed changes in the functional properties.

Multiplicative model

$$ML_d = \beta_0 X_{1d}^{\beta_1} \ldots X_{kd}^{\beta_k} \epsilon \qquad (2)$$

Taking logarithms converts this model into the linear form, equivalent to model (1).

The following nonlinear formulation of the multiplicative model was also tried:

$$ML_d = \beta_0 X_{1d}^{\beta_1} \ldots X_{kd}^{\beta_k} + \epsilon \qquad (2.1)$$

It should be observed that (2.1) cannot be transformed to the linear model above, i.e., (2.1) is intrinsically nonlinear.

"Metric" model

$$ML_d = \left[v_1 |X_{1d}|^\rho + \ldots + v_k |X_{kd}|^\rho\right]^{1/\rho} + \epsilon \qquad (3)$$

$v_i \geq 0$; $\rho \geq 1$. $v_1, \ldots, v_k, \rho$ are the unknown parameters to be estimated. In model (3), moisture loss changes are described as linear functions of distances in some metric space of unknown geometry. The space is composed of the k different functional properties (or some subset thereof). In the formulation above, the metric model is intrinsically nonlinear and requires the application of iteration techniques for parameter estimation.

To account for potential interactions between "dimensions," a number of generalizations of the "metric" model was also tried. Since no significant improvement in goodness of fit could be obtained, further elaborations of this model are omitted here.

Goodness of fit measure and test statistic. Let D_{est} denote the estimated (predicted) change in moisture loss properties obtained by the regression model under consideration, and D the corresponding experimentally observed change. A convenient test statistic may be calculated from the residuals $(D_i - D_{iest})$ i = 1, ..., N for the N observed differences according to the following:

$$Z = (D_i - D_{iest})^T \left[\mathrm{Var}(D_i - D_{iest})\right]^{-1} (D_i - D_{iest})$$

Var( ) denotes the variance, and $(\ )^T$ the transpose of the $(D_i - D_{iest})$ – vector.

According to the distribution assumptions of the above models, and the additional assumption that the covariance, $\mathrm{Cov}(D_i - D_{iest})(D_j - D_{iest}) = 0$; i,j = 1, ..., N; i = j; the distribution of Z is asymptotically a noncentral chi-square distribution. The degrees of freedom are given as r = N – p, where p is the number of estimated parameters of the applied regression model.

The decentralization parameter λ may be written as:

$$\lambda = \left[E(D_i - D_{iest})\right]^T \left[\mathrm{Var}(D_i - D_{iest})\right]^{-1} E(D_i - D_{iest})$$

E( ) denotes the expected value of the residual vector. E(Z) = r + λ and Var(Z) = 2(r + 2λ). Since λ = 0 for a "true" unbiased model, a reasonable goodness of fit measure is given by:

$$A = Z - r$$

In the subsequent calculations the test statistic Z and the goodness of fit measure A were used for two purposes:

(1) to compare different regression models with respect to goodness of fit when tested for all possible combinations of regression variables (functional properties) in order to select the most efficient regression model; and

Table 1—Calculations on percentages of protein incorporated in meat systems.

Exchanged meat protein[a] (%)	Protein added to the meat (%)	Protein added to the meat mixture (%)
10	2.2	1.7
20	5.0	3.3
30	8.5	5.0
40	13.2	6.7
50	19.8	8.3

[a] Added protein based on the protein content

Table 2—Effect of experimental conditions on moisture loss of raw meat systems (ML_c)[a]

Wt (g)	ML_c (%) A	ML_c (%) B	Centrifugation speed (X G)	ML_c (%) A	ML_c (%) B	Time (min)	ML_c (%) A	ML_c (%) B
15	41.0	37.3	12 500	34.0	30.5	10	39.0	35.0
20	40.8	37.0	27 500	37.5	34.5	15	39.8	36.0
25	40.8	37.2	40 000	40.0	36.5	30	41.5	37.0
			59 000	42.0	40.0	45	42.0	37.8

[a] The meats used in this study were from a sampling other than those used in the following experiments; A = beef brisket; B = pork shoulder.

(2) to compare different combinations of regression variables, for a specific regression model, with respect to goodness of fit, in order to select the most efficient combination(s).

Selection of regression variables (functional properties)

The selection of regression variables (functional properties) to be included in the above models was carried out by means of a computational procedure involving the calculation of all possible combinations of variables (Schatzoff et al., 1968; Draper and Smith, 1966). The main reasons for using such a "complete enumeration technique" rather than a conventional stepwise procedure were the following: (a) At least some of the potential variables were interacting in complicated ways and, as a result of this, confusing and contradictory outcomes were obtained when stepwise elimination procedures were tried (Efroymson, 1962); and (b) It could safely be assumed that there existed a number of equivalent, or almost equivalent, solutions for the different cases under consideration. Only by investigating all possible combinations could such sets of solutions be delimited.

The computational process included the following steps:

(1) Specification of the regression model to be used and calculation of maximum likelihood estimators of the parameters. (For the intrinsically nonlinear cases only approximate maximum likelihood estimators could be obtained.)
(2) Estimation of goodness of fit in terms of the measure defined above (the A-measure).
(3) Ordering of all possible outcomes with respect to goodness of fit. (With k different variables there are 2^k possible outcomes to be ordered.)
(4) Listing of the outcomes satisfying some preset criterion of goodness of fit (the others were deleted).

For each case under consideration, the set of moisture loss data was partitioned into two equivalent and statistically independent subsets ("split-half" method). Step (1) was carried out on subset I and steps (2)–(4) on the independent subset II, using the parameter estimators calculated from subset I as fixed parameter values. In this way, the maximum likelihood estimators for the different cases were calculated on one subset and the resulting goodness of fit was estimated and tested on another subset. Thus, the risk of overfitting the models could be reduced and the capacity for screening variable combinations having only "ad hoc validity" improved.

Experimental design

On each level (% exchanged protein or temperature), the three possible differences in moisture loss with respect to the three proteins were formed and correlated with the corresponding differences in functional properties. In order to identify possible deviations from one of the proteins, the following subsets were studied as well: $Diff_{P-W} + Diff_{P-C}$, $Diff_{P-W} + Diff_{C-W}$ and $Diff_{P-C} + Diff_{C-W}$. (P = Promine-D; C = caseinate; W = WPC.) As the two meat systems gave similar results they were pooled in the statistical analysis. The functional properties were classified into the following groups: solubility, swelling, viscosity and gel strength properties.

The outcomes of the regression analysis, satisfying the preset goodness of fit conditions, were evaluated according to the following:

(A) The most efficient regression variable (functional property) of each group was identified.
(B) The optimal solution, using all functional properties as potential variables, was identified.
(C) The solutions obtained when the variables of (A) were constrained as first variables in the different equations were identified.

In the following, the solutions of (B) are referred to as free choice solutions since no constrained conditions are imposed. Analogously, the solutions of (C) are called constrained choice solutions.

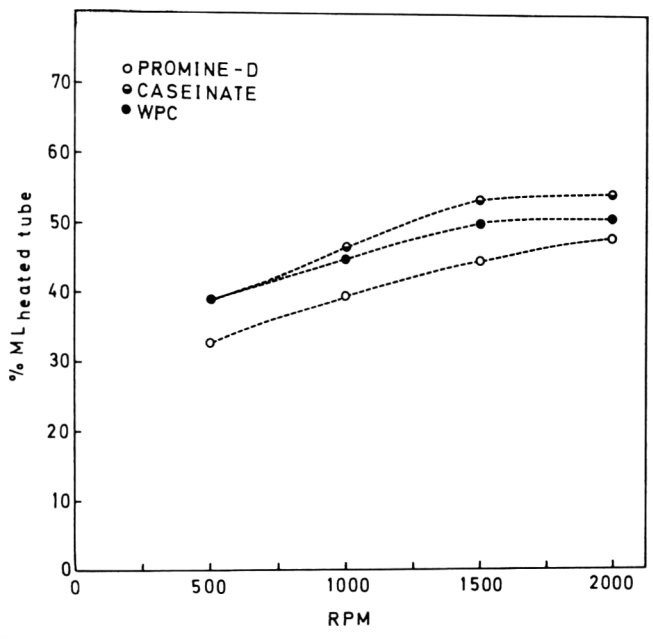

Fig. 1—ML_{ht} as a function of centrifugation speed for beef brisket systems with 50% exchanged protein.

RESULTS & DISCUSSION

Functional properties

The parameters used for correlation with moisture loss properties can be divided into solubility (group I), swelling (group II), viscosity (group III) and gel strength parameters (group IV) (Table 3). These groups are not independent, and the expected relationships are qualitatively illustrated by the Venn diagram shown in Figure 2. Swelling, when defined as

Table 3—Functional properties of Promine-D, caseinate and WPC

Group	Property	Temp[a] °C	Promine-D	Caseinate	WPC
I	Solubility of	25	52.9	80.8	78.3
	1% dispersions	70	67.0	78.5	83.5
	(% extractable	80	67.6	77.7	85.0
	nitrogen)	90	70.9	78.0	83.3
		100	80.7	76.4	83.5
II	Swelling	25	9.6	7.5	1.8
	(μl/mg)	70	16.7	6.4	2.9
		80	20.0	6.0	4.1
		90	17.2	6.8	4.3
		100	14.2	7.1	4.4
III	Viscosity (cp)				
	10% 42 s^{-1}	25	290	21	5
	10% 1142 s^{-1}	25	67	19	4
	12% 42 s^{-1}	25	1045	75	7
	12% 1142 s^{-1}	25	125	58	6
IV	Viscosity (cp)	70	3620	23	8
	10% 15 s^{-1}	80	7490	23	440
		90	5280	21	860
		100	1410	21	730
IV	Brookfield (p)	70	2320	0	0
	10%	80	3680	0	150
		90	2770	0	1050
		100	2130	0	1520
IV	Brookfield (p)	70	4950	0	3
	12%	80	7610	0	3700
		90	8680	0	6460
		100	7690	0	8630

[a] Measurements were made at 25°C after heat treatment except swelling measurements which were made at 20°C.

the spontaneous uptake of water, is the first step in the solvation process. If swelling is unlimited, the proteins will solvate; if not, swelling will proceed until it is limited by various intermolecular forces in the swollen sample. At 25°C the swelling

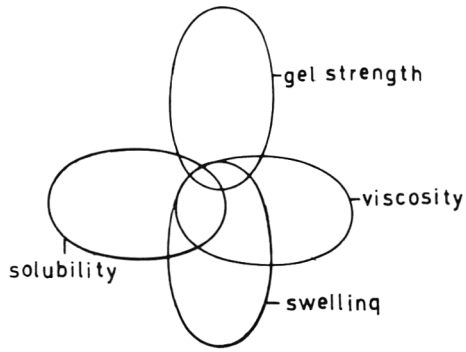

Fig. 2–Expected relationships of functional properties.

of Promine-D is regarded as limited and the swelling of caseinate and WPC as unlimited (see Hermansson, 1972). Heat treatment might cause gelation of concentrated dispersions (10%), which was the case for Promine-D and WPC, but not for caseinate. When the gels were freeze dried and ground, the protein network formed by heat treatment resulted in increased swelling ability and reduced solubility. As seen from Table 3, the swelling ability of heat-treated Promine-D samples was very high. The effect of heat treatment was not reflected by the solubility data listed in Table 3, since they were measured on 1% dispersions, where possibilities for intermolecular interactions were not sufficient to reduce the solubility.

The viscosity properties are influenced both by solubility and swelling. Highly soluble nonswelling proteins have low viscosity, which is the case for many globular proteins, such as those in WPC. Soluble proteins with high initial swelling, such as caseinate, show a highly concentration dependent viscosity, probably due to the amount of swelled, not fully solvated particles. In this case swelling and viscosity are highly intercorrelated (Hermansson, 1972; Hermansson, 1974). Promine-D, with its high limited swelling, shows a relatively high viscosity also at lower concentrations (4–8%) in distilled water.

The ability of proteins to form gels by heat treatment cannot be predicted from swelling, viscosity or solubility data

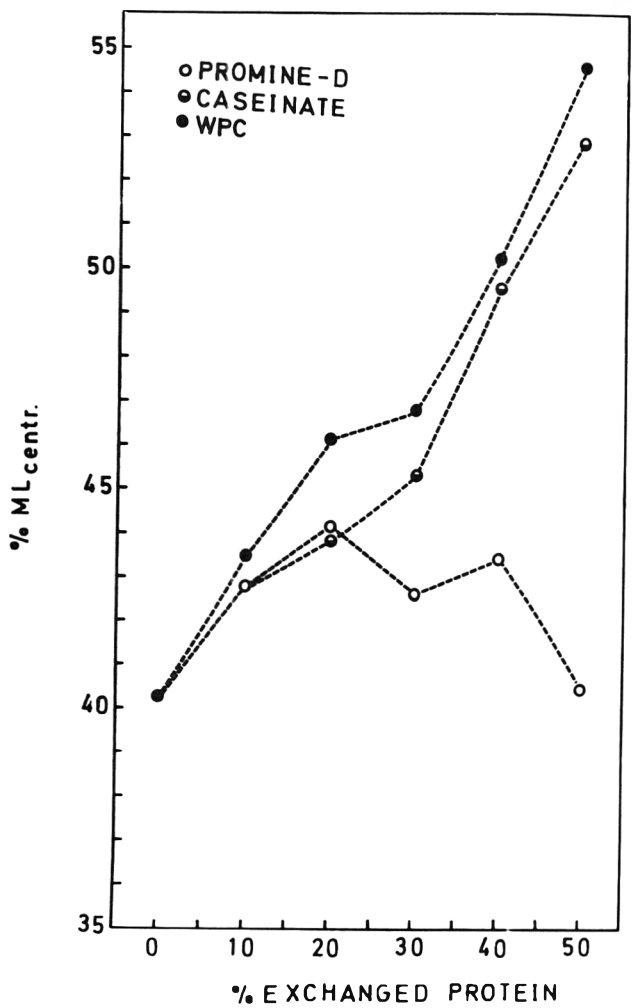

Fig. 3–ML_c of beef brisket systems as a function of % exchanged protein.

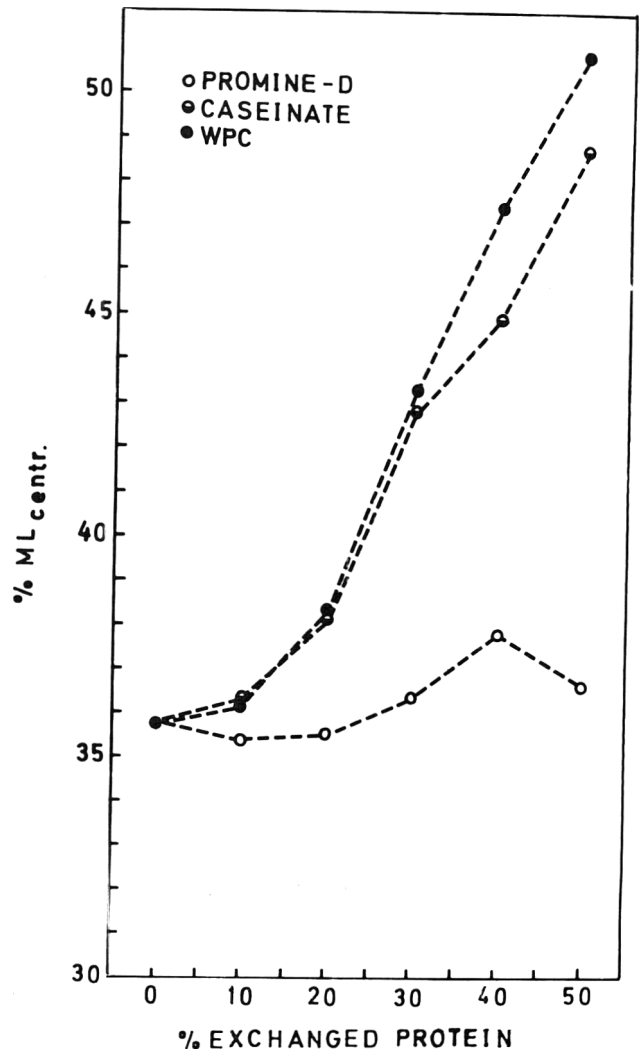

Fig. 4–ML_c for pork shoulder systems as a function of % exchanged protein.

Table 4—Penetrometer depth (mm) after 5 sec on beef brisket systems[a]

Exchanged protein %	Promine-D	Caseinate	WPC
10	3.8	3.8	5.5
20	4.6	5.2	7.6
30	6.5	7.0	9.8

[a] With no exchanged protein the penetration depth was 3.2 mm.

given at 25°C. As discussed above, gel strength and swelling measured after heat treatment are intercorrelated. Both the Brookfield data and the viscosity at 15 s^{-1} are qualitative measures of gel strength. In the Brookfield method, the structure of an undisturbed gel is determined, and in the viscometer a partially broken-down structure is measured. From Table 3 it can be seen that Promine-D formed a stronger gel in distilled water than WPC, with a maximum in gel strength (Hermansson, 1972). The effect of salt is discussed in the following paper (Hermansson and Åkesson, 1975).

Properties of meat systems

Raw meat systems. Figures 3 and 4 show the effects on ML_c of the incorporation of the three proteins in the two meat systems. The effects are evident, and the changes were similar in the two meat systems at levels ⩾ 30% (5.0% of the total mixture). Exchange of Promine-D resulted in the lowest moisture loss which did not differ much from that of pure meat. Increasing levels of caseinate and WPC caused a pronounced increase in ML_c, and was somewhat greater for WPC. Of the three proteins, Promine-D had the lowest solubility, highest viscosity and swelling ability. Both caseinate and WPC were highly soluble, but caseinate showed a higher swelling ability and viscosity than did WPC. These properties seem to be of great importance for the water-binding properties of meat systems, and the statistical correlations will be discussed later.

As much interest has been focused on the correlation between water-binding properties and texture, it was of interest to determine whether the differences in ML_c caused by incorporation of proteins could also be reflected in texture measurements. As seen from Table 4, WPC > caseinate > Promine-D with respect to penetration depth in meat systems, which is in accordance with the observed ML_c differences. The penetration data were too few for statistical evaluation and will not be further interpreted.

Heat treated meat systems. *Effect of temperature.* When pure meat is heated, moisture loss has been shown to increase to about 90°C. At 100–110°C there is usually a decrease in moisture loss, probably due to changes in collagen (Hamm, 1972). In order to prevent weight losses, processes are there-

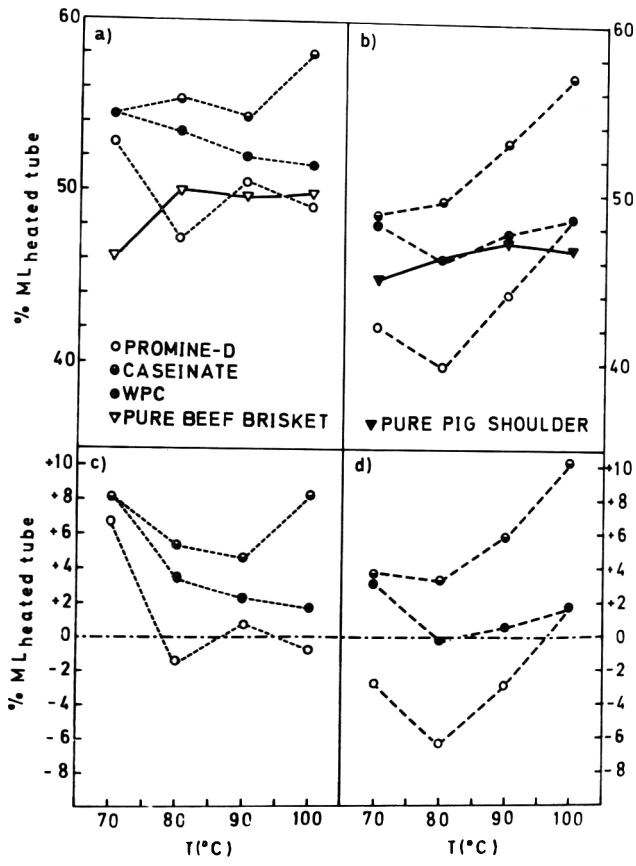

Fig. 5—ML_{ht} as a function of temperature: (a) beef brisket and (b) pork shoulder systems without exchanged protein and with 50% exchanged protein, respectively; (c) beef brisket and (d) pork shoulder systems respectively where the effect of 50% protein exchange is plotted relative to the pure meat systems.

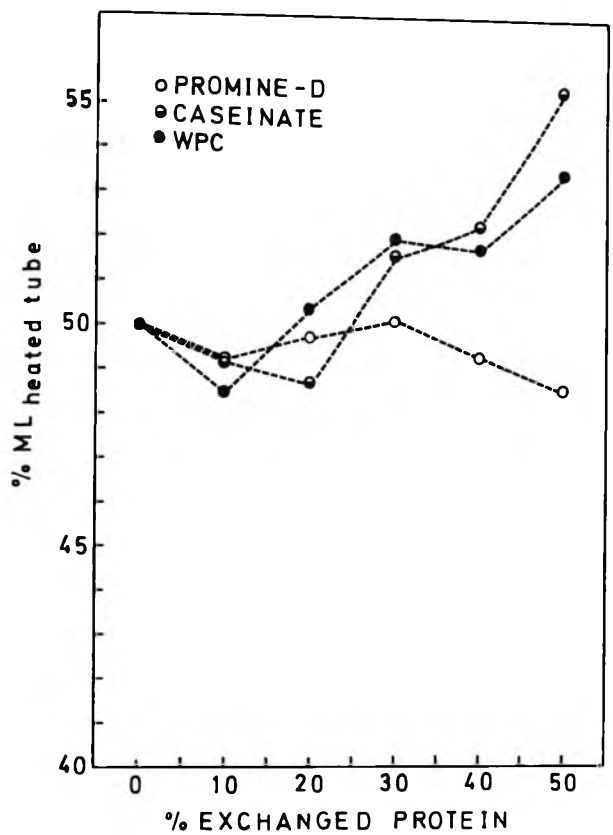

Fig. 6—ML_{ht} of beef brisket systems as a function of % exchanged protein (heated to 80°C).

fore often carried out at the lowest microbiologically acceptable temperature, i.e., 70°C.

The temperature dependence of moisture loss when 30% and 50% of the meat proteins were exchanged was studied. The results on the 50% level are depicted in Figure 5, which shows that 70°C is not the optimal temperature when proteins are added. Both WPC and Promine-D formed gels when heated. From Table 3 it can be seen that treatment at 70°C is not sufficient for WPC to gel, and that there is a maximum in gel strength for Promine-D at 80°C. When proteins like Promine-D and WPC are to be added to meat systems, 80°C is preferable to 70°C with respect to moisture loss, because gelation properties can be used to reduce moisture loss. The added proteins can then form gel structures within themselves or interact with the meat protein network (Hermansson, 1975). The differences found on the 30% level were of the same kind but smaller than on the 50% level, with the exception that caseinate showed a pronounced minimum at 80°C. The results on the 30% level will be discussed later.

Effect of levels of incorporation. Figures 6 and 7 show the effect on ML_{ht} when the meat systems were heated to 80°C. The changes in the two meat systems were similar in character, and at levels ⩾ 30% meaningful correlations with functional properties seemed possible. If the results on heat-treated meat systems are compared with the corresponding measurements on raw meat at levels ⩾ 40% (Fig. 3 and 4), there is one fundamental difference in that caseinate shows a higher moisture loss than WPC after heat treatment. This is probably due to the gelation of WPC, as discussed above. When heated, the lowest moisture loss was still obtained by the addition of Promine-D, and in the pork shoulder system there was even a decrease in ML_{ht} relative to the pure meat system when Promine-D was added.

Statistical correlation between functional and moisture loss properties

Results from Figures 3–7 show that the incorporation of proteins into meat systems has an impact on moisture loss properties that seems to be dependent upon the functional properties of the added protein. When the goodness of fit for the different types of regression models used was estimated, the results obtained by the linear additive model turned out to satisfy the preset conditions (defined in terms of significant levels of the test statistic) for most cases. In view of the limited amount of data and the large number of regression equations involved, this type of model was considered to be an acceptable compromise between parametric simplicity and descriptive power. The results presented in Tables 5–7 are those obtained by linear additive models.

Effect of exchanged proteins

ML_c. Levels of 30, 40 and 50% exchanged protein were used in the analysis. The total outcomes from the experimental design are shown in Table 5. As is usually the case when multiple regression procedures are applied to multivariate systems, there is no unique solution, but rather a set of almost equivalent ones. The solutions must then be judged on the basis of factual meaningfulness and interpretability.

The first point of interest in Table 5 is the sign of the

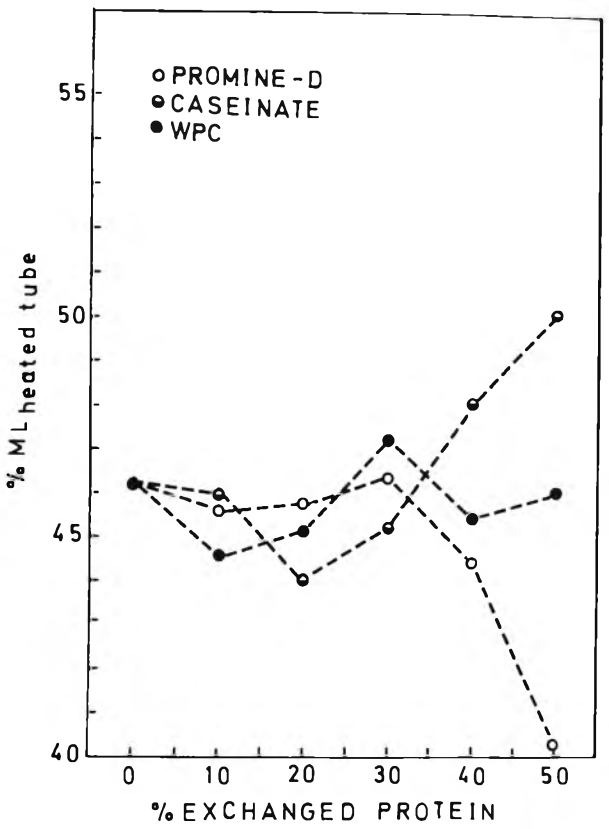

Fig. 7—ML_{ht} of pork shoulder systems as a function of % exchanged protein (heated to 80° C).

Table 5—Correlation of changes in functional properties with changes in moisture loss of raw meat systems as a function of percent exchanged protein

Group	Test material	Variables[a]	Corr coeff	Explained variance
Solubility	P-W/P-C	1+	0.90	0.81
	P-W/C-W	1+	0.91	0.83
	P-C/C-W	1+	0.92	0.85
	Total	1+	0.89	0.79
Swelling	P-W/P-C	3–	0.53	0.28[c]
	P-W/C-W	3–	0.55	0.31[c]
	P-C/C-W	3–	0.38	0.14[c]
	Total	3–	0.25	0.07[c]
Viscosity	P-W/P-C	7–	0.97	0.94
		8–	0.973	0.005[b]
	P-W/C-W	7–	0.96	0.92
	P-C/C-W	7–	0.95	0.91
		8–	0.96	0.02[b]
	Total	7–	0.95	0.91
Free choice	Total	1+	0.89	0.79
		3–	0.94	0.10
		7–	0.99	0.10
7 constrained as first variable	Total	7–	0.95	0.91
		3–	0.97	0.03[b]
8 constrained as first variable	Total	1+	0.89	0.79
		8–	0.95	0.11

[a] Functional parameters measured without preheat treatment were tested. They were: 1 = solubility, 3 = swelling, 5 and 6 = viscosity of 10% dispersions at 42 s^{-1} and 1142 s^{-1}, 7 and 8 = viscosity of 12% dispersions measured at 42 s^{-1} and 1142 s^{-1}. Units are given in Table 3.

[b] No significant contribution (95% significance level)

[c] Not significantly correlated (95% significance level)

parameters, i.e., positive for solubility and negative for the other parameters. This means that solubility has a negative influence on the water-binding properties, in contrast with the other parameters.

When tested individually, solubility and viscosity were highly correlated, but not swelling. Of the four viscosity parameters, No. 7 was responsible for 91–94% of the variance. The second best parameter (No. 8) gave a small nonsignificant contribution. This kind of outcome can either be due to the fact that the second variable is of no importance for the dependent variable (moisture loss), or that the two variables are highly intercorrelated. In the latter case, when the statistically best parameter has been chosen, the second best can give no additional contribution.

In the statistically best solution by free choice, solubility together with swelling and viscosity gave the very high correlation coefficient of 0.99. As discussed above, Promine-D had the lowest solubility and gave the lowest ML_c when added to meat. Solubility, as well as ML_c, was high both for caseinate and WPC. The differences in ML_c between caseinate and WPC may be explained by the differences in swelling and viscosity.

The reason for the nonsignificant contribution of the swelling parameter when individually tested can be found in the great differences in ML_c between Promine-D and caseinate, both of which showed high swelling ability. The results can, however, be somewhat misleading as the swellings of caseinate and Promine-D were of quite different characters. Perhaps limited and unlimited swelling should be tested separately.

The second best solution was given by viscosity parameter No. 7 alone, and the differences within this parameter were in accordance with differences in ML_c. When viscosity parameter No. 8 was constrained as the first variable, however, a solution was obtained with solubility in the first and parameter No. 8 only in the second place, giving a rather small contribution. Although variables No. 7 and No. 8 were measurements from the same dispersions, they correlate very differently with ML_c differences. The reason is the deviation from Newtonian flow for Promine-D (Hermansson, 1974), which at low shear rates (variable No. 7) results in a high apparent viscosity better correlated with the moisture loss differences.

ML_{ht}. Table 6 shows the free and constrained choice solutions of meat systems heated to 80°C with 30, 40 and 50% exchanged protein. The correlation coefficients were high but not as high as those of ML_c. The reason for the relatively low correlation coefficients is probably to be found in the change of position for caseinate between 30% and 40% exchanged protein (See Fig. 5 and 6). The solutions obtained were very similar with respect to correlation coefficients. Two of them contained swelling and solubility after heat treatment, the third gelation and viscosity, and the fourth viscosity alone.

Effect of temperature

Table 7 shows the free and constrained choice solutions of meat system on the 50% and 30% level as a function of temperature. As data from several temperatures were involved, the results in Table 7 probably give a better picture of the temperature dependence than those in Table 6. The correlation coefficients of three out of five solutions were as high as 0.97–0.98. As expected, the gelation ability was shown to be of importance for heat-treated meat systems. The best solution by free choice was given by parameter C (viscosity at 15 s^{-1}) alone. When solubility (A) was constrained as first variable, the same solution resulted with parameter C in the first place, and solubility not even giving a significant contribution. The second best had parameter E (Brookfield) in the first place and swelling in the second. The Brookfield parameter D, when constrained as first variable, gave a similar solution. Parameters C, D and E are all measures of gel strength. As discussed above, swelling measured after heat treatment is intercorrelated with gel strength properties, and swelling gave together with parameters C and E a rather good correlation.

Table 7 also includes solutions calculated from differences on the 30% level (5.0% of the total mixture). The variables

Table 6–Correlation of functional properties with changes in moisture loss of heated meat systems as a function of % exchanged protein

Group	Test material	Variables[a]	Corr coeff	Explained variance
Free choice	Total	4–	0.82	0.67
		2±	0.84	0.07
		9–	0.85	0.002[b]
2 constrained as first variable	Total	2+	0.79	0.64
		4–	0.83	0.05
5 constrained as first variable	Total	5–	0.77	0.60
		4–	0.80	0.04[b]
9 constrained as first variable	Total	9–	0.76	0.58
		4–	0.80	0.06
		1+	0.81	0.01[b]

[a] Functional properties measured without heat treatment and after heat treatment to 80°C were tested. 2 = solubility after heat treatment, 4 = swelling after heat treatment, 5 = viscosity of 10% dispersions at 42 s^{-1}, 9 = viscosity at 15 s^{-1} after heat treatment.
[b] No significant contribution (95% significance level)

Table 7–Correlation of functional properties with changes in moisture loss of heated meat systems as a function of temperature

Group	Level %	Test material	Variables[a]	Corr coeff	Explained variance
Free choice	50	Total	C–	0.97	0.94
			B–	0.98	0.01[b]
			A±	0.982	0.003[b]
A constrained as first variable	50	Total	C–	0.96	0.93
			A±	0.98	0.04[b]
B constrained as first variable	50	Total	B–	0.73	0.54
			C–	0.81	0.12
			E–	0.84	0.05
D constrained as first variable	50	Total	D–	0.77	0.60
			B–	0.85	0.13
E constrained as first variable	50	Total	E–	0.90	0.81
			B–	0.97	0.14
Free choice	30	Total	C–	0.71	0.49
			B–	0.79	0.13
			A±	0.83	0.02[b]
B constrained as first variable	30	Total	B–	0.47	0.22
			C–	0.53	0.06
			E–	0.57	0.04
E constrained as first variable	30	Total	E–	0.54	0.30
			C–	0.60	0.07
			B–	0.64	0.05

[a] Functional parameters measured after heat treatment to 70, 80, 90 and 100°C were tested: A = solubility, B = swelling, C = viscosity at 15 s^{-1}, D = Brookfield values of 10% dispersions, E = Brookfield values of 12% dispersions.
[b] No significant contribution (95% significance level)

involved were the same as on the 50% level, and the best solution by free choice on the lower level gave a correlation coefficient of 0.83. Parameter C contributed to the variance with 49% and swelling with 13%. The other two solutions were poorly correlated, as less than 50% of the variance could be explained. The fact that the same solution patterns as on the 50% level could be found in three out of five solutions on the 30% level is, however, further positive evidence for the validity of the solutions.

CONCLUSIONS

THE RESULTS of this study have shown that very good correlations can be obtained between differences in functional properties and moisture loss differences of model meat systems. Without heat treatment the best correlation was obtained from a combination of solubility, swelling and viscosity, where solubility explained the greatest part of the variance. Solubility was found to be positively correlated with moisture loss, whereas the other parameters were negatively correlated. In the studies of heat treated systems, properties measuring gelation were shown to be highly negatively correlated with moisture loss.

The present study can be characterized as explorative in nature, and the main purpose of using regression models was to obtain some general description of the main patterns of correlations between functional properties of proteins and moisture loss properties of model meat systems, rather than to test specific hypotheses about such correlations. The good correlations obtained by simple linear models strongly indicate that functional properties can be used as reliable predictors of changes in real food systems. In industrial applications for the control and optimization of real food systems, however, it seems obvious that far more complicated statistical models might have to be used.

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FUNCTIONAL PROPERTIES OF ADDED PROTEINS CORRELATED WITH PROPERTIES OF MEAT SYSTEMS. Effect of Salt on Water-Binding Properties of Model Meat Systems

INTRODUCTION

IN THE PREVIOUS paper (Hermansson and Åkesson, 1975), it was shown that good statistical correlations could be obtained between functional properties of added proteins and water binding properties of meat systems consisting of meat, water and added protein. However, a meat product often contains other components, such as salts, spices and carbohydrates as well. Of the components mentioned, salt is known to influence not only the meat itself, but also the functional properties of the proteins added (Hermansson, 1972; 1973a; 1974). Studies made on the effect of salt on meat systems have been reviewed by Hamm (1972) and later discussed by Shults et al. (1972) and Hamm (1973). The increase in water-binding properties on the addition of salt is regarded as an effect of the binding of chloride ions to the structure-forming meat proteins. When chloride ions are bound to proteins at pH's above the isoelectric point, the net negative charge is increased, and thereby the repulsive forces, meaning that more water can be imbibed in the protein network. The effect on raw meat systems is generally very large up to about 4% NaCl, where a maximum in water-binding ability is reached.

Properties of protein systems such as Promine-D (a soy protein isolate), caseinate and whey protein concentrate (WPC) are affected very differently by the addition of salt. A meat system in which part of the meat protein is exchanged by one of these protein systems can therefore be expected to show complex behavior with respect to water-binding properties. The aim of this study was to determine if meaningful correlations could be obtained between functional properties and water-binding properties, when increasing amounts of salt were added.

In this study more data on functional properties than cited in the previous research (Hermansson and Åkesson, 1975) were used, which made possible a quantitative analysis of existing interrelations between functional properties previously illustrated by the Venn diagram. A hierarchical cluster analysis, briefly described in this paper, was used for the quantitative calculations.

MATERIALS & METHODS

THE METHODS used for characterizing functional properties and moisture loss (ML) properties, as well as analysis data on the protein systems Promine-D, caseinate and WPC, have previously been given (Hermansson and Åkesson, 1975).

Statistical methods

Hierarchical clustering method. Since no specific assumption about the exact nature of the possible interrelations could be made, a general metric hierarchical clustering technique (Sokal and Sneath, 1963; Ward, 1963) was tried. To carry out the analysis, a modified version of the BMDP 1 M clustering program was used. A full description of the algorithms used is found in the program manual. Standardized differences of functional properties were used as input data ($Diff_{P-W}$, $Diff_{P-C}$, $Diff_{C-W}$, where P = Promine-D, C = caseinate and W = WPC). Similarity between variables (functional properties) was defined in terms of the Euclidean distance. The grouping principle was the following: The two variables with the closest distance to each other were amalgamated into one cluster. This cluster was regarded as a new variable and the distances to other variables were calculated. The two closest variables were then again clustered and so on. The procedure continued, reducing one variable at a time, until all variables were clustered and ordered in a tree-like manner. The total data set, as well as subsets of differences, were analyzed.

Multiple regression analysis. Regression models, goodness of fit measure and test statistic, selection of regression variables, and experimental design were described in the previous paper (Hermansson and Åkesson, 1975).

Selection of measurements of regression variables with respect to salt concentration. As shown in Table 1, the measurements of the functional properties are highly influenced by variations in salt concentration. Since it was not possible to determine, in an unambiguous way, the correspondence in ionic strength between the protein systems and the meat systems (beef brisket and pork shoulder systems) for different salt concentrations, the problem of selecting the most efficient set of measurements of functional properties to predict the changes in moisture loss turned out to be a difficult one. The problem was approached by a systematic trial and error procedure according to the following. The relation between salt concentration of the meat systems and the ionic strength (M NaCl) of the protein systems was assumed to be approximated by at least one of the combination rules $F_1 - F_4$ shown in Table 1.

Functional properties to be included in the regression equations were selected according to each of the combination rules $F_1 - F_4$. When the outcomes were compared, the differences obtained in terms of goodness of fit turned out to be quite small, indicating that the different combination rules were almost equally efficient with respect to predictive power. However, when the overall goodness of fit was compared, the combination rule F_2 was somewhat more efficient than the others for the pork shoulder system. For the beef brisket system, combination rule F_4 gave slightly better results.

Table 1—Tested combination rules between percent added salt to meat systems and ionic strength for measurements of functional properties

Added salt (% NaCl)	Combinations of ionic strengths (M NaCl)			
	F_1	F_2	F_3	F_4
0	0.0	0.0	0.0	0.0
1	0.2	0.2	0.5	0.2
2	0.5	0.5	0.5	0.2
3	0.5	1.0	1.0	0.5
4	1.0	1.0	1.0	1.0
5	1.0	1.0	1.0	1.0

RESULTS & DISCUSSION

Functional properties

Effect of salt. The addition of salt may influence properties of solvated proteins in different ways: by specific ion binding, by influencing the ionic strength and by changing the properties of the solvent. From Table 2 it can be seen that the three proteins react quite differently to the addition of salt with respect to the properties tested. For Promine-D there was a marked decrease in all the tested properties on the addition of salt. This decrease can probably be ascribed to changes in the quaternary structure. The majority of the soy proteins have complex quaternary structures that easily undergo association-dissociation reactions. At very low ionic strengths and pH's outside of the isoelectric region, the quaternary structure is unstable and dissociates, probably due to prevailing intramolecular repulsion forces (Wolf, 1970; Koshiyama, 1968; Hermansson, 1972; 1973a, b; 1974). Caseinate reacted quite differently toward the presence of salt than Promine-D. Solubility, swelling and the absence of gelation were not influenced by the addition of NaCl. Viscosity, on the other hand, showed a marked increase with NaCl concentration, especially at the higher caseinate concentrations. The rheological properties of concentrated caseinate dispersions were studied, and it was found that very weak cooperative rather than strong interaction forces were responsible for the increase in viscosity (Hermansson, 1974).

The nonheat-treated WPC dispersions were little affected by the addition of salt. However, when heated to 80°C, solubility decreased and gel strength increased rapidly with NaCl

Table 2—Functional properties as a function of NaCl concentration for Promine-D, caseinate and WPC[a]

Protein systems	Functional properties				Symbols used	Group	M NaCl 0	0.2	0.5	1.0
Promine-D	Solubility	25°C			1	I	52.9	24.3	24.1	23.5
		80°C	(ht)[b]		2	I	67.6	30.2	34.9	37.1
	Swelling[c]	25°C			3	II	9.6	3.9	3.5	3.5
		80°C	(ht)		4	II	20.0	5.9	5.4	5.2
	Viscosity	25°C	10%	42 s^{-1}	5	III	290	34	22	34
			10%	1142 s^{-1}	6	III	67	19	15	18
			12%	42 s^{-1}	7	III	1045	98	75	134
			12%	1142 s^{-1}	8	III	125	36	35	43
	Viscosity	80°C	(ht)							
			10%	15 s^{-1}	9	IV	7490	420	330	100
	Brookfield data		10%		10	IV	3680	80	100	20
		80°C	(ht)							
			12%		11	IV	7610	1500	440	90
Caseinate	Solubility	25°C			1		80.8	84.3	83.8	84.3
		80°C	(ht)		2		77.7	84.4	84.3	82.9
	Swelling	25°C			3		7.5	5.0	5.6	5.4
		80°C	(ht)		4		6.0	5.2	6.4	6.6
	Viscosity	25°C	10%	42 s^{-1}	5		21	33	34	53
			10%	1142 s^{-1}	6		19	27	34	49
			12%	42 s^{-1}	7		75	143	254	680
			12%	1142 s^{-1}	8		58	93	152	405
	Viscosity	80°C	(ht)							
			10%	15 s^{-1}	9		23	35	47	61
	Brookfield data		10%		10		0	0	0	0
		80°C	(ht)							
			12%		11		0	0	6	20
WPC	Solubility	25°C			1		78.3	75.8	73.9	72.9
		80°C	(ht)		2		83.5	66.6	66.7	66.7
	Swelling	25°C			3		1.9	1.9	1.8	1.5
		80°C	(ht)		4		4.1	3.4	3.4	3.7
	Viscosity	25°C	10%	42 s^{-1}	5		5	5	5	5
			10%	1142 s^{-1}	6		4	4	5	5
			12%	42 s^{-1}	7		7	8	6	6
			12%	1142 s^{-1}	8		6	7	6	6
	Viscosity	80°C	(ht)							
			10%	15 s^{-1}	9		440	890	800	680
	Brookfield data		10%		10		150	2200	4390	3350
		80°C	(ht)							
			12%		11		3700	6600	7290	4990

[a] For measurements of functional parameters studied, see Hermansson and Åkesson (1975).
[b] (ht) = heat treated before measurements were made at 25°C (or 20°C).
[c] All swelling measurements were made at 20°C.

concentration. The presence of salt thus seems to favor protein-protein interactions, resulting in a decreased solubility for 1% dispersions and an increased gel strength for heat-treated 10% dispersions.

Relationships between functional properties. In the previous paper the expected relationships between solubility, swelling, viscosity and gelation properties were illustrated by a Venn diagram (Hermansson and Åkesson, 1975). Figure 1 shows the relationships between functional properties when calculated from hierarchical cluster analysis on the greater amount of data obtained when amount of salt was used as a variable. Results from the total data set and the three subsets used in the regression analysis are shown. Symbols are explained in Table 2.

The parameters are ordered with respect to degree of similarity, e.g., parameter 1 is most similar to 2 and least to 11. The branching points illustrate statistical subgroups, and the smaller the distance the more closely related are the properties. All clusters calculated from the subsets show great resemblance to that from the total data set, which is a strong indication of the reliability of the results.

The solubility parameters 1 and 2 and the swelling parameters 3 and 4 are placed next to each other with a low distance value of the branching point. The same is true in 3 out of 4 clustering trees for the viscosity parameters 5 and 6. In subset 2 the parameters 5 and 6 are not next to each other, but their branching point distance value is lower than in the other three clustering trees. The branching point distances of the parameters mentioned are in no cases > 0.5, and solubility, swelling and viscosity (5 and 6) seem to behave in a similar way, with viscosity and swelling closely related. This is in accordance with the conclusion reached from a previous study on flow properties (Hermansson, 1974).

The effect of salt on the viscosity of caseinate is concentra-

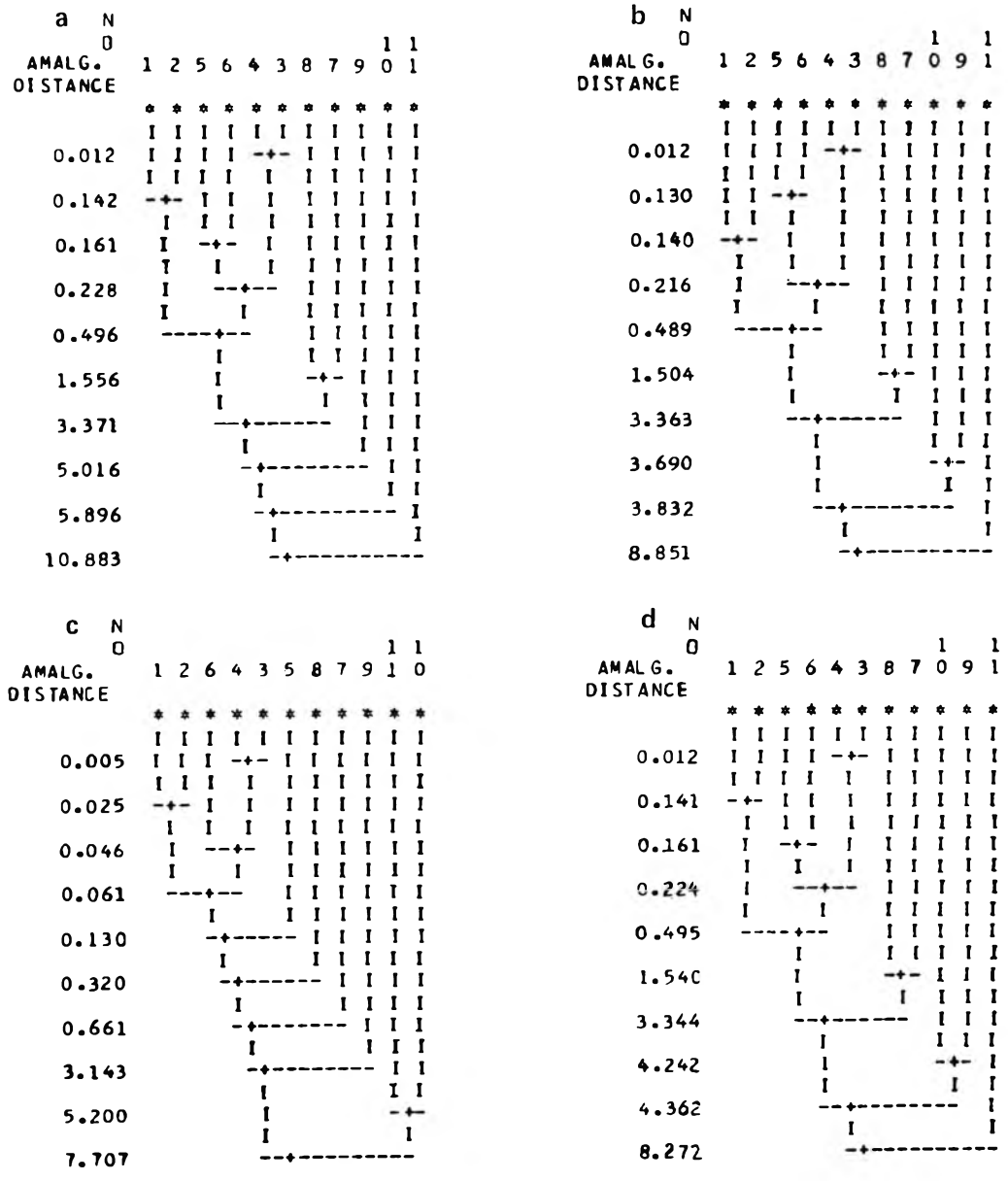

Fig. 1–Clustering trees obtained from (a) the total data set; (b) $Diff_{P\text{-}C}$ + $Diff_{C\text{-}W}$; (c) $Diff_{P\text{-}W}$ + $Diff_{C\text{-}W}$ and (d) $Diff_{P\text{-}W}$ + $Diff_{P\text{-}C}$.

tion dependent, and the increase in viscosity was marked at 12%. For Promine-D the addition of salt had a viscosity-decreasing effect and for parameter 7, the effect was enormous. The high variability of parameters 7 and 8 may explain the positions of these parameters in the clustering trees. Although the parameters were transformed into a standardized form, the variability of one parameter may influence its position. This effect is further illustrated by the clustering tree from the subset $Diff_{P-W}$ shown in Figure 2. As caseinate was not included, and the viscosity of WPC was not affected by NaCl, the variability of the viscosity parameters for Promine-D determines the position of the viscosity parameters. The flow of Promine-D deviated from Newtonian flow, and the relative differences in viscosities are greater when measured at low shear rates (see Hermansson, 1974). Consequently, parameters 5 and 7, measured at the lower shear rate, are placed to the right of the swelling data in this subset. The branching point distance for solubility, swelling and all viscosity parameters is also in this subset < 0.5.

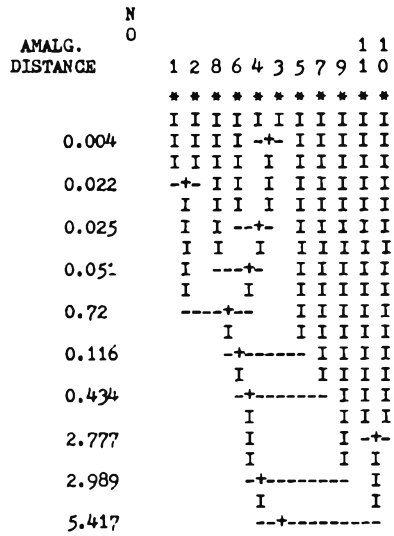

Fig. 2–Clustering tree obtained from the subset $Diff_{P-W}$.

The gel strength properties (9, 10, 11) are placed to the right in all the clustering trees with the highest amalgamation distances. It has previously been stated that gelation properties cannot be predicted from changes in the solubility, swelling or viscosity data measured at room temperature (Hermansson and Åkesson, 1975). The gel properties form a special group both with respect to their specific character and to the large variability of these parameters.

The calculated relationships illustrated by the clustering trees corresponded very well to the expected relationships previously discussed, and the functional parameters will also in this study be divided into solubility, viscosity, swelling and gel strength properties.

Properties of meat systems

Raw meat systems. ML_c as a function of salt concentration for pork and beef meat systems without and with 50% exchanged protein are shown in Figures 3 and 4, respectively. Salt had an enormous ML-decreasing effect on all the meat systems. For the pure pork shoulder system no ML could be measured at 2% NaCl. The decrease in ML was rapid below 3% NaCl in the beef brisket system, whereupon it declined. The

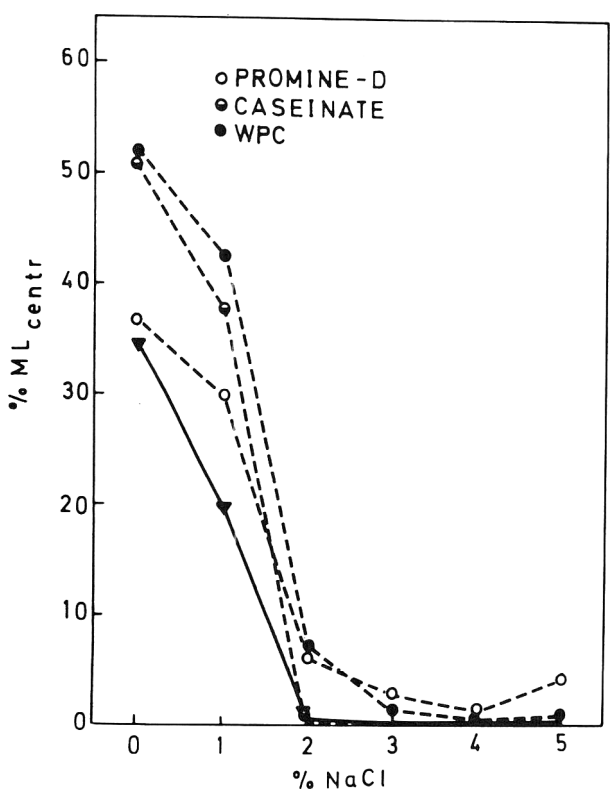

Fig. 3–ML_c of pork shoulder systems without and with 50% exchanged protein as a function of NaCl concentration.

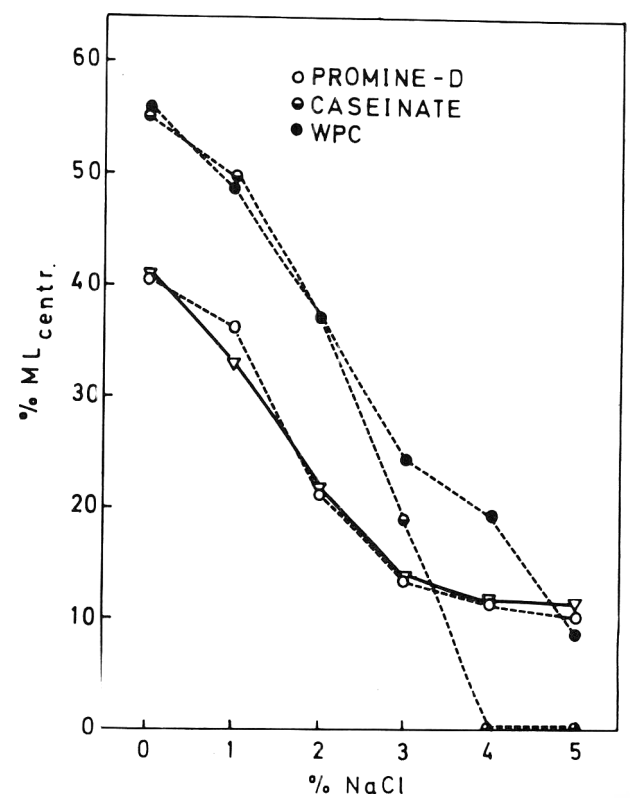

Fig. 4–ML_c of beef brisket systems without and with 50% exchanged protein as a function of NaCl concentration.

Table 3–Penetration depth (mm) after 5 sec on beef brisket systems

Protein incorporated	% exchanged protein	% NaCl			
		0	2	3	4
–	0	3.2	1.2	0.8	0.8
Promine-D	30	6.5	1.9	1.6	0.8
Caseinate	30	7.0	3.9	3.0	2.3
WPC	30	9.8	4.5	2.4	1.9

minimum reported by Hamm (1973) at I = 0.8 (3–4% NaCl in our systems) were not observed in raw meat. From the figures it is seen that the exchanged proteins influence the ML differently, in spite of the dominant salt effect. Most striking is the marked decrease in ML with increasing salt concentration for the caseinate-meat systems. Exchange of Promine-D in beef did not change the ML properties, as shown in Figure 4. In the pork shoulder system, exchange of meat proteins by Promine-D increased the ML_c and the meat-Promine-D system showed a minimum at 4% NaCl. The ML_c values for meat-WPC systems are high relative to those of the pure meat systems, but decreased with salt concentration, and was lower than that of the beef brisket system at 5% NaCl.

As in the previous study, ML_c data were compared to texture data measured by the penetrometer method. From Table 3 it can be seen that penetration depth decreased on the addition of salt in all the systems. Exchange of Promine-D resulted in the lowest penetration depth. The drastic change in the water-binding properties on the addition of salt for caseinate was not observed in the penetrometer study.

Heated meat systems. ML_{ht} for pork and beef meat systems with 50% exchanged protein is shown in Figures 5 and 6, respectively. In the pure beef brisket system a small minimum in ML_{ht} was observed at 4%, which is in accordance with the results of Hamm (1972) and Shults et al. (1972). In the pure pork shoulder system, however, ML_{ht} decreased rapidly below 2% NaCl, whereupon no changes were observed. The influence of exchanged proteins on heat-treated meat systems differed from their influence on raw meat systems. The drastic influence of caseinate disappeared on heat treatment, and the caseinate-meat systems showed the highest ML_{ht}. A reason for the difference in the caseinate-meat behavior can be that the addition of salt to the raw meat system caused increased swelling of the meat particles and increased the viscosity of caseinate. The highly viscous caseinate could then act as a paste between the meat particles. When heated the meat shrinks, water is pressed out, and the function of caseinate as a paste is partly lost. In contrast to the other two proteins, caseinate lacks the ability to form a protein network which can imbibe water and reduce moisture loss. The Promine-D and the WPC pork shoulder systems showed minima in ML_{ht} at 2% and 3% NaCl, respectively. In the beef brisket system, the relative order of the exchanged proteins with respect to ML_{ht} was the same as in the pork shoulder system, but the changes within the former system were much smaller.

Tests were also made on pork shoulder with only 30% protein exchanged (5.0% of the total mixture). As seen in Figure 7, the curves of the different meat systems are similar in shape, but the relative changes are much smaller than on the 50% level. Exchange of 30% meat proteins of Promine-D, WPC

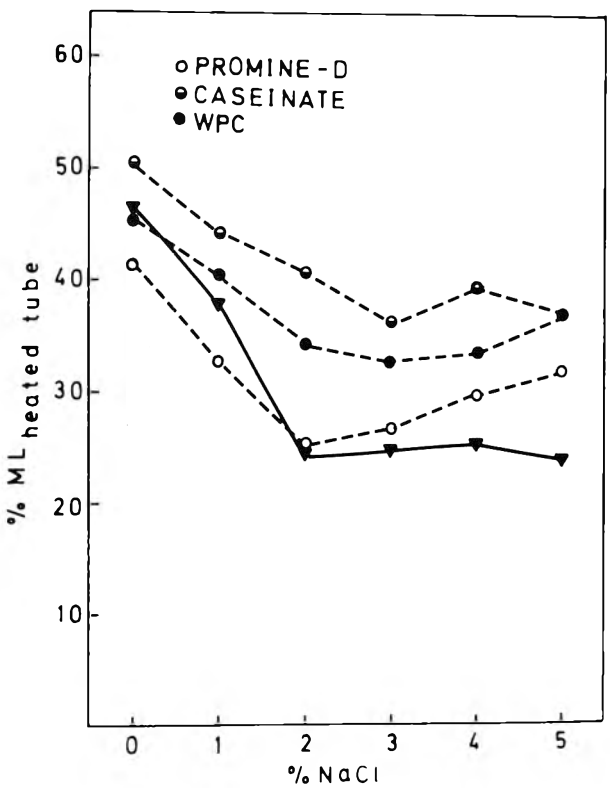

Fig. 5–ML_{ht} of pork shoulder systems without and with 50% exchanged protein as a function of NaCl concentration (heated to 80° C).

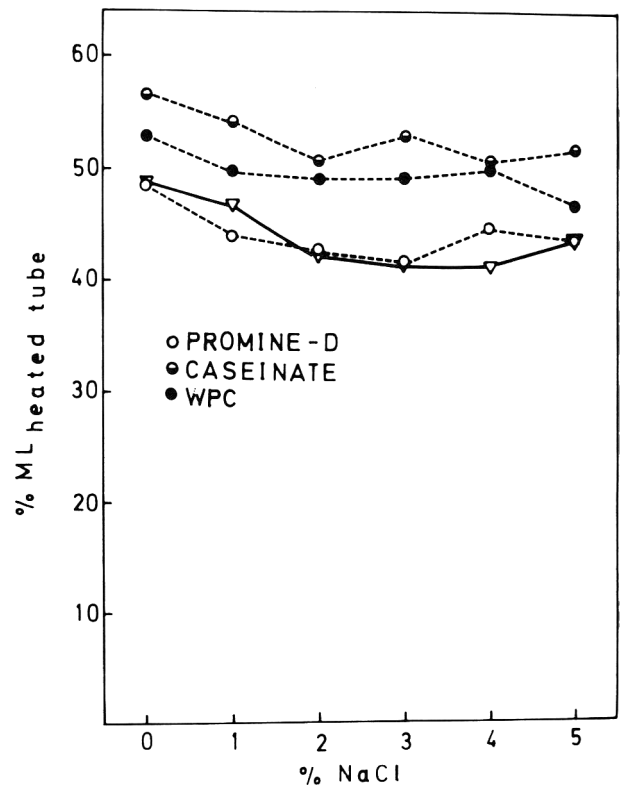

Fig. 6–ML_{ht} of beef brisket systems without and with 50% exchanged protein as a function of NaCl concentration (heated to 80° C).

or caseinate did not increase the ML_{ht} on the 3, 4 and 5% level of NaCl, and in some cases the ML_{ht} was even decreased. The statistical analysis will show whether significant correlations of the relative changes can be made with the functional properties listed in Table 2.

Statistical correlations between functional properties and moisture loss properties

In the previous study, very good correlations were obtained between changes in moisture loss and functional properties. In this study, somewhat different regression equations were expected, since salt had an influence both on the meat itself and on the functional properties.

Raw meat systems. Table 4 shows the results of the statistical analysis for the raw beef and pork systems. The solutions obtained from the two meat systems were similar in character. Rather high correlations were obtained, but as expected they were not as high as without salt, in which case the free choice model gave the very good correlation coefficient of 0.99, with solubility in the first place and significant contributions from swelling and viscosity. With salt the best solutions by free choice have a viscosity parameter in the first place and significant contributions from solubility and swelling in beef, and from swelling alone in pork systems. Also in all the constrained choice solutions, one of the viscosity parameters accounts for the largest part of the variance. The increase in viscosity for caseinate and the corresponding drastic decrease in moisture loss is probably responsible for the high correlation with viscosity.

In contrast to the systems without salt, neither solubility nor swelling gave high correlations in any of the studies. This was due to the fact that for caseinate none of these properties was affected by salt.

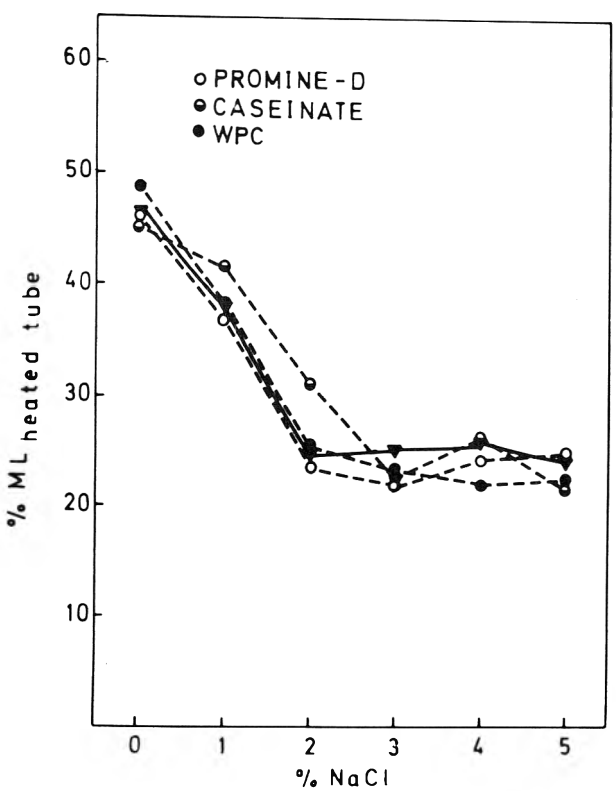

Fig. 7—ML_{ht} of pork shoulder systems without and with 30% exchanged protein as a function of NaCl concentration (heated to 80°C).

In the analysis made on the 30% level in pork systems (5.0% of the total mixture), the same variables were included as in the best solution on the 50% level, but with somewhat lower correlation coefficients.

Heated meat systems. Tables 5 and 6 show the statistical solutions for heat-treated beef and pork systems, respectively. The two meat systems were similarly correlated with functional properties.

The best solutions after heat treatment showed very high correlation coefficients. In the statistically best solutions, solubility was responsible for most of the variance. Significant contributions were also given by viscosity and gel strength properties. The contributions of swelling properties were small in the free choice as well as in the other models. With the solubility parameters 1 and 2 as first variables, very similar solutions were obtained. The similarity between the two parameters is in accordance with the results shown in the clustering trees. Also when other parameters were constrained as first variables, solubility explained much of the variance, and solubility seems to be an important property for heat-treated meat systems including salt. As seen from Table 2 and Figures 3 and 4, Promine-D showed the lowest solubility and caused

Table 4—Statistical solutions from moisture loss differences of raw meat systems

Test conditions	Meat	% exchanged protein	Variables	Corr coeff	Explained variance
Free choice	beef	50	7−	0.62	0.38
			1+	0.73	0.15
			3−	0.79	0.09
			6−	0.80	0.02[a]
1 constrained as first variable	beef	50	1+	0.39	0.15
			7−	0.70	0.34
			3−	0.72	0.03[a]
3 constrained as first variable	beef	50	3−	0.35	0.12
			7−	0.72	0.40
			6−	0.74	0.03[a]
Free choice	pork	50	5−	0.74	0.55
			3−	0.78	0.06
			6−	0.81	0.05
			1+	0.82	0.01[a]
1 constrained as first variable	pork	50	1+	0.29	0.08
			7−	0.64	0.33
			3−	0.69	0.07
			8±	0.72	0.04
3 constrained as first variable	pork	50	3−	0.41	0.17
			7−	0.66	0.27
			1+	0.70	0.05
			6−	0.72	0.03[a]
7 constrained as first variable	pork	50	7−	0.70	0.49
			3−	0.77	0.10
			6−	0.79	0.03
			1+	0.80	0.02[a]
Free choice	pork	30	5−	0.64	0.41
			3−	0.67	0.04
			1±	0.68	0.01[a]
7 constrained as first variable	pork	30	7−	0.59	0.35
			3−	0.64	0.06
			1±	0.67	0.04

[a] No significant contribution (95% significance level)

the lowest moisture loss properties when incorporated into meat, followed by WPC and finally by caseinate. The statistical solution cannot, however, be explained on a physico-chemical basis without some objections. When no salt was added the relatively low solubility, caused by aggregation during processing, is a plausible cause for the better water-binding properties of Promine-D-meat systems. The solubility in the absence of salt is correlated with high swelling, viscosity and gel strength data, as shown in Table 2. The decrease in solubility on the addition of salt is caused by protein association and correlated with reduced swelling and gel strength properties. The salt-induced decrease in solubility can therefore not be expected to give better water-binding properties of meat systems. In Figures 5 and 6 it is also shown that ML_{ht} values of the Promine-D meat systems relative to the pure meat systems were lower in the absence and with 1% salt and higher at salt concentrations above 2%. Even if salt has been shown to be a negative factor for the water binding of Promine-D meat systems, they showed still better water-binding properties than the other protein systems after the addition of salt.

The poor significance of the swelling properties was probably due to the decrease in swelling on the addition of salt for

Table 5—Statistical solutions from moisture loss differences of heated beef brisket systems

Test conditions	% exchanged protein	Variables	Corr coeff	Explained variance
Free choice	50	2+	0.91	0.82
		6–	0.95	0.08
		11–	0.97	0.04
		3–	0.98	0.01[a]
1 constrained as first variable	50	1+	0.86	0.74
		6–	0.91	0.08
		3–	0.92	0.02[a]
3 constrained as first variable	50	3–	0.39	0.15
		2+	0.67	0.30
		6–	0.79	0.17
		9–	0.87	0.13
4 constrained as first variable	50	4–	0.21	0.04
		2+	0.63	0.36
		6–	0.75	0.16
		9–	0.83	0.13
5 constrained as first variable	50	5+	0.31	0.10
		2+	0.73	0.43
		6–	0.77	0.06
		3–	0.79	0.03[a]
6 constrained as first variable	50	6–	0.61	0.37
		2+	0.83	0.32
		11–	0.86	0.05
7 constrained as first variable	50	7–	0.53	0.28
		2+	0.77	0.31
		9–	0.81	0.07
8 constrained as first variable	50	8–	0.31	0.09
		2+	0.66	0.34
		6–	0.79	0.19
		3–	0.83	0.07
9 constrained as first variable	50	9–	0.57	0.32
		2+	0.79	0.30
		6–	0.83	0.08
		3–	0.86	0.06
11 constrained as first variable	50	11–	0.52	0.27
		2+	0.69	0.20
		6–	0.77	0.12
		3–	0.83	0.09

[a] No significant contribution (95% significance level)

Table 6—Statistical solutions from moisture loss differences of heated pork shoulder systems

Test conditions	% exchanged protein	Variables	Corr coeff	Explained variance
Free choice A	50	2+	0.90	0.81
		6–	0.94	0.07
		9–	0.96	0.04
		3–	0.97	0.02[a]
Free choice B	50	1+	0.89	0.79
		6–	0.93	0.07
		9–	0.96	0.06
		3–	0.97	0.02[a]
3 constrained as first variable	50	3–	0.37	0.14
		2+	0.74	0.40
		6–	0.79	0.08
		11–	0.81	0.03[a]
4 constrained as first variable	50	4–	0.19	0.04
		2+	0.82	0.63
		6–	0.84	0.03
6 constrained as first variable	50	6–	0.63	0.40
		2+	0.77	0.19
		3–	0.81	0.07
		11–	0.83	0.03
7 constrained as first variable	50	7–	0.56	0.31
		2+	0.74	0.24
		9–	0.79	0.07
		3–	0.80	0.02[a]
8 constrained as first variable	50	8–	0.31	0.10
		2+	0.81	0.56
		6–	0.84	0.04
		3–	0.85	0.01[a]
9 constrained as first variable	50	9–	0.36	0.13
		2+	0.81	0.53
		6–	0.84	0.04
11 constrained as first variable	50	11–	0.45	0.20
		2+	0.76	0.38
		6–	0.79	0.04
		3–	0.80	0.02[a]
Free choice	30	2+	0.77	0.59
		6–	0.83	0.10
		9–	0.86	0.05
		3–	0.87	0.01[a]
1 constrained as first variable	30	1+	0.75	0.56
		6–	0.81	0.09
		9–	0.84	0.06
		3–	0.85	0.01[a]

[a] No significant contribution (95% significance level)

Promine-D and the higher swelling of caseinate relative to the other two protein systems in salt solutions. As previously shown, the swelling of caseinate was of another character than that of Promine-D and heat treated WPC (Hermansson, 1972). The viscosity parameters cannot explain 50% of the variance in any of the statistical models, but are responsible for more of the variance than solubility in some of the solutions (parameters 6 and 7). As caseinate showed a relatively high viscosity on the addition of salt but resulted in the highest moisture loss, a very low correlation was expected. The reason that this parameter still worked can be found in the very high viscosity data of Promine-D at I = 0 relative to the other two protein systems.

Gel strength properties when constrained as first variables contributed about 30% of the variance in the beef brisket system and only about 10% in the pork shoulder system. These low correlations were probably due to the enormous increase in gel strength with NaCl concentration for WPC, which was not reflected in the moisture loss properties.

The gels of WPC were, however, of another character than those of Promine-D. They were short and water could easily be pressed out. The gelation of whey proteins involves a very delicate equilibrium and heat treatment could sometimes result in a curd instead of a gel. The Promine-D gels, on the other hand, were smooth and it was impossible to press out water even under very high centrifugation forces. The gel strength methods used do not reflect these differences, and another way of characterizing the gels would probably give a higher correlation. The fact that WPC meat systems showed lower moisture losses than caseinate only after heat treatment is, however, a strong indication of the importance of the gelation ability. These results stress the importance of finding relevant parameters when studying model systems.

When correlations were made between functional properties and moisture loss data on the 30% level, solutions were obtained for the pork shoulder systems with exactly the same parameters as obtained in the best solutions on the 50% level.

CONCLUSIONS

SALT was shown to have a great influence both on meat systems and on the functional properties of the proteins added. The quantitative analysis of the relations between functional properties by hierarchical cluster analysis showed good resemblance to expected relationships. This method seems to be a useful tool for the structurizing of parameters for optimal processing. Valuable indications can also be obtained when attempts are made to explain the parameters on a physicochemical basis.

Good statistical correlations were obtained between functional properties and moisture loss properties, even if the addition of salt resulted in complex behavior. As salt influenced the functional properties very differently for the three protein systems, the statistical solutions were more difficult to interpret than in the absence of salt, and the very specific salt-induced changes of viscosity for caseinate and of gel strength for WPC influenced the general structure of the correlations.

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FUNCTIONAL PROPERTIES OF ADDED PROTEINS CORRELATED WITH PROPERTIES OF MEAT SYSTEMS. Effect on Texture of a Meat Product

INTRODUCTION

TEXTURE is regarded as one of the most important properties of meat products. Several studies have been made on the relationships between texture and water-binding properties (Galloway et al., 1973; Hamm, 1972; Bouton et al., 1973). In the previous two papers of this series (Hermansson and Åkesson, 1975a, b), it was shown that incorporation of the protein systems Promine-D, caseinate and whey protein concentrate (WPC) into model meat systems caused similar changes in both penetration data and in moisture loss data. Good correlations were shown between differences in functional properties of the added proteins and differences in moisture loss properties, and it was believed that the functional properties were also responsible for the differences in penetration data.

The model meat systems used contained meat, water, added proteins and in some cases salt, and were treated under controlled conditions. Apart from the components mentioned, a real meat product contains other components such as lipids, carbohydrates and spices, which might interact with the proteins and contribute to the final properties. Furthermore, a real meat product is processed quite differently than in the experimental design of the model systems.

The aim of this study was to determine whether texture changes in meat products with 4% external proteins added were of the same character as the moisture loss changes observed in the model meat systems, and to determine if functional properties of added proteins could be used as predictors of the observed changes. It was also of interest to know whether changes could be induced by pretreatment of the proteins to be added.

EXPERIMENTAL

PRETREATMENT of proteins was carried out by heating 10% dispersions of Promine-D, caseinate and WPC at 65°C, 80°C and 100°C for 30 min, whereupon the samples were freeze dried and ground (See also Hermansson, 1972). Analysis data on the untreated protein systems, as well as methods used for functional moisture loss and penetration properties have previously been given (Hermansson and Åkesson, 1975a).

A commercial meatball recipe was used including 50% meat (pork and beef), ca 20% potatoes and golden bread crumbs, 2.3% dried milk, ca 8% onions and spices and ca 10% water with 10% of the total mixture replaced by 4% protein and 6% water. Minced meat, potatoes and onions were mixed. A mixture of golden bread crumbs and proteins was added to the meat system alternately with water and spices. A total mixing time of 5 min was used. Meatballs of ca 20g were formed, fried in oil at 160°C for 1 min 45 sec, and then in a frying pan at ca 160°C for 1 min 15 sec. After cooling, the meatballs were frozen. Before tests were made they were fried in a frying pan for 10 min. They were served warm (at ca 55°C) to a panel consisting of 10 experimental judges for sensory evaluation of firmness and cooled to ca 22°C before instrumental tests were made. The texture measurements with five replicate runs were made in an Instron Universal Testing Machine.

Statistical design

The linear additive regression model described in the previous study (Hermansson and Åkesson, 1975a) was used to predict changes in texture properties, with functional properties as predictor variables. Because of the limited amount of data in the present study, the same regression equations as those obtained from the moisture loss studies were used in order to avoid overfitting of the models. The statistical analysis included the following steps:

(1) Application of all regression equations satisfying the goodness of fit conditions in the moisture loss studies to predict texture changes relative to the control samples. Before calculating regression coefficients the variables were normalized.
(2) Estimation of goodness of fit using the same chi-square measures as in the previous study.
(3) Listing the regression equations giving satisfactory outcomes.

Functional parameters measured in distilled water or in 0.5M NaCl were used as independent variables. The best fitting was obtained by functional parameters measured in distilled water and Table 4 contains a sample of the most efficient variable combinations. As in the previous work the results are given in terms of multiple regression correlation coefficients.

RESULTS & DISCUSSION

Functional properties

Table 1 shows the effect of preheat treatment on some functional properties. Both Promine-D and WPC formed gels

Table 1—Some functional properties of protein systems measured in distilled water

Protein systems	Functional properties[a]	Pretreated at 65°C	80°C	100°C
Promine-D	1	22.6	15.4	23.7
	4	14.0	20.0	14.2
	9	1920	7490	1410
	10	400	3680	2130
	11	2010	7610	7690
Caseinate	1	83.5	80.6	81.0
	4	7.4	6.0	7.1
	9	23	23	21
	10	0	0	0
	11	0	0	0
WPC	1	67.4	28.7	27.8
	4	2.1	4.1	4.4
	9	5	440	730
	10	0	150	1520
	11	0	3700	8630

[a] Same symbols as previously used: 1 = solubility of 1% dispersions after heat treatment of 10% dispersions at 25°C (% extractable nitrogen); 4 = swelling at 20°C after heat treatment (μl/mg); 9 = viscosity at 15 s^{-1} of heated 10% dispersions after cooling (cp); 10, 11 = Brookfield data at 25°C on gels at 10% and 12%, respectively (Brookfield "poise").

Table 2—The effect of pretreatment on moisture loss of heated meat systems

Protein system	Meat	% Exchanged protein	% Salt	Untreated	65°C	80°C	100°C
Promine-D	pork	30	0	46.2	47.0	44.2	44.6
	pork	50	0	40.2	42.7	38.1	37.7
	pork	30	3	21.8	20.3	18.7	19.2
	pork	50	3	26.6	24.8	19.8	21.0
	beef	50	0	48.6	46.5	45.1	44.9
WPC	pork	30	0	47.2	44.8	47.9	47.9
	pork	50	0	46.0	44.1	49.8	50.7
	pork	30	3	23.2	23.4	26.9	26.8
	pork	50	3	32.6	28.7	32.5	32.8
	beef	50	0	53.1	50.8	53.7	55.0

by heat treatment, whereas the properties of caseinate were unaffected. No changes in functional properties could thus be induced by preheat treatment of caseinate dispersions.

Promine-D formed strong gels in distilled water, and the formation of the network induced high swelling and reduced solubility properties in the dried product. WPC formed a weaker gel than Promine-D in distilled water. The solubility of the dried product was reduced, but the network formed by WPC had a much lower swelling ability than that of Promine-D.

The relative changes in gel strength parameters 10 and 11 are somewhat different from those of parameter 9 for Promine-D with respect to treatment at 100°C. At this high temperature, the gel structure of Promine-D is chemically broken down and there is a characteristic change in the rheological properties of the gel, which is not well reflected by gel strength parameters 10 and 11. These parameters measure the resistance to penetration of an undisturbed gel structure by a T-shaped spindle, whereas parameter 9 measures the apparent viscosity after 5 min of shearing and is thus a parameter for a partly broken down structure (Hermansson, 1972; Catsimpoolas and Meyer, 1970).

Functional properties used for untreated protein systems have previously been discussed (Hermansson and Åkesson, 1975a, b).

Properties of meat systems

Moisture loss properties. Table 2 shows the effect of preheat treatment on ML_{ht} under various conditions. As before, 30% exchanged protein corresponds to 5.0% and 50% to 8.3% of the total mixture. For Promine-D, the greatest difference, and a decrease in ML_{ht}, was found between 65°C and 80°C preheat treatment.

The moisture loss for WPC increased with penetration temperature. This result was not in accordance with the expected effect of gelation and swelling properties.

Texture properties. Table 3 shows the effect of protein incorporation on the texture of meatballs. Although only 4% proteins were added, significant changes were obtained both by instrumental and sensory evaluation. The relative differences between the controls and products containing untreated proteins were in accordance with the previously observed differences in ML_{ht} with Promine-D having the highest and caseinate the lowest values both in the extrusion test and the sensory evaluation (ranking) (See Hermansson and Åkesson, 1975a).

The effect of pretreatment differed between Promine-D and WPC. For Promine-D the changes in texture data were in accordance with the differences in functional properties shown in Table 1, and the increase in firmness between 65°C and

Table 3—Texture measurements on meatballs with 4% added proteins

Meat system	Instrumental measurements: Extrusion force (kp) MV	Extrusion force (kp) $\sqrt{S^2}$	Compression work (g cm) 1st cycle MV	1st cycle $\sqrt{S^2}$	2nd cycle MV	2nd cycle $\sqrt{S^2}$	Sensory measurements on firmness: Scoring[a]	Ranking[a]
Control I								
Without added proteins	34.9	3.4	420	36	305	33	4.8	3.3
With untreated Promine-D	38.1	3.0	319	16	237	13	3.7	2.4
With Promine-D Heat treated at 65°C	38.5	1.3	384	25	283	17	5.2	3.7
With Promine-D Heat treated at 80°C	48.4	2.8	427	35	305	20	5.3	3.9
With Promine-D Heat treated at 100°C	39.2	2.2	342	30	228	19	3.2	1.7
Control II								
Without added proteins	38.6	2.2	430	16	277	30	5.3	4.8
With untreated WPC	37.5	1.0	291	32	196	12	4.3	3.4
With WPC Heat treated at 100°C	31.6	1.1	349	39	218	19	3.5	2.6
With untreated caseinate	29.1	1.6	325	46	201	14	2.5	1.1

[a] Least significant difference 1.2 (95% significance level)

80°C was also in accordance with the corresponding decrease in moisture loss shown in Table 2. For WPC, on the other hand, preheat treatment had a negative effect on the extrusion, and no significant changes were obtained in compression work or sensory evaluation. The WPC pretreated to 100°C did, however, cause significantly lower values than the control sample in the sensory evaluation, which was not the case for the untreated WPC. This negative effect is in accordance with the observed increase in ML_{ht} between incorporation of untreated and samples pretreated to 100°C (See Table 2). A reason for the negative effect of pretreatment on WPC might be that the soluble untreated WPC interacts with the meat proteins when heated and thus improves the water-binding properties of the meat system. The interaction possibilities of WPC are partly lost by preheat treatment to 100°C because of denaturation and thereby reduced solubility. In contrast to Promine-D, the induced protein network of WPC has a poor swelling ability and cannot make any contribution to the water-binding properties of meat systems. Relative to caseinate, which did not undergo changes on heat treatment, both water-binding properties as well as extrusion force and scoring in sensory evaluation were higher for the untreated WPC systems.

Statistical correlations

A number of regression equations was previously calculated from differences in moisture loss and functional properties (Hermansson and Åkesson, 1975a, b). As moisture loss and texture changes were highly correlated, it was of interest to see whether any of the regression equations estimated on moisture loss of model systems had any validity for the texture differences of meatballs to which 4% proteins had been added. The sign of the differences calculated on extrusion and compression work had to be changed as comparisons were to be made with moisture loss differences.

Table 4—Correlations between predicted texture differences calculated from the previously estimated regression equations and observed texture differences

		Extrusion		Computer work		
Variables[a]	Proteins incorporated[b]	R	EV[c]	R	EV[c]	Origin of the regression equations
4–	A	0.69	0.48	0.47	0.22	ML_{ht} 50% (Hermansson and
2±		0.73	0.05	0.52	0.05	Åkesson, 1975a, Table 6)
9–		0.74	0.01*	0.54	0.02*	
4–	A + B + C	0.71	0.50	0.57	0.32	
2±		0.72	0.02*	0.59	0.03*	
9–		0.72	0.00	0.59	0.00	
4–	A + B	0.77	0.59	0.64	0.41	
2±		0.79	0.03*	0.68	0.05	
9–		0.79	0.00	0.68	0.00	
9–	A	0.61	0.37	0.40	0.16	ML_{ht} 30% (Hermansson and
4–		0.66	0.07	0.46	0.05	Åkesson, 1975a, Table 7)
2±		0.67	0.01*	0.48	0.02*	
9–	A + B + C	0.83	0.69	0.69	0.48	
4–		0.83	0.00	0.69	0.00	
2±		0.83	0.00	0.69	0.00	
9–	A + B	0.87	0.76	0.72	0.52	
4–		0.87	0.00	0.72	0.00	
2±		0.87	0.00	0.72	0.00	
9–	A + B + C	0.85	0.72	0.67	0.48	ML_{ht} 50% (Hermansson and
4–		0.86	0.02*	0.70	0.01*	Åkesson, 1975a, Table 7)
2±		0.86	0.00	0.70	0.00	
9–	A + B	0.87	0.76	0.73	0.53	
4–		0.88	0.01*	0.73	0.00	
2±		0.88	0.00	0.73	0.00	
2+	A	0.67	0.45	0.43	0.18	ML_{ht} 30% (Hermansson and
6–		0.70	0.04	0.47	0.04	Åkesson, 1975b, Table 5)
9–		0.73	0.04	0.49	0.02*	
2+	A	0.80	0.64	0.62	0.38	ML_{ht} 50% (Hermansson and
6–		0.81	0.01*	0.66	0.06	Åkesson, 1975b, Table 5)
9–		0.81	0.00	0.66	0.00	
11–	A	0.39	0.15	0.22	0.05	ML_{ht} 50% (Hermansson and
2+		0.63	0.25	0.39	0.10	Åkesson, 1975b, Table 5)
6–		0.67	0.05	0.43	0.03*	

[a] Symbols used: 2 = solubility after heat treatment, (2 = 1 for pretreated protein samples); 4 = swelling after heat treatment; 6 = viscosity at 1142 s^{-1} of 10% dispersions at 25°C; 9 = viscosity at 15 s^{-1} after heat treatment of 10% dispersions; 11 = gel strength (Brookfield) of 12% dispersions after heat treatment.

[b] A = untreated Promine-D, caseinate or WPC; B = pretreated Promine-D; C = pretreated WPC.

[c] R = correlation coefficient; EV = explained variance.

* No significant contribution (95% significance level).

Several fixed regression equations were chosen. Table 4 shows the correlations between predicted texture differences calculated from the regression equations and observed texture differences. Although the correlation coefficients may be somewhat overestimated due to the limited amount of data, some very high correlations were obtained, especially for extrusion data. The best model gave a correlation coefficient of 0.88. Due to reasons discussed above, only pretreatment of Promine-D gave positive contributions to the correlation coefficients. As expected, swelling and gel strength were two very important parameters and explained 59 and 76% of the variance, respectively, when they were constrained as first variables. Solubility also explained a high percentage of the variance when it was constrained as first variable on the 50% level. As already discussed, however, a low solubility alone is not a safe predictor for high water binding or texture properties. (Hermansson, 1972; 1973a, b; Hermansson and Åkesson, 1975a, b). The prediction of compression work data was not as good as extrusion data, and gel strength parameter no. 9 alone accounted for more than 50% of the variance.

CONCLUSIONS

THE RESULTS have shown that it was possible to predict changes in texture properties of a meat product from statistical models with functional properties as independent variables. As the regression equations used were estimated from changes in moisture loss, these changes and those of texture must be similar in character. Properties of great importance were swelling and gel strength. The effect of pretreatment was different for the various protein systems, probably due to interaction effects in the meat systems.

This is the last of three papers on correlation between functional properties and properties of meat systems. The following general conclusions can be made:

(a) Model systems where conditions are controlled are useful and important for the identification and estimation of relationships.
(b) Functional properties are highly correlated to changes in moisture loss properties.
(c) Even if only approximate models were used, the possibilities of using functional properties as predictors for the changes in properties of an industrially processed product containing as little as 4% added proteins are strongly indicated.

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VITAMIN B_6 CONTENT OF TURKEY COOKED FROM FROZEN, PARTIALLY FROZEN AND THAWED STATES

INTRODUCTION

VITAMIN B_6 is essential for metabolism of proteins, fats and carbohydrates. Since muscle meats are one of the best sources of vitamin B_6, losses should be minimized during preparation and cooking.

No information was found in the literature concerning vitamin B_6 retention in turkey muscle during defrosting and subsequent cooking. However, Larson (1956) noted defrosting losses of up to 50% of the thiamine, riboflavin and niacin content of frozen poultry. She suggested cooking poultry from the frozen state to minimize the defrosting loss of B vitamins. Spattering, sticking and uneven distribution of heat are encountered with chicken fried from the frozen state. Also more fuel and time are required when meats are cooked from the frozen state.

Kotschevar (1955) suggested that cooking partially thawed meat might circumvent some disadvantages of cooking frozen poultry, and still prevent defrosting losses of B vitamins. He observed that during the thawing process the rise in temperature to −1.7°C was fairly rapid, then a large amount of heat was needed to melt the ice crystals. Then the temperature began to rise again and drip was formed. He hypothesized that cooking meat when its internal temperature reached −1.7°C would result in a more nutritious product.

This study was designed to determine effects of roasting from frozen, partially frozen and thawed states on vitamin B_6 retention in turkey muscle and other selected measurements of turkey muscle.

EXPERIMENTAL

36 FROZEN paired halves of 18 turkey hens of similar weight (12–14 lb) were obtained locally. At each of nine evaluation periods (over a 2-mo period) four turkey halves from two turkeys were subjected to four treatments: (1) thawed, uncooked; (2) roasted from the frozen state; (3) roasted from the partially frozen state; and (4) roasted from the thawed state.

Turkey halves to be cooked from the partially frozen state were thawed (covered loosely with foil) at 25°C to −2°C before roasting. Halves to be cooked from the defrosted state were thawed at room temperature (25°C) for 16 hr to an approximate internal temperature of 0°C. Mercury filled thermometers (−10 to 110°C) were inserted into the breast muscle for recording temperatures. Holes were drilled in frozen samples for thermometer insertion.

Halves were placed on racks in aluminum pans and roasted in a preheated rotary hearth, electric oven maintained at 177°C to internal temperatures of 80°C in the pectoralis major (PM) muscle. The PM muscle and a composite of thigh muscles were removed and ground in a Kenmore Electric Food Grinder (1/8-in. plate) for objective measurements. Samples to be analyzed for vitamin B_6 content were freeze dried (Virtis Unitrap 10-102). Drip from thawing and roasting was collected, weighed and the volume determined by transferring to a graduated cylinder. The drip was held frozen until analyzed.

Total cooking time in minutes was recorded, and cooking time in min/kg was calculated based on weight of the uncooked half bird. Percentage total, drip and volatile losses, based on the weight of the uncooked half bird and of the cooked bird and drip, were calculated. Percentages of total moisture and ether extract were determined by AOAC (1970) methods.

Percentage ether extract of drip was determined by a combination of physical and chemical separations. The layer of lipid that collected at the top of the drippings on cooling was liquefied in a boiling water bath and removed. Collected lipid material was dried in a desiccator for a minimum of 16 hr before being weighed.

The remainder of the drip was extracted with petroleum ether (bp 30–60°C) to provide separate ether and water soluble fractions. The volume of petroleum ether used depended on the amount of lipid in the sample. Ether was distilled from the sample, using a Goldfisch extraction apparatus. After the ether was distilled, the residue was dried a minimum of 16 hr in a desiccator and weighed to determine lipid content of the extract.

Total ether extract was obtained by combining weights of lipid determined by both the physical and chemical separations. Percentage total moisture of the drip was determined by drying the water soluble fraction in a Brabender Moisture Tester at 121°C for 120 min.

Total vitamin B_6 content of muscle and drip was determined by the method of Toepfer and Polansky (1970) based on the growth response of the yeast *Saccharomyces uvarum*. No attempt was made to separate the three forms of vitamin B_6 during those determinations. Breast and thigh muscles and raw and cooked drip were sampled in duplicate, five dilutions of each sample were prepared, and vitamin B_6 in each dilution was determined in triplicate.

The experimental design was a balanced, incomplete block design with nine replications of each treatment. One turkey represented a block and each treatment appeared with every other treatment three times (Cochran and Cox, 1968).

Vitamin B_6 content of muscles was analyzed by analysis of variance:

Source of variation	DF
Blocks (unadjusted)	17
Treatment (adjusted)	3
Intrablock error	15
Total	35

Adjusted treatment means were calculated.

Cooking losses and time and the vitamin B_6 content of the cooked drip were analyzed by analysis of variance:

Source of variation	DF
Treatment	2
Error	24
Total	26

RESULTS & DISCUSSION

AVERAGES of thawing and cooking losses, cooking time and percentage total moisture and ether extract of breast and thigh muscles and drip collected during thawing and roasting are presented in Table 1.

Thawing and cooking losses were calculated on the weight of the uncooked half bird. Differences in thawing losses were not expected since both the uncooked half bird and the half bird cooked from the thawed state were subjected to identical thawing procedures. Cooking losses (total, drip and volatile) and thawing losses plus cooking losses were similar for the three treatments.

Meat cooked to 80°C from frozen and partially frozen states required about 20% longer ($P < 0.01$) cooking time than meat roasted from the thawed state. Fulton et al. (1967) reported a similar increase in roasting time (17%) for turkeys roasted from the frozen state to an internal temperature of 85°C.

As expected, uncooked breast and thigh muscle had more moisture ($P < 0.01$) than did cooked breast and thigh muscle. Muscles cooked from the frozen, partially frozen, or thawed state had similar amounts of moisture, but moisture contents varied ($P < 0.01$) among birds.

Percentage ether extract in thigh muscles was higher than in breast muscle. Percentage ether extract in breast muscle was not affected significantly by treatment, perhaps because variation in percentages of ether extract was small.

Raw thigh muscles had a lower ($P < 0.05$) percentage of ether extract than that cooked from the frozen, partially frozen, or thawed states, probably because moisture lost during cooking made the percentage of ether extract of cooked meat higher.

Large differences in composition between drip obtained on thawing and cooking were observed. Thawing drip was primarily water; cooking drip primarily lipids. Larson (1956) reported a mean of 6% solids from drip obtained from turkeys on thawing. In this study values were slightly lower (4.5% and 3.8% solids). Wide variation in individual values within a treatment may be attributable to turkey variation or to the method utilized for moisture and lipid analysis.

Adjusted means of vitamin B_6 contents of turkey muscles and drip on both wet-weight and moisture- and fat-free bases are presented in Table 2. Vitamin B_6, like niacin, but unlike thiamine and riboflavin (Cook et al., 1949), was concentrated more in the light than in the dark meat of turkey. Means obtained for vitamin B_6 ranged from 5.50–7.18 μg/g in the breast and from 2.97–3.51 μg/g in thighs. Bowers et al. (1974) found similar values (5–6 μg/g) for turkey breast muscle cooked in microwave or conventional ovens. No vitamin B_6 values for thigh muscles were found in the literature.

Vitamin B_6 contents of breast and thigh muscles were similar for all four treatments when calculated on a wet-weight basis. When calculated on a moisture- and fat-free basis, uncooked muscle usually had more vitamin B_6 than cooked muscle. Higher moisture content (Table 1) of raw muscle would explain the lack of difference in vitamin B_6 between cooked and raw muscles when calculated on a wet-weight basis.

Breast muscle cooked from the partially frozen state contained more ($P < 0.05$) vitamin B_6 (moisture- and fat-free basis) than did muscle cooked from the thawed state. Muscle cooked from the frozen state was intermediate in B_6 content. Vitamin B_6 content of raw thigh muscles (moisture- and fat-free basis) was higher ($P < 0.01$) than for muscle cooked from frozen, partially frozen or thawed states. Vitamin B_6 content varied more (on both wet and moisture- and fat-free bases) among birds than among treatments.

No clear trend is evident from those data. More vitamin B_6 in breast muscle cooked from the partially frozen state than from the thawed state was expected because there was no drip from the partially frozen meat before it was cooked. For the same reason vitamin B_6 content of meat cooked from the frozen state was expected to be higher than from meat cooked from the thawed state. We had postulated that vitamin B_6 would be transferred to the drip during thawing, so it would be lower in muscle that was cooked after thawing. That was not the case in breast or thigh muscle except for higher amounts of vitamin B_6 in breast muscle cooked from the partially frozen rather than the thawed state.

Differences in vitamin B_6 content between the raw and cooked treatments, in both breast and thigh muscle, indicate either that vitamin B_6 was transferred to the drip or was unstable during cooking. Hodson (1956) found that after extensive heat treatment, such as sterilization, some of the pyridoxal in milk was converted to pyridoxamine. Since the yeast used in the assay has a lesser growth response to pyridoxamine

Table 1—Cooking time and losses, moisture and ether extract of turkey muscle and drip

Factor	Raw	Cooked from frozen state	Cooked from partially frozen state	Cooked from thawed state	Significance of F-value[a]		LSD[b] for treatment differences
					Treatments	Birds	
Thawing loss, %	1.07	–	–	0.83	–	–	–
Total cooking loss, %	–	26.29	27.38	25.09	ns	–	–
Drip loss, %	–	5.05	5.71	4.47	ns	–	–
Volatile loss, %	–	21.19	21.67	20.62	ns	–	–
Cooking time, min/kg	–	65.38	63.03	53.09	**	–	6.34
Moisture, %							
Muscle							
Pectoralis major	74.05	68.65	68.93	68.49	**	**	1.61
Thigh	76.02	65.75	65.26	65.81	**	**	2.15
Ether extract, %							
Muscle							
Pectoralis major	1.59	1.74	1.83	1.51	ns	ns	–
Thigh	3.76	6.52	7.91	6.60	*	ns	2.32
Moisture, %							
Drip							
From thawing	94.5	–	–	96.2	ns	–	–
From cooking	–	8.21	19.95	15.15	ns	–	–
Ether extract, %							
Drip							
From thawing	0.17	–	–	0.06	ns	–	–
From cooking	–	80.1	64.9	71.0	ns	–	–

[a] **Significant at 0.01; *significant at 0.05; ns, nonsignificant
[b] LSD, least significant difference at 0.05

Table 2—Adjusted means of vitamin B_6 content (μg/g) of turkey muscle and drip

Factor	Raw	Cooked from frozen state	Cooked from partially frozen state	Cooked from thawed state	Significance of F-value[a]		LSD[b] for treatment differences
					Treatments	Birds	
Muscle							
Wet-weight basis							
Pectoralis major	5.96	6.35	7.18	5.50	ns	**	–
Thigh composite	3.51	3.27	2.97	3.14	ns	**	–
Moisture- and fat-free basis							
Pectoralis major	24.39	21.37	25.02	18.30	*	**	5.18
Thigh composite	17.31	11.87	10.97	11.73	*	**	4.23
Drip							
Wet-weight basis							
From thawing	2.09	–	–	1.59	ns	–	–
From cooking	–	1.99	3.03	2.88	ns	–	–
Moisture- and fat-free basis							
From thawing	3.70	–	–	43.7	ns	–	–
From cooking	–	23.0	34.6	26.6	ns	–	–

[a] **Significant at 0.01; *significant at 0.05
[b] LSD, least significant difference at 0.05

Table 3—Mean values of vitamin B_6 content (μg/100g of uncooked bird) of drip obtained from thawing and cooking

Factor	Treatments			
	Raw	Cooked from frozen state	Cooked from partially frozen state	Cooked from thawed state
Drip from thawing	2.54	–	–	1.37
Drip from cooking	–	6.75	10.59	8.92
Drip from thawing and cooking	2.54	6.75	10.59	10.29

than pyridoxal, lower total B_6 values in cooked meat may be caused partly by the conversion of pyridoxal to pyridoxamine during heating.

Differences in vitamin B_6 content of thawed or cooked drip were not significant calculated on either wet or moisture- and fat-free bases. When vitamin B_6 values of total drip loss were expressed as μg/100g of uncooked bird (to eliminate differences in bird weight) vitamin B_6 did not differ significantly among uncooked meat and meat cooked from each of the three states (Table 3). If vitamin B_6 were stable to the cooking process, more of it should have been in drip from a cooked bird because less was found in the cooked muscle. However that was not observed, so from those results it was assumed that vitamin B_6 may be unstable during roasting to the internal temperature used. The only exception was in breast muscle cooked from the partially frozen state.

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SURVIVAL OF SELECTED PATHOGENS DURING PROCESSING OF A FERMENTED TURKEY SAUSAGE

INTRODUCTION

WHOLE CARCASSES and pieces have traditionally been the primary mode of marketing poultry meat. Due to the increasing prices of red meat and in an attempt to expand the marketing of poultry meat, new products such as poultry burgers, poultry bologna and fresh and fermented poultry sausages have been developed (Baker et al., 1966; Dawson, 1970). A few investigators have reported on the microbiology of these products (Baran et al., 1973; Keller and Acton, 1974).

Turkey meat has commonly been implicated as a vehicle of foodborne disease. For example, 8.1 and 4.3% of the foodborne disease outbreaks reported in the United States in 1971 and 1972, respectively, were associated with turkey meat (CDC, 1972; 1973). Whole turkey carcasses have been reported to harbor various pathogenic microorganisms including salmonellae, *Staphylococcus aureus* and *Clostridium perfringens* (Walker and Ayers, 1959; Bryan et al., 1968). In addition, some pathogenic microorganisms have been detected in deboned turkey meat and products containing deboned turkey meat (Mercuri et al., 1970; Ostovar et al., 1971; Froning et al., 1971; Zottola and Busta, 1971). This report describes the survival of selected pathogenic bacteria during processing of a dry fermented turkey sausage.

MATERIALS & METHODS

SPICES and other typical sausage ingredients were added to a raw sausage mixture containing turkey meat and skin and fat. The entire process used for the production of the dry fermented turkey sausage was presented elsewhere (Baran and Stevenson, 1973; Baran et al., 1973). The pathogenic cultures were each inoculated into separate batches of the sausage formulation and held under green room conditions for 24 hr at 10°C. The samples labeled "before processing" were taken just prior to inoculation with a *Pediococcus cerevisiae* starter culture (0.62 g/kg). The sausage formulation was stuffed into 14-mm artificial collagen casing (Brechteen Co., Mt. Clements, Mich.) and the encased sausage was heated in a step-wise manner at 27°C for 3 hr, 32°C for 4 hr, and 46°C for 5 hr. The sausage was cooled to an internal temperature of 16–18°C by spraying with cold water. The sausage was dried at 10°C and 72% relative humidity (RH) for 8 days. For each sample, five separate 100-g sub-samples of meat were placed into 900 ml of 0.1% peptone diluent and blended for 2 min in Waring Blendors. Duplicate determinations were made on each sub-sample in the appropriate broth and agar media for the enumeration of the selected microorganisms. The bacterial strains used as inocula in this investigation and their sources are listed in Table 1. The cultures were maintained on nutrient agar (Difco) slants, except for *C. perfringens* which was maintained in cooked meat medium (Difco), and transferred to new media prior to use. Strains of salmonellae and enteropathogenic *E. coli* (EEC) were transferred to 250-ml Erlenmeyer flasks containing 100 ml of lactose broth and incubated on a gyrotary shaker (New Brunswick Scientific; New Brunswick, N.J.) at 37 and 45.5°C, respectively, for 48 hr. Salmonellae were enumerated using a 3-tube MPN procedure which consisted of an enrichment step in selenite-crystine broth (Difco), streaking plates of brilliant green sulfadiazine (BGS) agar (Difco) and presumptive identification on triple sugar iron agar (Difco) slants (Galton et al., 1968). In addition, a nonselective enrichment step, incubation in lactose broth (Difco) for 36 hr at 37°C, was used prior to inoculation into selenite-crystine broth for samples taken after processing. Confirmation of the results was accomplished with a micro-colony indirect fluorescent antibody technique (Thomason, 1971) using Salmonella 0 group D, factor 9, and Salmonella 0 group E4, factor 19 antisera (Difco) for *S. pullorum* and *S. senftenberg*, respectively, and anti-rabbit globulin labeled with fluorescine isothiocyanate (Difco). Fluorescence was detected using a Leitz-Ortholux fluorescence microscope system. Enteropathogenic *E. coli* strain 0125:B12 was enumerated using a 3-tube MPN procedure which consisted of incubation in tubes of lauryl sulfate tryptose broth (Difco) at 35°C for 24–48 hr followed by streaking on EMB agar (Difco) which was then incubated at 35°C for 24 hr. Typical *E. coli* colonies were picked from EMB agar and inoculated into tubes of EC medium (Difco) which were incubated in a water bath at 45.5°C for 48 hr. Tubes exhibiting gas production were sampled and slide agglutination tests using Difco OB poly A and OB poly B antisera were used for serological confirmation of a positive test for the inoculated strain. Strains of *C. perfringens* were enumerated using an anaerobic pouch system (Bladel and Greenberg, 1965) with basal SPS agar (Difco) containing D-cycloserine (Eli Lily and Co., Indianapolis, Ind.) which was added after autoclaving (0.4 mg/g). After inoculation the pouches were incubated for 48 hr at 37°C and typical black colonies were counted. Representative colonies were transferred to motility-nitrate medium and iron milk for presumptive identification. The colonies were presumed to contain strains of *C. perfringens* if they contained nonmotile, nitrate-reducing bacteria which produced a stormy fermentation in iron milk. For enumeration of *S. aureus*, 0.1-ml aliquots of the dilutions were spread onto surfaces of Vogel-Johnson agar (BBL) plates. The plates were incubated for 48 hr at 37°C and black colonies greater than 1 mm in diameter were transferred to BHI broth and a coagulase test was performed using coagulase plasma EDTA (Difco). A coagulase-positive test was considered to be presumptive identification of the culture. The microslide gel double diffusion assay for enterotoxin developed by Casman and Bennett (1965) was used with minor modification (Barber and Deibel, 1972) to determine if detectable amounts of staphylococcal enterotoxin type B (SEB) were produced during processing of the sausage. The anti-SEB was obtained from Makor Chemicals Ltd. (Jerusalem, Israel).

Total aerobic plate counts (APC) and counts of lactic acid bacteria were determined by pour plate procedures using Plate Count Agar (Difco) and LBS agar (Difco), respectively, followed by incubation for 72 hr at 30°C. Coliforms were enumerated by spreading 0.1 ml aliquots of appropriate dilutions over the surfaces of Violet Red Bile (VRB) agar plates and adding a thin overlay of VRB agar (APHA, 1966); the plates were incubated for 48 hr at 35°C.

Table 1—Organisms inoculated into the turkey sausage mixture

Organism	Strain	Source
Salmonella pullorum		MSU[a]
Salmonella senftenberg	775W	MSU
Escherichia coli	0128:B12	FRI[b]
Escherichia coli	0125:B12-HIC	FRI
Escherichia coli	026:B6	CDC[c]
Staphylococcus aureus	243	MSU
Clostridium perfringens	ATCC 3624	MSU
Clostridium perfringens	NCTC 8238	MSU

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RESULTS & DISCUSSION

MICROBIAL COUNTS are expressed on a dry weight basis (unless otherwise stated) in order to facilitate comparisons between the counts obtained prior to, and after processing. Data from control sausages, which were not inoculated with pathogenic bacteria, are presented in Table 2. The control sausages had a final pH of 5.3, total acidity expressed as lactic acid of ca 0.30%, and a moisture loss (weight loss during processing) of ca 35%. A 10- to 50-fold increase in lactic acid bacteria and aerobic plate counts occurred in the control sausages after addition of the starter culture. Coliforms, and yeasts and molds were present at low levels (10^2-10^3 cells/g and 10^3-10^4 cells/g, respectively) prior to processing, and they were not detected after processing.

In sausages inoculated with salmonellae, the concentrations of *S. pullorum* were reduced by ≥ 4.5 to ≥ 5.3 log cycles when the initial concentrations were $> 3.1 \times 10^5$ to 1.1×10^6 cells/g (Fig. 1A). Thus, *S. pullorum*, a contaminant of raw poultry products, was substantially reduced in numbers during processing. In contrast, the concentrations of *S. senftenberg* were reduced by only 1.5 to ≤ 2.0 log cycles when the initial concentrations were $> 6.3 \times 10^3$ to 1.7×10^5 cells/g (Fig. 1B). The greater tolerance of *S. senftenberg* to heat would not be expected to fully account for the difference in survival shown between *S. senftenberg* and *S. pullorum* in this investigation since the heating process was very mild. The apparent survival of the *S. pullorum* might have been increased, due to greater cell recovery, by using a selective medium which is less inhibitory than BGS agar. The survival of salmonellae during processing is not surprising since Takacs and Simonffy (1970) have reported that dry sausages contained viable salmonellae up to the time of consumption if the initial concentration was $> 2.0 \times 10^4$ cells/g (wet weight).

The concentrations of *C. perfringens* were reduced by 1.2–3.6 log cycles when the initial concentrations were 4.0×10^1 to 6.3×10^5 cells/g (Fig. 1C and 1D). The rate of survival appeared to be greater at the lower concentration; however, the plate count technique has a relatively high degree of error at the lower concentrations measured in this investigation. Since we did not distinguish between spores and vegetative cells, there is a possibility that the survival shown may have been influenced by sporulation during the initial stages of processing. Nonetheless, the results indicated that *C. perfringens* can survive the process, even when vegetative cells are present at low concentrations in the sausage mixture.

The initial concentrations of *E. coli* 0128:B12 ranged from 2.8×10^4 to 3.6×10^5 cells/g, and after processing the numbers were reduced by ≤ 1.4 to 2.1 log cycles (Fig. 1E). In preliminary experiments, results obtained from sausages inoculated with enteropathogenic *E. coli* strains 026:B6 and 0125:B12 indicated these strains were more readily destroyed during processing than strain 0128:B12. However, in further investigations we found the fecal coliform incubation conditions suitable for strain 0128:B12, but 45.5°C in EC broth, did not yield consistent results for quantitative recovery of strains 026:B6 and 0125:B12. Thus we, along with other investigators, have cited the problems associated with utilizing an incubation temperature of 45.5°C for routine analysis of enteropathogenic strains of *E. coli* (Baran and Stevenson, 1973; Mehlman et al., 1974).

Table 2–Microbial counts during production of dry fermented turkey sausage controls

Sample	Aerobic plate count (cells/g)[a]	Lactic acid bacteria (cells/g)[a]
Turkey meat and fat mixture	3.7×10^4	$< 10^2$
Sausage mixture	7.7×10^4	$< 10^2$
Sausage after greenroom incubation	3.7×10^7	2.4×10^7
Sausage after addition of *P. cerevisiae* and processing	1.4×10^9	1.0×10^9

[a] Dry wt basis

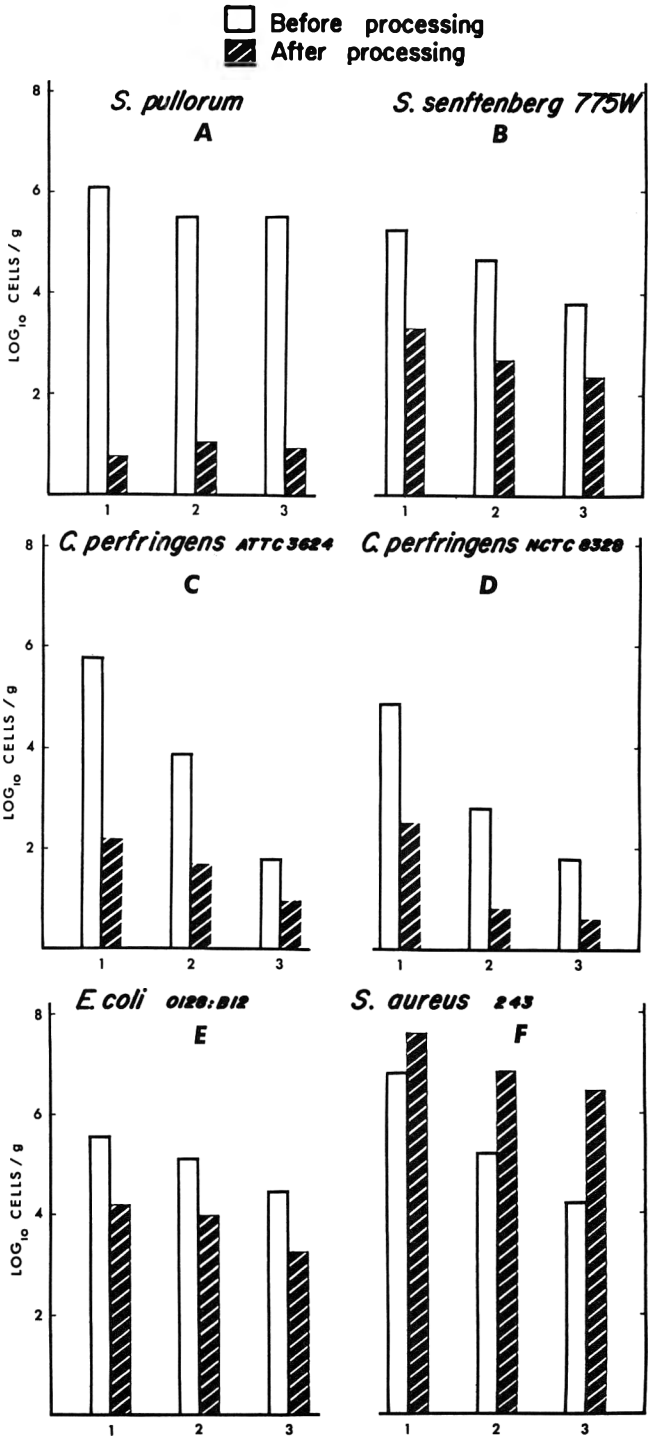

Fig. 1–Effect of processing on the survival of selected pathogens in separate batches of a dry fermented turkey sausage. (Numbers indicate batches for each organism.)

When cells of *S. aureus* were inoculated into the sausage mixture, growth occurred (Fig. 1F). The growth of *S. aureus* in the sausage, in contrast to the other pathogens, may be due to its ability to grow at a lower water activity than the other organisms. Final populations of 2.8×10^6 to 3.6×10^7 *S. aureus* cells/g were found, and SEB was not detected in any of the samples. Barber and Deibel (1972) similarly were unable to detect SEB in sausage mixtures unless *S. aureus* populations of ca 10^9 cells/g were present.

None of the pathogens utilized in this investigation were detected in analyses conducted on control sausages. The turkey meat used for the sausages was carefully processed in our own facilities under stricter conditions than those which generally prevail during continuous commercial processing. The highest populations were used in this investigation to determine the results for grossly contaminated products and except for *S. pullorum*, attempts were made to vary the the levels of inocula. The populations of pathogens used for inoculation of the experimental sausages are obviously greater than those which would be expected in commercial products, but, they are within the range of populations which could be found under poor processing conditions. The results presented herein indicate that certain pathogenic bacteria, which might be present in turkey meat, may survive in fermented dry turkey sausages which receive a "low heat" process. Since this investigation deals with an experimental product which is not marketed commercially, no standard procedures for thermal treatment and ripening have been adopted. Low heat processes, such as the one used in this investigation, may be adopted by manufacturers since processing fermented turkey sausages at ca 71°C yields undesirable product characteristics (Baran et al., 1973; Keller and Acton, 1974). Thus, manufacturers should be cognizant of the potential health hazards associated with the production of such products.

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GRILLED FREEZE-DRIED STEAKS. Effects of Mechanical Tenderization Plus Phosphate and Salt

INTRODUCTION

A TOUGH WOODY TEXTURE has long been associated with freeze-dried beef steaks and pork chops (Bird, 1965; Penny et al., 1963). Various attempts have been made to increase the tenderness of raw and precooked freeze-dried beef and pork slices. Tuomy et al. (1963) indicated that prolonged and/or higher internal cooking temperatures would result in a more tender freeze-dried beef steak. Hinnergardt and Burger (1975) investigated this theory and found that this was in fact, the case. However, the extra cooking necessary to produce an observable increase in tenderness also imparted an undesirable dry-mouth feel to the precooked freeze-dried steak. Hinnergardt and Burger (1975) did find greatly improved rehydration and rehydrated meat texture when roast beef was sliced 1.6 mm thick. Penny et al. (1963) reported an improvement in beef texture when the beef had been treated with a pre-slaughter injection of adrenaline. The adrenaline-treated samples had a high ultimate pH of 6.7 and were more juicy and less woody than the control samples with an ultimate pH of 5.6. They also observed that the dehydrated beef with a high ultimate pH showed less deterioration of texture, flavor and color during storage at 37°C. Improved hydration of meat was also found by Wismer-Pedersen (1965), to be associated with high pH values. Bouton et al. (1973) stated that beef tenderness increased linearly with increasing pH, particularly, when the ultimate pH is over 6.0. With pH values less than 6.0, they felt there were too many factors affecting tenderness to always find a fixed relationship between tenderness and pH values.

Since pre-slaughter treatment of beef to attain a high ultimate pH is frequently difficult or impossible, some workers have attempted to raise the ultimate pH of post-slaughter muscle to increase both tenderness and water-holding capacity. Shults et al. (1972) summarizes some of the work done using condensed phosphates on pH adjustment of fresh beef. This summary indicated a combination of salt and phosphate produced the most water retention. Their study not only confirmed this fact, but indicated Na tripolyphosphate and tetra sodium pyrophosphate had the most effect on water retention and pH adjustment. However, a combination of NaCl and 0.5% TPP resulted in the least amount of shrink during 180 min of heating at 60°C. They concluded that the synergistic effects of TPP with small amounts of NaCl (0.5–1.5%) presented the possibility of obtaining precooked beef items with a low shrink and no, or only a slight, salty taste. This could be an important observation for preparation of cooked beef for freeze-dehydration because more juice could be retained during cooking to prevent the dryness often observed with meat cooked for freeze dehydration. Wismer-Pedersen (1965) injected pork loins with ethylenediaminetetraacetate (EDTA) and pyrophosphate. He found the EDTA facilitated water penetration while the pyrophosphate improved the water-binding capacity of the meat. Warner-Bratzler shear values showed an improved texture of the treated samples.

Hamdy et al. (1959) tried to improve rehydration of freeze-dried meats by rehydrating them in solutions of 0.2M NaCl, 0.01M KCl and 0.05M sodium ascorbate. These treatments seemed to improve the texture and water-holding capacity (WHC) of the freeze-dried meats. Calcium and magnesium chlorides added to the reconstitution water increased the WHC but added an objectionable taste to the meat. The effect of the infused modifier solution added to the meat prior to freeze drying was also carried over to the rehydrated meat samples.

Table 1—Technological panel and penetrometer results of grilled, freeze-dried steaks treated with and w/o mechanical tenderization, and with and w/o a solution of PO_4 and NaCl

	No mechanical tenderization or PO_4 [a]	With PO_4/NaCl	With mechanical tenderization	With mechanical tenderization and PO_4/NaCl
Tenderness[c]	2.87d	4.22c	5.05b	5.95a
Penetrometer[b]	10.86 ± 2.06	9.02 ± 2.06	6.36 ± 1.20	5.15d ± 0.95
Cutability[d]	2.67c	4.55b	5.97a	6.55a
Residue[e]	5.57a	4.60ab	4.22b	3.87b
Juiciness[f]	3.20c	4.80a	3.97bc	4.40ab
Percent rehydration[g]	50.00c ± 5.36	56.61a ± 5.07	53.61b ± 3.68	55.96ab ± 3.46

[a] Means in a row not followed by a common letter differ significantly at the 1% level of probability as determined by the Neuman-Keuls test.
[b] Penetration was accomplished according to the method of Hinnergardt and Tuomy (1970). Shear force values = lb force required to penetrate a 1.27 cm thick beef steak.
[c] Tenderness is the overall effort required to chew a sample on the first and subsequent chews. (1 = extremely tough; 9 = extremely tender).
[d] Cutability is the degree of difficulty or ease one experiences in biting through a sample on one or more tries when the sample is initially put in the mouth. (1 = extremely difficult; 9 = extremely easy).
[e] Residue is the amount of material remaining in the mouth just before swallowing the sample. (1 = none; 9 = greatest amount).
[f] Juiciness is the degree to which one can feel free liquid in the mouth. (1 = extremely dry; 9 = extremely juicy).
[g] $\frac{\text{Rehydrated wt} - \text{dry wt}}{\text{rehydrated wt}} \times 100 = \%$ moisture.

Even though the treatments described resulted in improved texture of freeze-dried meat, apparently a great deal of variation was still observed in the rehydration and texture of treated freeze-dried meat. It appeared to the authors of this paper that use of a Bettcher needle tenderizer might reduce the variation in texture and rehydration of cooked freeze-dried meats without altering the rehydrated cooked meat appearance. The authors have been unable to find literature regarding the use of the Bettcher mechanical tenderizer. However, its use to mechanically tenderize wholesale cuts of beef is known.

The following study was undertaken to determine the effect of a phosphate and salt solution in combination with mechanical tenderization of meat prior to cooking and freeze-dehydration on the juiciness and tenderness of precooked freeze-dried beef.

MATERIALS & METHODS

Thirty-two USDA Choice top rounds, 7 to 10 days postmortem were obtained from a commercial source. The rounds were individually wrapped in freezer paper, and stored at −23°C for 1 wk. The rounds were then randomly divided into four lots, by random selection equilibrated to a temperature of 4.4°C, and the semimembranosus muscle was excised.

One lot was mechanically tenderized by passing the muscle three times through a Bettcher Industries Tend-R-Rite Model TR-2, at the slowest speed. Another lot was mechanically tenderized in like manner, and a solution consisting of 3.0% sodium tripolyphosphate (TPP) and 7.5% NaCl was pumped into the muscles to 10% of their weight. Pumping was done using a Koch Tenderizer Injecter (8127) equipped with four stainless steel needles 2.2 cm apart. A constant pump gauge pressure of 13.6 kg was maintained during pumping. The third lot received no mechanical tenderization, but was pumped with the TPP-NaCl to 10% of their raw weight. The fourth lot received no mechanical tenderization and no phosphate or salt solution. The treated roasts (semimembranosus muscles) were frozen to a temperature of −23°C and 5 slices 1.27 cm thick were obtained from the center portion of each roast by cutting across the grain with a meat saw. A 6.35 cm diameter die was used to cut individual steaks from the slices in each treatment. All steaks were grilled at a temperature of 176°C for 3 min per side. The grilled steaks were frozen at −23°C prior to dehydration in a Stokes freeze dehydrator at a plate temperature of 51.6°C and a chamber pressure of 0.3–0.5 mm Hg (0.047–0.067 k Pa). All treatments were sealed with a nitrogen flush in No. 2-1/2 cans and stored for one month.

After storage, the steaks were rehydrated in 48.9°C water for 10 minutes and rehydration percentages were determined by weight difference. Penetration measurement of tenderness was accomplished according to the method of Hinnergardt and Tuomy (1970) using an Allo-Kramer shear press modified with a five-needle penetrometer head. The trim from each raw steak was composited for each treatment and analyzed for % moisture, % fat, % NaCl, P mg/100g, % Ash, pH and % protein by Official AOAC (1970) methods.

Immediately following the needle penetrometer measurement, each steak was submitted to subjective evaluations. The subjective attributes evaluated were cutability, tenderness, juiciness and residue. A nine-category bipolar type scale was used to estimate the magnitude of each attribute. Judges were 20 food technologists and food chemists selected on the basis of previous experience in the sensory assessment of food texture, including meats. Two replications of the experiment were conducted: one in the morning and the other the afternoon of the same day. Samples were presented in a balanced random order. Judges were given an instruction sheet containing definitions of each attribute and criteria for making judgments.

Table 2—Variance of the Allo-Kramer penetrometer data for grilled, freeze-dried steaks with and w/o mechanical tenderization, and with and w/o a solution of PO_4 and NaCl

Treatments[a]	Variance
Control (no mechanical tenderization or PO_4 and NaCl	4.24
PO_4 and NaCl solution only	4.24
Mechanical tenderization only	1.44
Mechanical tenderization plus PO_4 and NaCl	0.90

[a] N = 40 observations/treatment. Five slices were selected from the center portion of each roast and there were eight roasts per treatment.

Table 3—Chemical analysis of raw steaks treated with and w/o mechanical tenderization and with and w/o a solution containing P and NaCl[a]

Treatments	H_2O	Fat	NaCl	P (mg/100g)	Ash	pH	Protein
No mechanical tenderization or PO_4 and NaCl solution	70.77	5.44	0.19	210	1.06	5.4	21.97
PO_4 and NaCl solution only	73.56	2.28	0.84	248	2.06	5.5	20.74
Mechanical tenderization only	71.69	3.98	0.23	217	1.11	5.3	23.46
Mechanical tenderization and PO_4 and NaCl solution	73.45	4.72	1.14	275	2.28	5.7	19.62

[a] Solution concentrations were calculated on a weight/weight basis of tripolyphosphate, NaCl and H_2O. The analysis reports elemental phosphorus and % NaCl found in the samples.

RESULTS & DISCUSSION

MECHANICAL tenderization of USDA Choice top rounds prior to cutting into 1.27 cm thick steaks resulted in grilled, freeze-dried steaks that were significantly more tender than the control as well as those samples that were injected with TPP-NACl solution. When considering those steaks that were mechanically tenderized, it should be noted in Table 1 that their tenderness was significantly superior to the treatments that were not mechanically tenderized. The penetrometer results in Table 1 also reflected the differences in tenderness found by the sensory panel, and these results agree with one another. It is also interesting to note that the steaks which were mechanically tenderized had less residue than the steaks that were not mechanically tenderized. Mechanical tenderization in the authors' opinion produced a freeze-dried steak which resembled a normal steak in appearance and texture.

Another effect of mechanical tenderization can be noted in Table 2. Comparison of the variances by means of an "F" test reveals that mechanical tenderization resulted in highly significant ($P < 0.01$) reduction in the variation of the tenderness of rehydrated, grilled, freeze-dried beef steaks. This resulting increase in uniformity of tenderness of the freeze-dried steaks should add greatly to their acceptability. Mechanical tenderization accounted for 69% of the variance components when tenderness was measured by the penetrometer and 40% as measured by the sensory tenderness panel. The TPP-NaCl solution had a small but significant effect on the tenderness of the freeze-dried steaks. The solution accounted for 8.9 and 12.2% of the variability components of tenderness for the penetrometer and taste panel evaluation respectively, as calculated by the method of Hicks (1956). The sensory attribute of cutability did not reflect the difference between addition or nonaddition of the TPP-NaCl solution to the mechanically tenderized samples.

The TPP-NaCl solution treatment had the most notable effect on sensory juiciness and percent rehydration of the grilled freeze-dried steaks (Table 1). A two-way analysis of variance showed the injected solution and not mechanical tenderization alone to be the responsible factor for the difference in juiciness. Table 1 does not indicate a clear difference between the treatment of mechanical tenderization and the treatment of mechanical tenderization with the injected phosphate solution due to the one-way analysis of variance and the multiple range test used to separate the means. Even though this difference failed to be statistically significant, the panelists rated the solution-injected samples higher in juiciness than the noninjected samples. The higher tenderness ratings for the TPP-NaCl injected samples over the control samples probably resulted from the improved rehydration and juiciness observed for the phosphate-salt treated samples.

The phosphate-salt solution contributed a significant (16%) of the variance component of rehydration. Mechanical tenderization also significantly effected rehydration, but contributed only 4.4% to the variance components of rehydration. Another 7% of the variance was attributable to the interaction between the injected solution and the mechanical tenderization treatment.

The chemical analysis reported in Table 3, reflected the changes in moisture, NaCl, P, ash, pH, fat and protein caused by the injected solution. Injection of the solution increased moisture content, NaCl, P, ash and pH. Undoubtedly, most of the difference in NaCl, P and pH between the steaks with TPP-NaCl solution only and those with mechanical tenderization plus the solution was due to the problem of achieving a uniform injection, even though the raw roasts were allowed to equilibrate at 4.4°C for 2 hr before freezing, slicing, cooking and dehydration.

It was concluded that while addition of TPP-NaCl solutions improved the texture of freeze-dried steaks, the most satisfactory textural quality resulted from mechanically tenderizing the roast and then injecting with TPP-NaCl solution.

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FRESHLY COOKED AND COOKED, FROZEN REHEATED BEEF AND BEEF-SOY PATTIES

INTRODUCTION

VEGETABLE PROTEINS are being used increasingly as fresh meat extenders. Gallimore (1974) reported that soy-ground-beef blends accounted for 26% of the ground beef sales of three grocery chains during 30 wk in 1973. The Food and Nutrition Service (FNS, 1971) allows a meat and textured vegetable protein combination (a maximum of 30% hydrated textured vegetable protein with 70% ground meat) as an acceptable meat alternative for the National School Lunch Program.

Little information concerning eating quality and other characteristics of meat with allowable (30% rehydrated textured soy) vegetable protein is available. However, some characteristics of meat blends with less than 30% soy protein added have been studied. Judge et al. (1974) found that soya flour and soya protein concentrate in ground beef patties decreased cooking shrinkage with no effect on bacterial number after 7 days storage. Huffman and Powell (1970) added 2% soya bits to ground beef patties and found that they received higher tenderness scores than those with no soya.

Adding soy to meat may reduce the stale or warmed-over flavor in cooked, reheated meat because certain vegetable extracts are effective antioxidants. Sato et al. (1973) found that adding one of several cereal protein products to ground meat loaves reduced rancidity (warmed-over flavor). Pratt (1972) and Sangor and Pratt (1974) reported lower TBA values for beef slices covered with soy extract solutions than for controls.

This study evaluated effects of pre-cooking, frozen storage and reheating on eating quality, cooking losses, percentages of moisture and fat, and TBA values of ground beef and beef-soy blends (15 and 30% soy).

EXPERIMENTAL

Materials

Ground beef (approximately 25% fat) was obtained from the meat laboratory of the Animal Science & Industry Dept., Kansas State University. Textured soy protein (Ultra Soy, Far-Mar-Co, Hutchinson, Kansas) was rehydrated with water and added to the ground beef as follows:

Percentage rehydrated soy	Ground beef	Water	Ultra soy
0%	4000 g	0	0
15%	3400 g	400 g	200 g
30%	2800 g	800 g	400 g

Blends were mixed with a Hobart mixer (Model A-200) for 2 min at #2 speed (113 rpm).

Preparation

20 patties (180g, 9.5 cm in diam) of each mixture were molded and thermometers inserted. 10 patties of each mixture were placed on wire racks 7 cm high in a shallow pan in a rotary-hearth electric oven at 177°C and heated to 75°C internal temperature. Total cooking losses were calculated. Patties were cooled at room temperature 15 min, then packaged in foil and frozen (−17°C). Ten additional patties of each mixture were frozen raw. After 8–9 wk frozen storage, raw and cooked patties were thawed 15 hr at 6°C and 2 hr at 25°C. Raw patties were placed on racks in shallow pans and heated to 75°C. Previously cooked patties were placed (two at a time) in an Amana Radarange (Model RR2) and heated 3 min (to approximately 55°C). Two patties comprised an experimental unit–one was cut diagonally to six wedges for sensory evaluation; the other was used for panel evaluation of appearance, then ground for moisture, fat and TBA determinations. The border outlines of the cooked patties were traced and their diameters measured to determine shrinkage during cooking.

Sensory evaluation

Cooked or reheated samples were transferred to warm, coded glass snifters, covered with watch glasses, and presented to the six panel members for sensory evaluation in individual booths. Before the evaluation, panelists had been trained to identify selected flavors and aromas. Intensities of flavors and aroma components were scored (1, absent, to 7, very intense); juiciness (1, very dry, to 7, very juicy); texture (1, crumbly, to 7, firm); acceptability also was scored.

Chemical measurements

Duplicate measurements for moisture, fat and TBA values were made on ground meat samples.

Ground 10-g samples were dried at 121°C for 60 min in a C.W. Brabender semi-automatic moisture tester. Ether extracts were determined by the AOAC method (1970). The 2-thiobarbituric acid values were determined by the method of Tarladgis et al. (1960). Slurries were prepared from approximately 10-g samples; optical density was read (Beckman DU Spectrophotometer), then values were adjusted for sample size to convert to mg of malonaldehyde per 1000g tissue.

Analysis of data

Data were subjected to analysis of variance as follows:

Source of variation	DF
Meat blend (M)	2
Heating treatment (H)	1
M × H	2
Error	24
Total	29

When F-values were significant for "meat blend" or "M × H" interaction, LSD's were calculated.

RESULTS & DISCUSSION

MEAN VALUES of five replications for the six treatment combinations, along with significance of F-values and LSD's, are presented in Table 1.

Cooking losses and chemical measurements

Cooking losses (including initial cooking and reheating) were affected ($P < 0.01$) by both percentage of textured soy added and heating treatment. Adding textured soy decreased cooking losses–30% additions more than 15% additions. Reheating increased cooking losses of all beef and beef-soy combinations; however, the difference was greater between the cooked-reheated beef and freshly cooked beef (approximately 10%) than between the cooked-reheated 30% soy-beef blend and the freshly cooked 30% soy-beef blend (approximately 5%). It generally is thought that soy additives bind some of the moisture during the heating so cooking losses are reduced.

Diameters of the cooked patties did not differ significantly. Other workers have reported that soy added to meat decreased shrinkage (Judge et al., 1974).

Table 1—Means of cooking loss, chemical measurements and sensory evaluations of freshly cooked and cooked-reheated beef and beef-soy patties

Factor	Freshly cooked			Cooked-reheated			Significance of F-value			
	0% Soy	15% Soy	30% Soy	0% Soy	15% Soy	30% Soy	Meat blend (M)	Heat treatment (H)	M X H	LSD*
Total cooking loss (Cooking + reheating), %	33.95	29.94	26.05	43.06	37.56	31.33	**	**	**	1.42
Total moisture, %	52.23	54.93	55.53	50.50	54.02	55.81	**	ns	ns	1.58
Ether extract, %	20.58	16.12	14.14	20.67	15.07	13.48	**	ns	ns	0.78
TBA value	0.371	0.171	0.141	0.346	0.119	0.110	**	ns	ns	0.05
Sensory evaluation[a]										
Meaty:										
aroma	4.0	2.0	1.5	2.8	2.8	1.4	**	ns	*	1.0
flavor	5.0	2.4	1.4	3.5	2.4	1.6	**	*	**	0.7
Stale:										
aroma	2.2	1.6	1.6	3.6	1.7	1.9	**	**	*	0.7
flavor	1.6	1.6	1.6	4.0	1.7	1.6	**	**	**	0.6
Cereal-like:										
aroma	1.4	4.4	4.9	1.0	4.5	5.4	**	ns	ns	0.6
flavor	1.3	4.1	5.3	1.1	4.0	5.2	**	ns	ns	0.5
Juiciness	4.8	4.2	4.1	3.4	3.8	3.4	ns	**	ns	–
Texture	3.5	4.5	4.8	4.0	4.0	4.6	**	ns	ns	0.5
Overall Acceptability	5.1	3.4	2.9	3.6	3.6	2.6	**	*	*	0.8

[a] Intensity scale of 1–7
* $P < 0.05$
** $P < 0.01$
ns Nonsignificance

Percentage moisture was less ($P < 0.01$) in beef patties than in beef-soy blend patties. Neither the amount of rehydrated soy added (15 or 30%) nor the reheating process affected moisture content. Percentage ether extract was greater ($P < 0.01$) in beef than soy-beef blend patties and the patties with 15% soy contained more ether extract than those with 30% soy. The textured soy that was used contained only approximately 1% ether extract and was rehydrated with 2 times its weight of water, so we expected the beef-soy blend to contain less ether extract and more water than the beef patties.

TBA values were determined, as an indication of oxidative rancidity. Heating treatment had no effect on TBA values; however, beef-soy blends (15 or 30%) had lower ($P < 0.01$) TBA values than beef. The difference may result from the reported antioxidant effects of soy (Pratt, 1972; Sangor and Pratt, 1974; Sato et al., 1973), or from less fat in the soy beef blend.

Sensory evaluation

Meaty aroma ($P < 0.05$) and flavor ($P < 0.01$) of freshly cooked beef patties were scored higher than when beef patties were reheated. However, meaty aroma and flavor of reheated and freshly cooked beef-soy patties did not differ. Generally, meaty flavor and aroma decreased with increased soy. After frozen storage and reheating, beef and beef-soy blends differed less in meaty flavor and aroma than when patties were freshly cooked.

Stale flavor ($P < 0.01$) and aroma ($P < 0.05$) of reheated beef were greater than for the other samples. Other samples received scores indicating stale flavor and aroma were nearly absent. Apparently, if a stale aroma or flavor developed during storage and reheating of the beef-soy blend, other flavor or aroma components masked the staleness.

Cereal-like flavor and aroma, as expected, increased ($P < 0.01$) as amount of soy increased. Heating had no effect on cereal-like flavor or aroma. Although the panel did not score sweetness, several commented that the beef-soy blends were sweet—particularly their browned surfaces.

Even though soy-beef blends contained more moisture than beef, adding soy did not affect juiciness. However, reheated patties were less ($P < 0.01$) juicy than freshly cooked patties. Increased soy added to ground beef increased ($P < 0.01$) pattie firmness (texture). Based on overall acceptability scores, freshly cooked beef was more ac ceptable than any other sample. The more soy added, the less acceptable were freshly cooked patties. However, the difference was significant ($P < 0.05$) for reheated patties only when 30% soy was added. There was no significant difference in acceptability between reheated beef and freshly cooked 15 and 30% beef-soy patties. Panel members agreed that shape uniformity of patties was not affected by adding soy.

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INFLUENCE OF SODIUM NITRITE ON THE CHEMICAL AND ORGANOLEPTIC PROPERTIES OF COMMINUTED PORK

INTRODUCTION

THE EFFECTS of sodium nitrite on the organoleptic and chemical properties of meat products have been studied in several investigations. Brooks and co-workers (1940) reported that the characteristic cured flavor of bacon is a result of the action of nitrite on the meat. More recent work has been done to establish the difference in flavor between meat products processed with or without sodium nitrite. Cho and Bratzler (1970) found that pork loins cured with sodium nitrite were correctly differentiated by a taste panel from pork loins processed without sodium nitrite. In an investigation by Wasserman and Talley (1972), it was reported that a significant number of panel members could distinguish between the flavor of frankfurters processed either with or without sodium nitrite. Simon et al. (1973) observed that higher taste panel acceptance was experienced for all meat frankfurters as the amount of nitrite initially added to the formula was increased from 0 to 156 ppm.

Watts (1954) stated that lipid oxidation (TBA value) is considerably delayed in cured meats. Cross and Ziegler (1965) concluded from their comparison of the volatile fractions of cured and uncured hams that nitrite interferes with the oxidation of unsaturated lipids.

This study was undertaken to further develop the present knowledge of the action of sodium nitrite in meat. The objectives involved an examination of organoleptic and chemical differences occurring between cooked, canned pork emulsions processed with or without added sodium nitrite. This examination was performed in samples with or without added salt (NaCl).

EXPERIMENTAL

THE STUDY was undertaken in two phases. The first phase involved organoleptic analyses, including a triangle taste test for differences in flavor between comminuted pork processed with or without sodium nitrite. Additional sensory panel evaluations included preference tests as well as examination of the degree of cured pork flavor. A gas chromatographic separation of the headspace vapor above each product was performed to determine if differences in the volatile compounds occurred between treatments. Data from proximate analyses and residual nitrite analyses were collected from comparable samples at a later date.

The second phase of this experiment involved aroma evaluation and thiobarbituric acid (TBA measures the quantity of an intermediate in fat oxidation) evaluation of the products stored under refrigerated and frozen conditions.

Product production

Meat emulsions were prepared from boneless pork shoulders (approximately 16% fat) using normal industry practices. Approximately 470g of the prepared emulsions were stuffed into No. 2 cans (409 × 307). The cans were sealed and the product was cooked in a 74°C water bath until internal product temperature reached 71°C. Four different treatments were prepared using the basic formulations shown in Table 1. Sodium nitrite was added at 20, 156 and 200 ppm in Phase I and at 156 ppm in Phase II of this experiment. Within each treatment (e.g., 20 ppm nitrite with 0% salt) samples from the same emulsion were used for all panel evaluations involving that specific treatment. No spices, sweeteners, or extenders were used in any of the formulations.

Flavor panel evaluation

In Phase I, testing for differences in flavor between products processed with or without nitrite was performed by using a triangle taste test with a blindfolded, untrained laboratory panel. The taste panels consisted of 7–9 people selected from a group of 16 members. Throughout the entire study, the position of the odd sample in the triangle test was assigned on a random basis to each of the three sample positions. Following the triangle test the panel members were asked to indicate which sample they preferred. This evaluation was followed by a paired comparison test to determine which sample had more cured pork flavor. The above tests were performed and comparisons were made between treatments 1 and 2 (0% salt) and between treatments 3 and 4 (2% salt) as described in Table 1.

The aroma evaluation (rancid odor) in Phase II was performed by a blindfolded panel on products stored 0, 1, 3 and 5 wk at refrigerated (3 ± 2°C) temperatures and on products stored 0, 1, 4, 8 and 14 wk at frozen (−29 ± 2°C) temperatures. A nine-point scale was used to score the odor of each sample (low intensity rancid odor = 1). Samples processed with nitrite were formulated at the 156 ppm level in this phase.

Headspace analysis

In Phase I duplicate headspace samples of the vapor generated by each product submitted to the taste panel were evaluated. Cooked product samples of 6.0g were placed into 10 ml freeze-drying vials and a split rubber septum was placed into the vial opening. The cap (with sampling port) was then screwed on tightly and the samples were heated for 10 min in a 60 ± 2°C water bath. The needle of a 10.0 cc gas-tight syringe filled with 4.0 cc of nitrogen was then inserted through the sampling port in the cap and into the vial headspace. The 4.0 cc of nitrogen was exhausted from the syringe and a 4.0 cc sample of the headspace gas was drawn up into the syringe. The 4.0 cc sample was then injected into a 150 cm glass U-tube column (6 mm o.d.) packed with 10% Carbowax 20M on Diaport S (60–80 mesh). The column was maintained at 80°C in a Hewlett-Packard model 402 gas chromatograph with hydrogen flame ionization detector. Flow rates of the gases were 300 ml/min for air, 35 ml/min for hydrogen and 300 ml/min for nitrogen. The volatile compounds in the heated meat samples were found to be eluted within 75 sec after injection. Exploratory experimentation revealed that no additional compounds were eluted when the chromatograph was allowed to run for an additional hour. Representative peaks were recorded on the recorder chart of the unit for later comparison by the peak area method.

Chemical analysis

Proximate composition (fat, protein, moisture and ash) and residual nitrite level were determined for each product submitted for taste panel

Table 1—Product treatments

Treatment	Ingredients
1	Boneless pork, 5% added moisture (control product)
2	Boneless pork, sodium nitrite (20, 156, or 200 ppm), 5% added moisture
3	Boneless pork, salt (2%), 5% added moisture
4	Boneless pork, salt (2%), sodium nitrite, (20, 156, or 200 ppm), 5% added moisture

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evaluation to assure product uniformity. Modified AOAC (1970) methods were used for the above analyses.

In Phase II the 2-thiobarbituric acid (TBA) method of Tarladgis et al. (1960) was used to determine the extent of lipid oxidation in the four different products.

Statistical analysis

Statistical significance in the flavor tests performed in Phase I was determined by reference to the published tables of Amerine et al. (1965). Statistical analysis of the remaining data was completed using the Least Squares Maximum Likelihood General Purpose program of Harvey (1968). Significance of treatment effects on aroma panel scores and TBA values in Phase II was based on individual F-test (analysis of variance) made on each of the comparisons.

RESULTS & DISCUSSION

Phase I

Sensory panel. Table 2 shows the results of each triangle test conducted at the various nitrite levels. Panel results are grouped at each nitrite level on the basis of whether or not salt (NaCl) was present in the samples being prepared. Each triangle test performed by the total panel is referred to as one attempt at differentiation. A statistically significant differentiation occurred when a significant number of panelists (Amerine et al., 1965) correctly identified the odd sample in the triangle test.

Panel members were able to distinguish samples with nitrite from samples without nitrite at the levels of nitrite examined (20, 156, 200 ppm). There was a greater incidence of significant differentiation at the higher levels of nitrite (156 and 200 ppm) than at the lowest level of nitrite (20 ppm). At 20 ppm nitrite there was a greater tendency to correctly identify the odd sample when salt was present in the samples being compared.

The summarized results for all preference tests are presented in Table 3. Due to the design of the panel test only the responses of those panel members correctly identifying the odd sample in the triangle test are recorded. A few panel members indicated no preference and their responses were thus excluded from the tabulation. The tabular values show the number of individual panel members preferring the samples with nitrite per total number of panelists who participated in the preference tests. At every level of nitrite the pooled responses show that the panel members indicated a significant preference for the sample containing nitrite. Simon et al. (1973) reported that panel members assigned higher acceptance scores to samples of all meat frankfurters processed with nitrite than to samples processed without nitrite.

Results of the two sample tests for cured pork flavor are summarized in Table 4. At all levels of nitrite, panel members indicated the samples with nitrite had more cured pork flavor ($P < 0.001$). This is in agreement with the findings of Cho and Bratzler (1970) in their study with pork loins.

When evaluating individual panel members responses, it was found that there was wide variation in the ability of individuals to differentiate flavor of samples processed with nitrite from samples processed without nitrite. Most panel members were usually successful at distinguishing the odd sample in the triangle test; however, a few panel members had practically no success at differentiation.

It should be noted at this point that the fat percentage of the products in this study was approximately 17%, a level which is somewhat below the 30% level used in other studies (Simon et al., 1973).

Table 2–Taste panel results for the triangle test

Total added nitrite (ppm)	Salt in samples (%)	Panel results[a,b]	No. of significant panels/total no. of panels conducted[c]
20	0	5/8 NS, 2/9 NS, 5/8 NS, 3/8 NS, 4/9 NS, 3/8 NS, 6/9*, 3/8 NS, 5/8 NS, 7/9*	2/10
	2	5/8 NS, 5/9 NS, 6/8*, 5/8 NS, 6/9*, 4/8 NS, 6/9*, 7/8**, 6/8*, 7/9**	6/10
	Total		8/20
156	0	4/7 NS, 5/7*, 5/7*, 2/7 NS, 6/7**, 5/7*	4/6
	2	6/7**, 5/7**, 6/7**, 4/7 NS, 4/7 NS, 4/7 NS	3/6
	Total		7/12
200	0	6/8*, 7/9**, 5/8 NS, 5/8 NS, 5/9 NS, 4/8 NS	2/6
	2	7/9**, 9/9***, 5/8 NS, 6/9*, 6/9*, 7/8**	5/6
	Total		7/12

[a] Tabular values in this column are the number of correct selections/number of panelists

[b] NS–not significant (P = 0.05); *significant at P = 0.05, **significant at P = 0.01, ***significant at P = 0.001. (Amerine et al., 1965)

[c] Number of times that panel differentiations were significant/total number of panels conducted. Significance was at the P = 0.05 level or higher.

Table 3–Summarized results of preference tests

Total added nitrite (ppm)	Salt level (%)	No. of panel members preferring sample with nitrite	Total no. of panelists[a]
20	0	31 *	43
	2	34 NS	57
	Total	65 **	100
156	0	18 **	25
	2	24 **	28
	Total	42 ***	53
200	0	21 NS	32
	2	26 NS	40
	Total	47 *	72

[a] NS–not significant ($P > 0.05$); *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 4–Results for a two-sample test for more cured pork flavor

Total added nitrite (ppm)	Salt (%)	The no. of nitrite samples which were selected as having more cured pork flavor	Total no. of responses[a]
20	0	32 **	41
	2	29 *	42
	Total	61 ***	83
156	0	18 ***	21
	2	19 ***	21
	Total	37 ***	42
200	0	12 NS	17
	2	15 **	17
	Total	27 ***	34

[a] NS–$P > 0.05$; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

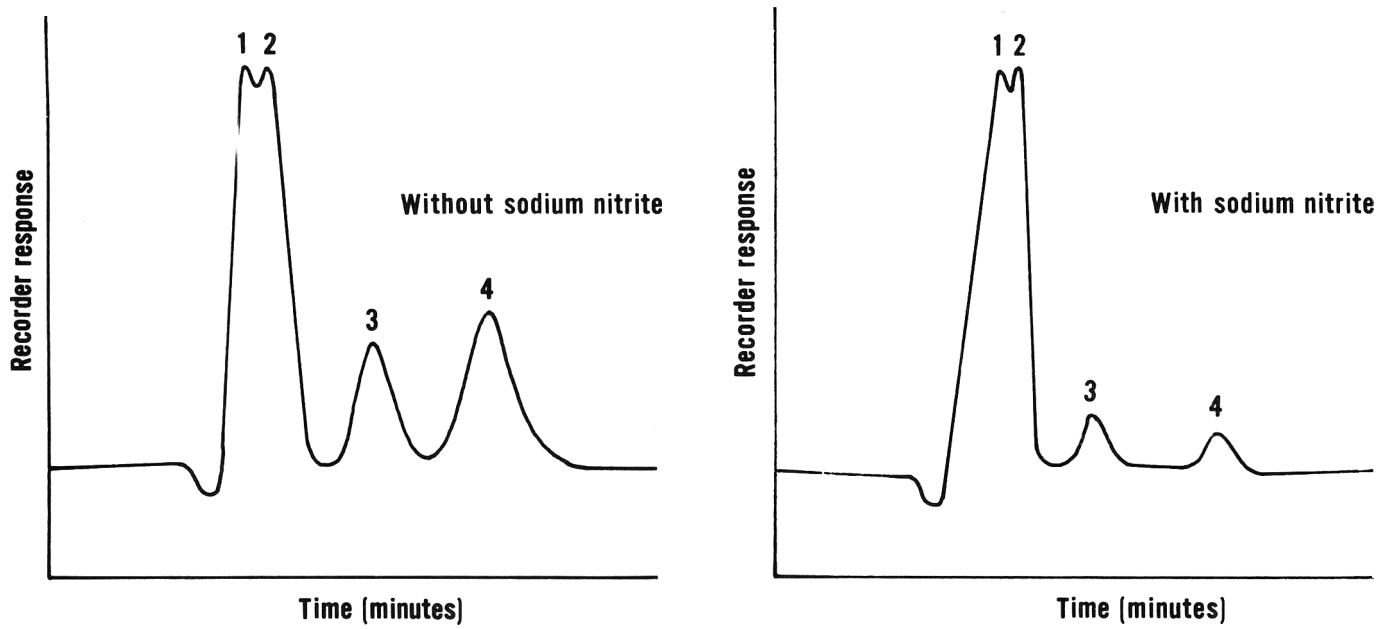

Fig. 1–Chromatograms of headspace vapors from samples with and without sodium nitrite.

Headspace analysis

Several taste panel members commented that there was a noticeable difference in the aroma between the samples processed with nitrite and those processed without nitrite. An objective evaluation of these products was needed to determine the nature of the aroma differences. Gas chromatography analysis of the headspace vapors above these products revealed that four major compounds were separated. Figure 1 shows sample chromatograms for the products evaluated in this study. All four peaks were found in each of the four treatments examined in this experiment. Peaks 3 and 4 were much less predominant in the samples containing nitrite than in samples without nitrite and this suggests that nitrite is reducing the quantity of some of the major volatile compounds. The presence of nitrite had a significant ($P < 0.01$) effect on the amounts of each compound existing in the headspace vapors above the meat. Salt significantly increased the quantity of compound 1 and significantly decreased the quantity of compound 4 but this influence was not as consistent as the nitrite effect. Cross and Ziegler (1965) reported that chromatograms of volatile carbonyl compounds from cured hams were observed to have lower quantities of valeraldehyde and hexanal than chromatograms from uncured hams.

Phase II

Products stored under refrigerated and frozen conditions were evaluated periodically for aroma (panel) and the extent of lipid oxidation (TBA method).

Refrigerated storage. Storage time at 3 ± 2°C significantly ($P < 0.01$) increased both aroma scores (more rancid odor) and TBA values. Figure 2 shows the change in aroma scores with storage time. Both the rate and final extent of development of rancid odor were observed to be the greatest in products processed without nitrite. In addition, the nonnitrited product containing salt exhibited the most pronounced rancid odor. No apparent difference in rancid odor was noted between the product containing only nitrite and the product containing nitrite and salt.

TBA values for the various products stored at 3 ± 2°C for different intervals are shown in Figure 3. The presence of nitrite in a product significantly ($P < 0.01$) reduced TBA values. At all storage intervals, products containing nitrite had much lower TBA values than products not containing nitrite. Even after only 1 wk of storage there was a very noticeable difference in TBA value between products with nitrite and those without nitrite. These data support Cross and Ziegler (1965) who concluded that nitrite interferes with the oxidation of unsaturated lipids. A possible explanation for this occurrence

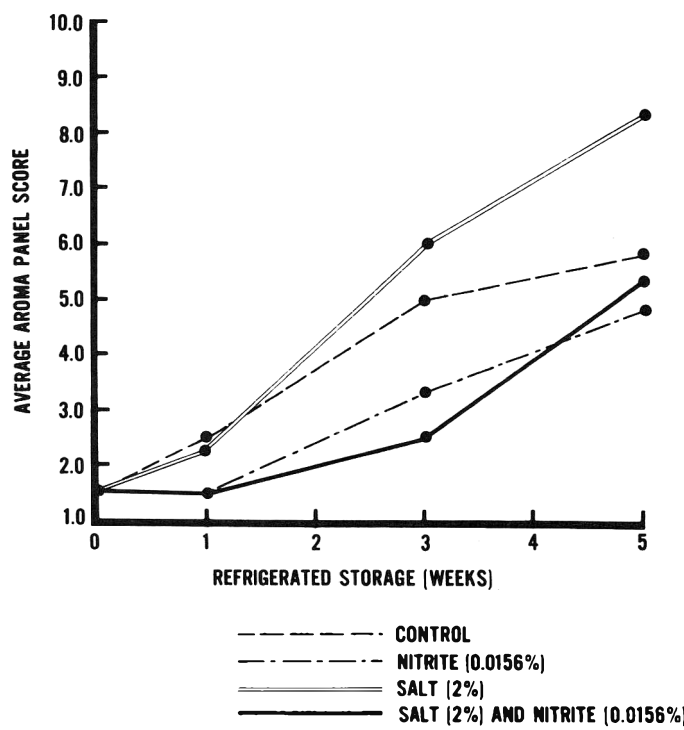

Fig. 2–Average aroma scores at various intervals of refrigerated storage.

has been offered by Tarladgis (1961) who stated that ferric heme compounds (Fe^{+3}) are active as catalysts in lipid oxidation. Cooked cured meat pigment is in the ferrous (Fe^{+2}) form which is inactive as a catalyst of lipid oxidation, whereas pigment of cooked meat without added nitrite is in the ferric (Fe^{+3}), catalytically active state.

The effect of salt in the product without nitrite can be seen after the first week of refrigerated storage. At 3 and 5 wk this product had considerably higher TBA values than all other treatments including the control.

Frozen storage

The aroma of products stored at −29 ± 2°C was not significantly affected by nitrite, salt or storage time. Figure 4 shows the aroma scores of products at various intervals of frozen storage. Samples containing nitrite had less rancid aroma than samples without nitrite at all storage intervals except at 8 wk.

The TBA values of products stored under frozen conditions were significantly ($P < 0.01$) reduced by nitrite and significantly ($P < 0.01$) increased by time. Salt, however, had no significant influence. Figure 5 displays the average TBA values at various intervals of frozen storage. The effect of nitrite on TBA values can be seen very dramatically in Figure 5 where the TBA values of the products processed with sodium nitrite have not risen above 1.0, whereas products without nitrite have considerably higher TBA values. In samples without added nitrite the TBA values increased with time to a certain point and then began to decline with time. The TBA test analyzes for malonaldehyde, an unstable product of lipid oxidation. Tarladgis and Watts (1960) found that malonaldehyde does not accumulate as a stable end product of fat oxidation but reaches a peak at the same time that oxygen uptake begins declining. This may account for the observed increase and subsequent decrease in TBA values for products without added sodium nitrite.

The sample containing salt but no added nitrite had the highest TBA value at all frozen storage invervals except at 8 wk. Moskovits and Kielsmeier (1960) stated that salt has prooxidant effects in sausage stored at 0°C.

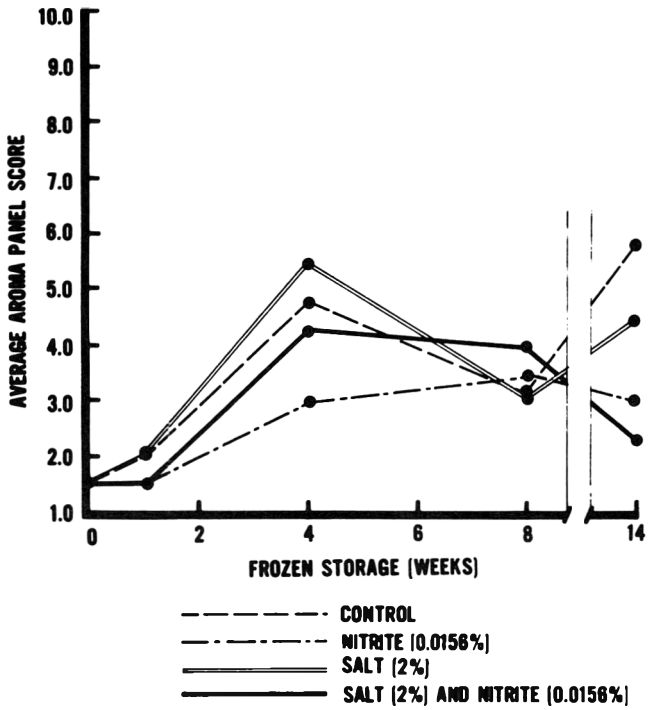

Fig. 4–Average aroma scores at various intervals of frozen storage.

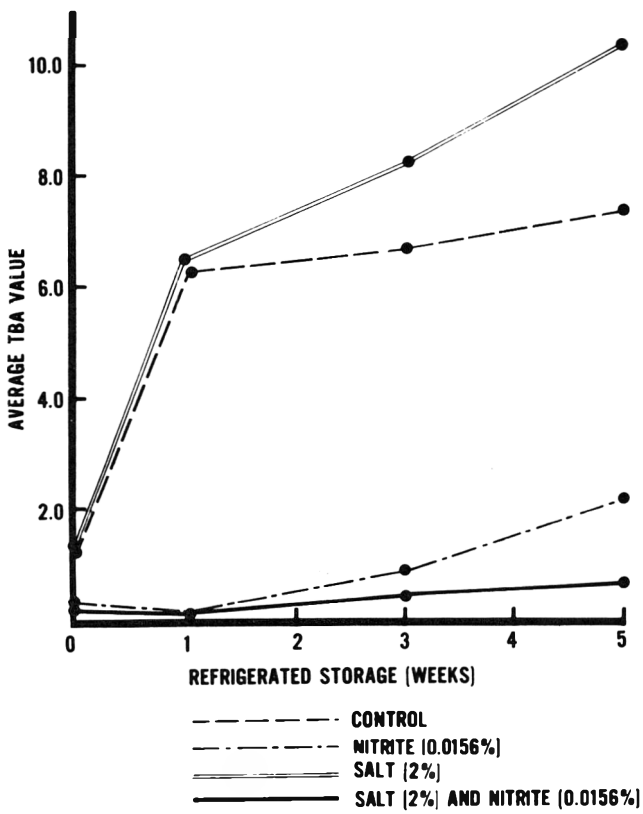

Fig. 3–Average TBA values at various intervals of refrigerated storage.

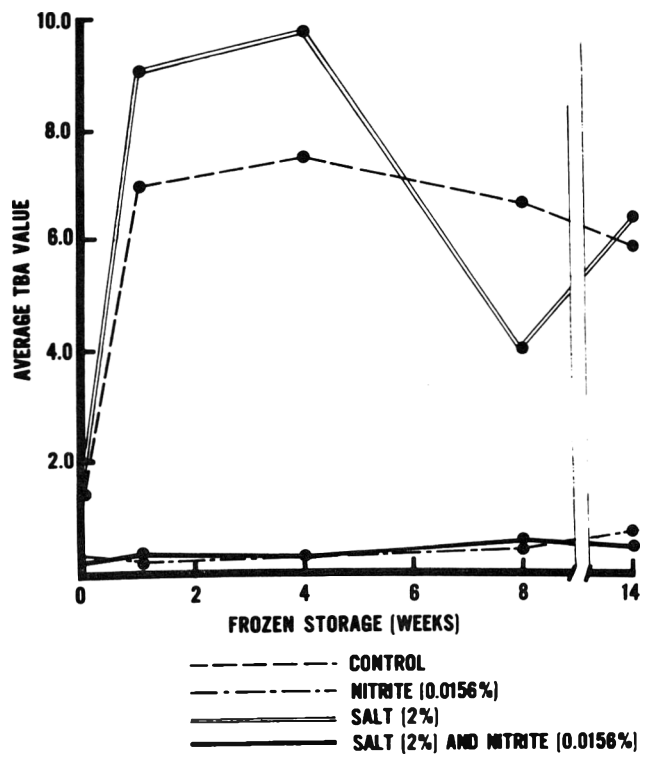

Fig. 5–Average TBA values at various interval of frozen storage.

CONCLUSION

FROM THE DATA collected in this study it appears that sodium nitrite added to cooked, canned comminuted pork plays a vital role in developing and maintaining cured pork flavor. Nitrite was found to retard the rate of oxidative rancidity (TBA value) in this model system. It is important to note that in the model system studied in this experiment, spices, sweeteners and extenders were excluded from the formulation. In addition the average fat level of the finished product was approximately 17% which is leaner than that used in most commercial formulations.

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EFFECTS OF FROZEN STORAGE, COOKING METHOD AND MUSCLE QUALITY ON ATTRIBUTES OF PORK LOINS

INTRODUCTION

MUCH WORK has been devoted to the determination of subjective and objective differences between three distinct types of pork muscle. Pale, soft, exudative (PSE) muscle tissue has been reported to exhibit a lower pH and a greater percentage of loosely bound water than either normal, or dark, firm, dry (DFD) muscle (Briskey, 1963; Wismer-Pedersen, 1959; Judge et al., 1960; Wismer-Pedersen and Briskey, 1961a, b; Bennett et al., 1973). Although Kauffman et al. (1964) reported that PSE musculature has less visual marbling than DFD or normal meat, this subjective finding has not always been upheld by tests for total lipid content of the muscles of the various pork types (Briskey, 1964; Sink et al., 1967; Sayre et al., 1964).

Taste panel comparisons of the cooked meats have generally shown PSE muscles to be more tender, but less juicy, than DFD meat (Sayre et al., 1964; Bennett et al., 1973). However, these differences were greatly affected by the cooking method.

Hardinge and Crooks (1958) reported the lipids of pork muscle to contain 40% saturated and 60% unsaturated fatty acids. It is generally assumed that this fairly unsaturated nature of pork fat contributes to the rapidity with which it develops a rancid taste in frozen storage. Hornstein and Crowe (1960) postulated that the most important flavor precursors in beef and pork are the fat soluble constituents. Because of the apparent importance of the lipid quality of meats, a comparison of the fatty constituents of three types of pork muscle might yield information which would correlate with taste panel results for those meats.

In this study, three types of pork muscle (PSE, normal, DFD) were evaluated after 9 months' frozen storage. Relative rates of rancidity development, palatability of pork cooked by two methods (oven-broiling, deep-fat frying), and quantity of lipid and major fatty acids of both raw and cooked meats are reported.

EXPERIMENTAL

10 UNBONED PORK LOIN ends each of PSE, normal, and DFD musculature were selected 24 hr postmortem from the lines of two commercial packing plants. Selections were made by visual judgement at the 10th rib by professors of Animal Sciences (Purdue University) experienced in distinguishing types of musculature, and were assigned a color score on the basis of Wisconsin standards (Forrest et al., 1963). The sections for this study were cut from an area posterior to the 3rd lumbar vertebra, and were chilled in a walk-in cooler at 0–4°C for an additional 24 hr (48 hr total postmortem aging); the meat was then frozen and stored in a walk-in freezer at -30 ± 4°C for 9 months. At the end of the storage time, each frozen loin was cut into five chops 3.2 cm thick, wrapped in freezer paper and held at −18°C in a household type freezer. One package (five chops) of each type of pork was thawed in a household type refrigerator at 4°C for 40–42 hr before testing. Only the longissimus muscle, trimmed of all external fat, was used for objective and subjective evaluations.

The method for oven-broiling was a modification of that used by Cover and Hostetler (1960). Chops were cooked at 177°C to an endpoint temperature of 77°C. The endpoint temperature (77°C) of the deep-fat fried chops was determined by a 15.2 cm standardized glass thermometer (made especially for meat research) inserted into the thickest part of the longissimus muscle; the chops were fried in corn oil preheated to 110°C.

A sample of raw, oven-broiled and deep-fat fried pork was finely ground and used for determination of rancidity according to the 2-thiobarbituric acid test (TBA) as modified by Tarladgis et al. (1960). Unfrozen (fresh) pork of each type of musculature was also tested in order to establish a TBA number (milligrams malonaldehyde per 1000g of sample) for the meat at zero storage time.

Total lipid of each raw and cooked sample was determined by a modification of the method of Folch et al. (1957). The lipid extracted with 2:1 chloroform-methanol (v/v) was washed once with a quantity of an 0.88% KCl solution in distilled water (w/w) equal to 0.2 of the extracted volume. An aliquot of the lower chloroform layer was carefully evaporated, and stored in a desiccator until a constant weight was obtained.

Percentages of major fatty acids in each raw and cooked sample were determined using the remaining chloroform layer from the total lipid measurement. Each sample was concentrated under nitrogen, and spotted on activated thin layer chromatography (TLC) plates, according to the method of Stahl (1965). A standard reference lipid, TLC Standard Neutral Lipid NL1, was obtained from The Hormel Institute, Austin, Minn. The developing solution contained the following mixture for separation on two plates: 87 ml hexane, 13 ml ethyl ether, 1 ml acetic acid. The plates were sprayed with 2,7-dichlorofluorescein (0.2% in methanol) for ultraviolet detection of the fractions obtained. The fatty acids of the phospholipid, free fatty acid, and triglyceride fractions were methylated and used for quantification by gas liquid chromatography (GLC). The instrument used in this study was a Varian Aerograph model 1200 gas chromatograph with a flame ionization detector. The 304.8 cm stainless steel column had an O.D. of 3 mm, and was packed with 10% by weight DEGS on 80–100 mesh Chromosorb AW. Column temperature was 175°C; nitrogen flow rate was adjusted to 40 ml per min.

Cooked samples were judged by a five-member experienced panel using a 5-point hedonic scale (5 = most desirable). 10 tasting sessions were conducted. One loin of each type was used per session; therefore, panel members judged six samples (oven-broiled normal, PSE, DFD; deep-fat fried normal, PSE, DFD) for a total of 10 replications. An analysis of variance was conducted on all data; the Newman-Keuls sequential range test was used to separate significant means (Steele and Torrie, 1960).

RESULTS

THE CONCENTRATION of malonaldehyde was found to be significantly ($p < 0.01$) different among types for the raw meat, with PSE musculature exhibiting the highest TBA numbers and DFD the lowest (Table 1). There was no significant difference for TBA numbers among types for the cooked meats, but all cooked pork contained significantly ($p < 0.01$) more malonaldehyde than the raw meats, and oven-broiled samples had higher numbers than the deep-fat fried muscles (Tables 2, 3). TBA numbers for unfrozen (fresh) meat are included in Table 1.

The taste panel detected differences due to cooking method and meat type (Tables 2, 3). Oven-broiled samples were judged significantly ($p < 0.01$) more tender, flavorful and acceptable than the fried meat. Significant ($p < 0.05$) differences in juiciness among types were also found, DFD being the most juicy and PSE the least. No significant differences were found among types for flavor, tenderness, or acceptability; furthermore, the method of cooking apparently had no significant effect on the juiciness of the meats.

The raw meats had significantly ($p < 0.01$) less extractable

Table 1–Mean TBA numbers[a] of three types of raw, oven-broiled and deep-fat fried pork

Treatment	Muscle type			Std error[b]	F value
	Normal (10 samples)	DFD (10 samples)	PSE (10 samples)		
Frozen					
Raw	1.2[c]	0.6[d]	3.0[e]	0.2	10.2**
Deep-fat fried	7.6	7.1	7.6	0.5	0.1
Oven-broiled	10.9	8.5	9.1	0.5	2.3
Unfrozen					
Raw	0.05	0.03	0.12	0.07	2.7
Deep-fat fried	0.7	0.5	0.6	0.2	0.7
Oven-broiled	0.7	0.6	0.7	0.2	0.2

[a] Milligrams malonaldehyde per 1000g of sample
[b] Based on error mean square from analysis of variance
[c,d,e] Means in same line bearing different superscripts are significantly different ($p < 0.01$)
** F value significant at 1% level

lipid than meat cooked by either oven-broiling or deep-fat frying (Table 2). No significant differences in percent lipid due to cooking method, meat type, or cooking method-type interactions were found; a similar lack of significant differences in fatty acid composition was noted for the variations (oven-broiled normal, PSE, DFD; deep-fat fried normal, PSE, DFD; raw normal, PSE, DFD).

DISCUSSION

THE TBA NUMBERS for the types of frozen-thawed raw meat indicate that the PSE pork contained the most malonaldehyde and DFD the least (Table 1). The lower pH which has been reported for this meat, combined with a larger amount of loosely bound water relative to DFD muscles, offer possible explanations for this marked difference (Briskey, 1963, 1964; Sayre et al., 1961, 1964). While the TBA numbers for the unfrozen meats are below estimated threshold levels for subjective detection of rancidity (Watts, 1961) they show a similar trend to that of the stored muscles. This trend supports the evidence that PSE pork is basically more susceptible to the development of malonaldehyde than are the other muscle types.

Cooking tended to elevate and even out the differences in

Table 2–Data for cooking methods only

Measurement	Cooking method			Std error[a]	F value
	Raw (30 samples)	Oven-broiled (30 samples)	Deep-fat fried (30 samples)		
TBA number[b]	1.6[c]	9.5[d]	7.4[e]	0.4	97.0**
Total lipid (%)	4.0[c]	7.7[d]	7.5[d]	0.004	135.7**
Tenderness[f]		3.4[c]	2.8[d]	0.1	41.9**
Juiciness[f]		3.0	2.9	0.1	0.0
Flavor[f]		3.3[c]	3.0[d]	0.1	10.0**
Acceptability[f]		3.2[c]	2.9[d]	0.1	12.0**

[a] Based on error mean square from analysis of variance
[b] Milligrams malonaldehyde per 1000g of sample
[c,d,e] Means in same line bearing different superscripts are significantly different ($p < 0.01$)
[f] 5-point hedonic scale: 5 = most desirable, 1 = least desirable
** F value significant at 1% level

Table 3–Combined data for cooked meats by muscle type

Measurement	Muscle type			Std error[a]	F value
	Normal (20 samples)	DFD (20 samples)	PSE (20 samples)		
TBA number[b]	6.6	5.4	6.5	0.4	2.6
Total lipid (%)	6.9	6.5	5.8	0.01	1.1
Tenderness[c]	2.9	3.2	3.1	0.2	0.7
Juiciness[c]	2.9[d]	3.3[e]	2.6[f]	0.1	4.7*
Flavor[c]	3.1	3.3	3.0	0.1	1.3
Acceptability[c]	3.0	3.3	2.8	0.1	1.8

[a] Based on error mean square from analysis of variance
[b] Milligrams malonaldehyde per 1000g of sample
[c] 5-point hedonic scale: 5 = most desirable, 1 = least desirable
[d,e,f] Means in same line bearing different superscripts are significantly different ($p < 0.01$)
* F value significant at 5% level

TBA numbers among muscle types; other workers (Younathan and Watts, 1959; Lundberg, 1962) have noted an increase in rancidity of meat following cooking. The TBA number has been used with taste panel evaluations to determine the extent of rancidity in foods (Tarladgis et al., 1960). Taste panel data obtained in this study support the pattern of the raw meat TBA numbers by trends in the scores for flavor and overall acceptability; these trends indicate DFD meat to be the most palatable of the three types, and PSE the least (Table 3). The panel members were not aware that they were judging pork believed to be rancid, and therefore the lack of significant differences in the taste panel scores for the muscle types may reflect evaluations other than that of rancidity in the meat.

The results reported in this study support evidence offered by Bennett et al. (1973) that oven-broiled pork tends to be rated more tender, flavorful and acceptable than fried muscle. Since none of the total lipid values or fatty acid percentages were found to be significantly different, it is assumed that factors other than amount of fat or its composition may contribute to the differences in palatability data for cooking method as well as for meat type.

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EFFECTS OF CURING INGREDIENTS AND HOLDING TIMES AND TEMPERATURES ON ORGANOLEPTIC AND MICROBIOLOGICAL PROPERTIES OF DRY-CURED SLICED HAM

INTRODUCTION

THE PRODUCTION of dry-cured hams has increased rapidly in the Southeastern United States during the last few years. An increasing percentage of the total hams being produced is being sliced and sold in vacuum packages rather than as whole hams (Varney, personal communication). The ham slices usually are kept under refrigeration; however, several retail outlets often display the packaged slices in nonrefrigerated areas to attract attention of the consumer.

The use of nitrate or nitrite as a curing agent was reported by Haldane (1901). As a result of work reported by Kerr et al. (1926) the USDA approved its use as a curing ingredient for pork products. The most noticeable effect of nitrate or nitrite has been its role in color development. Workers including Cho and Bratzler (1970) have shown that nitrite improved flavor of certain types of cured pork. Other workers, including Greenberg (1972), have pointed out the usefulness of nitrite as a bacteriostatic agent especially for the control of *Clostridium botulinum*. Dry-curing and aging were formerly done using ambient temperatures In recent years, however, most commercial dry-cured hams have been cured and aged under conditions of controlled temperature and relative humidity and with a decreased aging time (Cecil and Woodroof, 1954; Christian, 1960; Skelley et al., 1964; Varney, 1967). Varying ingredients, holding times and holding temperatures may affect color, microbial population and organoleptic properties. This project was designed, therefore, to test the effect of five curing mixtures and two holding temperatures on the color, general appearance, organoleptic properties and microbial population of sliced vacuum-packed country-style ham.

EXPERIMENTAL

FIVE CURING MIXTURES were used by a commercial ham curer (Harper Hams, Clinton, Ky.) in a ratio as follows (Table 1):

Cure 1: 100 lb salt, 10 lb sugar and 5 oz sodium nitrite;
Cure 2: 100 lb salt, 10 lb sugar and 44 oz potassium nitrate;
Cure 3: 100 lb salt, 10 lb sugar, 5 oz sodium nitrite and 32 oz potassium nitrate;
Cure 4: 100 lb salt, 10 lb sugar and 20 lb Prague Powder (A commercial mixture containing 6.25% sodium nitrite, 4.25% sodium nitrate and 89.5% salt);
Cure 5: 100 lb salt and 10 lb sugar.

8–9 lb of mixture were used for each 100 lb ham. The curing mixtures were applied in two equal applications at 7-day intervals and hams were held in cure 35 days at 3°C. They were then held 14 days at 16°C for salt equalization and aged 30 days at 26°C with a relative humidity of 65–68%. They were not smoked. Hams were sliced and vacuum packed with two center slices per package. Fifteen packages of each treatment were shipped to the University of Kentucky Animal Sciences Dept. and arrived 1 day after shipment. The packages were divided into three groups of five packages per group. One group was sampled soon after arrival. The second group was held as described below at 1°C for 1 month. The third group was held at 24°C (room temperature) for 1 month.

The slices were subjectively evaluated on arrival for color. Scores used were 3, dark red; 2, red; and 1, light red. Five slices of each group were broiled and evaluated organoleptically by a trained panel using a 9-point hedonic scale for flavor, tenderness, juiciness and over-all satisfaction. The samples were served six at a time with random distribution among treatments but with three of each temperature group served at each sitting. One slice of each pair was trimmed of fat, and the lean separated into three portions: (a) a 2.5-cm strip on the outside portion of the semimembranosus; (b) a 2.5-cm portion from the center of the slice; and (c) a 2.5-cm portion adjacent to the outside fat. Each section was analyzed for nitrite and NaCl (AOAC, 1970). A random slice from each treatment group was used to enumerate and establish base values for the various microorganisms noted below.

Five packages from each treatment group were held at room temperature (24°C) under normal fluorescent light for 1 month. An additional five packages from each treatment group were held at approximately 1°C under soft white lights (to simulate store conditions) for 1 month.

After storage, both temperature groups were evaluated visually and organoleptically, and analyzed for salt and nitrite as noted above. One slice from each package was examined for total, anaerobic, streptococci, enterococci, lactobacilli and staphylococci counts. Counts were determined using the procedures described by Langlois and Kempt (1974).

Table 1—Experimental design

Curing treatment[a]	Holding time and temp after slicing	
Nitrite,[b] salt, sugar	1 day,	1°C
Nitrate,[c] salt, sugar	1 mo,	1°C
Nitrite[d] + nitrate, salt, sugar	1 mo,	24°C
Prague Powder,[e] salt, sugar		
Salt-sugar only (control)		

[a] 8–9 lb mixture per 100 lb meat
[b] Approx 1 oz per 100 lb meat
[c] Approx 4 oz per 100 lb meat
[d] Approx 4 oz nitrate and 1/2 oz nitrite per 100 lb meat
[e] Approx 1.3 lb per 100 lb meat

Table 2—Effect of cure ingredients and time and temperature of storage[a] on color of dry-cured hams

Treatment	Period		
	Fresh	1 mo, 1°C	1 mo, 24°C
Nitrite	2.0 a,x	2.0 a,x	2.0 a,x
Nitrate	2.2 a,x	2.0 a,x	1.2 b,y
Nitrite + Nitrate	2.0 a,x	2.0 a,x	1.6 a,y
Prague Powder	2.0 a,x	2.0 a,x	1.2 b,y
Control	1.6 b,x	1.4 b,x	1.0 c,y

[a] Data within vertical columns with different letters (a, b, c) are significantly different ($P < 0.05$); Data within horizontal rows with different letters (x, y) are significantly different ($P < 0.01$).

Table 3—Effect of curing ingredients and time and temperature of storage[a] on organoleptic[b] properties of dry-cured hams

	Flavor			Tenderness			Juiciness			Over-all satisfaction		
Holding time	0	1 mo	1 mo	0	1 mo	1 mo	0	1 mo	1 mo	0	1 mo.	1 mo
Holding temp	–	1°C	24°C	–	1°C	24°C	–	1°C	24°C	–	1°C	24°
Cure treatment												
Nitrite	7.05 a,x	6.52 a,y	5.28 z	6.48 a,x	5.66 a,y	4.51 z	6.57 a,x	5.86 a,y	4.84 z	6.83 a,x	6.24 a,y	4.77 z
Nitrate	6.71 a,x	6.14 a,y	4.61 z	6.45 a,x	5.80 a,y	4.51 z	6.57 a,x	5.75 a,y	4.62 z	6.68 a,x	5.82 a,y	4.42 z
Nitrite + Nitrate	7.14 a,x	6.16 a,y	5.01 z	6.80 a,x	6.52 a,y	5.17 z	6.74 a,x	5.87 a,y	4.85 z	7.02 a,x	6.28 a,y	4.55 z
Prague Powder	6.96 a,x	6.36 a,y	4.73 z	6.60 a,x	5.79 a,y	4.58 z	6.82 a,x	5.79 a,y	4.83 z	6.60 a,x	5.98 a,y	4.60 z
Control	6.14 b,x	5.86 b,y	5.03 z	5.43 b,x	5.40 b,x	4.95 z	6.22 a,x	5.52 a,y	5.03 z	5.57 b,x	5.50 b,x	4.88 z

[a] Data within vertical columns with different letters (a, b) are significantly different ($P < 0.05$); Data within horizontal rows with different letters (x, y, z) are significantly different ($P < 0.01$).
[b] Based on 9-point hedonic scale with 1 being dislike extremely and 9 like extremely.

Data were analyzed using the Statistical Analysis System (SAS) program of Barr and Goodnight (1972).

RESULTS & DISCUSSION

Color

Color was evaluated subjectively as noted in Table 2. In the fresh group any curing treatment containing nitrite or nitrate brought about improved color ($P < 0.05$). This color generally was maintained when the slices were stored at 1°C. However, when slices were stored at room temperature (24°C) there was a significant decrease ($P < 0.01$) in color scores in all cure treatment groups except the nitrite group. The control group, cured with salt-sugar only and held at room temperature for a month, was especially off-color. The average score (1) was light red. However, in many instances slices also showed grey areas.

Flavor

Flavor was affected by both curing treatment and storage conditions (Table 3). Among the fresh samples, flavor was more desirable ($P < 0.05$) in any group where nitrite or nitrate was used than in the controls. The same trend continued after holding slices a month at 1°C. Flavor decreased ($P < 0.01$) during 1 month at 1°C. Scores were still above the hedonic scale of 6 (like slightly), however, and were very acceptable. The scores were much better ($P < 0.01$) when the slices were held at 1°C than at 24°C. At the latter temperature all had decreased and averaged below the "like slightly" (6) category with no significant difference among cure treatments.

Tenderness

Tenderness scores also are noted in Table 3. In the fresh group, all treated samples had higher average scores ($P < 0.05$) than the control group. This also was true after a month at 1°C. There was a significant decrease in tenderness as storage progressed. The difference was highly significant ($P < 0.01$) when the fresh or 1°C storage groups were compared with the 24°C group. Since some panelists tended to relate one organoleptic property to another the difference may be somewhat biased.

Juiciness

Juiciness scores averaged higher for the treated groups, although the differences were not significant. There was a significant ($P < 0.01$) decrease in juiciness, however, when the fresh group was compared with the 1°C group or the 24°C group or when the 1°C group was compared with the 24°C group.

Over-all satisfaction

Over-all satisfaction scores followed the same trend as flavor and tenderness scores. Treated groups were more desirable than the control group except at the 24°C storage temperature. Again, there was a decrease in over-all satisfaction scores with 1 month storage at 1°C, but the big decrease ($P < 0.01$) was due to temperature. This indicates that hams cured with some form of nitrate or nitrite were more desirable than untreated hams when sampled either shortly after cutting or after a month's storage at 1°C. It also shows that ham slices became undesirable after a month's storage at room temperature regardless of cure treatment.

Effect of curing ingredients

Percent salt by treatment groups (Table 4) ranged from 5.2

Table 4—Effect of curing ingredients and time and temperature of storage on percent salt in dry-cured hams

Portion[a]	Time 0	Temp 1°C		Time 1 mo	Temp 1°C		Time 1 mo	Temp 24°C	
Treatment[b]	a	b	c	a	b	c	a	b	c
Nitrite	6.1	7.0	6.6	5.5	6.1	5.8	6.4	6.6	7.2
Nitrate	5.2	6.5	5.9	5.9	6.1	5.9	6.6	6.8	6.6
Nitrite + Nitrate	5.8	6.3	5.2	6.1	6.4	6.2	6.2	6.6	6.3
Prague Powder	7.0	6.8	6.1	7.0	7.5	5.7	6.2	7.1	7.1
Control	6.9	7.5	6.6	6.5	6.9	6.3	6.8	7.0	6.9

[a] Portion: "a" Surface sample; "b" Intermediate sample; "c" Deep sample.
[b] Avg all "a" = 6.28; all "b" = 6.74; all "c" = 6.29.

Table 5—Effect of curing ingredients and time and temperature of storage on residual nitrite in dry-cured hams

Portion[a]	Time 0	Temp 1°C		Time 1 mo	Temp 1°C		Time 1 mo	Temp 24°C	
Treatment	a	b	c	a	b	c	a	b	c
	Residual nitrite, ppm								
Nitrite	62.2	7.1	14.0	40.0	18.6	16.9	12.5	8.3	7.2
Nitrate	10.2	43.3	25.4	13.5	30.0	23.6	9.0	11.2	9.3
Nitrite + Nitrate	42.6	72.2	46.4	21.1	18.9	18.6	12.5	14.0	14.4
Prague Powder	142.6	8.0	7.9	76.8	11.1	12.1	44.5	13.5	11.0
Control	6.1	3.9	6.4	6.0	5.2	5.4	7.1	8.4	7.4

[a] Portion: "a" Surface sample; "b" Intermediate sample; "c" Deep sample.

Table 6—Effect of curing ingredients and time and temperature[a] of storage on microbial counts (log 10) of dry-cured hams

	Total			Anaerobic			Streptococci			Lactobacilli			Staphylococci			Enterococci		
Holding time	0[b]	1 mo	1 mo	0[b]	1 mo	1 mo	0[b]	1 mo	1 mo	0[b]	1 mo	1 mo	0[b]	1 mo	1 mo	0[b]	1 mo	1 mo
Holding temp	–	1°C	24°C	–	1°C	24°C	–	1°C	24°C	–	1°C	24°C	–	1°C	24°C	–	1°C	24°C
Cure treatment																		
Nitrite	5.2	4.4 x	8.0 y	3.1	2.6 c,x	7.5 y	3.1	3.6 x	7.5 y	2.0	1.9 c,x	3.4 c,y	2.4	2.0 x	2.9 y	2.1	1.4 c	1.2 c
Nitrate	4.1	5.4 x	7.8 y	3.3	3.8 d,x	7.8 y	3.4	3.5 x	7.6 y	2.2	2.3 d,x	5.1 d,y	2.0	1.4 x	2.7 y	2.1	1.0 d	1.0 d
Nitrite + Nitrate	5.9	4.8 x	7.7 y	4.0	3.6 d,x	7.9 y	3.4	3.5 x	7.7 y	2.3	2.2 d,x	4.3 d,y	2.4	1.4 x	2.2 y	1.8	1.3 c	1.8 c
Prague Powder	5.6	4.6 x	8.2 y	3.6	3.1 d,x	7.6 y	3.0	3.2 x	7.6 y	2.2	1.6 c,x	3.5 c,y	2.4	1.1 x	2.4 y	1.0	1.0 d	1.0 d
Control	4.8	4.5 x	7.8 y	3.7	3.0 d,x	7.6 y	3.4	3.5 x	7.5 y	2.0	1.8 d,x	2.4 d,y	1.3	0.9 x	2.8 y	1.0	1.0 d	1.0 d

[a] Data within vertical columns with different letters (c, d) are significantly different ($P < 0.01$); Data within horizontal rows with different letters (x, y) are significantly different ($P < 0.01$).
[b] One sample only

in the 0 time nitrate "a" portion to 7.5 in the fresh control "b" portion and 1°C Prague Powder "b" portion. Averages of all a, b and c portions, respectively, were 6.28, 6.74 and 6.29%, showing that salt had equalized. Although there were a few significant differences among groups their validity is questioned or not explainable.

There was considerable variation in nitrite levels due to treatment and storage (Table 5). As expected, the highest levels occurred in the "a" portion of the 0 time sample. The Prague Powder group was considerably higher, followed by the nitrite group, nitrite + nitrate group and then the nitrate group. Even the control group had a small amount of nitrite. This could be due to cross contamination as all hams were cured in the same cooler by the same workers, it could be due to experimental error, or it could be a natural occurrence as Kemp et al. (1974) obtained similar results. In the treated groups at the fresh period, with the exception of the nitrite group, the "b" portion contained more nitrite than the "c" portion, showing a gradation as absorption is achieved. After a month's exposure to light at 24°C, the "a," "b" and "c" portions, with the exception of the Prague Powder group, were more nearly equal. The average was much lower. also than in the 0 time group or samples stored at 1°C, indicating that nitrite had been absorbed or broken down. This agrees with the work of Hill et al. (1973) who found decreased levels of nitrite in prepackaged processed meat with increased storage time.

Microbiological counts

Microbiological counts are noted in Table 6. Temperature of storage had a greater effect on microbial counts than did curing treatment or storage time. There were no significant differences in total count due to cure treatment. Holding temperature effects were significant ($P < 0.01$), however, as counts for the 24°C group were much higher, indicating the importance of refrigeration for this type product.

Salt treatment resulted in some of the counts (anaerobic lactobacilli) being significantly lower than the other counts. However, while differences are statistically significant, the differences appear to be too small to have any real importance. No significant effect was noted due to holding temperature for enterococci counts, which is different from all other microbiological results obtained.

SUMMARY & CONCLUSIONS

HAMS were cured using salt and sugar only (control) or salt and sugar plus (a) nitrite, (b) nitrate, (c) nitrite + nitrate or (d) Prague Powder. After curing and aging, the hams were sliced and vacuum packaged. Five packages of each cure group were evaluated without holding while five from each treatment were held 1 month at 1°C and another 5 were held one month at 24°C.

Color scores were more desirable for any treated group than for the controls when evaluated at 0 time. After 1 month at 1°C all treated groups retained their color while color of the control group had deteriorated. After 1 month at 24°C all groups except those treated with nitrite had poor color.

Organoleptic scores (flavor, tenderness, juiciness and overall satisfaction) favored the treated groups at the 0 time state and a month at 1°C. After a month at 24°C, average scores of all groups had decreased.

Salt levels were affected very little by cure treatment or time. Nitrite levels were variable especially in the outside lean portion but tended to decrease during storage. After a month at 24°C, the levels were low except in the Prague Powder surface sample.

Microbiological counts were affected significantly by storage temperature. Of the counts made (total, anaerobic, streptococci, lactobacilli, staphylococci and enterococci) only lactobacilli was appreciably affected by cure treatment as nitrate appeared to enhance growth. High storage temperature accelerated growth in all cure groups.

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FACTORS AFFECTING SHOWCASE COLOR STABILITY OF FROZEN LAMB IN TRANSPARENT FILM

INTRODUCTION

FOR ECONOMIC REASONS, the meat industry likely will move toward centralized cutting. Frozen, rather than fresh, retail cuts may fit more logically into a central cutting system. Retail markets. especially those in less-populated areas, could stock frozen lamb cuts in demand in that area. Consistent and acceptable meat quality, especially as manifested by acceptable color is a primary determinant of product salability (Naumann et al., 1957). Attractive frozen meat color and an absence of frost and blood in packages are important since first impressions of meat are usually visual (Kropf, 1971). Consumers prefer bright red fresh meat color which results from the muscle pigment, oxymyoglobin. Production of the same bright red color in frozen meats seems feasible, although recommended ways to increase shelf life of fresh products might not apply to frozen meat.

The purpose of this study was to investigate the effect of freezing temperature, display temperature and lighting, packaging film and marbling level on color stability, weight losses and thaw drip of frozen lamb chops displayed in transparent film.

MATERIALS & METHODS

LAMB CARCASSES were purchased from a commercial packing company. Each of three replications consisted of three carcasses with identical slaughter dates, representing three quality levels based on visual longissimus muscle marbling score at the 12th rib: namely, moderate or slightly abundant, slight or small and practically devoid or devoid. Beef marbling standards as described by Walters (1969) were used as a guide.

Sixteen loin and rib chops from each carcass, each 2.54 cm thick, were cut and randomly assigned to one of 16 treatments, which consisted of all possible combinations of two freezing temperatures (−40°C liquid nitrogen vapor versus −26°C circulating air blast), two display temperatures (−29°C versus −21°C), two packaging films (Saran, oxygen permeability about 150–200 cc/m²/24 hr, 0.033 mm thick versus Cryovac L-300, oxygen permeability about 4,000–5,000 cc/m²/24 hr at 1 atm and 23°C, 0.051 mm thick) and two lighting systems (deluxe cool white fluorescent versus incandescent with a Holophane Prismatic Reflectance lens (Holophane Co., Inc., New York, N.Y.). Relative spectral energy distribution of light sources and transmission of the Holophane lens is presented in Figure 1.

Chops were bloomed at about 21°C at least 30 min, individually vacuum packaged, clip sealed and heat shrunk by dipping for 2 or 3 sec in 88°C water. Chops frozen at −40°C were placed in a NCG liquid nitrogen simulator freezer in an upright position for maximum exposure to the nitrogen vapor. The freezing chamber had been chilled and was programmed to hold 0°C for 10 min and −40°C for 25 min. End-point internal temperature of −29°C was reached in 35 min. Chops frozen at −26°C were placed upright in a blast freezer 10 hr.

All chops remained in the dark at either −29°C or −26°C until displayed under predetermined conditions. Air temperatures of −29°C and −21°C at top surface of chops were maintained in open-topped display cases, except for defrost cycles twice daily. Product temperature never rose above −11°C during the defrost cycle, and prior temperature was restored in 1.5 hr. Cases were in an air-conditioned room (22°C), where humidity extremes were avoided. A light intensity of 1.076 lumens/m² (100 ft-c) at product level was maintained 24 hr/day for each lighting system; extraneous lighting was held at a minimum. Extreme care was taken so the same meat surface was evaluated and exposed to light throughout the study.

Subjective color scores, objective color measurements and weight losses were recorded at nine times: fresh unpackaged (bloomed), fresh packaged, immediately post-freezing (day 0), and after frozen display for 1, 7, 21 and 42 days. Day 43 evaluations were made immediately after unwrapping the frozen chops and day 44 observations were after thawing 24 hr at 14°C with relative humidity about 90%. Visual color was evaluated by two panelists under the assigned display lighting system to the nearest 0.5 point on this scale: 1 = very bright, 2 = bright, 3 = slightly dark, 4 = dark and 5 = extremely dark. A Bausch & Lomb 600 Spectrophotometer with reflectance attachment, calibrated for 100% reflectance with a $MgCO_3$ block was used to obtain reflectance spectra from 400–700 nm at a recording speed of 250 nm/min. A black rubber gasket (slightly larger than the reflectance aperture) was placed between the package and the sphere to minimize effect from thawing during the color scan. Reflectance readings to the nearest 0.1% were determined at 474, 525, 572 and 630 nm. Ratios of reflectance readings (474/525 and 572/525) were calculated as suggested by Snyder and Armstrong (1967). Weights were determined to the nearest 0.01g; weight loss was expressed as % of fresh packaged weight and drip loss as % of frozen unpackaged weight at day 43.

Either a split-plot or split-split-plot design, with carcass as the whole plot and treatment combinations as subplots, was used according to Cochran and Cox (1957). Analysis of variance and least significant differences were used to test treatment means.

RESULTS & DISCUSSION

Packaging film

Mean visual score and % reflectance at 630 nm for all fresh, unpackaged chops were 1.81 and 28.8%, respectively. Skin tight packaging in either oxygen permeable L-300 film or less permeable Saran film resulted in darkening of bloomed color (Table 1); however, the L-300 packaged chops possessed more desirable visual scores and reflectance values post-packaging for fresh chops than did Saran-packaged chops. Vacuum packaging has been reported to cause darkening of fresh meat (Landrock and Wallace, 1955; Rikert et al., 1957a, b; Dean and Ball, 1960) with regeneration of redness after 2–4 days' storage. Frozen lamb chops did not follow such a color regeneration pattern under frozen display conditions.

Chops packaged in L-300 film were more desirable in visual color after 1 and 42 days of frozen display than chops packaged in Saran. Lower values ($P < 0.01$) for reflectance ratio 474/525 were recorded for the L-300 film compared with Saran which indicates more reduced myoglobin (Mb) with Saran-packaged muscles. Data by Snyder (1965), transformed from absorbance to reflectance, indicate that MbO_2 (oxymyoglobin) and Mb^+ (metmyoglobin) predominate when the ratio 474/525 was about 1.03; and that Mb predominated when the value was about 1.6. Because Mb^+ reflects less light at 630 nm (Hansen and Sereika, 1969) than either MbO_2 or Mb, the lower reflectance for the L-300 film (Table 1) at days 7, 21, 42 and 43 suggested the presence of more Mb^+ in the L-300 packages. Additional evidence for more Mb^+ in the L-300-packaged chops was found in significantly ($P < 0.01$) larger values of reflectance ratio 572/525 at days 7, 21, 42

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and 43 as larger values for 572/525 ratio indicate samples contain proportionately more Mb^+ (Snyder, 1965).

Saran-packaged chops possessed more desirable visual and reflectance values after the thaw-bloom period than chops in L-300 film. Similar reoxygenation of myoglobin was observed by Pirko and Ayres (1957).

Spectral transmission characteristics of both films were nearly identical. Therefore, films used in this study probably did not decrease meat discoloration by absorbing various light wavelengths as some films apparently do (Kraft and Ayres, 1954).

Surface desiccation could explain the marked decrease in % R at 630 nm reflectance of thawed chops (day 44).

Freezing temperature

Effect of freezing temperature on visual scores and reflectance is given in Table 2. Brighter visual appearance resulting from freezing at the lower temperature of −40°C agreed with data of Ramsbottom and Koonz (1939, 1941); Pearson and Miller (1950) and Brissey (1963). Severe "bleaching" noted with −46°C plate freezing by Robertson (1950) was not observed in this study.

Improved visual scores for samples frozen at −40°C were confirmed by higher reflectance readings (more red reflectance) at 630 nm but not by the reflectance ratios.

Visual scores for thawed, day 44 samples indicated a slight beneficial visual color advantage fpr −40°C frozen chops; however, reflectance at 630 nm indicated no significant color difference, and tended to support Costello (1964), who found no color differences in thawed beef steaks that had been frozen at "conventional" vs "rapid" freezing rates.

Display temperature

Displaying chops at −21°C (air at meat surface level) resulted in more desirable visual scores ($P < 0.05$) at day 1 (Table 3), compared with display at −29°C but reflectance at 630 nm indicated a color advantage for display at −29°C.

Visual color stability of lamb chops displayed 21 days was not different for display temperatures of −29 or −21°C. However, a color advantage for −29°C display was apparent at day 42. The more desirable visual score at day 44 for −29°C display suggested that a less oxidized state of myoglobin was maintained at the colder temperature.

Reflectance data at 630 nm for display temperature (Table 3) supported research by Ramsbottom and Koonz (1941); Ramsbottom (1957); Snyder and Ayres (1961); Brown and Dolev (1963); Snyder (1964) and Cutaia and Ordal (1964) that lower storage temperatures decreased oxidation of myoglobin in fresh and frozen meat. Lower reflectance values at 630 nm plus lower values for ratio 474/525 at day 7, 43 and 44 (P all < 0.01) suggest more Mb^+ in chops displayed at −21°C a trend apparent at all frozen time periods and after thawing, but not confirmed by reflectance ratios measured at 572/525 nm.

Product-surface temperature for chops displayed at −29°C ranged from −26 to −15°C and for chops at −21°C, from −18 to −11°C. Display-temperature fluctuations, especially if thawing temperature was reached, have been found to affect frozen-meat color critically (Townsend and Bratzler, 1958), although this was reportedly not true for temperature fluctuations below −18°C (Hustruld et al., 1949) and from −18 to −10°C (Winter et al., 1952).

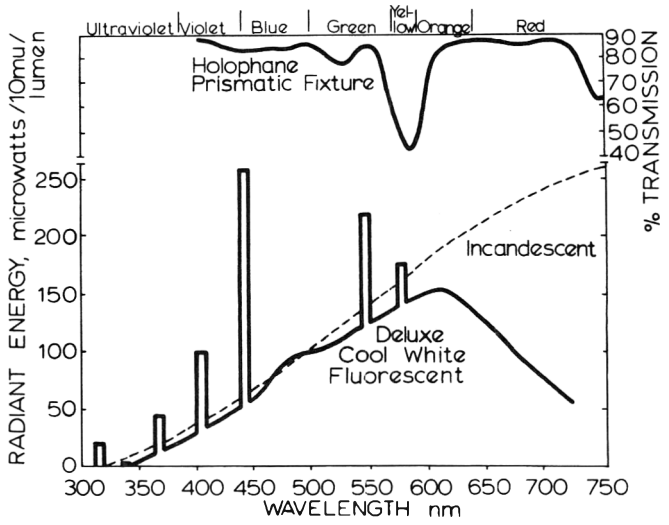

Fig. 1—Approximate energy distribution of deluxe cool white fluorescent and incandescent lamps and percent transmission of light by Holophane Prismatic Reflectance Fixture (used with incandescent) (General Electric, 1968).

Lighting

Chops displayed under incandescent lighting had lower, more desirable, visual scores at all frozen-display periods except day 1 (Table 4). However, because appraisal was only

Table 1—Effect of packaging film[a] on mean visual score and reflectance (% R) of frozen lamb chops at several time periods

	Visual Score[b]			630 nm % R			% R 474/525 nm			% R 572/525 nm		
Time	L-300		Saran	L-300		Saran	L-300		Saran	L-300		Saran
Fresh packaged	2.40	**	2.61	26.1	**	24.2	1.21	**	1.31	0.81	**	0.83
Frozen, day												
0	2.42		2.52	25.9		25.3	1.12		1.12	0.92		0 92
1	2.42	**	2.63	24.8		24.4	1.06	**	1.22	0.93		0 92
7	2.62		2.68	23.2	*	24.5	1.08	**	1.21	0.98	**	0.94
21	2.70		2.79	22.0	**	24.0	1.10	**	1.17	1.01	**	0.96
42	2.81	*	2.97	22.4	**	23.8	1.09	**	1.14	1.00	**	0.98
43[c]	2.98		2.88	24.0	**	25.7	1.12	**	1.19	1.01	**	0.97
Thawed-44[c]	3.32	*	3.19	15.6	**	16.6	1.10		1.10	1.00	**	0.94

[a] L-300 = oxygen permeable; Saran = oxygen impermeable
[b] 1 = very bright, 5 = Extremely dark
[c] Unpackaged
* ($P < 0.05$)
** ($P < 0.01$)

Table 2–Effect of freezing temperature on mean visual score and reflectance (% R) of frozen lamb chops at several time periods

	Visual score[a]			630 nm % R			% R 474/525 nm			% R 572/525 nm		
Time	−40°C		−26°C	−40°C		−26°C	−40°C		−26°C	−40°C		−26°C
Frozen, day												
0	2.29	**	2.66	29.0	**	22.6	1.12		1.13	0.91		0.92
1	2.31	**	2.74	27.6	**	21.6	1.13		1.14	0.92		0.93
7	2.37	**	2.93	26.8	**	21.0	1.13	**	1.16	0.95		0 95
21	2.48	**	3.01	24.7	**	21.3	1.13	*	1.15	0.99		0.98
42	2.65	**	3.13	24.0	**	22.2	1.11		1.13	1.00	**	0.98
43[b]	2.67	**	3.13	26.8		23.0	1.13	**	1.18	1.00	**	0.98
Thawed-44[b]	3.18	*	3.34	16.2		16.0	1.10		1.10	0.98		0.96

[a] 1 = Very bright; 5 = Extremely dark
[b] Unpackaged
* ($P < 0.05$)
** ($P < 0.01$)

under display lighting type, visual scores possibly did not reflect true color deterioration or change in pigment state. More desirable visual scores recorded for chops under incandescent light compared with fluorescent light could have resulted from the larger proportion of wavelengths greater than 600 nm (Fig. 1).

Lower values for ratio 474/525 were recorded for incandescent lighting at days 7 ($P < 0.05$), 21, 42 and 43 ($P < 0.01$); indicating the presence of more MbO_2 and Mb^+ combined, but lower reflectance values at 630 nm for chops under incandescent lighting indicated proportionately more Mb^+. Significantly higher values for ratio 572/525 recorded for incandescent lighting at days 42, 43 ($P < 0.01$) and 44 ($P < 0.05$), also suggest that incandescent lighting caused more Mb^+ to form in frozen lamb chops than did deluxe cool white fluorescent lighting. Determining visual score under the incandescent light may have masked color deterioration resulting from increasing Mb^+.

Random inspection of the bottom side of chops not exposed to light revealed consistently brighter visual color than

Table 3–Effect of display temperature on mean visual score and reflectance (% R) of frozen lamb chops at several time periods

	Visual Score[a]			630 nm % R			% R 474/525 nm			% R 572/525 nm		
Time	−29°C		−21°C	−29°C		−21°C	−29°C		−21°C	−29°C		−21°C
Frozen, day												
1	2.58	*	2.47	25.4	**	23.8	1.14		1.13	0.93		0 92
7	2.67		2.62	24.5	**	23.3	1.16	**	1.13	0.97		0 96
21	2.71		2.78	23.9	**	22.1	1.15		1.13	0.99		0 98
42	2.80	**	2.98	24.8	**	21.4	1.12		1.11	0.99		0.99
43[b]	2.80	**	3.01	26.2	**	23.6	1.17	**	1.14	0.98	**	1.00
Thawed-44[b]	3.19	*	3.32	16.3		15.9	1.11	**	1.09	0.97		0.96

[a] 1 = Very bright; 5 = Extremely dark
[b] Unpackaged
* ($P < 0.05$)
** ($P < 0.01$)

Table 4–Effect of display lighting[a] on mean visual score and reflectance (% R) of frozen lamb chops at several time periods

	Visual Score[b]			630 nm % R			% R 474/525 nm			% R 572/525 nm		
Time	I		F	I		F	I		F	I		F
Frozen, day												
1	2.40		2.65	24.5		24.7	1.13		1.14	0.92		0 93
7	2.52	**	2.77	23.6		24.2	1.14	**	1.15	0.96		0 97
21	2.65	**	2.84	22.8		23.2	1.12		1.16	0.98		0.99
42	2.82	*	2.96	22.4	**	23.8	1.10	**	1.14	1.00	**	0.98
43[c]	2.82	*	2.99	24.3		25.5	1.13	**	1.19	1.00	**	0.97
Thawed-44[c]	3.16	**	3.35	15.5	**	16.8	1.09		1.11	0.98	*	0.95

[a] I = Incandescent; F = Fluorescent (deluxe cool white)
[b] 1 = Very bright; 5 = Extremely dark
[c] Unpackaged
* ($P < 0.05$)
** ($P < 0.01$)

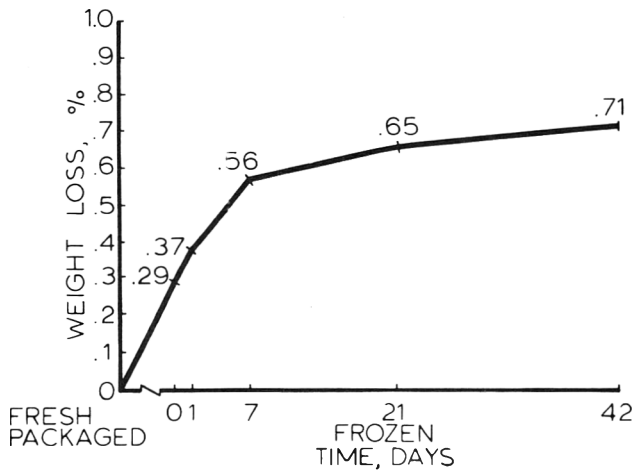

Fig. 2—Percent weight loss of fresh packaged and frozen lamb chops at various time periods.

did the side exposed to light, which agreed with findings of Townsend and Bratzler (1958) and Marriott et al. (1967).

Marbling

Degree of marbling in the longissimus muscle did not significantly affect any subjective or objective measurements of color, weight or drip loss.

Weight loss

Data in Figure 2 reveal weight losses of 0.29% due to freezing and 0.42% due to frozen display (days 0–42), totaling 0.71% after frozen display of 6 wk. Total weight loss from fresh unpackaged to frozen unpackaged (day 43) was 0.90%. The average weight loss, from day 1 to day 42, of the frozen chops packaged in the L-300 film was 0.23% compared with 0.44% for chops packaged in Saran; however, weight loss observed from one period to the next was not significantly affected by any of the 16 treatment combinations. High oxygen permeability and high water-vapor transmission properties of packaging films do not necessarily coincide. However, more frost accumulated in the L-300 packages; hence, more water vapor may have passed into these packages, condensed as frost and "could have been manifested" as less weight loss for chops packaged in the L-300 film. Certain packaging films have been observed to cause product weight gain (Hustruld et al., 1949).

Drip loss

Mean drip loss was 5.34%. Chops frozen at −40°C exhibited less drip loss (4.93%) than those frozen at −26°C (5.76%). Ramsbottom and Koonz (1939) stated that drip loss was a function of ice crystal size; that smaller crystals resulted from faster freezing rates, caused less tissue disruption and subsequently reduced drip loss. In our study, display temperature, packaging film and lighting did not significantly affect drip loss.

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IMMOBILIZED CATALASE REACTOR FOR USE IN PEROXIDE STERILIZATION OF DAIRY PRODUCTS

INTRODUCTION

THE USE OF chemical sterilization of cheese milk is permissible in the United States with no more than 0.05% H_2O_2. The effect of H_2O_2 on the bacteriological contents of milk has been reported by several investigators (Luck, 1956; Roundy, 1958; Amin and Olson, 1967; Naguib and Hussein, 1972). After the sterilization process, the enzyme catalase is added to remove the residual H_2O_2.

Catalase was first immobilized by ionic attachment to diethylaminoethyl (DEAE) cellulose by Mitz (1956), and covalently to a polymer by Brandenberger (1956). Schejter and Bar-Eli (1970) prepared an insoluble catalase by crosslinking purified catalase with glutaraldehyde. This technique was modified by Ferrier et al. (1972) by treating glutaraldehyde-crosslinked catalase with bisulfite to form an insoluble enzyme. Chang (1971) and Chang and Poznansky (1968) immobilized catalase in microencapsules with glutaraldehyde.

The possible application of immobilized enzyme in the removal of H_2O_2 from milk for cheese making was studied by Balcom et al. (1971) in a continuous packed-bed reactor with catalase coupled to cheesecloth with glutaraldehyde. A rapid inactivation of the catalase in the first 2 hr was observed. O'Neill (1972) showed theoretically that the inactivation of catalase by H_2O_2 may be reduced by using a stirred-tank reactor rather than a packed-bed reactor if the reaction is zero order. The inactivated catalase in a catalase-collagen reactor was regenerated to recover its activity by Wang et al. (1974). The mechanism of inactivation and regeneration is not thoroughly known; however, the phenomenon of regeneration of immobilized catalase activity offers encouragement for further investigation.

The bacteriological effectiveness of H_2O_2 in milk was determined. A catalase-collagen reactor was used to study the efficiency of the reactor in removing residual H_2O_2 from milk. The results of these studies are herein reported.

EXPERIMENTAL

Materials

Liver catalase was purchased from the Worthington Biochemical Corporation, Freehold, N.J. The enzyme, which was used without further purification, was bound to cattle hide collagen obtained from the USDA Eastern Regional Research Center. Vexar mesh sheet, used as a film spacer, was obtained from E.I. duPont de Nemours and Co., Inc. Glutaraldehyde, as a 50% solution, and H_2O_2, as a 3% solution, were obtained from Fisher Scientific Co.

Raw milk for the bacteriological studies was obtained from the All Star Dairy, Perth Amboy, N.J. The pasteurized milk used for the catalase reactor studies was from the Rutgers Dairy Plant.

Bacteriological examination

To 100g of raw milk H_2O_2 was added in concentrations of 0.01, 0.02, 0.03, 0.04 and 0.05%. The H_2O_2-milk samples were allowed to react for 30 min at various bath temperatures, and 1 ml of 0.06% (w/v) catalase solution was added to decompose the residual H_2O_2. The effect of the H_2O_2 on the bacteria counts of the raw milk was determined according to standard methods (APHA, 1972).

Preparation of catalase-collagen reactor

6g of collagen were suspended in 600 ml of distilled water and stirred while the pH was adjusted to 3.5 with 85% lactic acid solution. The dispersion was homogenized in a Waring Blendor. After adding 200 mg of catalase, the dispersion was again homogenized. The homogenized dispersion was then cast on a Mylar sheet and air dried at room temperature for 2 days. After drying, the film was about 0.08 mm thick. The dried film was then dipped in a 0.2% glutaraldehyde solution for 2 min, washed with distilled water and air dried. The catalytic activity of the catalase-collagen complex was equivalent to 8.3 μg of free catalase activity per g of catalase-collagen complex.

The complex was then layered on Vexar plastic netting and wound to form a spiral multipore module. The module was then fitted into a 2.54 cm plexiglass cylinder to form a reactor. Three reactors were made with different packing lengths, 7.62 cm with 2.23g of complex; 15.24 cm with 4.45g of complex; and 22.86 cm with 6.82g of complex.

Determination of residual H_2O_2

All tests were made at room temperature, 25 ± 1°C. Pasteurized milk samples containing 0.05% H_2O_2 were pumped through the reactors with a peristaltic pump at flow rates of 10, 25, 50 and 100 ml/min. The milk samples were collected from the reactors and the residual H_2O_2 was determined directly by means of a Sargent-Welch Polarograph XVI with a dropping mercury electrode. 10 ml of the milk sample were transferred to a test tube containing 1 ml of 2% H_2SO_4 solution which stopped the catalase-H_2O_2 reaction. 10 ml of 0.05M phosphate buffer at pH 7.0 were added as a supporting electrolyte. The oxygen level was reduced by flushing about 20 ml of the mixture with nitrogen for 5 min in the Sargent-Welch electrolysis vessel. By using a calibration curve, residual H_2O_2 in the milk samples was calculated in percent by comparing the diffusion current of the sample with responses from reference samples.

Initial reaction rate period

For the preliminary experiments on initial reaction rate, a catalase-collagen complex was used which had not been crosslinked with glutaraldehyde. This complex, which had an enzyme loading equivalent to 29.0 μg of free enzyme per g of complex, showed a stable decomposition of 97% of the H_2O_2 in milk which had been treated with 0.02% H_2O_2 when pumped through the reactor at 100 ml per min for 30 min. After this period, an unstable activity was observed which apparently resulted from the leaching of loosely bound catalase.

All succeeding tests in this study were made with a catalase-collagen complex which has been crosslinked with glutaraldehyde. Data were recorded after the reactors had been stabilized for 24 hr.

RESULTS & DISCUSSION

Bacteriological studies

The effect of H_2O_2 at different concentrations and temperatures on the bacteriological contents of raw milk is shown in Table 1. The results indicate that a slight variation of temperature had a definite effect on the bactericidal efficiency of the H_2O_2 solution. Using a H_2O_2 solution at a concentration of 0.02% at 45°C for 30 min resulted in a 99.99% reduction of the bacteria counts.

Reactor studies

The results of the reactor studies are plotted in Figure 1, where P is the percent destruction $(S_o - S_e)/S_o$; S_o is the ini-

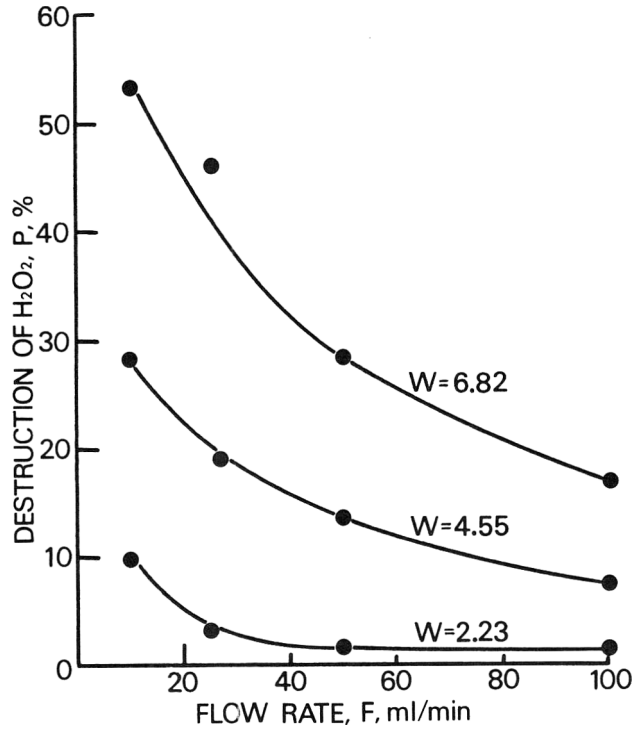

Fig. 1–Effect of flow rate on destruction of H_2O_2 in three catalase-collagen reactors; W = weight of complex, g.

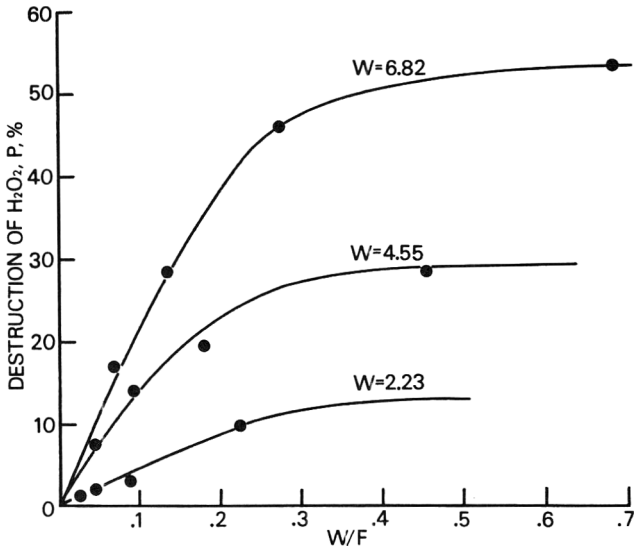

Fig. 2–Effect of space time on the destruction of H_2O_2 in three catalase-collagen reactors; W/F = space time, complex weight–flow rate ratios; W = weight of complex, g; F = flow rate, ml/min.

tial H_2O_2 concentration; S_e is the H_2O_2 concentration at reactor outlet; F is the flow rate in ml/min; and W is the weight of catalase-collagen complex g, packed in the reactor. This figure shows that the percent of H_2O_2 destruction decreases with an increasing flow rate.

In Figure 2, the space time term W/F, weight of complex divided by flow rate, is plotted against destruction, P. At a given W/F the percent of destruction changes in each reactor, which indicates the importance of diffusion resistance in the reactors.

Mass transfer coefficient

The rate of mass transfer from the bulk liquid phase to the surface of the catalase-collagen complex in the plug flow reactor is (Levenspiel, 1972):

$$r = \frac{dS}{d(W/F)} = k_L a(S-S^*) \qquad (1)$$

Table 1–Effect of H_2O_2 concentration on bacteria counts[a]

Bath temp °C	H_2O_2 concentration, %				
	0.01	0.02	0.03	0.04	0.05
	Bacterial reduction, %				
22	58.33	61.64	73.81	89.66	97.20
35	63.49	88.73	94.76	95.23	99.92
45	99.97	99.99	99.99	99.99	99.99
54	99.90	99.90	99.99	99.99	99.99

[a] Reaction time: 30 min; Control count per ml: 168×10^3.

Where r = rate of mass transfer, g mole/min/g complex; k_L = mass transfer coefficient, cm/min; a = external area of collagen complex, cm^2/g of complex; S = H_2O_2 concentration, g mole/ml; and S^* = H_2O_2 concentration at complex surface, g mole/ml. Integral from the inlet of the reactor to the outlet

$$\int_0^W dW = \frac{F}{k_L a}\int_{S_o}^{S_e}\frac{dS}{(S-S^*)} \qquad (2)$$

Assuming that an instantaneous reaction occurred at the complex surface,

$$S_o^* = S_e^* = 0$$

then,

$$k_L a = (F/W)\ln(S_o/S_e) \qquad (3)$$

The relation between the mass transfer coefficient, $k_L a$, calculated from Eq 3, and flow rate, F, is shown in Figure 3 with W/F as parameter. If the Reynold's number is defined as

$$Re = LV\rho/\mu$$

where L is complex length, cm; V is milk velocity, cm/sec; ρ is milk density, g/ml; μ is milk viscosity, g/cm sec, a relation can be derived from Figure 3,

$$k_L a \propto Re^{0.77}$$

In Figure 3 it is shown that an apparent increase in mass transfer coefficient could be obtained merely by increasing the flow rate at a high W/F. The thickness of the stagnant diffusion film around the catalase-collagen complex will be de-

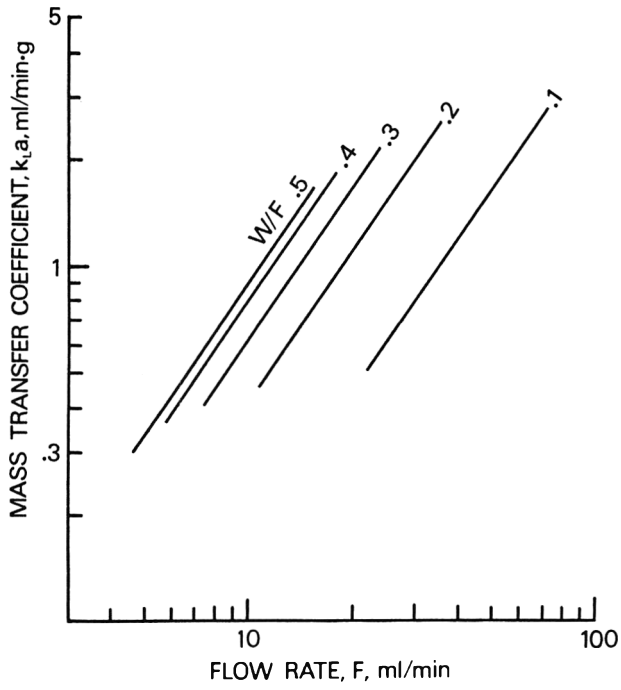

Fig. 3—Mass transfer coefficients vs flow rate in catalase-collagen reactors with various space time; W/F = space time, complex weight—flow rate ratios; W = weight of complex, g; F = flow rate, ml/min.

creased as the Reynold's number is increased. At a low W/F, conversion can be increased by increasing W/F or F. This indicates that it is necessary to consider both space time and flow rate in scaling up a catalase-collagen reactor for the most efficient activity.

The data in Figure 3 further indicate that it is possible to maximize the destruction rate of H_2O_2 as a function of space time and flow rate, and that only minimal improvement in mass transfer coefficient can be obtained by increasing the complex mass per unit volume of reactor at this enzyme load. The destruction rate of H_2O_2 in the reactor can be increased by increasing the enzyme load on the complex, improving the enzyme stability, and by increasing the efficiency of substrate contact by means of improved reactor design. The destruction rate can also be optimized with respect to the temperature and other operational conditions.

The data provide some estimate of the physical design parameters. For example, at 50% destruction of 0.05% H_2O_2, the optimum flow rate is 10 ml/min with 6.82g of complex. This can be extrapolated to a ratio of one volume of substrate flow per two volumes of reactor capacity, a ratio which is not considered to be desirable for dairy plant operation. The data further indicate that an increase in stable enzyme activity of 5 to 10 times that achieved in this investigation is necessary for any industrial use of a catalase reactor. Since there has been achieved at least a fivefold increase in the loading of lactase by improvements in forming complexes, and at least a twofold decrease in the bulk diffusion resistance in pilot reactors, it is hoped that these goals may be met in future work with the catalase reactor.

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STORAGE STABILITY OF DRIED SWEET CHEESE WHEY

INTRODUCTION

DRIED CHEESE WHEY has become an increasingly important product in the United States where production has increased dramatically within recent years. Reasons for increased production include new pollution regulations, improved processing methods, and economic incentives (Groves, 1972).

The value of dried whey as a human food supplement is partially dependent on its storage stability. Dried milk products have been found to be especially susceptible to storage browning which can result in changes in the physical properties of the product and the loss of palatability and nutritive value (Patton, 1955). However, few studies have considered the effect of storage on dried whey. Ferretti and Flanagan (1971a) subjected dried whey to accelerated browning and determined that nonenzymatic browning of whey powder under accelerated conditions produced many of the products which were observed in the lactose-casein model system. In a subsequent study they identified some volatile components of the nonenzymatic browning reaction in dried whey which had been stored for 3 yr at 4°C (Ferretti and Flanagan, 1971b). Mavropoulou (1970) found that whey which had been stored in sealed plastic bags for 2–8 wk at 22°C darkened in color and underwent flavor deterioration. Poor correlation was found between the degree of product lipid oxidation and organoleptic flavor evaluation.

The purpose of this research was to determine the effects of storage on some chemical, physical and functional properties of dried sweet cheese whey.

EXPERIMENTAL

Whey source and storage procedures

Two 50-lb bags of sweet dried cheese whey were obtained from Monticello Dairies, Charlottesville, Va. The whey had been manufactured by Foremost Dairies, Ohio, within the previous 10 days. The whey was prepared for storage by three methods: packaged in heat sealed plastic bags, packaged in glass jars and packaged in 8-in. square pans without a covering. Half of the samples of all packaging methods were stored in a refrigerator at 4°C and half in a nonair-conditioned room (average temperature 19.5°C), between July 15, 1973 and Oct. 16, 1973. The whey was evaluated after 0, 30, 60 and 90 days of storage.

Analysis of whey composition

Whey composition was evaluated on the day on which it was received. Lactose content was determined by the phenol-sulfuric acid method of Dubois et al. (1956). Total crude protein was conducted by the micro-Kjeldahl method using a factor of 6.38 × total nitrogen (AOAC, 1970). Moisture was determined using a Brabender Moisture/Volatiles Tester, Model SAS-692. A 10-g sample was dried at 100°C for 5 hr.

Chemical analysis of stored whey

Sample color was evaluated by a Hunterlab Model D25 Color and Color-Difference Meter. The instrument was standardized against a white tile, standard number 5191, L = 91.8, a_1 = −0.7, b_1 = −0.7. Moisture content was determined using a Brabender Moisture/Volatiles Tester as described above. Protein solubility was determined following the procedure of Morr et al. (1973) using a pH 4.6 buffer. The pH of a 5% whey solution in distilled water was determined using a Coleman Model 12 pH.

Baking properties of whey stored at room temperature

The effect of storage on the baking properties of whey stored at room temperature was evaluated by incorporating the whey in cakes. Plain white cakes were prepared using whey stored at room temperature for 30, 60 and 90 days by each of the three packaging methods. The whey was substituted for nonfat dry milk (ndm) by weight using the following cake formula:

Ingredient	Amount
Fat, hydrogenated vegetable shortening	63g
Sugar	205g
Eggs	101g
Ndm or whey	12.7g
Water	124ml
Baking powder	9.7g
Cake flour	208g
Salt	1.8g
Vanilla	2.7ml

The volume of the cakes was measured by rapeseed displacement.

The cakes were subjectively evaluated by a trained six-member taste panel. The panel members were instructed on the use of the scorecard and were given no information about the variables. Samples were arranged in randomized order and presented by a paired comparison method. Six pairs of samples were presented. The pairs represented all possible combinations of the samples identified in Table 1 (1-2, 1-3, 1-4, 2-2, 2-3, 3-4). Panel members received 1 × 1 × ¼-in. cake slices which included the top crust. Panelists were asked to judge the following characteristics on a relative basis within each pair of samples: crust and crumb color, cell uniformity, velvetiness, moistness, sweetness and preference. Two replications of each pair were completed. Sensory testing was conducted in individual sensory booths located away from the area of cake preparation.

Statistical analysis

Statistical analysis of objective measurements from the study were analyzed by analysis of Variance and Duncan Multiple Range using a IBM/360 computer. Sensory results were tested for significance by Student's t test (n = 12).

RESULTS & DISCUSSION

THE INITIAL analysis of the whey found it to contain 10.3% protein, 78% lactose and 3.5% moisture.

The most apparent visible change in the whey after storage was the browned color of several of the samples stored at room temperature (Fig. 1). All room temperature stored samples significantly discolored after 30 days of storage. The

Table 1—Identification of samples presented to the taste panel

Sample no.	Cake prepared from
1	ndm
2	whey stored open
3	whey stored in bags
4	whey stored in jars

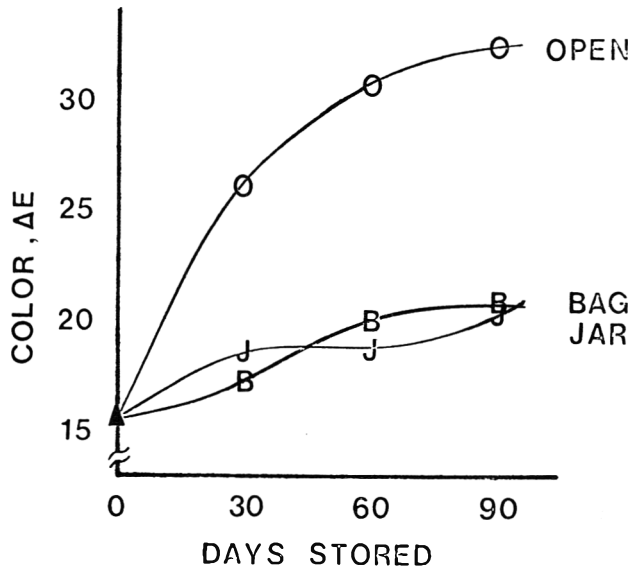

Fig. 1—Hunter ΔE color values of dried whey stored at room temperature.

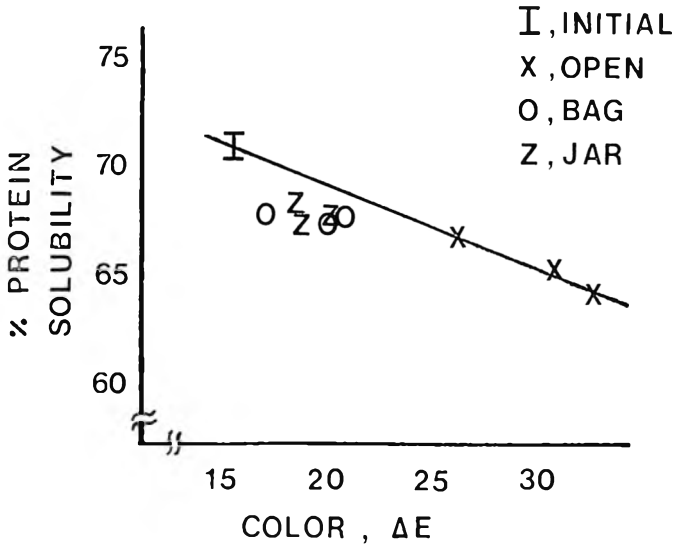

Fig. 2—% protein solubility vs Hunter ΔE color values of dried whey stored at room temperature.

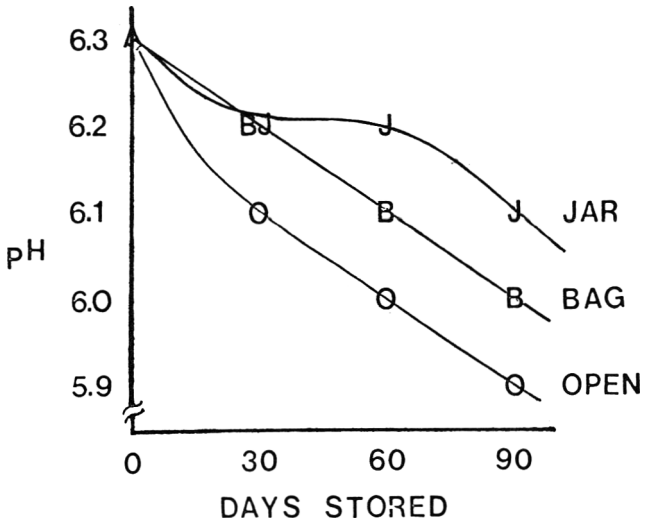

Fig. 3—pH values of dried whey stored at room temperature.

Table 2—Percent moisture content for stored dried cheese whey[a]

Storage location	Packaging method	Length of storage (Days) 30	60	90
			n = 6	
Room	Open	4.9*	4.6*	5.5*
Room	Bag	3.7	3.9	4.7*
Room	Jar	3.6	3.8	4.5*
Refrigerator	Open	16.7*	9.9*	5.6*
Refrigerator	Bag	3.6	3.6	4.4*
Refrigerator	Jar	3.7	3.6	4.2

[a] Initial moisture content = 3.5%
* Significant change from original moisture content ($p < 0.05$) as determined by Analysis of Variance

greater browning in the open-packaged samples than in the bag- and jar-packed samples stored at room temperature was attributed to greater moisture availability for the open-packed whey. Doob et al. (1942) found that the browning of dried whey increased with increased moisture content. Browning was attributed to the Maillard reaction.

The samples stored at room temperature were significantly darker than those stored in the refrigerator at all test periods. The influence of temperature on the rate of browning of dried whey was demonstrated by Doob et al. (1942). The bag- and jar-packed samples stored in a refrigerator showed no significant color change after 90 days of storage. Although the open-packed samples which were stored in a refrigerator had darkened significantly after 30 days of storage, the color lightened with continued storage. It is probable that the changes in ΔE for open-refrigerated samples were caused by changes in the moisture content which affected the light reflectance.

The changes in moisture content are shown in Table 2. The uptake of moisture was mainly attributed to the hygroscopic nature of the lactose. The degree of browning of room-temperature stored samples generally increased with increasing moisture content. Discoloration occurred in samples with moisture levels as low as 3.6%. Since browning occurred at a low moisture level and since lactose is very hygroscopic, low initial moisture levels are considered of little value in the prevention of browning of room temperature stored samples. The moisture content of refrigerated bag- and jar-packed samples increased slightly during storage. No significant browning occurred in these samples even after 90 days of storage, apparently because of the low temperature of storage.

All stored samples, with the exception of refrigerated jar-packed samples stored for 30 days, decreased in protein solubility during storage (Table 3). The greatest change in solubility occurred in open-packed refrigerated samples. There was no correspondence between color change and solubility of the refrigerated open-packed sample. The large solubility decrease was attributed to moisture up-take by the whey components during storage.

The decreased solubility of the open-packaged room temperature stored samples was related to color change (Fig. 2). The insolubility was attributed to the Maillard reaction. Decreased solubility due to the Maillard reaction was reported in storage studies of ndm (Henry et al., 1948; Patton, 1955). It is possible that the bag- and jar-packed samples stored at room temperature underwent some of the earlier stages of the Maillard reaction which caused decreased solubility, but the reaction did not proceed to the pigment forming stages during the storage period.

The pH of room temperature stored samples decreased with the length of storage (Fig. 3). These same samples darkened in

Table 3—Percent protein solubility of stored dried whey[a,b]

Storage location	Packaging method	% Solubility of pH 4.6 Length of storage (Days) 30	60	90
		n = 6		
Room	Open	66.8*	65.3*	64.1*
Room	Bag	67.8*	67.3*	67.7*
Room	Jar	68.2*	67.2*	67.8*
Refrigerator	Open	65.3*	66.6*	61.7*
Refrigerator	Bag	67.6*	66.5*	68.0*
Refrigerator	Jar	70.1	66.2*	68.2*

[a] Expressed on a dry weight basis
[b] Initial protein solubility = 70.9%
* Significant change from initial value ($p < 0.05$) as determined by Analysis of Variance

Table 4—Volume of cakes made from stored whey and ndm[a]

Cake	Length of storage (Days) 30	60	90
	Volume (cm^3) n = 6		
ndm	850a	925a	925a
Open whey	813a	863c	863b
Bag whey	837a	900a,b	875b
Jar whey	825a	888b,c	913a

[a] Values in vertical columns with different letters are significantly different ($p < 0.05$)

Table 5—Panel evaluation of crumb color of ndm and whey cakes[a,b]

Length of whey storage	Cake			
0	ndm	Whey		
30 and 60	ndm	Jar	Bag	Open
90	ndm	Jar	Bag	Open

[a] n = 12
[b] Continuous line indicates no significance at $p < 0.05$.

Table 6—Taste panel evaluation of cake tenderness[a,b]

Length of whey storage	Cake variation			
0	ndm	Whey		
30 and 60	ndm	Jar	Bag	Open
90	ndm	Jar	Bag	Open

[a] n = 12
[b] Continuous line indicates no significance at $p < 0.05$.

Table 7—Taste panel results of cake preference[a,b]

Days	Taste preference			
0	ndm	Whey		
30 and 60	ndm	Jar	Bag	Open
90	ndm	Jar	Bag	Open

[a] n = 12
[b] Continuous line indicates no significant difference at $p < 0.05$.

color during storage. The pH change was attributed to the bonding of amino groups by the lactose in the Maillard reaction. Coulter et al. (1951) suggested amino group binding as the cause of pH decrease during the storage of ndm.

Refrigerated samples showed little pH decrease during storage. The method of storage packaging had no effect on the pH of the samples.

The volume of cakes made from stored whey increased with the length of storage and degree of browning (Table 4).

Panel members could find no difference between the ndm and whey cakes initially. After storage the whey cakes had darker crust and crumb than the ndm cakes (Table 5). The cake darkening paralleled the browning which took place in the whey; darkest crumb and crust color occurred in open-packaged whey at all test periods.

The cakes made from whey stored for 90 days were judged by the panel to be less tender than ndm cakes (Table 6). The decreased tenderness was attributed to a reduced tenderizing effect of the lactose due to a lowered ability to pick up moisture. Lactose was reported to be a tenderizing agent in baked products because of its ability to compete with gluten for water (Webb and Whittier, 1970).

Table 7 shows the sensory results of cake preference evaluation. The taste deterioration of cakes made from stored whey was attributed to two effects. First, off flavors may have been picked up by the whey during storage. Reger (1958) noted the ability of lactose to carry flavors. A second cause of lower preference ratings was attributed to the Maillard reaction. preference of cakes made from stored whey decreased with increased cake discoloration. Off flavors due to Maillard reaction products have been characterized as 'caramelized' or 'burnt-sugar' in flavor (Henry et al., 1948).

Under conditions of this study, the results indicated that browning and associated changes occurred during the storage of dried sweet whey. These changes resulted in decreased functionality of the product. The results of this study indicated that storage deterioration may be limited by moisture-resistant packaging, low storage temperature and short length of storage.

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RESEARCH NOTES

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A Research Note

A REFLECTANCE METHOD FOR THE ENUMERATION OF SURFACE BACTERIA

INTRODUCTION

THE RECOVERY of microorganisms has been studied for many years. Seven basic, nondestructive surface sampling methods have evolved. They are:

1. Swab contact (APHA, 1960)
2. Plate contact (APHA, 1960)
3. Rinse (APHA, 1948; Winter et al., 1971)
4. Replicate-transfer (Tresner and Hayes, 1970; Tengerdy et al., 1967)
5. Agar Overlay (Hughes et al., 1968)
6. Agar dip
7. Vacuum probe (Whitfield et al., 1969)

At the same time, bacterial population estimation methods have increased from manual dilution, plating and counting to:

1. Automated dilution, plating and counting (Sharpe et al., 1972a, b; Schoon et al., 1970)
2. Membrane filter–Plate count
3. Membrane filter–Fluorescence microscopy
4. Photoelectric measurement of turbidity (Fujita and Nunomura, 1968)
5. Measurement of light scattering (Merek, 1969)
6. Radiometry (^{14}C glucose → $^{14}CO_2$ metabolism) (Previte, 1972)

In comparing relative merits of combinations of the above sampling/estimation methods, varying degrees of accuracy can be traded for varying amounts of analysis time, equipment investment and skilled personnel labor.

Current interest in extensive microbiological sampling programs dictates the need for a rapid, inexpensive method for determining surface bacterial populations.

The reflectance method has been developed and used for estimating viable bacterial contamination on surfaces. With this method, contact plates are used to remove microorganisms from surfaces. After incubation, the loss of reflectance of the medium is related back to numbers of bacteria. The main advantages of this method are:

1. Useful for testing heavily contaminated surfaces such as carcasses, floors, etc.
2. Minimal investment in microbiological equipment.
3. No need for skilled personnel to perform tests.
4. Minimal labor time per test.

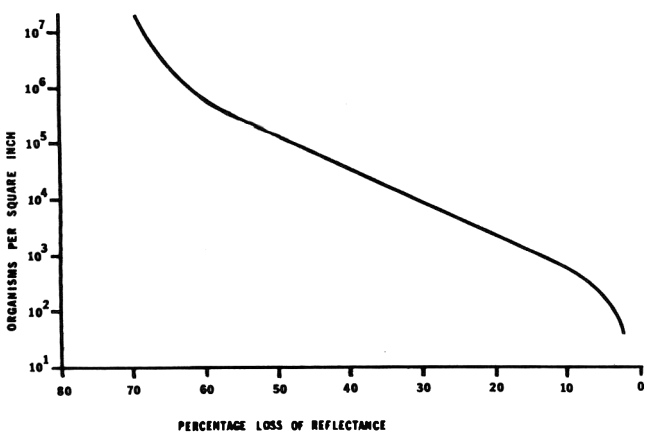

Fig. 1–Standard curve for determining bacterial levels.

MATERIALS & METHODS

Physical description

Rodac (Falcon) plates pre-poured with reflectance medium (could be prepared at a central location or purchased from a supplier)

Brain Heart Infusion	37g
Agar	15g
Titanium Dioxide	10g
TTC*	10.0 ml
H_2O	1000 ml

*1.0% filter sterilized solution of 2,3,5-Triphenyl-tetrazolium chloride

All ingredients except the TTC are mixed, dissolved and autoclaved. After cooling to 50°C, the TTC is added and 17 cc of media are dispensed per Rodac plate. The media must be agitated during dispensing to keep the TiO_2 suspended. The plates should then be covered and left at room temperature overnight to dry. Use of titanium dioxide insures an opaque medium, and provides a uniform white color. TiO_2 is inert and nontoxic.

The colorless 2,3,5-triphenyl-tetrazolium chloride is reduced to a red color triphenyl-formazone by reductases present in bacteria. This

reaction is entirely nonspecific, eliminating differences due to species of bacteria (Tengerdy et al., 1967).

Incubator. Any laboratory incubator capable of maintaining 30° ± 1°C is appropriate. Due to the small size of the Rodac plates, many plates may be incubated at once in a relatively small incubator.

Reflectometer. Any reflectometer capable of measuring whiteness and reflectance should be suitable. The one available for use in our laboratory was a Hunterlab Model D40 (Hunter, 1960).

Each Rodac plate is numbered and has its initial reflectance measured. If the plates have been carefully prepared, a standard initial reflectance may be determined for each lot of plates. This initial reflectance will approach 100% due to the whiteness of the TiO_2 and the opacity of the medium. The plates are then contacted with the surface to be tested. After approximately 48 hr of incubation at 30°C, the reflectance of each plate is again measured. Every bacterial colony on the plate will be colored red, thus lowering the reflectance (whiteness) of the medium. The difference in reflectance can be related back to organisms per square inch by using the standard curve.

Two methods were used for determining the standard curve shown in Figure 1. Initially, sterile stainless steel plates were inoculated with known numbers of pure and mixed bacteria cultures. The inoculated plates were allowed to dry and were then tested using the reflectance method. Organisms used were *E. coli* and *S. faecalis* in dilutions from 100 to 10^8 (100,000,000) organisms per square inch. The second method consisted of pipetting known dilutions of bacteria onto the media surfaces, spreading with sterile glass "hockey sticks," incubating and reading. The standard curve shown in the figure was derived using these methods.

RESULTS & DISCUSSION

COMPARISONS of the reflectance method with standard swab techniques on surfaces at the laboratory level show similar results. Work to date has shown no problems with spreading or clumping colonies. Tests at the plant level are currently underway. There is indication that other standard curves would need to be evolved in order to use the reflectance method on items other than equipment, such as food, water, or air.

The primary advantage of the method is the ease of enumerating higher levels of bacteria which would involve many dilutions in the swab techniques, or would be impossible to count on the standard Rodac plate. Additional advantages are minimal investment on equipment and labor.

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A Research Note

EFFECT OF PRECOOKING ON COPPER CONTENT, PHENOLIC CONTENT AND BLUEING OF CANNED DUNGENESS CRAB MEAT

INTRODUCTION

SEVERAL INVESTIGATIONS have sought to determine the cause of blueing of canned crab meat since periodic outbreaks of blueing are a serious quality control problem. The blueing discoloration, ranging from blue-gray to black, predominates on the surface of the meat and in the coagulated blood that is released from the meat. The blueing has been observed in king crab (Groninger and Dassow, 1964), blue crab (Waters, 1971), queen crab (Dewberry, 1970), and Dungeness crab (Elliott and Harvey, 1951).

It is well documented that copper (Elliott and Harvey, 1951; Inoue and Motohiro, 1970) and iron (Waters, 1971) are involved in the blueing of canned crab meat. Several studies have demonstrated a relationship between the presence of crab blood and the occurrence of blueing in canned crab meat. These studies have led several investigators to suggest procedures for the reduction of residual blood prior to or during processing and the addition of citric acid to the canned crab meat (Elliott and Harvey, 1951; Farber, 1953). To prevent the inclusion of copper-bound hemocyanin of the crab blood with crab meat, Osakabe (1957) proposed precooking the crab at 60°C prior to cooking. Thus, the crab meat is coagulated while the hemocyanin can be washed free of the meat preventing graying in canned Kegani crab.

The copper content of "blue" crab meat has been found to be much higher than "white" crab meat (Babbitt et al., 1973b; Inoue and Motohiro, 1970). However, the role of copper may be that of a catalyst since it can be freed from the blue-pigment by dialysis against deionized water (Babbitt et al., 1973b) and EDTA (Inoue and Motohiro, 1971). The blueing of canned Dungeness crab meat may be related to the presence of polyphenoloxidases in crab (Babbitt et al., 1973a) and the subsequent oxidation and polymerization of phenols to colored melanins particularly in the presence of metals (Cu and Fe) and under alkaline conditions (Babbitt et al., 1973b).

The purpose of this investigation was to investigate the effect of precooking Dungeness crab on the copper content, phenolic content and blueing of canned crab meat.

EXPERIMENTAL

Analytical methods

Procedures used in the determination of total phenolic content, pH and copper have been described previously (Babbitt et al., 1973b).

Handling and processing of crab

Live crab in good condition were purchased from local processors, covered with wet towels and held at 1–3°C. At 0, 2 and 4 days, 60 crab were backed, cleaned and split in sections. The hind legs of only 15 crab (right section), including the body meat, were removed for raw tissue analysis. After precooking 60 sections (30 left and 30 right) at 60.0° ± 0.5°C for 20 min, the precooked sections were washed well with a spray of cold water. Again, the hind legs of only 15 crab (right section), including the body meat, were removed for sampling. All the precooked sections were then cooked in a similar manner as raw crab sections in boiling water for 12 min. After cooking, the crab sections were cooled with a spray of cold water and sampled as mentioned above. The cooked sections were picked by hand and thoroughly mixed. 142g of the picked meat were packed with 40 ml of 2.5% brine (NaCl) containing: (1) no additive; (2) $CuCl_2$ (20 μg Cu^{+2}/ml); or (3) citric acid (4.5 mg/ml) in 307 × 113 C-enamel cans, sealed in a vacuum and retorted for 55 min at 116°C (10 psi). After water cooling, the cans were held at 30°C. The cans of crab meat were opened and examined after 3 wk.

RESULTS & DISCUSSION

THE COPPER CONTENT in the raw crab meat increased during refrigerated storage (Table 1). The increase in copper content, calculated on a dry weight basis, cannot be readily explained. Perhaps copper was being freed from the shell and carapace or the alteration of cellular tissue as monitored by the formation of phenolic compounds resulted in a drastic change in the solids composition of the muscle. Precooking the crab sections reduced copper and phenolic levels, but was less effective after 2 and 4 days of refrigerated storage.

As reported earlier (Osakabe, 1957; Babbitt et al., 1973b) precooking prior to cooking the crab sections improved the over-all quality of the canned crab meat, but only when the crab were freshly caught and in good condition (Table 2). No

Table 1—Copper content[a] and phenolic content[b] of raw, precooked and cooked crab meat

	Days held at 1–3°C					
	0		2		4	
Treatment	Copper	Phenol	Copper	Phenol	Copper	Phenol
Raw tissue	21.50 ± 0.26	203.8 ± 11.2	23.26 ± 0.28	340.5 ± 8.3	29.06 ± 2.09	389.6 ± 9.4
Precooked	9.66 ± 1.29	190.1 ± 14.1	12.04 ± 0.01	267.7 ± 7.7	21.97 ± 0.14	295.3 ± 6.9
Precook-cooked	14.21 ± 1.99	159.8 ± 4.4	14.59 ± 0.33	185.5 ± 3.1	17.42 ± 0.87	219.7 ± 2.0
Cooked	20.20 ± 0.06	176.2 ± 2.6	23.91 ± 1.61	194.3 ± 36.3	28.57 ± 0.34	262.7 ± 7.8

[a] μg Cu/g sample (dry wt); mean of duplicate samples for two determinations
[b] μg Phenol/g sample; mean of duplicate samples for two determinations

Table 2—Effect of precooking on extent of blueing, pH and quality of canned crab meat[a]

Days	Treatment	pH	Visual evaluation	
			Blueing	Quality
		Cooked		
0	No additive	7.10	Slight	Good
	+ Copper	7.00	Slight	Good
	+ Citric acid	6.70	Very slight	Good
2	No additive	7.20	Slight	Good
	+ Copper	7.00	Moderate-gray tint	Fair
	+ Citric acid	6.75	Very slight	Fair
4	No additive	7.40	Slight-moderate	Fair
	+ Copper	7.20	Moderate-extreme	Poor
	+ Citric acid	6.80	Very slight	Poor
		Precooked, then cooked		
0	No additive	7.40	Very slight	Very good
	+ Copper	7.10	Slight	Very good
	+ Citric acid	6.70	Very slight	Very good
2	No additive	7.60	Slight	Good
	+ Copper	7.30	Slight-moderate	Fair
	+ Citric acid	6.70	Slight	Fair
4	No additive	7.70	Slight-moderate	Poor
	+ Copper	7.40	Moderate-extreme	Poor
	+ Citric acid	6.80	Slight-moderate	Poor

[a] Average of two cans per treatment

difference in blueing was observed in the cooked or precook-cooked canned crab samples when the crab were processed immediately. Holding the crab under refrigeration for 2 and 4 days progressively resulted in poor canned crab meat. Citric acid partially reduced blueing but caused the canned crab meat to become very chalky particularly when the crab were held 4 days before processing. This was especially true when the crab sections were precooked prior to cooking. When copper was added to canned crab meat from freshly processed crab no blueing was observed in the cooked or precook-cooked samples. However, when the crab were held for 2 or 4 days before processing the added copper resulted in blueing in the canned samples regardless of whether the crab were cooked or precook-cooked. The phenolic content of cooked and precook-cooked crab meat was related to the blueing observed in the canned crab meat. The copper added to the canned crab meat may increase blueing by catalyzing or forming complexes with the phenolic derivatives, particularly under alkaline pH's (Mathew and Parpia, 1971).

The results indicate that precooking can greatly reduce the copper content and only slightly reduce the phenolic content of crab meat. Precooking followed by the addition of citric acid to the canned crab meat will only help prevent blueing when the crab are in good condition and handled promptly after harvesting.

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A Research Note

A STUDY OF THE RATE-LIMITING FACTORS IN THE RESPIRATORY OXYGEN CONSUMPTION OF INTACT POST-RIGOR BOVINE MUSCLE

INTRODUCTION

OXYGEN UPTAKE by intact post-rigor muscle has been measured by Brooks (1929), Urbin and Wilson (1961), Bendall and Taylor (1972) and DeVore and Solberg (1974). In each of these investigations, the rate of oxygen consumption decreased during the measurement period. Urbin and Wilson (1961) attributed the falling off of oxygen uptake to diffusional limitations. Bendall and Taylor (1972) reported that mitochondrial respiration was the main element determining post-rigor oxygen consumption, and that the decline of oxygen consumption rate occurred as a result of a deterioration of mitochondrial structure. Other investigations have implicated enzyme degradation (Grant, 1955) and substrate depletions (Atkinson et al., 1969) as the factors limiting post-rigor oxygen uptake.

DeVore and Solberg (1974) studied the relationship between respiratory oxygen consumption and total oxygen uptake in post-rigor bovine muscle. The rate of nonrespiratory oxygen uptake remained constant up to 30 hr. The respiratory oxygen consumption rate, however, decreased exponentially during the 30-hr exposure time. The rate decline appeared to be partially related to a reduction in respiratory enzyme activity. Substrate depletions were also indicated.

In the present investigation, studies were initiated to determine the relationship between the decline in oxygen consumption in post-rigor muscle and coincident changes in cytochrome c reductase activity and tissue NADH concentration.

EXPERIMENTAL

OXYGEN UPTAKE by post-rigor bovine semimembranosus muscle was measured manometrically using a differential respirometer (Gilson Model GR-20, Gilson Medical Electronics, Middleton, Wisc.). Experiments were conducted at 5°C and at a constant oxygen headspace pressure of one atmosphere. Fresh top round of beef from 5–7 days postmortem was obtained locally. Muscle slices from the excised semimembranosus muscle were prepared aseptically in a nitrogen atmosphere, as described previously (DeVore and Solberg, 1974). Sample discs, 13-mm thick and with a surface area of 12.5 cm^2, were prepared aseptically and placed and sealed in specially fabricated glass sample cells measuring 15-mm high and 4.0 cm in diameter (Belco Glass Co., Vineland, N.J.). The samples were temperature-equilibrated for 30 min (5°C) on the differential respirometer. During this time, the headspace system, exclusive of the sample cell, was flushed with oxygen. Measurements of oxygen uptake were then initiated. All times reported in the study are based on a zero time at this point. Oxygen uptake was recorded at hourly intervals. Duplicate samples of muscle discs were removed sequentially from the respirometer at hourly intervals and analyzed for cytochrome c reductase activity and NADH concentration.

Succinate/NADH cytochrome c reductase activity was measured spectrophotometrically using a modification of the method described by Tappel (1960) and Kaniuga et al. (1968). Two reaction systems were used: one containing tissue homogenate alone as the substrate source (endogenous substrate) and the other containing succinate as an additional substrate source (exogenous substrate). Tissue homogenates were prepared by adding finely minced muscle to three times the sample weight of cold 0.9% KCl. Enzyme activity was assayed spectrophotometrically by measuring the absorbance increase at 550 nm. The pro-

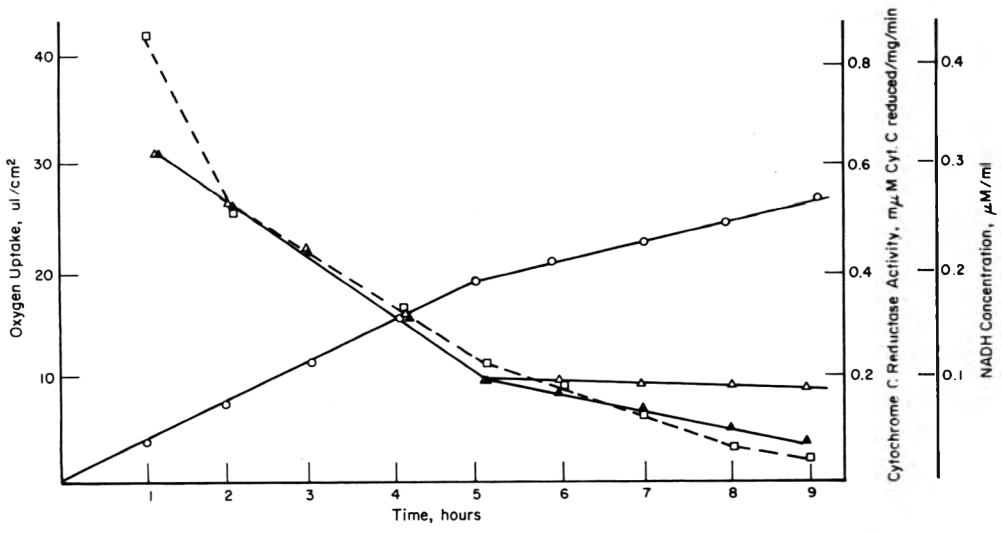

Fig. 1—The reduction of tissue cytochrome c reductase activity in tissue homogenates containing added succinate (△-△-△) and in tissue homogenates alone (▲-▲-▲) and the reduction of tissue NADH concentrations (□-□-□) during the time course of oxyten uptake (○-○-○) in post-rigor bovine semimembranosus muscle at 5°C and one atmosphere of oxygen pressure.

tein content of the dilute tissue homogenate was determined by the Biuret reaction.

NADH concentration was determined using the enzymatic method described by Klingenberg (1965). Absorbance was measured at 340 nm.

Three sets of experiments were conducted with duplicate samples utilized for each sampling period. Each point on the curves showing changes in cytochrome c reductase activity and NADH concentration represents the average of six samples. On the curves showing oxygen uptake, the 1-hr point represents the average of 54 samples, the 2-hr point, 48 samples, and so forth to the 9-hr point, which represents the average of six samples.

RESULTS & DISCUSSION

FIGURE 1 shows the time course relationship between oxygen uptake and cytochrome c reductase activity and NADH concentration in postmortem bovine semimembranosus muscle at 5°C and one atmosphere of oxygen pressure. Cytochrome c reductase activity with added succinate dropped rapidly for 5 hr before leveling off at approximately 0.2 mμ moles cytochrome c reduced/mg protein/minute. If exogenous succinate were not added, the enzyme activity continued to decrease with time. The rate of oxygen uptake remained constant for 5 hr and then decreased to approximately 50% for the period from 5–9 hr. The concentration of NADH dropped rapidly during the initial period of oxygen uptake and continued to decrease to nearly zero after 9 hr.

The decline in oxygen consumption rate appeared to be a function of respiratory enzyme activity and substrate availability. Both tissue NADH concentration and in situ cytochrome c reductase activity decreased during the decay of the oxygen consumption rate. A reduction in the activity of cytochrome c reductase indicates that the functionality of the electron transport system (ETS) is impaired. The correlatable changes in enzyme activity and oxygen consumption rate also indicate that the rate of oxygen consumption is directly related to respiratory enzyme activity.

Adding exogenous succinate to the reaction mixture resulted in a significant increase in cytochrome c reductase activity (Fig. 1). This suggests that the reduced enzyme activity was somewhat limited by substrate availability.

NADH production in pre-rigor tissue is primarily a function of the mitochondrial NAD+ linked oxidation of pyruvate. Cheah and Cheah (1971) reported that after 6 days of postmortem storage, this reaction was reduced to 30% of the pre-rigor value. Thus, the depletion of tissue NADH content, in part, occurs due to a reduction in the NAD+ linked oxidation of pyruvate. This reaction, furthermore, may be limited by other enzyme deterioration or depletion in pyruvate.

While both substrate depletion and enzyme deterioration were indicated from these studies, the specific factor or factors limiting the rate of respiratory oxygen consumption were not fully elucidated, and it might be realistic to attribute the observed rate decay to a general phenomenon, such as irreversible impairment of mitochondrial function as suggested by Bendall and Taylor (1972). However, the stability of the mitochondrial structure during postmortem storage has been documented by Cheah and Cheah (1971) as long as appropriate pH requirements are maintained. It appears that additional knowledge might be gained from experiments that would directly correlate the ultrastructure and respiratory function of isolated mitochondria concurrently with the observed decay in respiratory oxygen consumption.

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