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ABSTRACTS:

IN THIS ISSUE

WATER IN BIOSYSTEMS. E. KARMAS. *J. Food Sci.* **38,** 736–739 (1973)–It has been widely accepted that the structural properties of water can serve as a key to understanding and explanation of biological phenomena and processes. A qualitative account in the form of a literature review is given on biological significance of water, biological activity as a function of temperature, the structure of water, solute effect on water structure and the state of water in biological systems. The functional role of water in biosystems is discussed.

PROTEIN STRUCTURE AND STABILITY: CONVENTIONAL WISDOM AND NEW PERSPECTIVES. D.B. WETLAUFER. *J. Food Sci.* **38,** 740–743 (1973)–The conventional wisdom on protein structure and stability is reviewed. With occasional exceptions, the conventional wisdom works quite well. However, the thermodynamic basis of the conventional wisdom fails to qualify it for addressing time-related questions such as: "How rapidly can three-dimensional structures form, and how long can specific native structures persist in vivo, and under practical in vitro conditions?" A more inclusive, kinetic perspective is required for addressing such questions. Experimental approaches and some practical consequences of the broader kinetic perspective are outlined.

SOME RECENT IDEAS ABOUT THE NATURE OF THE INTERACTIONS BETWEEN PROTEINS AND LIQUID WATER. R. LUMRY. *J. Food Sci.* **38,** 744–755 (1973)–The phenomenological pattern of thermodynamic behavior called "linear enthalpy-entropy compensation" which has been found to characterize many small-solute processes in water solution has been found with protein reactions and provides an experimental pathway for studying the role of water in determining the chemical, physical and specific functional properties of proteins and other biological macromolecules. The pattern is closely related to the source of inhibitor, pH and species specificity in protein reactions. Attempts to determine whether the source is water, the protein or some combination using experimental values for structural parameters of protein suggest that proteins have unique and remarkable properties which must be understood before direct questions about water participation can be studied with any promise of success.

PROTEIN-LIPID INTERACTIONS. M. KAREL. *J. Food Sci.* **38,** 756–763 (1973)–A number of significant processes in food and biological systems involve interactions between proteins and lipids. Mechanisms by which proteins and lipids interact, the nature of the forces involved and factors which affect the interactions are reviewed. Examples of particularly important interactions in food and biosystems are presented, including biological membrane interactions, protein-oxidizing lipid interactions and coalescence and inversion of emulsions.

A STANDARDIZED REVERSED PASSIVE HEMAGGLUTINATION TECHNIQUE FOR THE DETERMINATION OF BOTULINUM TOXIN. G.M. EVANCHO, D.H. ASHTON, E.J. BRISKEY & E.J. SCHANTZ. *J. Food Sci.* **38,** 764–767 (1973)–The use of reversed passive hemagglutination (RPHA) for the rapid detection of *Clostridium botulinum* type A toxin in foods and culture filtrates was investigated. Detection of 0.0008 μg of toxin per ml of sample (27 mouse LD_{50} per ml) was possible with cells sensitized with antitoxin globulin. Hemagglutination results compared well with those obtained by mouse assay but were available within 1.5 to 3 hr. No cross-reactions were observed between the A antiglobulin sensitized cells and the other botulinum toxin types. Titers were identical using either crystalline toxin or toxoid prepared from the crystalline toxin, making standardization of the test possible, so that results from different laboratories can be made comparable.

CONTRIBUTION OF ADSORPTION TO VOLATILE RETENTION IN A FREEZE-DRIED FOOD MODEL CONTAINING PVP. J. CHIRIFE & M. KAREL. *J. Food Sci.* **38,** 768–771 (1973)–Retention of ^{14}C-labeled n-propanol was studied in a freeze-dried system containing polyvinylpyrrolidone (PVP). The major fraction of n-propanol retained after freeze drying of aqueous solutions of PVP and propanol is held by entrapment in microregions. Some n-propanol can also be entrapped by the polymeric aggregates of PVP after sorption of the alcohol from the vapor phase, even in the absence of plasticizing action of water, apparently because of the ability of propanol to act as solvent for PVP. In addition to entrapment, there is a small but significant contribution of adsorption to n-propanol retention in freeze drying which could not be determined exactly. However, dry layer adsorption experiments on rapidly frozen PVP show readsorption amounting to approximately 10% of the total retention.

PEA LIPIDS AND THEIR OXIDATION ON CARBOHYDRATE AND PROTEIN MATRICES. M. HAYDAR & D. HADZIYEV. *J. Food Sci.* **38,** 772–778 (1973)–The total lipids of pea seeds were isolated and fractionated by column and two dimensional thin-layer chromatography, and their fatty acid composition determined. The polar lipid fraction revealed up to 10 individual components consisting of phospho-, glyco- and sterol-lipids. The neutral lipid fraction consisted mainly of triglycerides, small amounts of diglycerides, free fatty acids and esterified sterols. Oxidation of lipids coated on pea carbohydrate and protein matrices depended on both the matrix used and the polar or neutral lipid classes being oxidized. The neutral lipids coated on cellulose were oxidized at a higher rate than lipids on cellulose precoated with amylose, amylopectin or pectin. The pectin matrix induced a 26% retardation. The amylopectin matrix induced the highest retardation of 36%. The oxidation rate of polar lipids was four times higher than that of neutral lipids. All carbohydrate precoats promoted oxidation relative to cellulose, with pectin exerting the greatest effect. The effect of pea albumins and globulins was low in promoting oxidation of neutral lipids but high for polar lipids. In the latter case, globulins were exerting a rate of oxidation 2.8 times higher than that of albumins after 20 hr, while after 40 hr the results were not comparable by direct manometric readings. From these results it was concluded that lipid polarity rather than degree of unsaturation has the primary influence on lipid oxidation.

CALCIUM ACTIVATION OF SOYBEAN LIPOXYGENASE. F. RESTREPO, H.E. SNYDER & G.L. ZIMMERMAN. *J. Food Sci.* **38,** 779–782 (1973)–Lipoxygenase is known to be activated by Ca^{2+} but only under special circumstances. This study confirms that Ca^{2+} activation depends on the addition of Ca^{2+} to the reaction mixture before or concurrently with enzyme. We have found that two isozymes of lipoxygenase differ in their response to added Ca^{2+}, lipoxygenase 1 is inhibited while lipoxygenase 2 is activated. Also, Ca^{2+} activation of soybean extracts is eliminated by a procedure for phytate removal, but addition of phytate does not restore the activation. A study of the Ca^{2+}, lipoxygenase and linoleic acid concentrations on lipoxygenase activity showed that maximum Ca^{2+} activation varies with linoleic acid concentration but not with lipoxygenase concentration. The mechanism by which Ca^{2+} activates lipoxygenase is still obscure.

ABSTRACTS:
IN THIS ISSUE

INFLUENCE OF INGREDIENTS UPON EDIBLE PROTEIN–LIPID FILM CHARACTERISTICS. L.C. WU & R.P. BATES. *J. Food Sci.* 38, 783–787 (1973)–Pure and mixed systems of aqueous slurries from soybean, peanut, cottonseed and milk have been used as substrates for protein-lipid film formation. Film strength, yield, formation rate and protein incorporation efficiency indicated that soymilk, soy protein isolate or cow's milk formed ideal films. Full fat peanut and cottonseed milks required upward adjustment of the protein-lipid ratios for optimal film strength and quality. Whey protein concentrate demonstrated excellent film-forming ability whereas cottage cheese whey did not. Protein-lipid film formation represents a practical technique for both partially concentrating and texturizing protein from dilute solutions while controlling lipid composition and nutritive value.

FORMATION OF A POTATO CHIP-LIKE FLAVOR FROM METHIONINE UNDER DEEP-FAT FRYING CONDITIONS. S-C. LEE, B.R. REDDY & S.S. CHANG. *J. Food Sci.* 38, 788–790 (1973)–A model system was developed for treating the various components of potatoes under deep-fat frying conditions. The system involved the deep-fat frying of cotton balls moistened with an aqueous solution of either amino acids or sugars or their combinations. When methionine was treated under deep-fat frying conditions, its reaction products imparted an odor and flavor reminiscent of that of potato chips to the oil. By observing the aroma generated by different homologs and analogs of methionine under deep-fat frying conditions, it was observed that a certain chemical structure is necessary for the production of the potato chip-like flavor.

DISTRIBUTION OF DIELDRIN IN MILK FRACTIONS. C.Y.W. ANG & L.R. DUGAN JR. *J. Food Sci.* 38, 791–795 (1973)–Dieldrin was found to be distributed in a similar pattern in milk containing added pesticide and milk containing physiologically incorporated pesticide. The relative amounts of dieldrin in various fractions closely resemble the relative amounts of total lipids in these fractions. Comparable levels of residue, on a fat basis, were found in whole milk, skim milk, cream, washed cream, butter and butteroil. Lower levels were observed in buttermilk and butter serum, and lowest values were found in refined buttermilk, refined butter serum and fat globule membrane pellets. An inverse relationship between the dieldrin concentration and the level of phospholipid and/or high-melting glyceride content apparently exists. The slightly higher concentrations of dieldrin, on a fat basis, observed in refined skim milk might be due to slight solubility of this pesticide in the milk serum. This study suggests that dieldrin has a tendency to be distributed more favorably with the neutral or free lipids in milk regardless of whether it is the original deposition or is in an altered orientation as a consequence of separation.

ACID PRODUCTION BY Streptococcus lactis IN LOW-LACTOSE SKIM MILK. R.L. RICHTER, G.A. REINECCIUS & L.L. McKAY. *J. Food Sci.* 38, 796–798 (1973)–Limiting lactose content of milk was examined as a means of controlling dairy fermentations. Low-lactose milk (< 0.2%) was prepared from 11% reconstituted nonfat dry milk by cycling it several times through Sephadex G-25 in a basket centrifuge. Acid production by lactic streptococci was restricted in the low-lactose milk, and supplementation with lactose did not restore normal acid production. Supplementation with yeast extract, metals and lactose was required to regain acid production comparable to the untreated milk. Corresponding increases in acid production were observed when increasing amounts of lactose were added to treated milk supplemented with yeast extract and metals.

POLYPHENOL OXIDASE OF ROYAL ANN CHERRIES: PURIFICATION AND CHARACTERIZATION. N.D. BENJAMIN & M.W. MONTGOMERY. *J. Food Sci.* 38, 799–806 (1973)–Polyphenol oxidase (*o*-diphenol: O_2 oxidoreductase, EC 1.10.3.1) was purified from freeze-dried Royal Ann cherries by extraction with polyethylene glycol and acetone. The acetone powder was extracted with 0.05M acetate (pH 5.6) and the polyphenol oxidase was precipitated with acetone. Chromatography on DEAE-cellulose and Sephadex G-100 partially separated the acetone precipitate into two and three fractions, respectively. This suggests that the polyphenol oxidases had different molecular sizes. The two fractions from DEAE-cellulose had similar sensitivities to inhibitors, pH optima, V_{max} and K_M, but differed in substrate specificity and heat stability. Polyacrylamide-gel electrophoresis separated the polyphenol oxidases into three groups containing five, three and one components. All three groups possessed similar substrate and inhibitor specificities. The polyphenol oxidase system of Royal Ann cherries appears to be composed of three enzymes. Two of these enzymes have isozymes.

NONVOLATILE ACIDS OF STRAWBERRIES. W.A. SISTRUNK & J.N. CASH. *J. Food Sci.* 38, 807–809 (1973)–A study was initiated to determine nonvolatile acids in different maturities and varieties of strawberries. The only major difference between the two maturities (firm-ripe and full-ripe of the four varieties (Surecrop, Earlibelle, Sunrise and SIUS 253) was the higher total acidity in greener fruit. Malic and citric were responsible for most of the difference. Concludes that (1) the nonvolatile acids in these varieties did not vary qualitatively but there were quantitative differences between varieties and maturities; and (2) it is possible the ratio of citric to malic acid in different genetic lines and maturities could affect color.

ANTHOCYANINS OF ROSELLE (Hibiscus sabdariffa, L.). C.T. DU & F.J. FRANCIS. *J. Food Sci.* 38, 810–812 (1973)–The anthocyanin pigments of Roselle were extracted with acidic methanol and purified by conventional chromatography. The major pigment which is primarily responsible for the reddish-violet color of Roselle was identified as delphinidin-3-sambubioside. Cyanidin-3-sambubioside was found to be the pigment present in second largest concentration. Two minor pigments, delphinidin-3-glucoside and cyanidin-3-glucoside, were also identified. Several other trace pigments were present in Roselle in too low a concentration for identification. Total pigment was approximately 1.5g/100g dry weight expressed as delphinidin-3-glucoside.

OXIDATIVE CHANGES IN OXYMYOGLOBIN DURING INTERACTION WITH ARGININE LINOLEATE. C. KOIZUMI, J. NONAKA & W.D. BROWN. *J. Food Sci.* 38, 813–815 (1973)–Interaction of MbO_2 with linoleate was studied with regard to the oxidative changes of MbO_2. When MbO_2 was added to give a final concentration of 0.07 mM in a buffer solution of linoleate of concentration higher than 1.32 mM at pH 6.28, the MbO_2 was rapidly oxidized to the ferric form, accompanied by some degradation of the heme moiety of Mb. Accompanying the oxidative changes of MbO_2, the linoleate also underwent catalytic oxidation. Addition of 0.2% serum albumin to the reaction system effectively inhibited both rapid oxidation of MbO_2 and of linoleate.

EFFECT OF ALTERING ULTIMATE pH ON BOVINE MUSCLE TENDERNESS. P.E. BOUTON, F.D. CARROLL, A.L. FISHER, P.V. HARRIS & W.R. SHORTHOSE. *J. Food Sci.* 38 816–820 (1973)–Preslaughter injections of adrenaline have been used to produce bovine muscles with ultimate pH values ranging from 5.4 to about 7.0. Measurements of water-holding capacity showed a highly significant relationship

with pH. Shear force and taste panel measurements on the longissimus dorsi muscles from sides hung from the Achilles tendon showed maximum toughness at pH 5.8–6.0. Most objective and subjective measurements on other muscles indicated that tenderness increased linearly with increasing pH. Aging reduced fiber tensile strength until it was no longer dependent on pH. Adhesion values were related to pH when meat samples were cooked at 80°C but were independent of pH when cooked at 60°C.

EFFECT OF FEEDING A PROTECTED SAFFLOWER OIL SUPPLEMENT ON THE COMPOSITION AND PROPERTIES OF THE SARCOPLASMIC RETICULUM AND ON POSTMORTEM CHANGES IN BOVINE SKELETAL MUSCLE. R.P. NEWBOLD, R.K. TUME & D.J. HORGAN. *J. Food Sci.* **38**, 821–823 1973)–Supplementing the diet of steers with safflower oil which had been protected from ruminal hydrogenation by formaldehyde-treated protein led to substantial changes in the fatty acid composition of the phospholipids of the sarcoplasmic reticulum. However, it did not affect the rate or extent of Ca^{2+} uptake, the rate or extent of Ca^{2+} release on cooling, or the basal or extra ATPase activities of the sarcoplasmic reticulum. Nor did it affect the rate and extent of pre-rigor changes such as cold shortening, thaw shortening and fall in pH. In addition the postmortem rates and patterns of change in the concentrations of adenine nucleotides, glycolytic products and intermediates were unaffected. Thus the meat from animals fed a protected polyunsaturated oil supplement does not appear to need different prerigor handling to that from unsupplemented animals.

MYOFIBRIL FRAGMENTATION IN BOVINE LONGISSIUMUS DORSI AS AN INDEX OF TENDERNESS. A.J. MØLLER, T. VESTERGAARD & J. WISMER-PEDERSEN. *J. Food Sci.* **38**, 824–825 (1973)–Fragmentation of bovine longissimus dorsi myofibrils exposed to standard mechanical treatment was investigated as an index of tenderness. 20 young bulls were chosen at random from a group of Danish Black Pied cattle reared at a progeny testing station. The degree of fragmentation was measured in a phase contrast microscope as well as by emission spectrophotometry on myofibril suspension. Significant correlations were found between Warner Bratzler shear value and number of sarcomers per fibril as well as between Warner Bratzler and emission. The relationship between the nitrogen concentration in the suspension and the emission values was linear. However, the effect of nitrogen concentration on emission values differed and was related to the level of shear force. When the spectrophotometric values were corrected to the same nitrogen contents they accounted for about 60% of the variation in tenderness.

AN EXPERIMENTAL STUDY OF THE OPTIMAL FEEDBACK CONTROL OF A FREEZE DRYER. D. MEO III & J.C. FRIEDLY. *J. Food Sci.* **38**, 826–830 (1973)–The optimal feedback control of a freeze dryer has been experimentally investigated using a 50-g sample of reconstituted instant nonfat milk as a food product. Constraints on the maximum temperatures of the product surface and the ice core are maintained to insure product quality. To decrease the drying time the radiator can be at full power until the maximum surface temperature is approached, after which the radiator can be controlled in a feedback fashion based on the surface temperature. This intuitive control strategy is used in a series of constant total pressure runs to establish the relationship between the drying chamber pressure and the time required to freeze dry the sample to a fixed moisture content. An analysis of the constant total pressure series is used to develop feedback control loops capable of automatically maintaining the experimentally indicated optimal drying chamber pressure during the entire freeze-drying cycle. It is expected that this policy constitutes a near optimal feedback control.

ACCELERATED PORK PROCESSING. Fresh-Frozen Pork Chops. L.C. HINNERGARDT, R.W. MANDIGO & J.M. TUOMY. *J. Food Sci.* **38**, 831–833 (1973)–60 pork carcasses were split and alternate sides designated for accelerated and conventional processing under packing house conditions. The loins were used for this study. Accelerated is defined as fabricating to finished fresh primal pork loin prior to initial chilling of the carcass. Ten 1.27-cm thick boneless pork chops from each loin were evaluated for tenderness with an Allo-Kramer shear press and an organoleptic panel. The organoleptic panel also evaluated the pork chops for color, aroma, flavor and appearance. Proximate analysis, percent cooking loss, water-holding capacity and pH were determined for each loin processing method. Accelerated processing of pork loins under packing house conditions failed to have any major effect on fresh-frozen boneless pork chops when compared to chops made from corresponding conventionally processed pork loins. Minor differences were found by the taste panels for tenderness in two of the three studies.

ACCELERATED PORK PROCESSING. Freeze-Dried Pork Chops. L.C. HINNERGARDT, R.W. MANDIGO & J.M. TUOMY. *J. Food Sci.* **38**, 834–836 (1973)–Ten 1.27-cm thick pork chops were removed from accelerated processed pork loins with their counterparts being removed from conventionally processed pork loins for freeze drying at 51.7°C plate temperature and a chamber pressure of 0.3–0.5 mm mercury. Accelerated is defined as fabricating to finished fresh primal pork loins prior to initial chilling of the carcass. The dry weights, rehydrated weights and rehydration ratio of freeze-dried pork chops were not affected by the accelerated processing method. A 10-member organoleptic panel noted no significant differences in flavor, aroma, color and appearance due to accelerated processing. Tenderness was acceptable for freeze-dried pork chops from accelerated processed pork loins.

FEASIBILITY OF ADDING FREEZE-DRIED MEAT IN THE PREPARATION OF FERMENTED DRY SAUSAGE. J. LU & W.E. TOWNSEND. *J. Food Sci.* **38**, 837–840 (1973)–Fermented dry sausages were prepared by mixing freeze-dried meat with fresh pork at the ratio of 1:4, 1:5, 1:6, 1:9 and 0, respectively. Chemical and physical changes were recorded during the 35-day drying period. Results indicate a shorter drying period, less weight loss, slower rate of decrease in pH during the fermentation period, lower peroxide value, higher fatty acid value, softer consistency and the development of a brownish-red color with the increase in proportion of freeze-dried meat. Flavor was good in all sausages. Comparison of all of the quality attributes, indicates that the best ratio is between 1:6 and 1:9. The practical application of these findings is contingent on several economic factors.

FREEZE DRYING OF BEEF: THEORY AND EXPERIMENT. H.J. HOGE & M.N. PILSWORTH JR. *J. Food Sci.* **38**, 841–848 (1973)–Six spherical specimens of raw beef were freeze dried under controlled conditions. A theory of freeze drying is developed and tested with the experimental data. Satisfactory agreement is obtained between theory and experiment. Values obtained for the thermal conductivity of freeze-dried beef are in reasonably good agreement with previously published work. Drying times of the specimens were measured and a convenient method of determining the drying time by extrapolating the curve of rate-of-change of mass is described. Heat transfer to the specimen is broken down into its radiative and conductive components and equations are given from which the surface temperature of the specimen can be calculated.

EFFICACY OF PROTEIN ADDITIVES AS EMULSION STABILIZERS IN FRANKFURTERS. G.C. SMITH, H. JUHN, Z.L. CARPENTER, K.F. MATTIL & C.M. CATER. *J. Food Sci.* **38**, 849–855 (1973)–Frankfurters of four different fat contents were prepared using only meat (control) or meat plus 3.5% of 11 kinds of protein additives. Protein additives were of low (LNS) or high (HNS) nitrogen solubility and included: soy protein isolates (LNS and HNS), soy protein concentrates (LNS and HNS), soy flours (LNS and HNS), glandless cottonseed flours (LNS and HNS), cottonseed protein concentrate (LNS), nonfat dry milk (HNS) and fish protein concentrate (LNS). None of the functional properties or characteristics of the protein additives was closely related to their performance in stabilizing emulsions when utilized at the 3.5% level. Nitrogen solubility index was significantly correlated with water-holding capacity but was not related to other functional properties of protein additives. Higher emulsion stability was not always indicative of more desirable appearance in frankfurters. The protein additives had little or

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no effect on emulsion stability among frankfurters of low fat content (24–26% fat). When frankfurters were prepared to contain 34–35% fat, soy protein concentrate (LNS) and fish protein concentrate (LNS) increased emulsion stability and decreased fatting-out. Microscopic studies suggested that these latter two protein additives affected emulsion stability by their contribution in forming three-phase emulsions in which the protein additives, as solid particles, attached to oil droplets and lowered the interfacial contact between the internal and external phases.

BATCH DRY RENDERING: AN INVESTIGATION OF HEAT TRANSFER TO BOILING WATER/TALLOW EMULSIONS. L.S. HERBERT & H. MAMERS. *J. Food Sci.* 38, 856–859 (1973)–Laboratory scale experiments have been performed to determine the changes in heat transfer coefficient which occur when water is evaporated from a boiling water/tallow emulsion. The coefficients for a water-continuous emulsion were found to be higher than for a tallow-continuous emulsion. However, for a stabilized emulsion, the transition in coefficients from a water-continuous state to a tallow-continuous state was not abrupt and was preceded by a period of declining heat transfer rates to the water-continuous emulsion. The results have been used to interpret the heat transfer processes occurring in a batch dry rendering cooker.

HOT WATER AND MICROWAVE ENERGY FOR PRECOOKING CHICKEN PARTS: EFFECTS ON YIELD AND ORGANOLEPTIC QUALITY. J.T. CULOTTA & T.C. CHEN. *J. Food Sci.* 38, 860–863 (1973)–Precooking chicken parts in hot water and by microwave energy were studied. Precooking times and temperatures, effects of microwave oven load, percentage yield and organoleptic quality were determined. All chicken parts precooked by water at 85.0, 87.8 and 90.6°C had significantly lower cooking losses than those precooked by the microwave oven. Volatile fraction accounted for the greatest amount of total cooking loss for parts precooked in the microwave oven. Precooking treatments significantly influenced taste panel rating for flavor, juiciness and tenderness of both dark and white meat. Microwave cooked white meat received lower acceptability scores (higher values), and dark meat for both precooking methods received the higher palatability ratings (lower values).

AUTOLYSIS AS A FACTOR IN THE PRODUCTION OF PROTEIN ISOLATES FROM WHOLE FISH. W.W. MEINKE & K.F. MATTIL. *J. Food Sci.* 38, 864–866 (1973)–An earlier paper cited data relative to the influence of some factors–species of fish, frozen vs. iced, pH temperature, salt concentrate–on the extraction of protein from whole fish and the ultimate recovery of a protein isolate from such extracts by the mean isoelectric pH precipitation method. Subsequent studies have indicated that the autolytic (proteolytic) enzymes of fish also may influence the yield of isolate obtainable from both whole and eviscerated fish by the mean isoelectric pH precipitation approach employed in this investigation. Proteolytic enzymes of both the viscera and flesh enhance the extraction of protein from whole fish at pH 3 and 10. The native autolytic enzyme activities increase crude isolate (curd) yield on whole fish basis by the mean isoelectric pH precipitation method. Curds produced from whole fish, as well as fillets of certain fish, are creamy rather than granular. Catheptic enzymes of flesh and viscera are more active during acidic extractions. Other enzymes (proteolytic) of the viscera are more active during alkaline extractions. Variations in enzyme activities between different fishes are indicated.

SKIM MILK PROTEIN RECOVERY AND PURIFICATION BY ULTRAFILTRATION. Influence of Temperature on Permeation Rate and Retention. C. POMPEI, P. RESMINI & C. PERI. *J. Food Sci.* 38, 867–870 (1973)–Ultrafiltration experiments were carried out on pasteurized skim milk at 5 and 50°C, using a pilot plant with a tubular membrane configuration. The permeation rates and retentions are reported with particular reference to the behavior of nitrogen compounds of both the protein and nonprotein fractions. It is concluded that the operation is more economical and more effective on protein purification when carried out at 50°C. At this temperature permeation rates are four- to fivefold higher than at 5°C, retention of low molecular weight contaminants such as lactose and salts is nearly zero; and minor loss of whey proteins, especially α-lactalbumin, takes place accounting for only about 1% of the total protein content. When operating at 5°C higher retentions were obtained for the soluble constituents and the loss of whey proteins was negligible. Disc electrophoresis showed no detectable qualitative changes or denaturation of proteins at either temperature.

REDUCTION OF CHILLING INJURY OF CITRUS FRUITS IN COLD STORAGE BY INTERMITTENT WARMING. P.L. DAVIS & R.C. HOFMANN. *J. Food Sci.* 38, 871–873 (1973)–Intermittent warming of Marsh grapefruit in cold storage reduced the incidence of pitting and brown staining, two symptoms of chilling injury. 1-wk intervals of warming at 70°F for 1 day (8 hr) were more beneficial than warming at 2-wk intervals. Brown staining was eliminated in Temple oranges by intermittent warming. Valencia oranges developed less decay when warmed at weekly intervals which may indicate that the rind is weakened by chilling during continuous cold storage even though visible symptoms do not appear. Internal quality, as measured by analysis of juice for ethanol, acetaldehyde, solids, acid and pH, was not affected by intermittent warming.

CHANGES IN TITRATABLE ACIDITY, °BRIX, pH, POTASSIUM CONTENT, MALATE AND TARTRATE DURING BERRY DEVELOPMENT OF THOMPSON SEEDLESS GRAPES. T. PHILIP & J.R. KUYKENDALL. *J. Food Sci.* 38, 874–876 (1973)–Changes in titratable acidity, °Brix, pH, potassium content, malate and tartrate during berry development of Thompson Seedless grapes are reported. A positive relation between increasing pH and °Brix; a negative relation between increasing °Brix and log titratable acidity; and a negative relation between increasing log titratable acidity and pH are shown. The potassium content increases linearly with °Brix. The possible role of potassium in the control of pH is shown.

COMPACTION BEHAVIOR OF GROUND CORN. M. KUMAR. *J. Food Sci.* 38, 877–878 (1973)–The compaction behavior of ground whole corn and endosperm was described by an exponential expression containing two parameters namely: compressibility factor and packing factor. Packing factor could be related to densification during filling, densification due to individual particle movement and preloading of specimen. Compressibility factor was a measure of the ability of powder foods to densify by plastic deformation. The compressibility factor increased with an increase in moisture content, indicating that deformation at higher moisture contents was plastic.

HOT AIR TOASTING AND ROLLING WHOLE WHEAT. Effect on Organoleptic, Physical and Nutritional Quality. A.P. MOSSMAN, W.C. ROCKWELL & D.A. FELLERS. *J. Food Sci.* 38, 879–884 (1973)–Whole wheat batches tempered to 10 and 20% moisture contents are treated at high temperature for short times in a continuous hot air grain popper and then rolled. The flakes have an attractive toasted flavor when prepared with boiling water to make a hot breakfast cereal. The effect of processing conditions on color, flavor, flake integrity, thiamine, phytic acid, starch damage, water absorption index, soluble solids, digestibility and protein efficiency ratio are reported. The toasted whole wheat can also be reduced to a flour or grit suitable for use in formulating precooked high-protein porridge or beverage-type products.

INHIBITION OF STAPHYLOCOCCAL ENTEROTOXIN PRODUCTION IN CONVENIENCE FOODS. R.J. HEIDELBAUER & P.R. MIDDAUGH. *J. Food Sci.* 38, 885–888 (1973)–Presents data showing the effects of combinations of environmental conditions (temperature of incubation, initial pH, NaCl concentration and an atmosphere of CO_2, N_2 or air) on staphylococcal enterotoxin B production in cheese, ham and shrimp slurries. Enterotoxin production in cheese slurries was limited at 44°C and optimum at 37°C. A pH of 5.5 inhibited toxin production. Fluorescence preceded enterotoxin detection in the supernatant fluid by up to 4 hr. Growth conditions at pH 7 and 9 with 2% NaCl (37°C) gave best cell fluorescence, with fair fluorescence at pH 7 and 9–6% NaCl. Fluorescence was limited to 2% NaCl with $\leqslant$ 50% added CO_2. 6% NaCl inhibited cell fluorescence and enterotoxin production in CO_2 or N_2. Results with shrimp slurries were similar except pH change was insignificant.

EFFECT OF ROASTING ON AFLATOXIN CONTENT OF ARTIFICIALLY CONTAMINATED PECANS. F.E. ESCHER, P.E. KOEHLER & J.C. AYRES. *J. Food Sci.* 38, 889–892 (1973)–Dry roasting at 191°C for 15 min reduced the concentration of aflatoxin B_1 and G_1 in artificially contaminated seedling pecan halves (500 halves per lb) by 80%, in Stuart pecan halves (240 halves per lb) by 45%, and in pecan meal by 60%. Roasting of these nutmeats in coconut oil at 375°F for 6 min or in margarine at 107°C for 60 min lowered the concentration by 60%. 3% of the quantity of toxin initially introduced was found in the oil or margarine bath after roasting. No survival of mold was observed on halves after any of the three treatments.

COMPARATIVE EFFECTS OF ETHYLENE OXIDE, GAMMA IRRADIATION AND MICROWAVE TREATMENTS ON SELECTED SPICES. M. VAJDI & R.R. PEREIRA. *J. Food Sci.* 38, 893–895 (1973)–This study involved microbiological, chemical and physical effects of gamma irradiation, ethylene oxide and microwaves on six ground spices (black pepper, paprika, oregano, allspice, celery seeds and garlic) and their effects on the quality of garlic sausage containing such spices. In general, gamma irradiation was most effective for the destruction of bacterial flora in spices. Ethylene oxide treatment affected oil content of the spices and color of paprika, while gamma irradiated spices indicated no change following radiation. With respect to garlic sausage, microbiological examinations indicated the highest increase in sausages made with untreated spices followed by the ethylene oxide treated and gamma irradiated spices during the storage at different conditions of incubation. Flavor analysis indicated no sigificant difference among the sausages. However, preference analysis indicated that the panel preferred sausages made with the gamma irradiated spices to ethylene oxide treated samples.

REACTION PRODUCTS OF HISTIDINE WITH AUTOXIDIZED METHYL LINOLEATE. R.B. ROY & M. KAREL. *J. Food Sci.* 38, 896–897 (1973)–Histidine was reacted with autoxidizing methyl linoleate: (a) in stirred anhydrous mixture; (b) dispersed on filter paper; and (c) in aqueous dispersion. Analysis of the reaction products led to the tentative identification of histamine, ethylamine and aspartic acid. An unidentified histidine-derived compound was also observed. Different reaction conditions resulted in different reaction products.

STABILITY OF BUTYLHYDROXYANISOLE (BHA) IN WATER UNDER STRESSES OF STERILIZATION. W.C. MONTE & J.A. MAGA. *J. Food Sci.* 38, 898–900 (1973)–Most antioxidants are incorporated into food systems at a concentration between 0.01–0.02% (based on lipid content of food moiety). Our study of the behavior of BHA under thermal processing conditions was at concentrations compatible with these systems. The most significant finding was the separation of two compounds representing dimer structures identical to those resulting from the photodegradation of BHA. Under normal circumstances of concentration and time and temperature of thermal processing, BHA undergoes extensive rearrangement and condensation. Further work is in progress to completely identify all reaction products and to investigate effects of pH and reactive food ingredients such as amino acids and reducing sugar.

A COMPARISON OF CHILLED-HOLDING VERSUS FROZEN STORAGE ON QUALITY AND WHOLESOMENESS OF SOME PREPARED FOODS. C. KOSSOVITSAS, M. NAVAB, C.M. CHANG & G.E. LIVINGSTON. *J. Food Sci.* 38, 901–902 (1973)–Experiments were conducted to determine, on a limited basis, whether the Swedish Nacka pasteurization treatment is adequate to destroy *Salmonella* or *C. perfringens* if present in the food after preparation; how the retention of thiamine, riboflavin and ascorbic acid in chilled samples compares with frozen samples; and how palatability of stored chilled foods compares with frozen controls. Refrigerated samples gave negative bacteriological results indicating that the preparation, handling and pasteurization procedures prescribed by the Nacka system effectively destroyed the organisms with which they were inoculated. Retention of vitamins varied: retention of ascorbic acid in frozen samples was significantly greater than in the chilled samples; comparable thiamine and riboflavin values were found in both before storage, but slightly higher in refrigerated samples after storage. After 15 days' storage, the panel detected no significant difference in appearance, flavor and consistency between frozen and refrigerated samples, but rated fresh controls superior to either. After 30 days' storage, refrigerated samples were no longer deemed acceptable by the panel; frozen samples were acceptable but inferior to fresh.

MICROBIOLOGICAL COMPARISON OF STEAM- (AT SUB-ATMOSPHERIC PRESSURE) AND IMMERSION-SCALDED BROILERS. H.S. LILLARD, A.A. KLOSE, R.I. HEGGE & V. CHEW. *J. Food Sci.* 38, 903–904 (1973)–A microbiological comparison was made between broilers from the bleeding line, after water scalding, and after scalding with steam at sub-atmospheric pressure. Lung samples were used as indicators of the degree of contamination resulting from each treatment. Lung samples were analyzed for *Clostridium perfringens*, which is found in water from commercial scald tanks, and for total number of aerobic microorganisms. No significant difference was found between lungs of broilers from the bleeding line and lungs from sub-atmospheric steam-scalded broilers. Counts from lungs of broilers scalded by sub-atmospheric steam were significantly lower at the 0.1% level than counts from lungs of water-scalded broilers.

THE RATE OF COAGULATION OF ZEIN. E. BALMACEDA & C.K. RHA. *J. Food Sci.* 38, 905–906 (1973)–The rate of coagulation of protein dope (zein in 95% alcohol) in HCl constant temperature coagulating bath (pH 3) was determined experimentally. Data were analyzed assuming diffusion controlled coagulation and values of a parameter called diffusion velocity, which adequately describes the boundary advancement, were obtained.

CHILLING INJURY OF GREEN BANANA FRUIT: KINETIC ANOMOLIES OF IAA OXIDASE AT CHILLING TEMPERATURES. N.F. HAARD. *J. Food Sci.* 38, 907–908 (1973)–Indole-3-acetic acid oxidase was isolated from green banana pulp and assayed at temperatures ranging from 5–30°C. The velocity of IAA oxidation was hyperbolic in the substrate range of 0.05–1.00 mM when the assay temperature was above 15°C. At assay temperatures below 15°C the velocity was preferentially decreased at low substrate concentrations such that sigmoid-like kinetics were observed. At an assay temperature of 5°C the enzyme was inactive with 0.2 mM IAA. It is suggested that the temperature-induced anomolous kinetics relate to the failure of banana fruit to acclimate to chilling temperatures and ripen.

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ANTHOCYANINS OF BLACK GRAPES OF 10 CLONES OF *Vitis rotundifolia*, **MICHX.** W.E. BALLINGER, E.P. MANESS, W.B. NESBITT & D.E. CARROLL JR. *J. Food Sci.*, 38, 909–910 (1973)–The anthocyanins (Acy) of homogenized hulls of ripe berries were extracted with 1% HCl in methanol, and separated and purified by paper chromatography using the solvents 1% HCl, BAW and 15% HAc in sequence. The purified Acy were identified using spectral analysis, partial- and complete-hydrolysis, and co-chromatography with known markers. Berries from each of the 10 clones, representing a wide range of genotypes, contained the same Acy, all 3,5-diglucosides of delphinidin, petunidin, cyanidin, malvidin and peonidin. None was acylated. These findings confirm and extend those of Ribéreau-Gayon which were based on an unspecified number or type of clones.

SPECTROPHOTOMETRIC DETERMINATION OF CAFFEINE IN NIGERIAN KOLA NUTS. O. SOMORIN. *J. Food Sci.* 38, 911–912 (1973)–The purpose of this investigation was to determine the caffeine contents of Nigerian kola nuts. By using gradient elution chromatography and by determining the caffeine spectrophotometrically, reliable quantitative results were obtained. Results show that Nigerian kola nuts have variable caffeine contents, for instance *Cola acuminata* has a caffeine content of 2.19% in contrast to *Cola verticillata* with a caffeine content of 1.04%. The variability in caffeine content also exists between varieties of the same species as exemplified by *Cola nitida rubra* with a caffeine content of 1.91% and *Cola nitida alba* with a caffeine content of 1.40%.

SPECTROPHOTOMETRIC ANALYSES OF ORANGE JUICES AND CORRESPONDING ORANGE PULP WASHES. D.R. PETRUS & M.H. DOUGHERTY. *J. Food Sci.* 38, 913–914 (1973)–The combined visible and ultraviolet absorption was obtained of alcoholic solutions of five midseason (Pineapple) and seven late season (Valencia) orange juices and orange pulp washes. The spectra for all pulp washes were much lower in visible absorbance but higher in ultraviolet absorbance than their corresponding orange juices. From analyses of the spectra, the orange juice content of the pulp wash was estimated. The orange juice contents of the pulp washes were found to vary between processors.

AMYLOSE CONTENT AND PUFFED VOLUME OF PARBOILED RICE. A.A. ANTONIO & B.O. JULIANO. *J. Food Sci.* 38, 915–916 (1973)–Results show equilibrium water content of steeped rice differed among varieties and lines tending to be highest in waxy rice followed by nonwaxy rice with low and intermediate amylose and then by those with moderately high and high amylose. Protein content was not related to equilibrium water content. Volume expansion during puffing differed among samples, being highest for waxy rice. Puffed volume was greater in steeped rices with water contents above 30% at 28–30°C. Samples parboiled at different moisture contents but puffed under the same conditions showed the water content of the rice on parboiling determined the puffed volume. Data indicate in addition to parboiling conditions that amylose content of rice influences the puffed volume of the resulting milled parboiled rice by affecting the degree of parboiling of grains processed under identical conditions.

Erratum Notice

J. Food Sci. 38(4): v (1973), K. Ostovar: "A study on survival of *Staphylococcus aureus* in dark and milk chocolate." On page v, Abstract, line 3 and following, change to: Dark and milk chocolate bars were inoculated with *Staphylococcus aureus* to establish an initial population of approximately 10^3, 10^5 and 10^7 cells per gram. Samples were stored at room temperature and examined for the survival of staphylococci at 2-day intervals for the first 6 days and every 8 days thereafter. Counts of less than 100 cells per gram were obtained after: (a) 2 days in dark and 14 days of storage in milk chocolate when the samples were inoculated with 10^3 cells per gram; (b) 38 days in dark and 86 days in milk chocolate when the bars were inoculated with 10^5 cells per gram; and finally (c) 86 days in dark and 110 days of storage in milk chocolate when the samples were inoculated with 10^7 cells per gram.

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A SYMPOSIUM . . . complete in this issue

PROTEIN INTERACTIONS IN BIOSYSTEMS
INTRODUCTION

FOODS are complex biosystems in which many constituent interactions can take place. Recent developments in food science have singled out protein as an important food constituent nutritionally as well as functionally. Proteins in foods undergo a multitude of interactions.

The constituents in food biosystems which can interact with proteins include water, lipids, carbohydrates, minerals, vitamins, pigments and others. It is, however, impossible to cover such a vast field of interactions. Therefore, the discussion is limited to the macroconstituents: water, lipids and carbohydrates.

In order to provide a better understanding of the protein interactions, the structure of water (Karmas, 1973) and protein (Wetlaufer, 1973) are also discussed to provide important background information. The protein interaction topics include protein-water (Lumry, 1973), protein-lipid (Karel, 1973) and protein-carbohydrate (Hodge, 1973) interactions. Unfortunately, the latter paper, discussing an extremely interesting and important field from the food science point of view, was not made available for publication by the author.

It should be noted that the interactions in biosystems mentioned above take place in aqueous medium and are thus intimately related to the water structures. The first paper of the symposium (Karmas, 1973) deals with the structural properties of water. It has been widely accepted that the structural properties of water can serve as a key to understanding and explanation of biological phenomena and processes. Biological significance of water, biological activity as a function of temperature, the structure of water, solute effect on water structure and the state and functional role of water in biological systems are discussed in this paper.

The second paper (Wetlaufer, 1973) reviews the protein structure and stability in biosystems. Recent research has shown that protein structures are frequently not as archtypical as described in the classical literature. For example, the α-helical and pleated sheet structural modes are frequently irregular; or, many proteins have a substantial fraction of their nonpolar groups exposed to water and charged groups are occasionally found in the protein interior. These irregularities do not necessarily violate the conventional principles. On the contrary, protein structures may be considered as compromises on the simultaneous optimization of many structural principles. The author points out that protein nucleation or self-assembly mechanism, directed by the local free energy minimum, appears to be the only plausible way of reducing the number of statistically possible protein structures.

Lumry (1973) discusses some recent ideas about the nature of the interactions between proteins and liquid water emphasizing many puzzling problems. The paper points out that the understanding of proteins as chemical species has to be improved before the ideas about the protein reaction mechanisms can be verified. The linear enthalpy-entropy compensation pattern has been found to provide an experimental pathway for studying the role of water in determining the chemical, physical and specific functional properties of proteins. Protein function may be linked to bulk water through volume change. Similarly changes in protein surface properties can be linked to bulk water.

Karel (1973) points out a number of significant processes in food and biosystems which involve interactions between proteins and lipids. Mechanisms by which proteins and lipids interact, the nature of the forces involved, and factors which affect the interactions are reviewed. Examples of particularly important interactions in food biosystems are presented, including biological membrane interactions, protein-oxidizing lipid interactions and coalescence and inversion of emulsions.

This symposium attempts to present some fundamental concepts on protein interactions which should contribute to the recognition of the complexities involved in food biosystems. Furthermore, it is hoped that this information will project potential applications in food science.

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SYMPOSIUM: Protein Interactions in Biosystems
WATER IN BIOSYSTEMS

Biological significance of water

Szent-Györgyi (1957) has stated that "water is not only the *mater*, mother, it is also the matrix of life." It is reasonable to think that life originated in water and is thriving in water, since biological reactions do not take place unless aqueous medium is present. Water is not only an inert medium, but an all-important biochemical, a chemically active substance participating in such essential reactions as photosynthesis and terminal respiration. Water also seems to have molded the various forms of life according to its own structure. Hence, it is appropriate to say that in the evolutionary stream of life in water, the shapes were formed and the kinds were multiplied. On the molecular level, the proteins were structured and molded by the structure of water. Finally, the aquatic plants and animals adopted themselves to the conditions of existence on land. However, water was still retained as the most important constituent in all forms of life, from relatively simple single-celled to the exceedingly complex multicellular organisms, amounting sometimes to as much as 95%, but seldom less than 60%.

The origin of life lies in the unique power of water to interact with biochemical solutes. The existence of life depends on controlled movement of biochemical solutes. Water itself is unable to exercise that control. The creation of proteinaceous framework was a primary step in the evolution of life, and this creation took place in water. In general sense, the tension at both sides of the membrane, termed as osmotic pressure, may be called life. In biological systems this tension—life—is maintained by metabolic action within the biosystem.

In every phase of the evolutionary process, water with its remarkable properties has played a leading and perhaps a dominant role. Proteinaceous structures enter so closely into the structure of water that they may well be considered as an intimate part of the biological structure of water.

Biological activity as a function of temperature

In the universe, the temperature ranges from near absolute zero to millions of degrees. However, biological activity is restricted to a narrow temperature interval. It is within the range in which water remains liquid at one atmosphere. For higher forms of life—man and other mammals—this range is extremely small being only some 15°C. For plants, the temperature range over which survival may occur is some 90°C.

The temperature range for major biological activity is illustrated in Figure 1. Much of the background for this illustration comes from the publications of Drost-Hansen (1965; 1971). He reviews the distribution of optimum temperatures for various biological activities and concludes that the temperatures near 15, 30, 45 and 60°C are usually avoided. These temperatures are thought to cause transitions in water structures.

Metabolic rates in complex biosystems may undergo notable changes at the temperatures which influence the transition in water structures. Based on this assumption, it is proposed that during evolution, biological systems have tended to avoid temperature regions associated with changes in water structures and, hence, have optimized the temperature of activity as far away as possible from these transition points. Were these transitions to occur at 30 and 45°C, the optimum temperature would be expected to fall near 37°C, which is the case in man and other mammals. This temperature also happens to be the point of the minimum specific heat capacity for water (Barnes and Jahn, 1934).

The traditional classification of microorganisms into psychrophiles, mesophiles and thermophiles may be an example of a tendency for these groups of organisms to

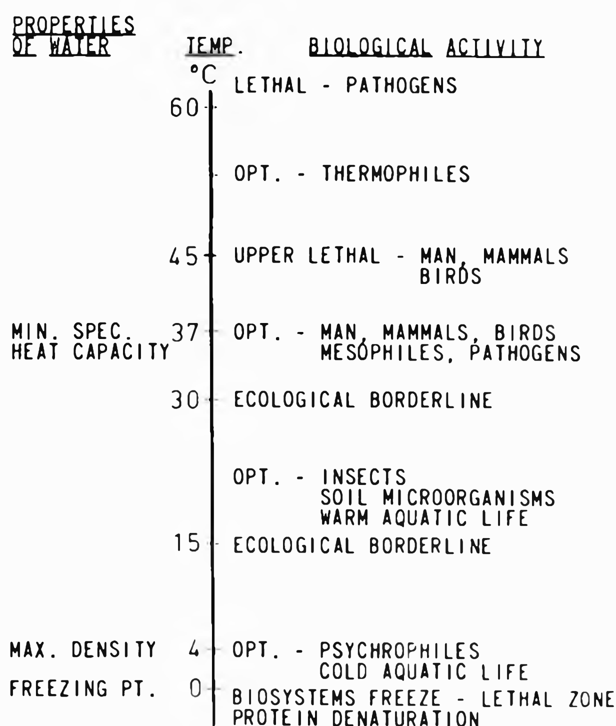

Fig. 1—A schematic presentation of major biological activity on the temperature scale with reference to properties of water.

exhibit optimal growth between various consecutive ecological borderlines. For example, a majority of thermophilic microorganisms are known to possess optima around 53°C. On the other hand, it is well known that pasteurization temperature for human pathogens starts at 60°C.

There are thermophilic organisms which survive at temperatures higher than 60°C. A few organisms may even survive at above the boiling point of water. Such life processes suggest that some living systems have been able, through evolution, to select and enhance the mechanism which has the greatest ability to protect the native structures.

An obvious transitional temperature for water structure is the freezing point. Biological systems freeze a few degrees lower than pure water. This is a most detrimental temperature to aquatic life and microorganisms as well as to the native proteins. Microorganisms die rapidly when kept at freezing point temperatures, whereas they may survive when rapidly frozen to, say, −70°C (Frazier, 1967). Proteins denature extensively at the freezing point temperatures, whereas denaturation may be negligible when the protein is kept at −20°C (Finn, 1932).

This discussion suggests a strong interaction between the structure of water and biological activity. Furthermore, it is meaningless to speak about water structures without reference to temperature. The properties of water are strongly related to the temperature. As the properties and the structures of the water change, the biological activity is influenced.

Water structure

Water is a puzzling substance. This most abundant, but most anomalous liquid on earth has challenged scientists for centuries. A number of monographs (e.g., Eisenberg and Kauzmann, 1969; Kavanau, 1964) and articles e.g., Buswell and Rodebush, 1956; Frank, 1970) have been written about "ordinary" water.

To understand the behavior of water in biological systems, the structure of pure water must be understood. The unusual properties of water are directly traceable to the complex structure of this remarkable liquid. Although improved instrumentation is rapidly becoming available, understanding which presently prevails regarding the structure of water, in general, and that in biological systems, in particular, is fragmentary and incomplete. In fact, contradictory and mutually exclusive theories are currently discussed in the literature. Safford and Leung (1971) have given a detailed overview of current water structure theories.

All models of water structure invoke the occurrence of hydrogen bonding to a greater or lesser degree. The best models also agree that water is a mixture of monomeric and hydrogen-bonded polymeric molecules. The models have been divided into three groups: continuum, cluster and specific structure models.

In continuum models, a hydrogen-bonded system penetrates throughout the water body without a definitive structure. One of such continuum models is proposed in the classic paper of Bernal and Fowler (1933). This was also the first time when hydrogen bonding, discovered by Latimer and Rodebush in 1920, was proposed as a basic framework of water structure.

Compared with liquid water, ice is 100% hydrogen-bonded and has a symmetrical, highly open hexagonal structure (Runnels, 1966). In the ice structure, each oxygen atom is tetrahedrally surrounded by four other oxygen atoms at a distance of 2.76Å. This ice structure is possible because the tetrahedral angle of 109.5° approximates rather closely to the water dipole angle of 104.5°. The hydrogen atoms in ice, however, are distributed asymmetrically. Each oxygen atom has two hydrogen atoms covalently bonded and two farther away hydrogen-bonded. The distinctive structural features of liquid water generally have been ascribed to its partial retention of the tetrahedrally-directed hydrogen bonding involved in the crystalline structure of ice.

Large more-or-less structureless ice-like polymers represent the idea of the cluster models. The most important cluster model was proposed by Frank and Wen (1957). The crystalline clusters are dispersed in a denser nonhydrogen-bonded monomeric water. The formation and collapse of hydrogen bonds is a cooperative phenomenon and short-lived or "flickering" (10^{11} times per second) ice-like clusters of varying size are mixing and exchanging with monomeric molecules. No specific size is assigned to the clusters. Némethy and Scheraga (1962) have done statistical-thermodynamic calculations for the flickering-cluster model. In their concept, the compact clusters are made up of four distinct species corresponding to molecules with one, two, three and four hydrogen bonds; a fifth species is the monomer which is in equilibrium with the clusters. Cluster size and monomeric fraction vary with the temperature.

Specific structure models are based on the notion that structurally identifiable ice-like units are present in liquid water. The most important representative of the structure models is the Pauling's clathrate model (Pauling, 1959). In Pauling's model, 20 water molecules lie at the corners of a pentagonal dodecahedron with an unbonded molecule at the center of the dodecahedron. The water is conceived to be a hydrate of itself with the formula of $H_2O \cdot (H_2O)_{20}$. The clathrate structures are similar to those of the gas hydrates (Stackelberg, 1949). The inside diameter of the clathrate cage is about 5Å. The significance of the pentagonal dodecahedron to molecular architecture is in part a result of the close approximation of the angles of 108° between its edges to the tetrahedral angle of 109.5° in ice. The necessary fluidity is introduced into the model by "flickering" of the clathrate hydrogen bonds (Frank and Quist, 1961). The dodecahedra may be arranged relative to one another in a large number of ways, forming additional hydrogen bonds with each other or fusing together to share pentagonal faces; they also may be joined through hydrogen-bonded water molecule bridges. In addition, monomeric water molecules may be present. Such labile structures give rise to a variety of voids within the structures.

The concept of structured water is now being taken seriously. It is remarkable that it has not been possible to firmly prove or disprove any of the various theories proposed for water structure. If the ordered water is conceived as a part of the highly differentiated and ordered three-dimensional macromolecular proteins which constitute the fabric of living systems, it is natural to assume that structured water—from the crystalline clathrate structures to the numerous other possible frameworks—is an integral part of the macromolecular structures.

Solute effect on water structure

Frank (1965) has classified the general effects of different types of solutes in terms of water "structure-making" and "structure-breaking." Four types of solute action may be recognized and analyzed with respect to the water structure: ionic, nonionic polar, nonpolar and polyfunctional.

The electric field in the vicinity of an electrically charged ion strongly polarizes the water dipoles and they become oriented as a shell around the ion. This causes a breakdown of the latent intrinsic structures of pure water. Through the torque of the ionic electric field on water dipoles, the structure equilibrium is shifted in the direction of a smaller degree of ice-likeness (Frank and Wen, 1957). An electric charge, whether carried by a free ion in solution or an ionic side chain of a protein molecule, generally does not differ appreciably in magnitude. Beyond the immobilized monolayer, restricted motional freedom is induced in other adjacent water dipoles. According to Frank and Wen (1957), three concentric regions surround an ion: (1) an innermost region of polarized and immobilized, (2) an intermediate region of random organization and (3) an outer region of normal tetrahedrally oriented liquid water molecules.

Nonionic polar groups—such as hydroxyl, amide and peptide bond—either participate in the hydrogen bond formation or exert only negligible effect on the normal water structures and, hence, they

are more-or-less neutral. Molecules with a large polar moment, such as tyrosine sidechain, tend to interfere with the symmetry required for the formation and stabilization of the clathrate framework, such that may otherwise shelter a phenylalanine sidechain (Karmas and DiMarco, 1970).

Nonpolar solutes have the structure-making ability by forming water clathrates in the Pauling's model sense. For example, the methane molecule is nonpolar, it does not ionize, nor does it accept hydrogen bonds; there is no obvious attraction between it and the water molecule. Methane, however is "soluble" in water, not in the classical sense, but through the formation of crystalline hydrate with water (Stackelberg, 1949). Pentagonal dodecahedral clathrate structures provide an array of voids that may be able to envelop smaller nonpolar sidechains. Drost-Hansen (1971) states that while methane, ethane, propane and isobutane form clathrate hydrates quite readily, pentane does not appear to form such compounds. This may be interpreted as due to the inability of the classically recognized clathrate cages to accommodate larger hydrocarbon chains. It is interesting to point out that the largest nonpolar aliphatic amino acid sidechain is isobutane. Karmas and DiMarco (1970) in a thermoanalytical study have demonstrated a strong interaction between water and nonpolar amino acids, particularly those of leucine and isoleucine.

The macrosolutes in biological systems are polyfunctional and the resulting interactions and water structures exceedingly complex. Szent-Györgyi (1957) stated that crystalline water structures can be built around dissolved molecules. These structures may be different according to the polar or nonpolar nature of the molecule and the mutual distance of these groups. It is believable that different spacing promotes different crystalline forms, possibilities being rich and relations complex.

The physical state of water in biosystems

Historically Szent-Györgyi (1951) was the first scientist to state that ordered water structures are important elements in biological functioning. He proposed that muscle contraction and relaxation involves collapse and reformation of crystalline water structures. Klotz (1958) suggested that the nonpolar sidechains of proteins could induce crystalline clathrate structures of water around protein with the added cooperative effects due to the presence of many such sidechains bound to the frame of the protein molecule. Ling (1965) concluded that all the water in the living cell exists in cooperatively polarized multilayers oriented by the electric charges of cell proteins and ions.

Until recently these concepts were only speculations. However, experimental evidence has now confirmed these predictions qualitatively. One of the most convincing pieces of evidence for ordered water in biological systems has come from the NMR studies by Hazlewood et al. (1969). They concluded that most of the skeletal muscle water has restricted motional freedom. At least two ordered phases of water have been observed. The major phase consists of water molecules that have lost considerable motional freedom relative to free water. The minor phase contains water molecules that have even less motional freedom than the major phase but more than solid ice. Heat denaturation of the muscle protein lessens the ordered phases significantly.

Similarly Cope (1969), employing NMR, indicated that the structure of biological water has a significantly greater degree of crystallinity than liquid water.

Szent-Györgyi (1951) stated that "heat can be expected to destroy water structures." Karmas and DiMarco (1969) found that heat denaturation of skeletal muscle tissue and egg albumin takes place in two irreversible phases. The calculations strongly suggest that collapse of water structures is involved. The native structures, stable at low temperatures, are not reformed after denaturation when the temperature is lowered. The possibility exists that the symmetry and ordered matrices of the native structures are destroyed irreversibly by high temperatures and thus cannot be restored.

Biological water activity and the functional role of water

There is plenty of evidence that the living cell is not simply a sac of biological solutes in ordinary water solution. Water, considering all its unusual properties, must necessarily accomplish a much more important and complicated role than that of an inactive solvent.

Water in biological systems is usually divided into two major phases: bound and free water. These terms seem to be inadequate. It would be more meaningful to use the term "*biological water activity*" which ranges from *biologically active water* to *structural water*. Biologically active water is needed by biosystems at the peak biological activity. The structural or protective water is vital for survival of the living organism and it cannot be frozen or evaporated.

Biological water activity is a function of the cellular water content, solute concentration and temperature. Thus, the biological water activity may be regulated by the nature in three ways: by changing the cellular water content, solute concentration and temperature. All these mechanisms are interrelated by a common denominator–change in biological water activity.

A grain of wheat at its peak growing period contains some 75% of water. When the biologically active water is no longer needed, it is released readily and the cereal grain is dehydrated to about 13% water content which is the upper level of structural water essential for survival. This mechanism may be considered as cereal grain "*spore*" formation. A grain of wheat at this water content may remain biologically dormant, its enzyme active sites "frozen" or protected by the structural water, for many years until the active water is replaced and a new biological cycle begins.

Similarly, the vegetative bacterial cells have a high biologically active water content. Bacterial spores, on the other hand, have given up their biologically active water and retained only the vital structural water necessary for survival and protection particularly at temperatures near, or even above, the boiling point of water. It has been found that the heat resistance of spores from six bacterial test species was at a maximum at water activity levels between 0.2 and 0.4 (Insalata, 1972) which may be correlated with a moisture content between about 7% to 10%.

The survival of woody plant cells at freezing temperatures is analogous to that of bacterial spores at high temperatures. The death of hardy plant cells invariably results when intracellular freezing of the intracellular water occurs due to rapid freezing rates (Weister, 1970). The winter acclimation of hardy plants has to take place slowly. First, the extracellular water freezes and rapid propagation of ice throughout the stem results. Due to the vapor pressure difference, the once active protoplasmic water migrates from the cell to the extracellular ice. The partial dehydration of protoplasm, or concentration of the protoplasmic solutes, prevents intracellular freezing and thus contributes to the survival of the plant. Farmers in the cold Nordic climates protect semi-hardy plants from freezing by supplying the plants, immediately before the cold season, with certain inorganic salts, such as potassium nitrate and phosphates, and plenty of water. This supposedly increases the intracellular solute content and helps the plant to survive the freezing weather.

However, not all phenomena of biological water activity need involve extreme conditions. It is known (Drost-Hansen, 1965; 1971) that in both man and many other mammals, 30°C is a temperature of considerable physiological importance. For example, in man there is a loss of consciousness at about 30°C combined with a loss of the ability to regulate body temperature. For mammalian hibernation, the critical temperature again is around 30°C. These phenomena are believed to be due to the transition in the water structures toward more ice-like states. Some water activity is "frozen" which, in turn, arrests the functional

activity of the delicate brain cells. The same effect can be accomplished by the anesthetic solutes which are believed to form ice-like hydrates (Pauling, 1961).

It is reasonable to believe that the structural water has a complex molecular architecture. Perhaps Pauling's pentagonal dodecahendron-like clathrates are surrounded by Némethy-Scheraga's ice-like clusters interwoven with Ling's multilayer interaction?

Western scientists were skeptical when the Russian scientists Deryagin and Churayev (1968) claimed to have produced a mysterious new substance, a new form of water, the so-called *polywater*. Made in thin capillaries of 1–50 μm in diameter, this "new" water boiled above 250°C. It did not freeze, though at −40°C it hardened into a glass-like substance. It was soon reported by Davis et al. (1971) and Everett et al. (1971) that the anomalously behaving water contained high concentrations of sodium, potassium, sulfate, chloride, nitrate and other impurities, but relatively little water.

At this time, when the final chapters of the story of the anomalous water have been written, I would like to revive this controversial subject matter once more to take another look at the anomalous water but from a different point of view.

One is tempted to picture the so-called anomalous water as a part of the biosystems. The conditions described by Davis et al. (1971) and Everett et al. (1971) may be found in nature. Living cells, particularly at the time of survival, contain relatively high concentrations of various inorganic salts in the structural water. Furthermore, the cellular diameter is in the range of that of the capillaries in which the anomalous water has been reported to form. Therefore, there is a strong possibility of the formation and existence of anomalous water in nature. Any point of a small pore or capillary may be the deposition site of anomalous water. Perhaps the difference in the height and cellular diameter between the Nordic and tropical plants, for example, is influenced by the difference in the biological water activity? Is it coincidental that temperatures around −40°C, the hardening temperatures of anomalous water, are particularly critical for plant survival in the cold climates?

The survival of bacterial spores when boiled at high temperatures could be explained if their structural water were a high-boiling substance such as the anomalous water. Animal cells, for example, do not have such a survival mechanism.

Is Deryagin water a combination of Pauling's, Némethy-Scheraga's, and Ling's concepts? Or may it be considered as a new concept of water in biosystems? These and many other questions remain to be answered regarding the state of water in biosystems.

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SYMPOSIUM: Protein Interactions in Biosystems
Protein Structure and Stability: Conventional Wisdom and New Perspectives

ONE OF THE basic perspectives of the conventional wisdom is that native proteins contain certain characteristic structural modes of peptide chain arrangement which are probably familiar to most of you.

Collagen structure

Since collagen is the most abundant protein in higher organisms, and surely is very important in determining the mechanical and textural properties of tissues, we will consider its structure first. The collagen twist is a three-stranded helix, with each strand a polypeptide chain. The three peptide chains are packed close together in a way that permits extensive interchain hydrogen bonding. (An up-to-date and readable refresher on this and other standard peptide chain structural modes can be found in the recent paperback by Dickerson and Geis, 1969.)

The second structural mode is the pleated sheet, which is formed from two to several lengths of extended peptide chain packed side by side in such a way as to produce a wavy (pleated) planar structure. There are two varieties of pleated sheet, depending on whether two adjacent peptide chains are pointing the same direction, looking from the N-terminal end of the chain segment (parallel), or pointing in opposite directions (antiparallel). In both parallel and antiparallel sheets, the adjacent segments of peptide chain are so closely packed that there is intersegment peptide-peptide H-bonding at every amino acid residue.

The third structural mode surely has the greatest fame of all—it is the α-helix. Its fame is so great, in fact, that I have been told by a few students that the α-helix is the characteristic structure of DNA. However, the conventional way of viewing the α-helix is that of a moderately pitched polypeptide chain wound round a cylinder so that one complete turn requires somewhat less than four amino acid residues with the residues in such a geometry that intrachain H-bonding occurs between the carbonyl oxygen of the nth peptide bond and the H-N of the (n+3)rd peptide bond.

In proteins whose three-dimensional structure has been determined, the above structural modes often account for no more than half the molecule. The remainder of the peptide chain is arranged in structures which have no discernible structural pattern. Earlier workers often tended to call these structures "random", or "random coil", in analogy with the unordered structures of synthetic polypeptides in good solvents. However, since these structures in proteins are usually the same in all the molecules of a particular protein, "random" is clearly inappropriate. There is at present no accepted generic term for these structures. We have suggested "remainder" (Saxena and Wetlaufer,1971) which can be recommended as operationally acceptable.

Elastic proteins

Another protein structural mode is found in the elastic proteins elastin (Mammal), resilin (Insect) and abductin (Scallop). Various investigations support the interpretation that these proteins have the rubber-like elasticity associated with a highly crosslinked random coil. In these structures, which maintain their elasticity only when hydrated, the peptide chain segments continually undergo random thermal motions, within the constraints of the covalent bonds and customary steric factors.

Protein structural principles

In addition to the structural modes sketched above, several structural principles have become part of the conventional wisdom about protein structure. We begin with the criteria for stable structures proposed by Pauling and Corey, (1951a–e; 1952; 1953a,b) and Pauling et al. (1951; 1952). From extensive X-ray crystallographic structural determinations on small peptides and peptide analogs, these investigators argued that stable protein structures should meet the following conditions: (A) the six atoms of peptide bond should be co-planar; and (B) the bond should have a trans conformation; (C) and (D) the bond angles and interatomic distances should be the same as in the low molecular weight model compounds; (E) all H-bond donor and acceptor potential is satisfied, mainly by peptide-peptide interactions; and (F) for maximum energy from H-bond interactions, co-linearity of peptide N-H→ O=C atoms should obtain.

The next group of structural principles concerns the disposition of the hydrophilic and hydrophobic parts of the protein. It is assumed that the most stable structure will result when the nonpolar sidechains (leucine, valine, tryptophan, etc.) are packed inside the protein molecule away from aqueous solvent, while polar sidechains (formally charged groups, plus hydroxyl groups, amide groups, etc.) are exposed to aqueous solvent. The clear and compelling presentation of these principles is due to Kauzmann (1959). In what appeared to be reasonable extension of these principles, globular proteins were thought to be compact, close-packed structures with no holes or channels.

CONVENTIONAL WISDOM VS EXPERIMENTAL DATA

WE WILL NOW briefly discuss how the conventional wisdom compares with experimental data.

Bond angles and distances

Bond angles and distances are generally in good agreement with those of model compounds. However, refinement of the lysozyme data appears to show that a few peptide bonds are out-of-plane, perhaps by as much as 30° (Phillips, 1972).

In general peptide bonds are trans in proteins, but a cis bond has been found in ribonuclease S and another in carboxypeptidase A (Phillips, 1972).

Structural modes

When the α-helical and pleated sheet structural modes are found in proteins, they are frequently distorted from the archtypical structure. Helices may be bent, and do not always show the H-bonding pattern of the α-helix. Pleated sheets are more frequently twisted or curved than planar, notable examples being found in chymotrypsin, carboxypeptidase A, and carbonic anhydrase.

Many proteins have a substantial fraction of their nonpolar groups exposed to water, although generally more than 2/3 are "buried." Charged groups are occasionally found in the protein interior, as ion pairs. As to the "no holes" rule, myoglobin has an unfilled cavity large enough to accept a Xenon atom, (Schoenborn, 1969), ribonuclease S has an empty channel (Lee and Richards, 1971), and chymotrypsin has 13 internal water mole-

cules, (Birktoft and Blow, 1972).

In carboxypeptidase A only ~60% of the peptide H-bonds are made to other peptides. An appreciable number of the other 40% peptide bonds are inside the molecule and may be associated with internal water molecules (Kuntz, 1972).

It is quite common to find internal H-bonds in proteins which depart strongly from the co-linearity principle.

How do we understand these breaches of principle? Are the principles wrong? No, they seem to be right, most of the time. But it seems likely that protein structures are compromises on the simultaneous optimization of a half-dozen or so structural principles. In a particular protein, principles A, B, D, E and F may be fulfilled with 85–95% fidelity, while principle C is only 65% satisfied. As yet we have no way of predicting what compromises will be made in a particular protein.

What we have already said about the structure and stability principles of single-chain proteins applies also to subunit proteins, except that we relax considerably the constraint "no holes or channels" for subunit assemblies. Turning to the question of function, "Why subunits?", let us begin by noting that the association of proteins reduces osmotic pressure. It is easy to calculate that the osmotic pressure of a cell would be excessive (the cell would rupture) if its contents were not largely associated. Beyond this, we will simply mention the more conventional functions ascribed to subunit systems: they afford an economy of the genetic information required by an organism, and they provide the possibilities for control, either by allosteric routes, or by altering the isozyme distribution (Sund and Weber, 1966).

The basic premise behind the structural and energetic principles sketched above is that a native protein is the most stable structure that a protein can assume. Put more formally, the conventional wisdom says, "The native protein is in its thermodynamically most stable structure." This is probably not so. Why? Because any reasonable estimates of the time it would take for even a small protein to randomly sample all possible structures is truly astonomical–much, much longer than the age of the solar system (Wetlaufer, 1973). But proteins do form their native, functional, 3-dimensional structures in biological time–in seconds or minutes. The most reasonable resolution of this disparity of time scales is that a self-assembling protein samples only a very small fraction of all its possible structures. Something must occur which limits the number of structures searched. We suppose, along with Levinthal, who first made the suggestion (Levinthal, 1968), that this "something" is nucleation. Of course, if not all structures are sampled, we have no guarantee that the native structure is the free energy minimum. An energy minimum, to be sure, but not necessarily the global minimum.

As convincing as the foregoing argument is, we must acknowledge that it is argument, not evidence. What, then, is the evidence?

To begin with, let us consider the idea that proteins self-assemble in vivo while the peptide chain is still undergoing synthesis on the ribosome. This is in some ways an attractive picture and may be true for some proteins. The supporting evidence for this view, however, is weak. It is possible to isolate ribosomes interrupted in the process of biosynthesis of a particular enzyme. These ribosomes have some of the particular enzymic activity firmly associated with them. But this does not necessarily mean that self-assembly of the enzyme occurs on the ribosomes in vivo. What it does mean is that enzyme self-assembly can take place on ribosomes in the time required to isolate the ribosomes, which requires hours. The experiments unfortunately tell us nothing about self-assembly on the time scale for the incorporation of amino acids into proteins, which is measured in seconds.

Rate of enzyme regeneration

Although we are ultimately interested in how self-assembly occurs during biosynthesis, we have not yet been able to devise suitable experiments directly to that point. Instead we have studied the in vitro rate of regeneration of a specific enzyme from a completely unorganized (denatured) polypeptide chain. We have chosen to do this employing enzymes containing disulfide crosslinks in the native state. Although this introduces possibilities of complication in the kinetics of regeneration, it also offers a significant advantage, which will appear shortly. The process whose kinetics and mechanism we have studied is shown schematically in Figure 1.

In studies that have been detailed elsewhere (Saxena and Wetlaufer, 1970), we

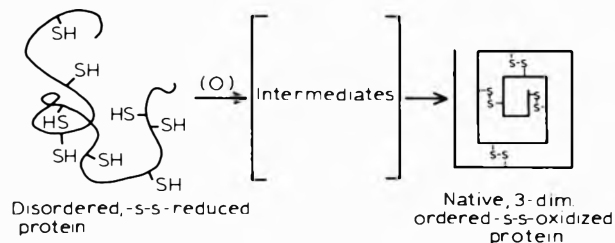

Fig. 1—Schematic representation of the in vitro system in which the kinetics of 3—dimensional structure formation were studied.

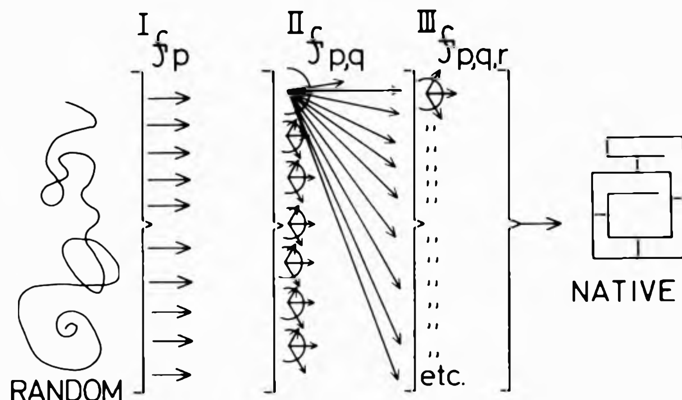

Fig. 2—Random-search mechanism for protein folding shows a very large number of parallel, intersecting and finally converging pathways. Roman numerals indicate the average number of disulfide crosslinks formed in the regeneration of a 4-disulfide protein. Subscripts to the Roman numerals indicate qualitatively the increasing complexity of the disulfide combinations as the stoichiometric number of disulfides increases from 1 to 3.

found that rapid, nonenzymic regeneration of hen eggwhite lysozyme could be achieved by using a mixture of oxidized and reduced glutathione. We were gratified to find that conditions for the most rapid regeneration (50% lysozome activity regained in less than 5 min) were physiologically feasible. In a first analysis of the kinetics of reactivation, we found that neither thiol oxidation nor disulfide re-shuffling appeared to be rate-limiting, but rather that rate-limitation appeared to be in peptide and sidechain folding steps. The system that promoted rapid regeneration of hen eggwhite lysozyme also gave rapid regeneration of bovine pancreatic ribonuclease and human lysozyme (Ahmed, 1969; Schaffer, 1970; Wetlaufer et al., 1973).

Now, using this lysozyme regeneration system, let us return to the question of nucleation. If exhaustive random trials are made of all possible peptide chain arrangements in the regeneration, then all of the possible pairings of protein sulfhydryls should exist in the intermediates between completely reduced and completely regenerated protein. The other extreme in the range of possibilities is that if nucleation occurs, only one folding pathway exists between the initial and final states, and only the four native sulfhydryl pairings will be found. An analysis of the intermediates early in the overall regeneration could readily distinguish between these two extremes. Figure 2 shows schematically the highly branched, but finally convergent set of folding pathways of the exhaustive random search model. Figure 3 shows the other extreme, a unique folding pathway.

The experiments outlined above have been carried out in my laboratory by Dr. Sandra Ristow (1972). Reduced lysozyme which has been regenerating for a short time is rapidly acidified and the unoxidized thiols blocked to further reaction by alkylation. The protein is then submitted to proteolysis, first by pepsin, then by chymotrypsin. The resulting mixture of peptides is then submitted to "mapping" by chromatography on paper followed by electrophoresis at right angles to the chromatography direction. The disulfide-containing peptides are visualized on the paper by a specific chromogenic reaction (Maeda et al., 1970). Control experiments with native lysozyme, which contains 4 disulfide bonds, gave a total of 10 -S-S- positive peptides under standardized mapping and detection conditions. The number of different intramolecular disulfide bonds that could form in a random search is 28. Since the proteolysis we used gave 10 peptides from 4 disulfides, we could reasonably expect 50-60 disulfide peptides from a random search mechanism. Lysozyme regenerated to an average disulfide content of 1/4, 1/3, 1/2, 1 and 1-1/3 -S-S- bonds showed, on mapping, 3, 3, 6, 10 and 7 disulfide peptides respectively. Thus the regeneration occurs by a limited search rather than by an exhaustive search of structures. A nucleation mechanism appears to be the only plausible way of reducing the number of structures searched. Figure 4 shows schematically how nucleation could lead to this result.

A reasonable next step is to identify the disulfide-containing peptides over the early time-course of regeneration, to locate if possible the nucleation region(s) and chart the course of the self-assembly. This work is currently in progress in my laboratory.

The approach just outlined appears to be applicable to the general class of -S-S- proteins to test whether nucleation is general in self-assembly. In a couple of years we may have extensive and substantial evidence to test this idea.

At this point the reader may be thinking, "Well, what of it? Would it really make any difference whether proteins self-assemble by nucleation or by random search?" It could make a great difference, both in the technology of protein isolation and purification, and in the physiological roles of proteins in vivo.

Technological issues

To help us discuss technological issues, consider Figure 5. On the far right of this figure, an exhaustive search of structures must arrive at the structure of lowest possible free energy (the global minimum). On the left, a nucleation event can restrict the number of structures searched, and lead along a particular folding pathway to generate a native structure which is in a local free energy minimum. Now this latter structure is metastable with respect to other structures which have lower free energy. However, metastability can have the following range of practical consequences. If there is a small activation energy the native structure will rapidly and spontaneously transform to a structure of lower free energy. In Figure 5 this corresponds to pathway (a) or (a′) and in the experience of the laboratory enzymologist it corresponds to an enzyme that rapidly loses activity. If there is a moderate activation energy, the enzyme may spontaneously lose activity over weeks or months [pathways (b) or (b′)]. If there is a very high activation energy for structural transformation [pathways (c) or (c′)], this corresponds to a very stable enzyme, which may undergo only a slight loss of activity over a period of years. It is worth noting that this last behavior is indistinguishable from that of a protein whose structure is in the global free energy minimum.

The practical protein chemist has had all too many experiences of spontaneous inactivation of a preparation he was working up. In his modesty, and under the influence of the conventional wisdom, he was inclined to believe that the activity was especially sensitive to trace contaminants or heat or alkali, or that some tissue protease was destroying his activity, or simply that he himself has been clumsy in some unknown way.

Now surely there have been numerous instances where one or another of these inactivation mechanisms was operative. But it is important to remember that for

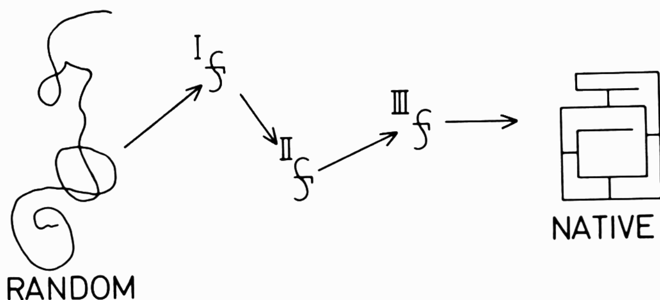

Fig. 3–Limited search mechanism for protein folding shows a single pathway for accumulating 3-dimensional structure and the set of native disulfide bonds. The symbol identity follows that of Figure 2.

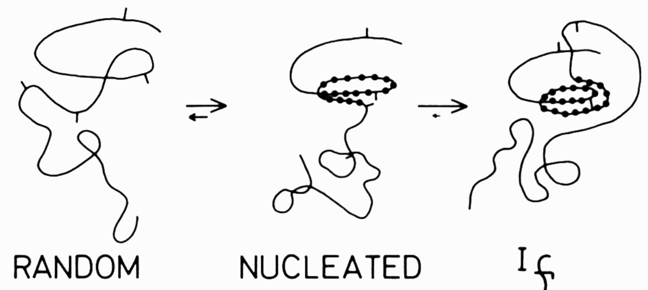

Fig. 4–Schematic mechanism showing how an early condensation (nucleation) event can juxtapose "native" cysteinyl residues and lead to a limited search of 3-dimensional structures and crosslink combinations.

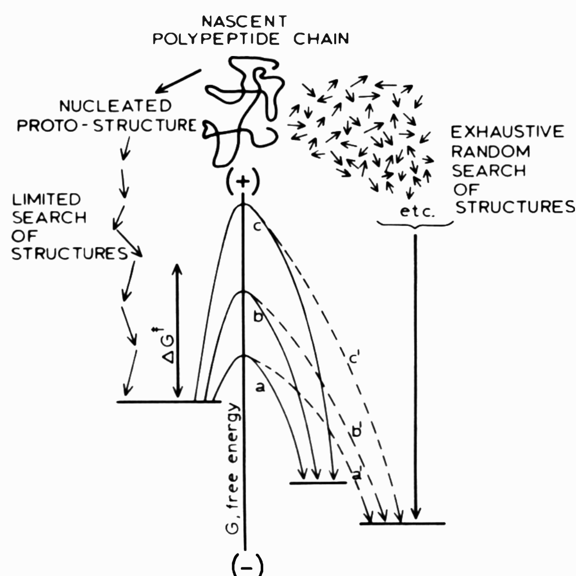

Fig. 5—Comparison of the random search mechanism (right) with the limited search mechanism (left) for protein folding. The vertical coordinate represents Gibbs free energy. The lower a state is on this coordinate, the greater the thermodynamic stability.

the perhaps two dozen proteins that have been reversibly denatured, many scores of protein preparations have steadfastly resisted attempts to revive a lost activity. (We emphasize that reversible denaturation is in no way evidence that the native structure is the most stable structure. When reversible denaturation occurs, we know that there is a favorable free energy change from the denatured to the native structure, and that kinetic barriers are sufficiently low for the transformation to occur in practical time. This is far short of knowing whether the free energy minimum of the native structure is local or global.)

In contrast with the conventional wisdom, our present perspective predicts that instability is the intrinsic character of many proteins. Under these game rules loss of activity is not always the responsibility of the investigator.

Physiological issues

Let us turn now to physiological issues. Recall, if you will, the watershed experiments of Schoenheimer (1942), who showed so clearly the rapid turnover of body proteins. This work, so elegantly confirmed and extended in recent years by Schimke and his associates (1969) makes it clear that most proteins (in mammalian tissues) are continually being degraded and resynthesized.

Since turnover is a fact, we should probably view a protein in vivo as having stability requirements only of the order of its characteristic half-time. What shall it profit an organism to build a protein for the ages if it will be degraded in a few days? There appears to be no survival advantage for the organism that has a particular protein in its global free energy minimum. On the contrary, such proteins might prove difficult for an organism to degrade, and excessive stability would be a liability.

SUMMARY

IN SUMMARY, I have tried to show (1) that the conventional wisdom has served us well for the questions it raised with only occasional exceptions; (2) that viewing one of the first functions of every protein to be its own self-assembly of 3-dimensional structure provides a coherent perspective for looking at more of the functions of proteins than we generally have with the conventional wisdom; (3) that we must recognize the requirement that self-assembly processes must occur in biologically feasible time; and (4) that we obtain a broader but still coherent view of the multiple functions of proteins after accepting that their synthesis and degradation operates under kinetic as well as thermodynamic constraints.

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SYMPOSIUM: Protein Interactions in Biosystems

SOME RECENT IDEAS ABOUT THE NATURE OF THE INTERACTIONS BETWEEN PROTEINS AND LIQUID WATER

MOST BIOLOGICAL macromolecules obtain their characteristics of maximum physiological importance on association with water. Proteins, for example, cannot be said to exist in the absence of a liquid water phase. It was shown many years ago (Reyerson and Hnojewej, 1960) that dry proteins are no more than a randomly crossed-linked dust through which water molecules readily diffuse when the water vapor pressure is low. As the vapor pressure is increased there comes a point at which enough water molecules are present to form a liquid water phase. When one measures the magnetic susceptibility of cytochrome c as a function of water, sharp transitions are seen to occur (Lumry et al., 1962) at critical vapor pressures when a stable water phase becomes possible. Water molecules move out of the polypeptide dust to form a bulk phase and oily groups are driven from the new water phase even though it is no more than a few layers deep around the protein and are forced to form clusters giving the familiar globular structure of proteins and the associated properties found in solution studies. Thus whenever we talk about "living" proteins we are, or should be, talking about water quite as much as about the polypeptide since the latter takes on its useful form only under the domination provided by liquid water. Much the same sort of thing must be true for nucleic acids and other biological macromolecules. This ubiquitous importance of water makes it a hub around which the wheel of biological research must be built. It makes water a dominant factor in the food industry so much so that a knowledge of the molecular details of the behavior of liquid water and its interactions with proteins, carbohydrates, lipids and nucleic acids will lead to steady improvement in the preservations of food and the maintenance of taste and texture as well as the synthesis of new food stuffs from inexpensive raw materials. Indeed one can anticipate a revolution in the entire food industry when these matters are understood in fundamental terms. Much progress has been made by ad hoc investigations and by a continuing high-quality but unsystematic attack on these problems. Unfortunately a very great amount of work on the most fundamental aspects of these problems is likely to be necessary before major qualitative changes in food processing and handling will become possible. Water itself is very far from being understood at any fundamental level and there are many controversies among water scientists about even the phenomenological behavior of liquid water. Since it is almost certain that the nature of the interactions of bulk and surface water with biological materials whether macromolecules or small solutes will only become clear after water itself is thoroughly mastered, there remains much work for those people interested in the biological roles of water. In the beginning we shall probably have to content ourselves with a set of phenomenological patterns of behavior and little detailed molecular description. One such pattern has been lurking in the background for many years (Lumry and Rajender, 1970). As we shall see, even its detection and confirmation has had associated several severe difficulties but there is no longer much doubt that the pattern exists and has a broad significance for water solutions.

Because the phenomenological pattern has thus far only been studied with proteins among the biological macromolecules, we shall concentrate on the protein-water interactions. Although x-ray diffraction studies of proteins have been an overwhelming success, one scarcely ever sees water molecules in the pictorial representations of such work. A few water molecules are found inside proteins bound through good hydrogen bonds to protein groups so that they become an integral part of the protein. X-ray evidence also suggests that water molecules occupy some cleft and hole areas in the absence of substrates or inhibitors so the energetics of the displacement of these water molecules must make important contributions to the thermodynamics of specific binding reactions. Still other x-ray evidence (A. Tulinsky, personal communication) demonstrates large regions of empty space not filled by water or anything else despite the fact that the normal opening and closing of protein conformations is relatively frequent. The absence of water in such holes and in regions where packing is poor is probably as interesting as the appearance of water in other regions but the understanding of their absence, which is a matter of thermodynamic stability rather than kinetics, must await a higher level of thermodynamic sophisitication.

The protein-water interface

The more general questions about the interactions which protein make at their surfaces with water are clouded by a confusion of experimental results only a few of which need be mentioned. Kauzmann (1959) has championed the idea that the nature of the water-protein interface is relatively simple, it being dominated by solvation of charged groups, accommodation of water over nonpolar areas in such a way as not to diminish the number of hydrogen bonds or the free volume upon which the entropy of the water strongly depends. To these we must add the filling of caves, i.e., surface irregularities into which water molecules must sit simply to fill up what would otherwise be a hole (Lumry and Eyring, 1954). But although this picture does seem to be that shown by Schoenborn's (1971) neutron-diffraction studies of myoglobin and x-ray diffraction studies of rubridoxin (Watenpough et al., 1971), it must be noted that there remain several basic problems about the degree to which we may think of a protein as the rock-like objects they appear to be in diffraction determination of structure. The picture of a rigid solid has classical authority which seems to be supported by x-ray diffraction results. However, the latter do show a considerable degree of diffuseness in scattering behavior near protein surfaces indicating that even in the crystal such side-chains as those of lysine or glutamic acid retain considerable freedom to move and thus to "mix" with water. In addition the index of diffuseness of the diffraction spots is very much higher for most protein crystals than for small crystals. These high values are probably not due to thermal effects per se but rather to the weak bonding of protein crystals, which allows considerable disordering of the lattice, and possibly to conformational variations of considerable significance. At present the

large values of the index can be attributed primarily to the disordering in the crystal since such few studies of the interiors of globular proteins as are available from nmr work, fluorescence and light absorption by buried protein groups or inhibitors bound to proteins indicate that indeed proteins in folded parts are rocklike or at least they have the mechanical consistency and rigidity of a hard wax or even stronger solids (*vide infra*). However, the possibility of considerable conformational variation from one molecule in the lattice to another cannot be eliminated.

Some protein structures like collagen fibers and muscle fibers may have a good bit of water actually built into the structure and as tightly packed as the polypeptide itself. The large interstitial hole in hemoglobin is too small to accommodate water in its normal liquid state. Water positions within this cavity must have been evolved in the same way that the remainder of the structure of hemoglobin was evolved. The rearrangements of this water accompanying function are thus likely to prove to be as important thermodynamically as the changes in the polypeptide so that the x-ray diffraction job will not be finished until this sequestered water and its changes during function have been described. Even then it is unlikely to be possible to attach numbers indicating the energetic importance of the changes for function any more than it is possible at current x-ray resolution to attach numbers for any type to change in the polypeptide.

Whether or not the conformational description of proteins provided by diffraction studies are adequate to the freely dissolved globular protein remains to be seen. Current opinion supported by some evidence suggests that the differences are slight. NMR relaxation studies, for example, show that most protein hydrogens and carbons are so firmly associated with neighboring groups that the rocklike character is retained as demonstrated by the existence of a slow tumbling time characteristic of a large firmly built molecule (Jardetzky and Wade-Jardetzky, 1971). Such studies on the other hand have yet to establish that there are no other folded conformations of about equal free energy. Proton-exchange studies not only provide some support for the latter, more complicated picture, but also show that unfolding and refolding are relatively frequent. Hence kinetic barriers to the interconversions among conformers are not usually restrictive (Woodward and Rosenberg, 1971a, b). Arguments based on similar reasoning were used long ago (Lumry and Eyring, 1954) to show that the folded conformations of proteins are the states of lowest free energy. Recent discussions of the need for nucleation and an ordered sequence of steps in folding do not seem to be required by the magnitudes of the negative entropy changes observed in polypeptide folding processes (Lumry, 1971). We can admit that the inside of the protein probably folds before the outside which is quite reasonable considering that the protein is rough lattice of polar and nonpolar regions and that the internal nonpolar regions are likely to form rather stable but distortable clusters which can undergo subsequent reorganization to satisfy hydrogen-bonding and other details of folding.

Water must be intimately involved in the folding process too. In fact, the major feature of the application of thermodynamic methods to proteins has been the revelation of a large heat capacity effect due to the interaction of oily side-chains and peptide groups with liquid water when the protein is unfolded (Brandts, 1969). About the only classification we now have for conformation changes is based on the amount of heat capacity change in the process (Lumry and Biltonen, 1969). A large positive heat capacity change is a clear indication of an increase in unfolding. A small overall heat capacity change but a slow reaction usually indicates a refolding process with little net change in exposure of oily groups and peptide groups to bulk water; a fast reaction without heat capacity change indicates a rearrangement of atoms without unfolding—the so-called "subtle change" which is probably of the type associated with specific function. The large heat-capacity effect in unfolding has several consequences of which the most interesting from the point of view of the food industry is that cooling is quite as effective in unfolding proteins as heating and produces the same unfolded state (Brandts, 1969). Thus those people interested in the preservation of food by low-temperature storage must bear in mind that unfolding and aggregation, which is essentially irreversible, can occur in low-temperature solutions and may even occur in ice as shown by studies of Brandts et al. (1970a) and Massey (1966) and their coworkers.

Returning to the protein-water interface and taking into account the possibility that the unfolded regions of proteins near the surface may be greater in solution than x-ray pictures of crystals indicate, we can examine the alternative to the relatively simple picture proposed by Kauzmann. This is the picture which surrounds the protein with a layer of icelike water. This view is an old one but is still accepted by some workers (e.g., Likhtenshtein, 1969; Likhtenshtein & Sukhorukov, 1965). Klotz (1958) has been the major spokesman for a more moderate view in which it is proposed that the protein induces a general ordering effect on some shells of water molecules around proteins. The x-ray diffraction data give no evidence for any extended icelike structures having any permanency but they do show in at least the case of carboxypeptidase A that there are slightly preferred positions for water oxygen atoms throughout the crystal lattice of this protein (Lipscomb et al., 1968). Thus even in the largest interstitial regions of the crystal the influence of the protein on water is felt. Similar influences appear to be exerted by the surfaces of clays and zeolites out to distances as large as 30Å (Berendsen, 1967) but in protein cases the thermodynamic consequences are probably minor since studies of the diffusion of small molecules and even polymers through the crystal (Low et al., 1956) demonstrate that diffusion is rapid. Such studies do not, however, exclude a few rigid layers of water next to the protein surface. The most interesting recent observations on the protein-water interaction due to Kuntz and coworkers (1969; 1970a, b) probably do provide information about these layers. Using nmr these investigators find that there is a considerable fraction of water about proteins and polypeptides which does not freeze at temperatures even below $-50°C$ in the sense that the nmr band widths are very much smaller than those expected for water protons held in a rigid lattice. The amount of water thus influenced by the protein is roughly 30% of the dry weight of the protein but it decreases with temperature as more water molecules are trapped in the true ice phase about the protein. Thus the protein or polypeptide prevents normal freezing rather than favors the formation of an icelike shell at least at temperatures below $0°C$. As indicated by band widths the amount of motion which remains at the low temperatures varies considerably with the hydrated solute but is always considerable relative to the motion in ice. Recently also Schachman and Edelstein (1967) studying the sedimentation behavior of hemoglobin as a function of salt produced data most easily interpreted to indicate a shell of water into which ions cannot penetrate so that the buoyant density of hemoglobin is less than would be predicted if the solvent composition in this shell were that of the true salt-containing solvent. However, such measurements like most indirect measurements of protein density and volume are complicated by the fact that changes in the density may be a result of changes in the populations of the two phenomenological species of water in the neighborhood of the protein. Most students of the behavior of water agree that for some strange reason water acts as though it exists in only two states, one of higher density than the other. This two-state picture of water (Davis and Jarzynski, 1972) is not quite thermodynamically sound but has nevertheless been verified repeatedly although each investigator appears to have

his own idea as to the description of the states. If the effect of the protein is to increase the local population of the lower-density phenomenological state (the same effect produced by lowering the temperature or adding monohydroxy cosolvents) the apparent reduction in density may be due to this change in population rather than to any change in the protein. All indirect studies of protein volume and volume change, many of which suggest a contraction of proteins on binding specific substrates, inhibitors or allosteric regulators, must be viewed in the same way since few methods distinguish this kind of change in water density from the change in protein density. Gurney (1953) has made the concepts of the A, B and C shells of solvent about solutes as well known as it is useful. However, for proteins, Figure 1, it is difficult to distinguish the A shell, those solvent moleulces dominated by the solute, from the B shell, which contains water molecules ambivalently attempting to respond to the demands of the protein and of the normal bulk water which exists in the C shell. Solvation of single charged groups may well correspond to A-type shells but the interesting aspects of protein hydration probably involve the B shell as suggested by the work of Kuntz (1972a, b) and Kuntz et al. (1969) even though these results are difficultly rationalized with the results of Schachman and Edelstein (1967). We can now turn to the phenomenon previously mentioned. It appears to be clearly associated with the distant limits of the B shells for small solutes and may lie in this solvent region for proteins.

Compensation of enthalpy changes by entropy changes

In the early fifties, Doherty and Vaslow (1952, 1953) observed that the binding of inhibitors to chymotrypsin produced negative changes in enthalpy and entropy. Closer examination of the enthalpy, ΔH°_{bind}, and entropy, ΔS°_{bind}, values obtained at different pH values (Lumry and Biltonen, 1969; Yapel, 1968) showed that the relationship was linear thus of the form of eq. (1)

$$\Delta H^\circ_{bind} = \alpha + T_c \Delta S^\circ_{bind} \qquad (1)$$

so that when the experimental temperature is equal to the slope constant, T_c, which we call the compensation temperature, exact compensation of enthalpy by entropy occurs leaving $\Delta F^\circ_{bind} = \alpha$. The intercept α in a plot of ΔH°_{bind} vs. ΔS°_{bind} is independent of pH so that at T_c the enzyme loses its ability to distinguish any pH effect in inhibitor binding. That is, although pH has a large effect on ΔH°_{bind} and ΔS°_{bind} and in this way reflects important interactions between inhibitor binding and pH variation, at an experimental temperature near T_c, the pH-dependent contributions to ΔH°_{bind} and ΔS°_{bind} compensate each other leaving ΔF°_{bind} pH independent. Not only was this found to be the case for the several inhibitors tested with α-chymotrypsin but all inhibitors appeared to have nearly the same linear relationship, eq. (1), (Yapel, 1968; Shiao, 1970; Shiao and Sturtevant, 1969) with similar T_c values. The ability of the protein to make quantitative distinctions among inhibitors becomes very poor at experimental temperatures slightly below room temperature since T_c is usually close to 13°C. We must note that at body temperatures the differences in ΔF°_{bind} are considerable and if we take a series of inhibitors in order of increasing affinity constant at 37°C, lowering the temperature to about 13°C produces a large loss in quantitative specificity followed by an inversion in the affinity order as we move to still lower temperatures. Yapel carried out his studies at high precision using a van't Hoff method and Shiao (1970) and Shiao and Sturtevant (1969) have confirmed most of his results calorimetrically, as is always desirable. A similar loss in pH dependence and specificity is indicated in inhibitor binding studies of ribonuclease A (Flogel et al., 1973; H. Ruterjans, personal communication), ribonuclease T_1 (H. Ruterjans, personal communication) and several pyridoxyl enzymes (Turano et al., 1971) to indicate the generality of the pattern. It must be realized that there is no thermodynamic basis for a linear relationship between experimental enthalpy and entropy changes and certainly none which requires that the proportionality constant T_c be the same or nearly the same from system to system. Likhtenshtein (1966) and Likhtenshtein and Sukhorukov (1963; 1965) have presented a large body of data of varying quality to suggest that this linear relationship is universal in protein reactions and Belleau (1966; 1967) has noted that similar behavior with a similar slope constant T_c occurs in small-solute reactions in water. Thus Belleau was led to suggest that the compensation pattern in protein systems was due to the same cause as that in the small-solute systems which appears to be some aspect of the behavior of water since linear enthalpy-entropy compensation has been clearly manifested only in water solutions; or to be more specific, in view of the extensive discussions of the phenomenon by Leffler and Grunwald (1963), it is only in water solutions that the linear pattern has been found with the same T_c value, which is usually 285°K plus or minus at most 15°. Thus Belleau's identification of water as the common source for compensation behavior in small solute and protein processes

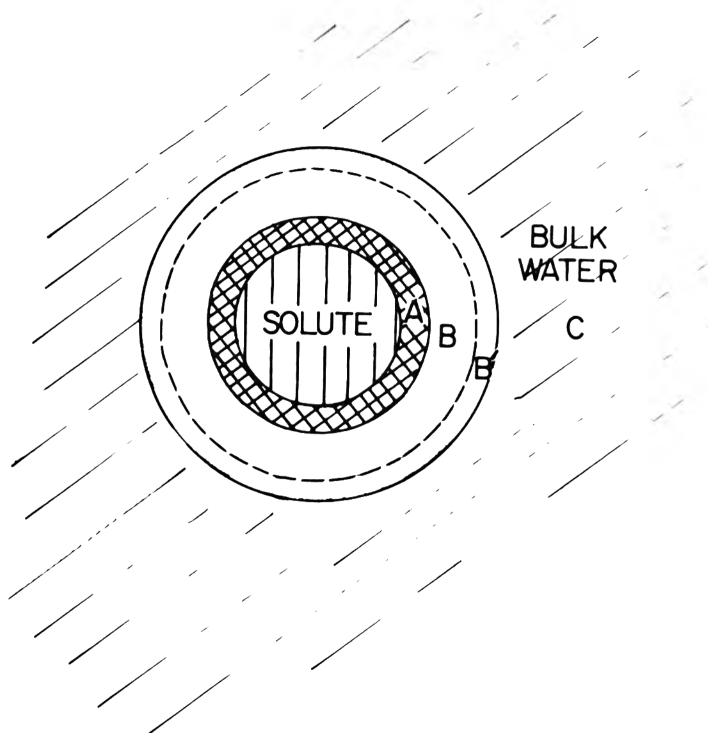


Fig. 1–Gurney's (1953) division of water into domains of different character. Usually applied to ions but the same picture now appears appropriate for all solutes in water solution including proteins. H_2O-to-D_2O transfer data collected by Arnett and McKelvey (1969) appear to demonstrate that compensation behavior is due to the outer part of the B shell, here labelled B'. (See text).

in water solution seems to be entirely reasonable. An examination of the literature about thermodynamic changes in small-solute processes in solution by Lumry and Rajender (1970) has shown that the pattern appears for many types of small-solute processes in water although it is often difficult to detect. Perhaps the most extensively studied examples are the ionization processes of congener series of weak acids in water solution. Consider as an example the series of substituted benzoic acids. Hepler (1963) and Ives and Marsden (1965) showed for series of this type that if one assumed the additional chemical contribution to the total free energy of ionization due to ring substitution to be a strictly inductive contribution to the enthalpy which they estimated using the Hammett σ parameter (Hammett, 1940), then the residual enthalpy contributions and the total entropy changes for the different substituted benzoic acids were linearly related with a T_c value of about 285°K. Thus far it is only in this type of experiment that the specific involvement of water has been included in the interpretation so that in a simple summary the linear compensation relationship is between solvation contributions to enthalpy and entropy. Thus for each member, i, of the congener family

$$\Delta H^\circ_{expt,\,i} = \Delta H^\circ_{induc,\,i} + \Delta H^\circ_{solv,\,i} \quad (2)$$

$$\Delta S^\circ_{expt,\,i} = \Delta S^\circ_{solv,\,i} \quad (3)$$

$$\Delta F^\circ_i = \Delta H^\circ_{induc,\,i} + \Delta H^\circ_{solv,\,i} - T\Delta S^\circ_{solv,\,i} \quad (4)$$

$$= f(\sigma_i) + \Delta H^\circ_{solv,\,i} - T\Delta S^\circ_{solv,\,i} \quad (4')$$

$$\Delta H^\circ_{solv,\,i} = T_c\,\Delta S^\circ_{solv,\,i} \quad (5)$$

The phenomenological compensation relationship is eq. (5). Then

$$\Delta F^\circ_i = f(\sigma_i) + (T_c - T)\,\Delta S^\circ_{solv,\,i} \quad (6)$$
$$= f(\sigma_i) \text{ at } T = T_c$$

T = experimental temperature

Many examples, especially transfer reactions in which small solutes are transferred from water to water made slightly dirty by the addition of cosolvents such as the monohydroxy alcohols or tetralkylammonium salts show the linear relationship without the complication presented by the need to accommodate the change in inductive effect, as above, or other "chemical" contributions. These have been reviewed elsewhere (Lumry and Rajender, 1970) but some general results are worth noting.

First of all, compensating pairs of ΔS and ΔH values can be obtained in small-solute processes by addition of cosolvents or by minor modifications in chemical structure to produce congener series. Second, the effect is seen as clearly in rate processes as in equilibrium situations, a very good example being the studies of the rates of hydrolysis of t-butylchloride studied by Robertson and Sugamori (1969) and the corresponding studies of hydrolysis of benzoylchloride by Hyne (1968). The latter author's data suggest that there is a characteristic linear relationship between volume change and entropy change when the linear enthalpy-entropy change appears but that is a matter for much additional study.

We should also note that in protein systems the linear pattern is seen in pH and salt variation and for chymotrypsin in the variation in competitive inhibitors. The most extensive studies of the phenomenon for protein systems are those of Beetlestone and Irvine (1965; 1969), Bailey et al. (1969; 1970a, b), Beetlestone et al. (1970) and Anusiem et al. (1968) who observed the pattern not only when pH or salt was varied in the ligand-binding reactions of a wide variety of ferrihemoglobins but also that species variations in these ligand-binding reactions disappear near 285°K regardless of ligand. Differences among binding enthalpies in hemoglobins on hydroxyl-ion binding at heme iron as great as 10 kcal/mole of ligand are nearly exactly balanced by corresponding entropy changes at 285°K. Despite the abnormality of the reactions being those of ferric rather than ferrous ion, the same pattern of behavior must attract our attention to some general basis of behavior. Just as with inhibition of chymotrypsin, species variations which are very marked at 37°C because enthalpy and entropy contributions do not balance, disappear just below room temperature and reappear in opposite order of affinity at lower temperatures.

The Beetlestone experiments are also discussed in detail by Lumry and Rajender (1970) although much has been added to the total picture since then. The linear relationship between enthalpy and entropy of binding of ligands (CN^-, N_3^- -SH, methylamine, imidazole, SCN^- and F^-) progresses toward more negative values of enthalpy and entropy as pH is increased up to a value near the isoelectric point. Further increases in pH produce a sharp turn-around behavior followed by decreasing negative values of ΔH° and ΔS° as pH is further increased. This "turn-around" behavior is obviously an important clue to the source of the phenomenon but it has not been explained in a satisfactory way as yet. Does turn-around behavior result from a maximum in total charge or a minimum in net charge? The effect although clearly involving charged groups of the protein, is still strongly present at even 1 M salt concentrations. Of even more importance the single change of a glutamate for a valine residue on the β chains of hemoglobin at points as distant from the heme groups as it is possible to get in hemoglobin produces large quantitative changes in ΔH° and ΔS° without loss of the compensation pattern even when salt concentrations are high. Clearly these observations indicate a close cooperation of the charged groups and make it most unwise to think of single charged groups as behaving with sufficient independence that they may be compared with their counterparts on small-molecule models. In short, among the many interesting consequences of the Beetlestone work is the conclusion that cooperation among charged groups, probably all of them on the protein, even at high salt concentrations is large and that single groups can never be safely treated as independent. This is a rather remarkable conclusion and one which may depend on linkage mechanisms extending through the protein conformation as much as electrostatic cooperation among charged groups. Whether or not it is general for globular proteins remains to be established.

Beetlestone and Irvine and their coworkers were led to conclude that the compensation pattern they observed with the several types of variation made in the ferrihemoglobin systems had its source in water since, like Belleau, they were familiar with the fact that the same pattern is observed in water solutions of small solutes. The quantitative basis for this identification is that the slopes of the compensation lines are about the same varying for small-solute systems and protein systems between extreme limits of 285°K ± 15°. Most examples lie between 293 and 270°K but experimental errors are almost always too large to make a precise distinction greater than ±5° in T_c.

Lumry and Rajender (1970) initially made a similar identification in their analysis of the compensation patterns produced by change in inhibitor or by pH variation. It is interesting to note that the rough data available at this time and shown in Figure 2 suggest that for the small family of ester substrates for α-chymotrypsin studied, both K_m and k_{cat} values appear to demonstrate the compensation pattern. Unfortunately the three substrates studied by Cohen et al. (1970) were studied with no attempt at high precision so that the lines shown may be accidental. The situation with substrates for chymotrypsin is complicated by the large errors involved. In their original paper Lumry and Rajender (1971) showed that all the rate and equilibrium constants for N-acetyl-L-tryptophanethylester obtainable by steady-state-kinetics study gave compensation

plots with mean slopes near 290°K. Corrections and improvements in statistical fitting of their data by Barksdale (private communication) have shown about the same T_c values for the E + S = ES and the ES = EA equilibria but the acylation and deacylation rate constants yield values well above 300°K which is consistent with neither the normal values of T_c nor the value of 520°K obtained by Martinek et al. (1972) from the compensation plot for K_{cat} in a systematic study of the N-acetyl-L-ester derivatives of tyrosine, tryptophan, phenylalanine, norleucine, norvaline and α-amino butyric acid. It is clear that we can say little more about this unique deviation from the normal T_c values until the systems have been more extensively studied. As recognized by Lumry and Rajender there has as yet appeared no basis for expecting compensation behavior in the bond rearrangements steps of chymotryptic catalysis or those supported by any other enzyme. The existence of compensation in such steps reaffirms once again the hazards associated with any attempt to assign the basis for enthalpy-entropy compensation a matter we shall now discuss in some detail.

Significance of the compensation pattern

Following the discussion of enthalpy-entropy compensation by Leffler and Grunwald (1963), Exner (1964) demonstrated that such patterns with apparent compensation temperatures near the lower experimental temperature were generally false but were to be expected to appear as errors because of the form of the relationship between ΔH and ΔS in terms of equilibrium or rate constants measured at two different temperatures. We believe Exner's analysis is extreme since standard chi-squares procedures for linearity testing should be entirely adequate to the problem but it is nonetheless true that many examples of compensation are false and appear for the reason given by Exner. In addition there are several other simpler types of error which give linear compensation plots with slopes about equal to the mean experimental temperature. Examples of the latter, some extreme, have been given by Likhtenshtein and Sukhorukov (1963) and by Eyring and Stearn (1959) but are undoubtedly very common. Good statistical testing is possible only with a small fraction of the examples of compensation behavior thus far found in the literature. However, there are some excellent examples, e.g., the studies of the solubility of argon in water containing various amounts of monohydroxy alcohols (Ben-Naim, 1972 and personal communication). Many of the protein examples also have the required precision level to make it certain that true examples of compensation occur. It is also to be noted that even when precision limits are lower than required for significant analysis, compensation may be present. In fact, one would have to conclude that a large fraction of the enthalpies measured in equilbria and rate processes in water solution are very badly in error if one is to explain away compensation as invariably due to errors of one type or the other. Thus we cannot take refuge in this easy solution and must examine the alternatives.

Chief among the alternatives, of course, is that the source lies in the most peculiar properties of water itself perhaps in readjustments of the concentrations of the two phenomenological species of water already discussed. In this connection it is important to note that very accurate data on the transfer of salts, noble gases, hydrocarbons, amino acids and several other types of chemical species from pure H_2O to pure D_2O manifests a compensation plot with slope of about 285°K (Arnett and McKelvey, 1969). This result establishes that H_2O and D_2O are quantitatively but not qualitatively different with respect to the existence of compensation but it also demonstrates, since ions, noble gases and pure hydrocarbons lie near the same average line without any adjustment for chemical differences, that compensation is due to water lying outside the range of the coulombic potential of the ions and thus no nearer than the outer part of Gurney's B shell (B′ in Fig. 1). Apparently specific solvation effects in the A shells are the same within error in the two kinds of water. It is observations such as these which have attracted the attention of water chemists to the compensation phenomenon and numerous attempts are being made to explain its occurrence in water at the moment. However, thus far all such theoretical attempts have produced results which suggest that compensation should occur but that the T_c value should be near the mean experimental temperature. This can be seen in a typical theoretical approach due to Ben-Naim (1972) but equivalent in fact with discussions given by Ives and Marsden (1965) and by Frank and Franks (1968). Consider a simple two-component system in which there is a change in one component. In this case A goes to B. Consider as an example that water exists in only two states and that the popula-

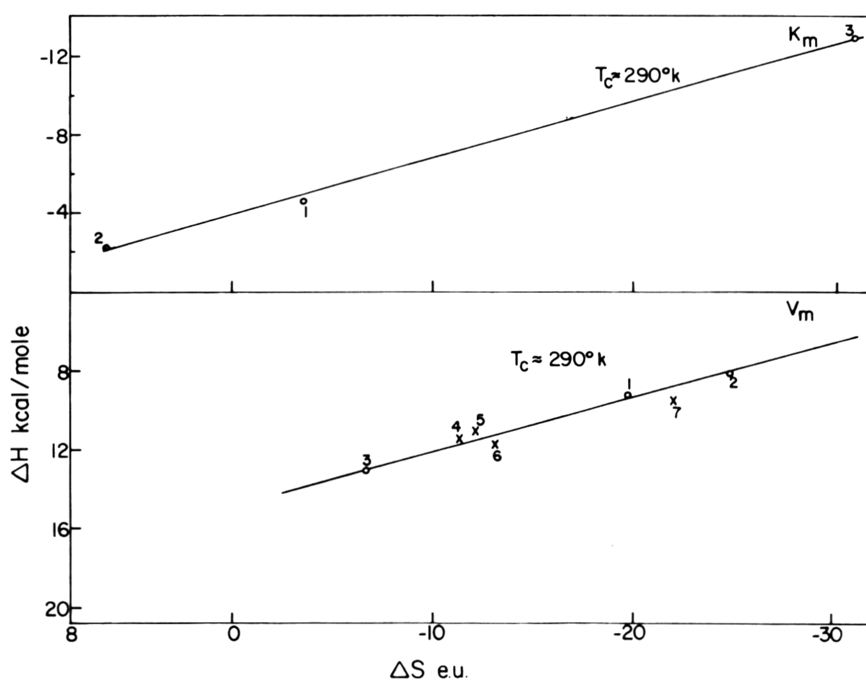
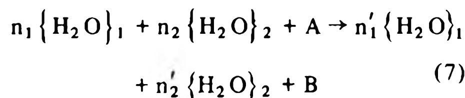


Fig. 2—Rough data suggesting that the "standard" linear enthalpy-entropy pattern appears in chymotryptic hydrolysis of some ester substrates and thus reduces the specificity to a minimum near 290°K in the same way that it reduces the specificity for competitive inhibitors. The substrates are: (1) N-acetyl-L-phenylalanine methyl ester (Cohen et al., 1970); (2) D-1-keto-3-carbomethoxy tetrahydroisoquinoline (Cohen et al., 1970); (3) Methyl-3,4-dihydro isocoumarin-3-carboxylate (Cohen et al., 1970); (4) and (5) N-acetyl-L-tryptophan ethyl ester (Rajender et al., 1971; Bender et al., 1964); (6) N-acetyl-L-tyrosine ethyl ester (Bender et al., 1964); (7) N-benzoyl-L-phenylalanine ethyl ester (Laidler, 1958); (8) N-benzoyl-L-tyrosine ethylester (Laidler, 1958). K_m and k_{cat} values for (1), (2) and (3) are reported by Cohen et al. (1970) as having low accuracy. Martinek et al. (Berezin, personal communication, to be published) find a linear compensation pattern but with slope near 400°K. (See text.)

tions of these two states are altered by the solute change.

Total Process:

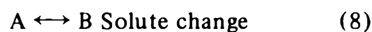


$$n_1\{H_2O\}_1 + n_2\{H_2O\}_2 + A \rightarrow n_1'\{H_2O\}_1 + n_2'\{H_2O\}_2 + B \qquad (7)$$

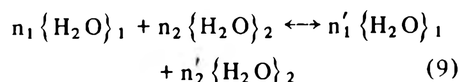
$\{H_2O\}_1$ = cooperative state 1 of water;

$\{H_2O\}_2$ = cooperative state 2 of water.

Part processes:



$$A \longleftrightarrow B \text{ Solute change} \qquad (8)$$



$$n_1\{H_2O\}_1 + n_2\{H_2O\}_2 \longleftrightarrow n_1'\{H_2O\}_1 + n_2'\{H_2O\}_2 \qquad (9)$$

Relaxation process of second component resulting from solute change, eq. (8) is eq. (9).

$F = F(n_1, n_2, n_A, n_B, T, P)$ but $n_1 + n_2 = n_1' + n_2' = n_w$, constant

and

$n_A + n_B = n_S$, constant. Hold T + P constant

$$\underbrace{\left(\frac{\partial F}{\partial n_B}\right)^{\circ}_{n_W,n_A,T,P}}_{\mu_B^{\circ}} - \underbrace{\left(\frac{\partial F}{\partial n_A}\right)^{\circ}_{n_W,T,P,n_B}}_{\mu_A^{\circ}}$$

$$= \underbrace{\left(\frac{\partial F}{\partial n_B}\right)^{\circ}_{n_1,n_2,n_A,T,P}}_{\mu_B^{*\circ}} - \underbrace{\left(\frac{\partial F}{\partial n_A}\right)^{\circ}_{n_1,n_2,n_B,T,P}}_{\mu_A^{*\circ}}$$

$$+ \left[\left(\frac{\partial F}{\partial n_1}\right)_{n_A,n_B,n_2,T,P} - \left(\frac{\partial F}{\partial n_2}\right)_{n_A,n_B,n_1,T,P}\right] \cdot \left(\frac{\partial n_1}{\partial n_B}\right)_{n_S,n_W,T,P} = -RT\ln\left(\frac{[B]}{[A]}\right)_{eq.}$$

$$- RT\ln\left(\frac{\gamma_B}{\gamma_A}\right)_{eq.\ cond.} = -RT\ln K_a \qquad (10)$$

(K_a = true activity equilibrium constant for total process)

Experimental quantity: $\overline{\Delta F}^{\circ}_{app}$

$$\overline{\Delta F}^{\circ}_{app} = -RT\ln\left(\frac{[B]}{[A]}\right)_{eq.} = \mu_B^{*\circ} - \mu_A^{*\circ} + (\mu_1^* - \mu_2^*)\left(\frac{\partial n_1}{\partial n_B}\right)_{n_S,n_W,T,P} + RT\ln\frac{\gamma_B}{\gamma_A}$$

For this system according to Gibbs, $\mu_1^* = \mu_2^*$; Hence

$$\overline{\Delta F}^{\circ}_{app} = -RT\ln\left(\frac{[B]}{[A]}\right)_{eq} = \mu_B^{*\circ} - \mu_A^{*\circ} + RT\ln\left(\frac{\gamma_B}{\gamma_A}\right)_{eq\ cond} \qquad (11)$$

$$\overline{\Delta H}^{\circ}_{app} = \left(\frac{\partial\left(\frac{\overline{\Delta F}^{\circ}_{app}}{T}\right)}{\partial\frac{1}{T}}\right)_{P,n_W,n_S}$$

$$= -\overline{H}_B^{*\circ} - \overline{H}_A^{*\circ} + R\left(\frac{\partial\ln\left(\frac{\gamma_B}{\gamma_A}\right)}{\partial\frac{1}{T}}\right)_{P,n_W,n_S} + (\overline{H}_1^* - \overline{H}_2^*)\left(\frac{\partial n_1}{\partial n_B}\right)_{P,n_W,n_S}$$

$$+ \frac{(\mu_1^* - \mu_2^*)}{T}\left(\frac{\partial\left(\frac{\partial n_1}{\partial n_B}\right)_{P,n_S,n_W}}{\partial\frac{1}{T}}\right)_{P,n_W,n_S}$$

$$+ \frac{1}{T}(\mu_{11}^* - 2\mu_{12}^* + \mu_{22}^*)\left(\frac{\partial n_1}{\partial n_B}\right)_{T,n_S,n_W,P} \cdot \left(\frac{\partial n_1}{\partial\frac{1}{T}}\right)_{P,n_W,n_S} \qquad (12)$$

$$+ \frac{1}{T}\left[\left(\frac{\partial\mu_B^{*\circ}}{\partial n_1}\right) - \left(\frac{\partial\mu_A^{*\circ}}{\partial n_1}\right) - \left(\frac{\partial\mu_B^{*\circ}}{\partial n_2}\right) + \left(\frac{\partial n_A^{*\circ}}{\partial n_2}\right)\right]_{n_S,n_W,T,P}\left(\frac{\partial n_1}{\partial T}\right)_{P,n_S,n_W}$$

$\overline{\Delta S}^{\circ}_{app}$ is found by using $\left(\frac{\partial\Delta F^{\circ}_{app}}{\partial T}\right)_{P,n_S,n_W}$.

By eliminating common terms from $\overline{\Delta S}^{\circ}_{app}$ and $\overline{\Delta H}^{\circ}_{app}/T$ we obtain $\overline{\Delta H}^{\circ}_{app}$ =

$$(\overline{H}_B^{*\circ} - T\overline{S}_B^{*\circ}) - (\overline{H}_A^{*\circ} - T\overline{S}_A^{*\circ}) + [(\overline{H}_1^* - T\overline{S}_1^*) - (\overline{H}_2^* - T\overline{S}_2^*)] \qquad (13)$$

$$\cdot\left(\frac{\partial n_1}{\partial n_B}\right)_{P,T,n_S,n_W} + RT\ln\left(\frac{\gamma_B}{\gamma_A}\right)_{eq\ cond} + T\overline{\Delta S}^{\circ}_{app}$$

Although $\mu_1^* = \mu_2^*$, $\overline{H}_1^* \neq \overline{H}_2^*$ and $\overline{S}_1^* \neq \overline{S}_2^*$.

However the condition $\mu_1^* = \mu_2^*$ requires that

$$\frac{\overline{H}_1^* - \overline{H}_2^*}{\overline{S}_1^* - \overline{S}_2^*} = T \qquad (14)$$

Rewriting the relationship between $\overline{\Delta H}^{\circ}_{app}$ and $\overline{\Delta S}^{\circ}_{app}$ using this requirement.

$$\overline{\Delta H}^{\circ}_{app} = (\mu_B^{*\circ} - \mu_A^{*\circ}) + RT\ln\left(\frac{\gamma_B}{\gamma_A}\right)_{eq\ cond} + T\overline{\Delta S}^{\circ}_{app} \qquad (13a)$$

The first two terms on the right are, of course, the experimental quantity, $\overline{\Delta F}^{\circ}_{app}$, and will in general vary with the variation in the independent variable used to produce the pairs of $\overline{\Delta H}^{\circ}_{app}$, $\overline{\Delta S}^{\circ}_{app}$ values required to test for compensation e.g., chemical change in a congener series, cosolvent additions, pH in an inhibitor-binding process. Consequently no compensation pattern will in general appear. We see, however, that the water relaxation process produces the relationship $(\overline{H}_1^* - \overline{H}_2^*) = T(\overline{S}_1^* - \overline{S}_2^*)$ which is a linear compensation relationship but is not usually detectable because of variations in $\overline{\Delta F}^{\circ}_{app}$. One must either be able to calculate the variation in $\overline{\Delta F}^{\circ}_{app}$ as Hepler (1963) and Ives and Marsden (1965) have for the ionization of weak acids or one must devise an experiment which will expose the contributions to the enthalpy and entropy from the water relaxation process. The latter appears to be the case for processes in which solutes are transferred from H_2O to D_2O without any chemical change. Such experiments are rare. Furthermore the value of T_c predicted by the theorem even in this case is the experimental temperature value which is not supported by the many experimental results which show T_c values near 285°K regardless of the experiment or the experimental temperature.

This theorem applies as written only to congener systems. Ben-Naim's own experiments used cosolvent additions and require a more complicated version. It is possible to suppose that the temperature behavior of the activity-coefficient term in $\overline{\Delta F}^{\circ}_{app}$ pulls the value of T_c to something lower than the experimental temperature in the theorem as given above. It is also possible to suppose that some of the terms in the more extensive expressions appropriate to Ben-Naim's own experiments have the same effect. Thus far these suppositions have not been examined in detail but we must not forget them.

As regards the relaxation part of the process the theorem is quite general so that although we have used the two states of water for the relaxation part of the total process, the theorem applies quite as well to any two component system and certainly to any three- or more component systems. As such it no longer restricts us to the consideration of water as the sole basis for compensation. Indeed we can substitute a change in the populations of protein conformers produced by inhibitor binding, pH or solvent modification for the relaxation of the water populations used in the above example of the theorem. This conclusion from the theorem immediately brings compensation due to the relaxation of one component under the influence of another into the family of the so-called "linear free-energy relationships." These have as their central characteristic that in a systematic change in structure of a parent molecule the ratio of change in enthalpy, $\Delta\Delta H$, for any process to the change in entropy, $\Delta\Delta S$, must equal the experimental tem-

perature (Leffler, 1965). It is thus possible that the true basis for linear free-energy relationships is the theorem given above or its variants rather than the usual explanation. According to the last, the nature of the partition function is such that changes in a process of one kind of degree of freedom for another will always produce a ratio $\Delta\Delta H/\Delta\Delta S$ about equal to the experimental temperature (Hepler, 1971).

Very few examples of compensation in nonaqueous systems with T_c values near 285°K have been reported but this may be simply due to the fact that water solutions have been so much more extensively studied. Transfer experiments which demonstrate compensation behavior in water-cosolvent systems and pure water are numerous but few such experiments have been carried out with all nonaqueous solvents. Those which we have found reported do not show compensation but Ben-Naim's theorem implies that the relaxation compensation phenomenon given by eq (14) should be as common in nonaqueous as in aqueous systems.

The more important matter is the fact that T_c values for compensation processes in water solutions usually fall below the mean experimental temperature (essentially the Hinshelwood "error slope") (Fairclough and Hinshelwood, 1937). It may prove that this behavior is also due to a shortage of data particularly for experimental temperatures well above room temperatures. A few examples exist in which experimental temperatures up to 70 and even 80°C have been used and these appear to show the same low value of T_c (see Lumry and Rajender, 1970). Unfortunately there are too few of this type of experiment and the precision is usually too low to draw positive conclusions.

For proteins recent developments have been interesting but not conclusive. In the first of these Weintraub et al. (1972) have found that additions of small monohydroxy alcohols to reaction mixtures of the enzyme $\Delta_{5,4}$-oxysteroidisomerase produce excellent compensation plots for the k_{cat} parameter, which is thought to measure proton transfer not involving water molecules. This is the first attempt to make comparisons between compensation patterns produced in small-solute reactions by addition of alcohol cosolvents with the effects of the same cosolvents on enzymic processes. The results are of considerable interest but the compensation temperature was always found to be 305 ± 5°K which is very close to the mean experimental temperature and considerably higher than T_c values for all reliable small-solute compensation processes we know of. The net result of these findings is that we cannot definitely relate compensation in the enzyme system to changes in bulk water even though other results of the experiments of Weintraub et al. (1972) suggest that the alcohol effect is an indirect one through the solvent rather than due to any influence of direct binding of alcohols on the k_{cat} process.

In the recent set of experiments Anusiem and Lumry (1973) confirmed by calorimetry the compensation pattern obtained with pH variation by Anusiem et al. (1968) for azide binding to human ferrihemoglobin using the van't Hoff method. Furthermore the compensation plot has been found to be closely correlated with the variation with pH of the magnetic susceptibility of the H_2O liganded (aquo) species. The susceptibility is found to be very sensitive to additions of t-butylalcohol. The results require the existence of at least two high-spin and one low-spin species for ferrihemoglobin in the aquo form and thus suggest that the compensation behavior may be associated with changes in the protein since the experiments show no direct binding of alcohols by the protein. Atanasov (1970; personal communication) has provided evidence for a similar complexity of substates or states in ferrimyoglobin but has not yet reported the existence of compensation behavior in this protein although Bailey et al. (1969) found such behavior with this protein. It should be noted that chymotrypsin has a number of substates and that recent evidence from studies of inhibitor binding increasingly suggests that at least one of

Table 1—Physical constants[a]

Substance	α Conditions and Ref (deg^{-1})	β Condition and Ref (atm^{-1})	$(\partial E/\partial V)_T$ From α and β (Atm)	
Water	≈ 2×10^{-4} deg^{-1} 20° (ICT)	≈ 5×10^{-5} 0 → 100°C (CRH)	1.2×10^5	20°
Ice	1.125×10^{-4} −7°C (ICT)	1.2×10^{-5} −7°C (ICT)	2.5×10^3	
Kerosene		7.7×10^{-5} 20° (ICT)		
Carbon tetrachloride (liquid)	1.27×10^{-3} 20° (CRH)	1.1×10^{-4} 20° (ICT)	3.5×10^3	
Ether (liquid)	1.66×10^{-3} 20° (CRH)	1.87×10^{-4} 20° (CRH)	2.8×10^3	
Pentane (liquid)	1.6×10^{-3} 20° (CRH)		3×10^3	
Hexane (liquid)		1.6×10^{-4} 25° (CRH)		
Amyl alcohol (liquid)	9.0×10^{-4} 20° (CRH)	8.9×10^{-5} 20° (CRH)	3×10^3	
Paraffin (solid)	5.9×10^{-4} 20°C (CRH)	9.0×10^{-5} 400 atm (ICT)	2.0×10^3	
Tin (solid)	6.9×10^{-5} 20°C (CRH)	2.0×10^{-6} 30° (ICT)	10×10^3	
Sodium chloride (solid)		4.2×10^{-6} 25° (ICT)	8.6×10^3	
Rock salt (solid)	1.2×10^{-4} 50° (CRH)			

[a] The very large values for the proteins must be in error if the compressibility estimate for RNase A is at all reliable. The heat capacity at constant pressure of RNase appears to lie between 0.35 and 0.42 cal/gram-deg or about 5300 cal/mole-deg. The relationship between C_p and C_v is $C_p - C_v = TV\alpha^2/\beta$. Using 5.7×10^{-4} deg^{-1} for α and the "upper limit" of 5×10^{-6} for β, $C_p - C_v = 5000$ cal/mole-deg so that C_v is about zero which is certainly very far from the case. We can nevertheless assume that C_v is very much smaller than C_p and this is consistent with the discussion of α given in the text.

the transitions between substates is involved in producing compensation behavior in inhibitor binding to this protein (Kim and Lumry, 1971; Fersht and Requena, 1971).

Quantitative characteristics of proteins

The most useful insight as to the true properties of substances are provided by quantitative values of such characteristics as the compressibility, $\beta = -(1/V)(\partial V/\partial P)_T$; coefficient of thermal expansion, $\alpha = (1/V)(\partial V/\partial T)_P$; internal pressure, $P_{int} = (\partial E/\partial V)_T$; heat capacity; C_p and C_v, Young's modulus, etc. Unfortunately this side of protein chemistry has been almost completely ignored. The few data which exist are quite uncertain but nevertheless deserve attention because they suggest that proteins are so different quantitatively from other substances as to be in a class by themselves. In the context of the current discussion the very tentative observations we can make are relevant to the role of water in determining the abnormal characteristics. It has long been a puzzle that proteins in reversible unfolding experiments produce practically no change in the volume of the total system although unfolding of the α-helix of poly-l-glutamic acid produces a volume contraction of 0.5–1 ml/mole of residues (Noguchi and Yang, 1963) and the immersion of oily side-chain groups in water produces contractions variously estimated from small-molecule models as 3–20 ml per mole of group (Kauzmann, 1959; Klapper, 1971). Instead of liters of contraction estimated from such models, the changes in unfolding processes are usually less than 100 ml per mole of protein and may be plus or minus. However, this is only one of the several puzzles which must be explained. Brandts et al. (1970b), have discussed many of these including several important ones we cannot go into here.

We shall list the factors which now seem to be implicated in establishing the physical characteristics of globular proteins in water solution, then using the data given in Table 1, we shall examine the peculiarities.

We have already mentioned the high density of proteins recently emphasized by Klapper (1971) and the question of a very large cohesive attraction as the basis for the solid-like character. To this we must add the interfacial free energy. In such cases as myoglobin which has a "surface" consisting almost entirely of polar groups, many of which are charged at pH 7, the interfacial free energies may be about equal to the water-water "interface" replaced by the protein-water interface. In general we expect the nonpolar groups in the "surface" to raise the interfacial free energy as will poor packing of water into cavities which produces an unusually large number of free hydrogen-bonding valencies or else leave holes which are also expensive in energy. The effects at the interface for proteins with such surface problems will follow the expectations from the LeChatalier Principle. Nonpolar groups will tend to be forced into the protein, cavities will tend to be squeezed shut and water molecules will tend to arrange themselves in such a way as to reduce hydrogen-bond rupture without undue loss in entropy. There is obviously a B sphere of the Gurney type for proteins in which water attempts to respond in an ambivalent manner to the demands of the interfacial region on the one hand and those of bulk water on the other. Major problems are to determine the size of this B shell, i.e., the distance out from the protein surface before true bulk-water properties are found, the nature of water in this shell and whether the size of the shell depends on protein volume or surface area as well as charge number and distribution, all formidable problems which have produced much controversy but little useful experimentation.

An additional water-dependent factor is provided by the work required to expand or compress bulk water and this effect, of course, has its counterpart in the work required to expand or contract the protein. Since we are concerned about equilibria at constant temperature and pressure, it is the free energy change in these processes which is important. Specifically for a change in volume ΔV in the protein the free energy change is $-\int_0^{\Delta V} \beta_p^{-1}\, d\nu$. For a change in interfacial area ΔA it is $+\int_0^{\Delta A} \gamma dA$ in which β_p is the compressibility of the protein and γ the interfacial free energy per unit area. The water compressibility, β_w, behaves in a remarkable fashion since it is nearly constant from 0 to 100°C, a property often attributed at least on phenomenological grounds to the change in populations of the lower-density (also low-temperature) species and the higher-density species.

When oily groups are inserted in water, there is usually a contraction in total system volume, an important characteristic first emphasized by Kauzmann (1959). This behavior, already mentioned several times, is especially important in showing that water, unlike nearly all other liquids, can accommodate a large fraction of the volume of small molecules in its free space (see Fig. 3). It is generally supposed on the basis of the somewhat

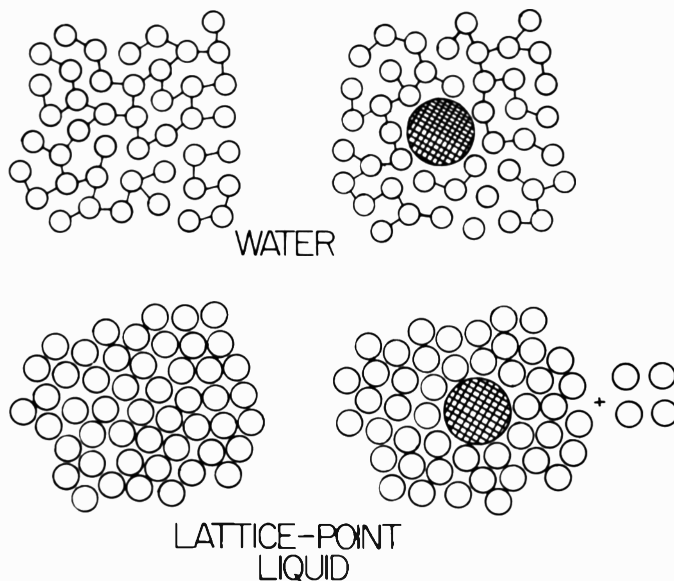


Fig. 3—How water accommodates nonpolar solutes. Weakly associating solvents like methane or carbon tetrachloride are called lattice-point liquids. To insert a solute it is necessary to make a hole by removing solvent molecules. In water, the most strongly three-dimensionally associated solvent, small bits of free volume are displaced to form a hole sufficient to accommodate the solute. At temperatures below 60°C few, if any, hydrogen bonds are broken, but the loss in free volume reduces the entropy of water. It is the entropy loss which restricts solubility. The picture has some generality. Nonpolar parts of molecules on insertion into water reduce the enthalpy of the system but reduce the entropy contribution to the free energy considerably more.

loosely defined concept of "structure-making and structure breaking" (Frank and Wen, 1957) that this free volume is available only in the lower-density state so that those solutes accommodated in water's free space favor the lower-density state. These rough ideas are well supported by the observations that small nonpolar, a-polar, polar-nonpolar molecules and even salts reduce the compressibility often by a considerable factor. This matter will appear again when we discuss the nature of the unfolded protein polypeptide in water. The question of interest at the moment is whether or not protein molecules, large and often very large compared with the small molecules studied for their effects on water behavior, can also expand into the free volume of the water or whether their expansion must show up as an increase in the total volume of the system. This question is not so simple as it may sound (cf. Fig. 4). If the change from state 1 to state 2 of water involves only a few water molecules, a position taken by Narton (1972) and Walrafen (1968), then cooperation among water molecules extends only a short distance in any direction and certainly no distance comparable with protein dimensions. On the other hand, if many water molecules are involved in the two-state process even though the effect per water molecule is very small, the range of cooperativity is significant with respect to protein size and part, perhaps a large part, of any protein expansion can be accommodated in the free volume of water without much increase in total system volume. Clearly it is necessary to establish which picture for the two states of water is the more nearly correct regardless of the fact that the two-state concept is a phenomenological construction. One also notes that proteins may prove very useful as probes in this study.

The most novel situation but not necessarily the wrong one would be that in which an expansion ΔV in the protein is entirely balanced by a contraction ΔV in the water. Then there would exist a simple Gibbs-Duhem relationship among the changes in γ, A, the free energy per unit volume of water and its total volume and the free energy per unit volume of the protein and its total volume (Lumry and Rajender, 1970). If part of ΔV produces an expansion of the total system, it must also be taken into account in this relationship. A rigorous relationship can be written in either case and suggests interesting possibilities. For example, addition of cosolvents to bulk water will produce changes in the free energy per unit volume of the water which must be balanced by changes in some or all of the other quantities listed above. Or the insertion of a substrate may change the free-energy per unit volume of the protein which would require another pattern of changes in the other quantities, a situation providing a means for direct participation of bulk water in the protein function and thus a potential water-based explanation for the enthalpy-entropy compensation process appearing in protein reactions. A number of possibilities inherent in the Gibbs-Duhem relationships have been discussed by Lumry and Rajender (1970) but it is impossible to examine them on a useful quantitative basis because of the paucity of values and the uncertainty of the values of the quantities which are involved. We can, for example, set an upper limit for the interfacial free energy by taking γ for the hexane-water interface (Chemical Rubber Handbook, 1969-70). This would give about 200 kcal/mole for the interfacial free energy of a mole of ribonuclease molecules but the estimate is probably much too high. Even a more realistic lower estimate would not be negligible but if the values of the physical quantities thus far estimated for the protein itself are taken into consideration, even very large changes in the value of γ or in the area of the interface are small relative to the free-energy changes associated with changes in protein volume. To see this we will use the data in Table 1 bearing in mind that our deductions are to be taken only as a tentative indication of the remarkable nature of proteins.

As shown in Table 2, the coefficient of thermal expansion, α, for the few proteins which have been studied is very large being similar in magnitude to those of very weak liquids like pentane and ether and very weak solids like paraffin but not at all like good solids, e.g., tin, NaCl or even ice (Table 1). Water itself has a considerably lower value which can be rationalized in terms of population changes in its two phenomenological states.

The compressibility of ribonuclease A on the other hand is that of a good solid, e.g., NaCl judging from the single estimate made by Brandts et al. (1970b) from the density measurements as a function of pressure carried out by Fahey et al. (1969). The latter authors could not detect a reduction in volume due to the ribonuclease A itself over their pressure range so that the compressibility value in Table 1 is based on their error estimate and is an upper limit. It is possible that some solid polymers have similar characteristics but we have not found data so indicating and the α values for solid polymers are considerably smaller than the protein values given in Table 2 (Polymer Handbook, 1966). It is also interesting that ice is quantitatively quite different from protein although it is dominated by hydrogen bonds, as to some extent proteins must be, and does not have peculiarities of changes among states of different volume such as water manifests.

The internal pressure is an interesting but not always directly useful parameter. It will be noted in Table 2 that the internal pressure computed from the α and β values for the protein using the equation

$$P_{in} = (\partial E/\partial V)_T = T\,\alpha/\beta - P_{atm}$$

is higher, possibly higher by a factor of ten, than the P_{in} values for tin and NaCl. Thus the α and β estimates suggest that as regards compression and expansion globular proteins resemble good solids but their volume response to temperature is that of very poor liquids. More specifically the free energy change with volume at constant T which is measured by $\beta^{-1} = -(\partial F/\partial V)_T$ indicates very good packing

Table 2–Physical constants for proteins in water solutions[a]

Compressibility			
β : Ribonuclease A (water solution 25°)		$<5 \times 10^{-6}$ atm^{-1}	Brandts et al., 1970
Thermal expansion coefficient			
α : Protein	t°C	$\alpha \times 10^{-3}$ deg^{-1}	References
Chymotrypsin	3.4	1.51	Zimmer, 1971
Dimethionine sulfoxide chrmotrypsin	3.6	1.12	Zimmer, 1971
Denatured chymotrypsin	3.4	1.81	Brandts, 1969
Ribonuclease A	20	0.65	Holcomb and Van Holde, 1962
Denatured RNase A	20	2.59	Holcomb and Van Holde, 1962
Ribonuclease A	20	1.15	Cox and Schumaker, 1961
Bovine plasma albumin	20	0.5	Hunter, 1966
Bovine mercap. albumin	20	0.5	Hunter, 1966
Human mercap. albumin	20	0.5	Hunter, 1966
Egg albumin	20	0.57	Pollard, 1964

[a] Internal Pressure, from $\beta = 5 \times 10^{-6}$ atm^{-1}; $\alpha = 5.7 \times 10^{-4}$ deg^{-1}; and $P_{int} = 3 \times 10^{4}$ atm.

with little free volume in the protein or at least with little ability to adjust to reduce any such free volume. The indication of tight packing is consistent with Klapper's (1971) discussion on the high density, the finding by Brandts and Kaplan (1973) that buried chromophores have only weakly broadened spectra compared with the spectra in liquid solution, and with the ideas of solid-like binding (i.e., extreme loss of vibrational and librational freedom as well as rotation and translation freedom) of substrates and inhibitors to proteins as recently examined by Page and Jencks (1971). Nevertheless new experiments are necessary to establish the nature of the protein as a solid, the generality of the solid model and the variability in degree of solidity, which is probably considerable, from point to point in any given protein.

For the proteins the low values for the coefficient of thermal expansion, which is equal to $-(1/V)(\partial S/\partial P)_T$, are also very intriguing since their source is also not at all obvious. Taken together with the low compressibility values the α values tend to suggest that some of the peculiarities may be due to water. An alternative explanation is that the internal pressure is so high that the usually weak force constants associated with van der Waal's contacts, i.e., those of the vibrational modes due to the bonding produced by weak secondary interactions, are forced to high values. Then small decreases in pressure producing small expansions will produce great weakening in these force constants which in turn produce large increases in entropy since the weaker vibrational modes are responsible for most of the entropy of proteins. The latter alternative does not explain why the internal pressure is so great and is thus as puzzling as the alternative based on water.

The compressibility is the most relevant quantity since it is the only one of the three physical parameters simply related to free energy and the values thus far estimated for proteins suggest excellent packing, low free volume and apparently little change in protein volume in protein processes. There are increasing numbers of reports of protein expansion and contraction on binding of substrates, inhibitors or "allosteric effectors." The actual volumes involved are invariably measured indirectly so that it is usually not possible to separate true changes in protein geometry from associated changes in nearby water volume. However, claims of actual change in protein volume are not readily consistent with the β value since if we make the poor but order-of-magnitude estimate that the compressibility of proteins is independent of volume for small enough volume changes, a value of β for RNase A of -5×10^{-6} atm means a change of 5 kcal of free energy per ml of volume change near the equilibrium volume and thus for a 3% change in volume of this protein the free-energy change would be 1500 kcal/mole. Even if the compressibility value is revised upward and the expansion of a protein found to fall off very rapidly with volume increase, some hundreds of kilocalories of free energy must be associated with changes in volume of only a few percent. As yet there has been no indication that such large free-energy changes are associated with specific or nonspecific binding processes of proteins. Nor, for that matter, is there any apparent way for other part processes of the total binding process to provide or utilize this much free energy.

Brandts et al. (1970b) have pointed out in their discussion of the effects of pressure on proteins that some factor of major importance is missing in our understanding. The high compressibility is the most direct manifestation of the missing factor. The high compressibility is also inconsistent with recent plotting of the internal spatial distribution of $-CH_2-$ and CH_3- groups and polar groups, mostly backbone, for carboxypeptidase A by Kuntz (1972c) which show large "puddles" and layers of hydrophobic materials distributed among hydrogen-bonded structures of the main chain. The density he calculated for these regions from x-ray coordinates is about 0.9g/ml and thus consistent with the provisional value of β but less consistent with the values for α given in Table 2. The α values suggest that the mechanical and thermal properties should be dominated by the soft hydrophobic regions of a protein. The small volume change on unfolding proteins in water, the very low β value, which appears to be a sound upper limit, and the density discussion given by Klapper clearly convey a completely different picture. The two pictures might be made consistent if the polar groups form an unusually strong skeleton. It is true that the hydrogen bonds formed by these groups have considerably higher force constants in media of low dielectric constant and that the dielectric constant at points well removed from the surface of the protein must be quite low, but the associated lower free-energy of the hydrogen bonding is exactly balanced by a positive free-energy contribution which occurs in folding due to the replacement of water molecules around the hydrogen bonds by the nonpolar groups; that is, by the very process of reducing the dielectric constant at the hydrogen-bonds. Thus we can explain a strong skeleton and a somewhat higher overall density but we cannot explain the low value of β since β^{-1} is a measure of the free energy change with volume and the density gain in polar regions is unlikely to be sufficient to overbalance the density of the nonpolar regions which Kuntz computes.

We have already mentioned that unfolding to the hydrated polypeptide also

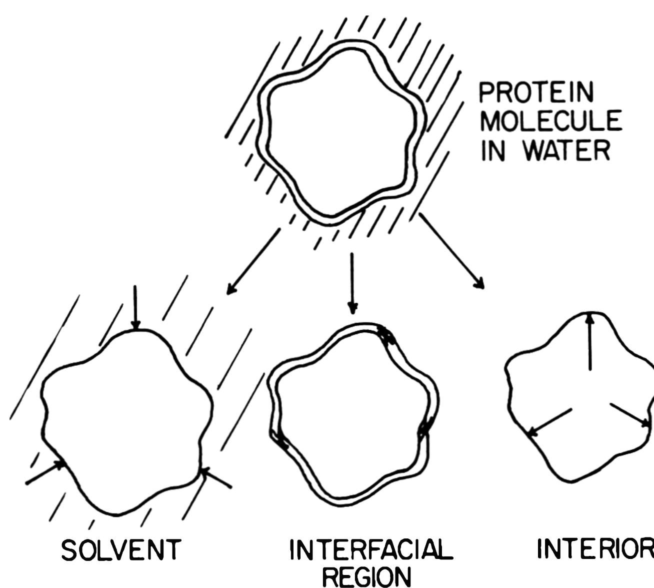


Fig. 4—The balance of forces of a protein in water. Compression by bulk water is shown on the left. Compression due to an unfavorable interaction of interfacial water and protein surface is shown in the middle. The cohesive and repulsive forces within the protein provide a balancing outward force shown on the right.

produces a rather remarkable situation. Brandts et al. (1970b) were able to obtain a reliable value for the increase in β in the reversible unfolding of RNase A and found it to be only $+1.5 \times 10^{-6}$ atm^{-1} so that the compressibility of the hydrated net of unfolded polypeptide is estimated to be less than $+6.5 \times 10^{-6}$ atm^{-1}. Hawley (1971) estimated a change of $+0.8 \times 10^{-6}$ atm^{-1} for the same process in reasonable agreement considering the assumptions and differences in conditions. For unfolding of chymotrypsinogen A, Hawley estimated $+1.24 \times 10^{-6}$ atm^{-1}. The high value of the compressibility for unfolded proteins is psychologically, but only psychologically, easier to accept since, as already mentioned, the addition of polar and nonpolar small molecules as well as salts reduces the magnitude of the compressibility of water and we are accustomed to accept water as abnormal. The small changes in volume reported for unfolding processes have been rationalized on the basis that there are errors in the use of small hydrophobic models for the desolvation of oily side chains of proteins but we see that although there may be such errors, it is really the abnormally low compressibility of the protein and the equally abnormal low compressibility of the hydrated polypeptide which are responsible. The small free-energy changes which appear in unfolding, e.g., 5–25 kcal for many small proteins are consistent with this view.

CONCLUSIONS

THESE OBSERVATIONS bring us back to water and point up the confusion which exists. The relatively high constant compressibility of water when combined with its high and highly variable thermal expansion coefficient suggests that water plays a minor role in determining the properties of proteins. Water appears to be too easily compressed itself to act as an effective source of compression on the protein and it clearly plays such a secondary role in the free-energy changes associated with protein volume changes, if the protein β and α estimates are roughly correct, as to be an almost negligible factor relative to the protein itself. In our RNase example the 3% expansion would produce only a 150 kcal/mole contribution to the free energy from the compression or expansion of the water if all the protein volume change is accommodated by change in water volume. However, this discussion has taken us rather far afield from the original problem which was the source of the compensation pattern. Even though we have had to assume that changes in protein volume involve large free energies and we cannot explain why this is so in terms of cohesive energy, compression by water or a high interfacial free energy, if volume changes do occur in protein function, they can, under the condition that the cooperative regions in water are large, produce changes in water of the same kind produced by changes in small solutes in water solution and thus produce the compensation pattern in the same way. Thus protein function may be linked to bulk water through volume change. Similarly changes in protein surface properties can be linked to bulk water.

In conclusion we observe that this discussion has covered many puzzling problems. Almost none of the alternatives given for explanations of compensation behavior or to rationalize the abnormal values of α, β and P_{int} is even remotely consistent with first impressions based on familiar small-solute systems but familiarity does not necessarily imply accuracy and it is probable that many traditional views about the nature and behavior of small-solute solutions in water will undergo dramatic change when water becomes truly understood. We may then be able to establish just how water fits into the biological picture. Are proteins really so insensitive to water as our discussion would suggest? It hardly seems possible but perhaps we have fixed our attention too much on the real or apparent abnormality of the protein. Compressive and interfacial effects of water, despite a relative unimportance in terms of thermodynamic changes as compared with the new picture of the protein which has developed, nevertheless could easily produce contributions to enthalpy, entropy and free energy of the size actually measured in protein experiments. We can suspect that this is the case and that the protein properties will turn out to produce less dramatic contributions than we have estimated. Even for such large structures as mitochondria, grana and lipid bilayer membranes with their associated surface material, there is much evidence to implicate water as a major determinant of properties and behavior. It seems quite possible that bulk and interfacial water determine the permeability, viscosity and density properties of these bodies but it is quite clear at present that we cannot casually assume this to be the case. In fact, one must work on a foundation of existing facts which suggests that these bodies like globular proteins may be so dominated by their cohesive forces as to make the role of water very secondary in importance. The purpose of this paper has been to show that water may play a direct role in the physical state and function of most biological macromolecules, a role revealed by the enthalpy-entropy compensation pattern. The message of the paper is obviously quite different. That message is that too many of the most important kinds of experiments have been put off too long. We can look at the x-ray diffraction pictures of proteins forever without improving our understanding of proteins as chemical species and we can never verify ideas about reaction mechanisms drawn from such pictures or from any other type of currently popular experimental approach until basic information on the physical properties and the quantitative characterization of the forces holding proteins in folded forms are determined in difficult experiments. Unfortunately such experiments still have very little popular appeal.

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SYMPOSIUM: Protein Interactions in Biosystems
PROTEIN-LIPID INTERACTIONS

INTRODUCTION

INTERACTION between proteins and lipids is a major field of biochemical research. Most of the work has concentrated on cellular and subcellular structures (especially membranes), on blood constituents, and on model system interactions in aqueous solution. The status of this research has been the subject of numerous conferences, reviews and books. A recent book edited by Tria and Scanu (1969) is particularly instructive.

This paper will present:

(a) Current ideas about mechanisms of lipid protein interactions in model systems, in tissues and in selected food systems;

(b) The role of protein lipid interactions in changes in food processes;

(c) The status of current research on interactions between oxidizing lipids and proteins.

NATURE OF LIPID PROTEIN INTERACTION

Types of bonding between protein and phospholipids

There is no single type of bonding by which lipids interact with proteins. In most isolated natural lipid protein complexes, the lipoproteins contained phospholipids in their lipid constituents; two types are shown in Figure 1. Phospholipid interactions with proteins may involve electrostatic forces. Electrostatic binding can arise from attraction between the negatively charged phosphate group and a positively charged protein group such as lysyl or guanidyl residue. It may also involve a positively charged group in the phospholipid (for instance choline) and a negatively charged residue (for instance aspartyl). A related mode of binding is the formation of salt bridges via divalent metals such as calcium as shown in Figure 1. Chapman (1969) suggested that simultaneous and cooperative occurrence of electrostatic binding and of salt bridges is operative in bacterial cell walls.

Characteristics of different types of intermolecular forces are shown in Figure 2. Of these, covalent binding is the excep-

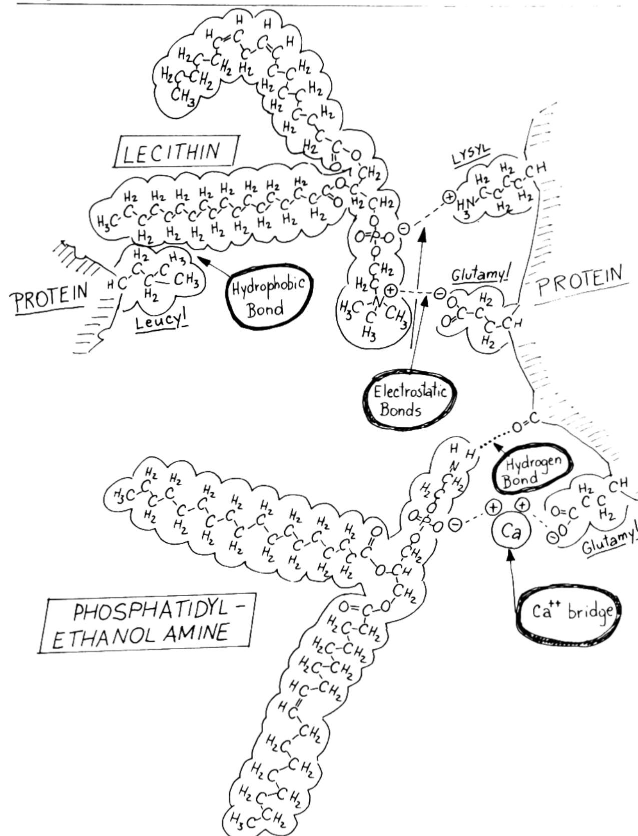


Fig. 1—Possibilities for phospholipid-protein bonds.

TYPICAL BOND ENERGIES FOR DIFFERENT LIPID PROTEIN BONDS

BOND	ENERGY (kcal/mole)	DEPENDENCE ON DISTANCE (r)
COVALENT	30 - 100	MAX. ATTRACTION AT 1-2 Å
ELECTROSTATIC	10 - 20	ENERGY ∝ r^{-1}
HYDROGEN BOND	1.5 - 6	MAX. ATTRACTION AT 2-3 Å
VAN DER WAALS	0.5 - 2	ENERGY ∝ r^{-6}

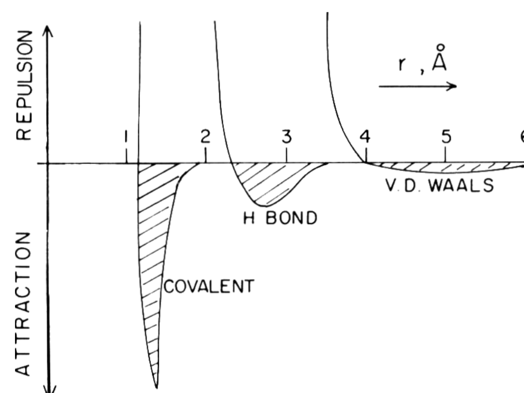


Fig. 2—Typical bond energies for different lipid-protein bonds.

tion rather than the rule and hydrogen bonding is also secondary in lipid-protein complexes, although it is indirectly important in hydrophobic bonding. Nonpolar dispersion or Van der Waal forces become important when interacting groups are near. These forces are weak, but the attraction between nonpolar groups is greatly increased in aqueous media where they are "pushed together" by water because the water-water interaction by hydrogen bonding is much stronger than the interaction between water and the nonpolar groups. This phenomenon based on "hiding" nonpolar groups from water has been called "hydrophobic bonding." The total standard free energy of formation of the hydrophobic bond is larger than the energy due to Van der Waal forces alone because changes in the water structure contribute to this free energy of formation (Scheraga, 1963).

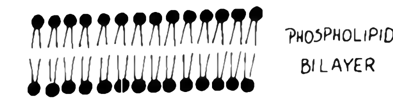
Interfacial energy in lipid-water systems

As a consequence of differences in attractive forces between molecules of different liquids, or differences in molecular density between phases, surface molecules possess extra energy, the free surface energy. Water surface in contact with its vapor or with air has a free surface energy (or as it is also called, surface tension) of 72 $ergs/cm^2$. The presence of amphipolar molecules including polar lipids and proteins lowers surface tension as shown in Figure 3, due to adsorption and orientation of these molecules in the surface; molecules of this type are termed surface active, or surfactants. Typically, this adsorption shows three phases with increasing concentration:

(a) Saturation and orientation in the surface;

(b) Region of constant surface excess (surface excess is approximately equal to surface concentration) but increasing bulk concentration. In this stage the decrease in free surface energy is proportional to the logarithm of the surfactant concentration;

(c) Region of micelle formation.



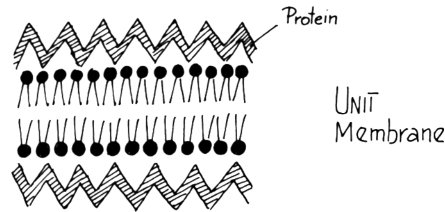


Fig. 4—The "unit membrane" concept of biological membrane structure.

The formation of micelles as well as orientation of amphipolar molecules at interfaces between water and air or between water and oil are of key importance in many colloidal phenomena, including emulsion stabilization, foaming and solubilization of otherwise insoluble compounds within micelles (Davies and Rideal, 1963). For each type of amphipolar molecule, a critical micelle concentration range exists, and a very comprehensive tabulation of these was published recently by the National Bureau of Standards (Mukerjee and Mysels, 1971).

Micelle formation and the adsorption of amphipolar molecules at oil-water interfaces are closely related to two important phenomena:

(a) Biological membrane models

(b) Membrane and films stabilizing food emulsions.

Evolution of concepts of biological membrane structure

The formation of phospholipid bilayers is a well established phenomenon in model systems, and the phospholipid leaflet or bilayer was the earliest widely

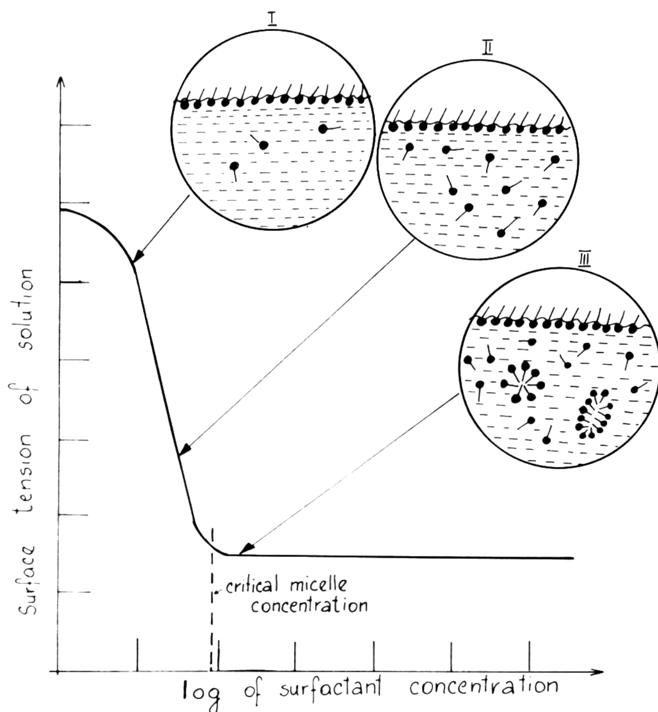


Fig. 3—Adsorption of amphipolar compounds at surfaces of aqueous solutions, and the formation of micelles at critical micelle concentration.

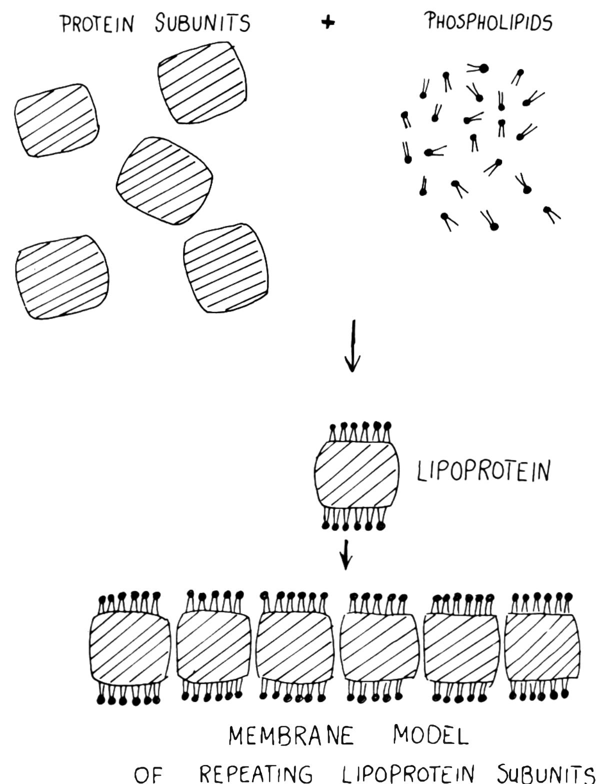


Fig. 5—The "globular" membrane model.

accepted model of cell membrane structure (Castleden, 1969). From it grew the "unit membrane" concept of Robertson shown in Figure 4. The unit membrane consists of a phospholipid bilayer with the polar groups of the phospholipids oriented outward and bound by polar interactions (probably electrostatic) to proteins in extended chain or β-configuration (Tria and Scanu, 1969; Pethica, 1969; Tria and Barnabei, 1969). Extensive work on myelin seemed to confirm this concept and it received wide acceptance in the fifties. In the sixties, however, the concept was severely attacked by many investigators who found major contradicitons between this model and new experimental findings. In particular, electron microscopic studies on a number of membranes were consistent with a globular rather than lamellar membrane (Sjostrand, 1969; Vanderkooi and Green, 1971). In addition, studies showed that the protein associated with the membranes is at least in part a α-helix conformation rather than in β-configuration (Singer and Nicolson, 1972).

These observations led to a globular membrane model advanced primarily by Green and his coworkers at Wisconsin and resulted in a decade of often heated scientific controversy (Tria and Scanu, 1969; Anonymous, 1970). The major features of the globular membrane model are shown in Figure 5. One should notice that in this model the membrane consists of repeating lipoprotein units, one layer thick, in which the association of lipids with the protein is a prerequisite for membrane formation, since it prevents the protein units from forming three-dimensional aggregates.

Some very recent thought has combined the bilayer and the globular concepts. Two versions of this concept are represented schematically in Figure 6.

The protein-liquid crystal (Vanderkooi and Green, 1971) proposes a double layer of globular protein subunits with phospholipid located between the globular subunits of protein in such a way that polar as well as nonpolar interactions are possible between the protein and the phospholipids. The thickness of the membrane is now determined by protein and the amount of lipid filling the spaces between the proteins can vary. The membrane is considered in constant thermal motion in the plane of the membrane.

The model currently most in vogue is the fluid mosaic model (Singer and Nicolson, 1972). In this model the phospholipid bilayer constitutes the continuum of the membrane forming, as it were, a "sea of lipid." Within the bilayer, however, various protein interactions can occur as shown schematically in Figure 6. Proteins can be present as globular, α-helix containing units, or in unfolded β-configuration. They may interact with lipids by polar or by hydrophobic bonds, and individual subunits, or their complexes may penetrate across the membrane. Singer and Nicolson consider the system to be a fluid mosaic and membrane properties may change drastically with various chemical and physical influences. In particular, this model allows the protein-lipid complexes to form either hydrophilic or hydrophobic "gates" allowing transport. This concept of a dynamic membrane is also stressed by Wolman (1970).

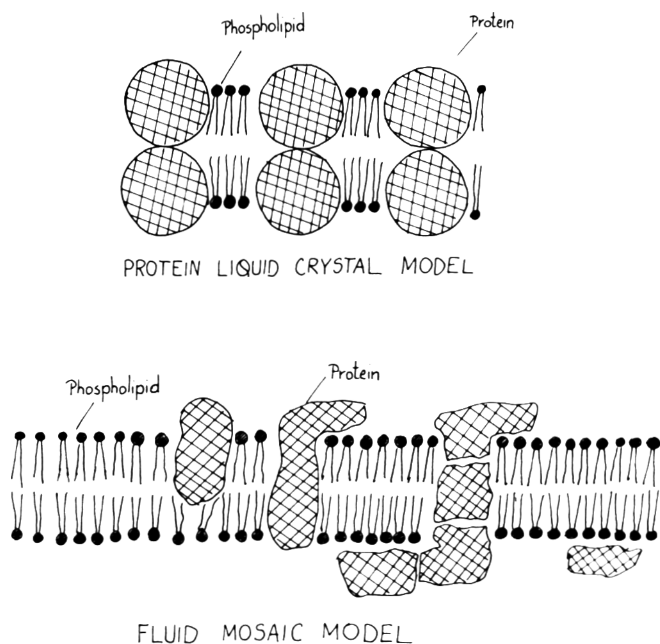


Fig. 6—Some recent models of membrane structure.

We should mention here a school of thought which denies the importance of membrane structure in transport in and out of cells. This group, represented by Ling et al. (1967), feels that transport is controlled by structure of water within the cell.

LIPID-PROTEIN INTERACTIONS IN FOODS

LIPID PROTEIN complexes are important in properties and behavior of many food types. The nature of the interactions involved, however, is only imperfectly understood. The present discussion will be limited to some selected examples.

Lipid-protein complexes in muscle-derived foods

Protein changes in meat and fish products cause physical changes which determine the eating qualities and functional properties of these products. Most workers sugest that lipid-protein complexes are involved in the following situations:

(a) In aging of meat, and in subsequent treatments the changes in the lipoprotein membranes, particularly with respect to their selectivity to ions, may noticeably affect various biochemical processes which result in the so-called resolution of rigor.

(b) In stored frozen and dried muscle-derived foods, peroxidation of lipids can lead to various interactions with muscle protein, in particular with myosin. This will be discussed in detail later.

(c) Fish is particularly sensitive to storage changes in frozen storage, and also poorly retains its quality of freeze-dehydration. Most workers believe that these changes are due to protein-protein interactions. However, a school of thought attributes the loss of protein solubility to changes in lipoprotein complexes.

Dyer and Fraser (1959) for instance, proposed that the effect may be due to greater sensitivity to denaturation of proteins after lipids associated with them are hydrolyzed, or alternatively to formation of insoluble complexes between free fatty acids and proteins.

Some facts which support these ideas include:

(a) Lipoproteins are very sensitive to freezing and drying.

(b) Fatty acids increase in stored frozen fish and this increase correlates with loss of protein solubility.

(c) Isolated muscle proteins are partially insolubilized when they react with linoleic and linolenic acids (Anderson and Steinberg, 1964).

(d) Free fatty acids are bound to proteins,

and can cause their denaturation (Bull and Breese, 1967).

Stability of food emulsions and foams

In cells and tissues, including muscle tissue used as food, membranes separate essentially aqueous compartments from one another. This is true of cell membranes and of membranes of subcellular particles.

In the case of emulsions we deal with a different system: a dispersion of two dissimilar phases, and, in foods, often with a dispersion of a nonpolar ("oil") phase in an aqueous phase ("water"). A full discussion of emulsion science is beyond this paper's scope, and we shall limit ourselves to the somewhat oversimplified statement that stability against droplet coalescence is a major requirement for most food emulsions. Figure 7 shows an equation which relates coalescence rate to various properties of the emulsion. The rate is increased by high volume of the dispersed phase and decreased by high viscosity, but the major way to stabilize emulsion is to develop an energy barrier, E, which prevents coalescence. The quantity ($e^{-E/RT}$) may be considered as equivalent to the fraction of all droplet collisions effective in coalescence and, in stable emulsions, is of the order of 10^{-6} to 10^{-3}, corresponding to activation energies of 4000–8000 cal/mole.

The mechanisms of the activation barrier common in foods are shown in Figure 8:

(a) Film formation at the oil-water interface with strong steric hindrance to oil coalescence.
(b) Electrostatic repulsion between charged groups located in the oil-water interface.
(c) Formation of hydration layers outside the oil droplet, because water-orienting hydrophilic groups are present at the surface.
(d) Low interfacial tension can stabilize emulsions by allowing large drop deformations.

Lipoproteins are potentially able to form strong films around oil droplets, and actually some lipoproteins, including those of egg, are excellent emulsifiers.

Films at oil-water interfaces depend strongly on various environmental and chemical influences, and emulsions may be stable under one set of conditions and unstable under others. In particular, chemical or physical changes may cause inversion in which the dispersed phase becomes continuous, or may produce coalescence and breaking of emulsion.

Foams are even more complex, and depend equally on the nature of films adsorbed at the interfaces between air and water. A major prerequisite for foam stability is the existence of films of surface active agents which attain equilibrium surface tensions at moderately low rates. A priori, lipoproteins belong to the class of amphipolar compounds which desorb from micelles and films slowly, and therefore attain equilibrium tensions slowly. Some lipoproteins, notably those of egg and milk, do seem to function as foam promoters, while under other circumstances lipoproteins may "kill" foams.

Milk-fat-globule membrane

Cow's milk is simultaneously a solution of low molecular compounds, a colloidal solution of globular proteins, colloidal dispersion of protein micelles and an oil-in-water emulsion. In fresh milk the emulsion is protected by the milk-fat-globule membrane, a lipoprotein complex which has been studied very extensively by many investigators. The concepts of this membrane have changed in parallel with concepts of biological membranes.

Among the early concepts of the fat globule membrane structure was one of King (Brunner, 1962) in which a layer of high-melting triglycerides in the periphery of the fat globule was associated with the surface layer of phospholipids, which in turn was connected to a closely oriented layer of protein.

Subsequent work, notably that of Brunner and Dowben, has demonstrated that the membrane, including the specific enzymes associated with it, originates in the mammary gland at the time of secretion, and that the membrane is similar to biological membranes, especially to

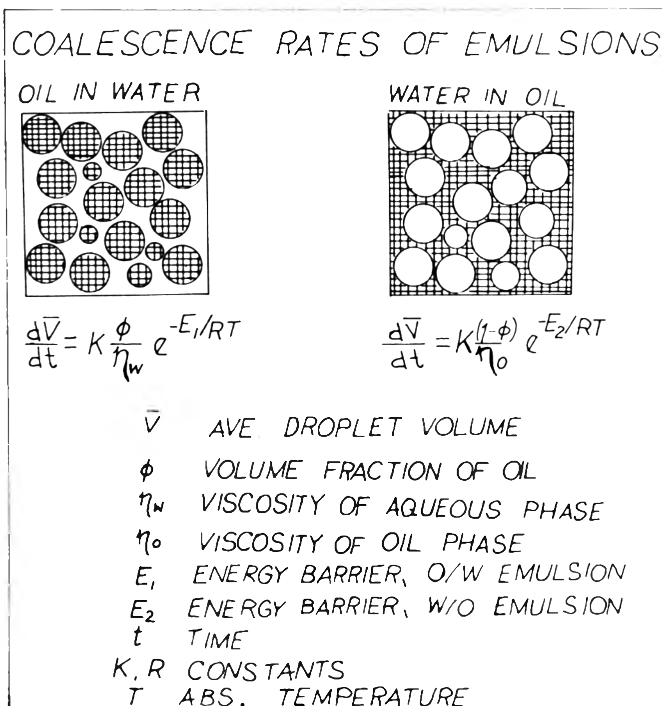


Fig. 7—Theoretical relations describing coalescence rates of emulsions.

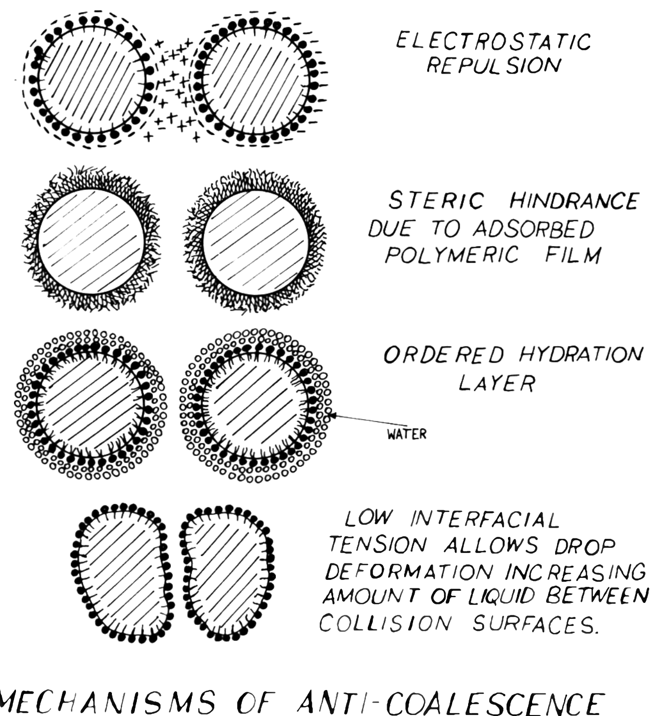


Fig. 8—Mechanisms of coalescence retardation.

erythrocyte ghosts. At the surface of the membrane, adsorbed lipoprotein particles are present (Brunner et al., 1969; Prentice, 1969; Brunner, 1969).

Modern views center on the following (Peereboom, 1969; Henson et al., 1971; Brunner, 1969):

(a) The fat globule is surrounded at the time of secretion by a lipoprotein membrane of glandular origin.
(b) Associated with this membrane are lipoprotein particles.
(c) Upon cooling, triglycerides crystallize first at the outermost layers of the fat, and in layers parallel to and associated with the membrane.
(d) As milk is stored, and treated in various ways, the membrane structure changes profoundly.

This last aspect is of most interest to the food technologist since it affects the functional properties of milk products.

Lipid-protein interactions in processing of milk products

Membrane changes occur in various milk processing operations. Fresh, unhomogenized, cooled milk is very susceptible to creaming due to globule clustering. The clusters are apparently held together by bridges formed by a globulin-like protein which interacts with the lipoproteins of the membrane. This process is similar to flacculation of bacteria, and may involve glycoproteins as well as lipoproteins.

Though globule clusters are undesirable in milk, they are desired in ice cream where a partial destabilization of the fat globule membrane system causes globule clustering around the air bubbles created during aeration. Most recent work indicates that destabilization of the membranes in ice-cream freezing actually leads to some spillage of free oil around the air-water interface, and this free oil may relate to mouthfeel of ice cream (Berger and White, 1971). Ice cream formation is a complex process in which the air-water and the oil-water interfaces apparently compete for adsorption of surface active components (Keeney and Maga, 1965).

Another important process in food technology involving this competition is buttermaking. In conventional batch churning, as well as in some, but not all, continuous processes, butter formation is due to a process akin to froth flotation (Brunner, 1965). The fat globules concentrate at an air-water interface (foam in batch churning, or film surfaces in some continuous buttermakers) and the lipoproteins change rendering at least part of the surfaces more hydrophobic. Whether desorption of parts of the membrane is a prerequisite for such changes is not clear. The increased hydrophobicity leads to spreading of oil at the air interface, suppression of foam, clumping of fat into what eventually becomes the complicated water-in-oil emulsion of butter. Peereboom (1969) proposed that the degree of desorption from the globule membrane during buttermaking depends on pretreatment of the cream and that the partition of heavy metals between butter and buttermilk is affected by heat denaturation of the lipoprotein complex during pasteurization of cream. This process is shown schematically in Figure 9.

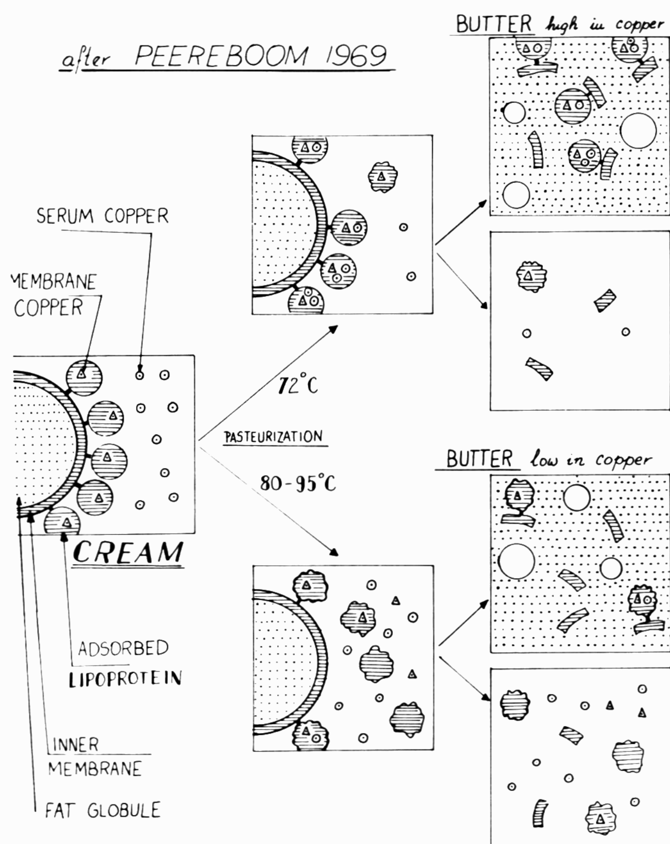


Fig. 9–Changes in milk fat globule membrane during cream pasteurization before buttermaking, and the effect of these changes on copper content of butter (Peereboom, 1969).

Significant changes occur during homogenization of milk. During homogenization the total globule surface increases several-fold, and a lipid-casein complex forms at the globule surface. This complex formation involves distortion of the casein micelles by homogenization pressure, as well as the increase in globule area (Fox et al., 1960).

In an elegant electron microscopic study, Henstra and Schmidt (1970) showed that casein particles and fat globules occur separately before homogenization but form clearly discernible complexes after the process. The distortion and partial dissociation of casein micelles due to pressure is apparently a necessary factor in the complex formation. The effects of pressure and of homogenization are shown schematically in Figure 10 which is based on electron micrographs published by Schmidt and Buchheim (1970) and those of Henstra and Schmidt (1970). In a related study, Schmidt et al. (1971) used freeze-etching to obtain electron micrographs of evaporated milk, and showed that during sterilization the casein micelles dissociate into casein subunits of 100 Å size, and these particles form a complex imbedding fat globules (Figure 10).

The "resurfacing" of the globules and new modes of association which exist as a result of various types of milk processing may very profoundly affect stability and properties of milk, including:

(a) Tendency to gel;
(b) Stability to metal-catalyzed and photo-catalyzed oxidations (Brunner, 1965);
(c) Susceptibility to enzyme-catalyzed changes. Most recent work shows clearly that disruption of normal lipid-protein associations existing in tissues in vivo may predispose the lipids of these tissues to peroxidation (Barber et al., 1970).

Lipid protein complexes in wheat flour, dough and bread

Wheat proteins are unique in that they are able to form the gas-retaining structure of bread. The rheological properties of dough, and to a large extent, the structure of bread as well, depend on the formation of a gluten network anchored upon the partially gelatinized starch granules. The proper development of this network depends partially on the proper balance of intermolecular and intramolecular -S-S- bonds in the proteins. The gluten network depends in addition on the existence of a lipoprotein complex which is essential to development of gas impermeability for good gas retention and adequate loaf volume.

The exact manner in which the lipoproteins participate in development of dough and bread is far from clear. Coppock and Daniels (1962) in a review consider gluten "an immature myelin" with protein subunits held together by lipids, and they retain in their discussion the Grosskreutz model in which the film of gas cells in dough consists of a lipoprotein layer with associated hydrogen bonded protein platelets (Grosskreutz, 1961). More recent work by Hoseney et al. (1969) and Finney (1971) also indicates that polar lipids able to form lipoprotein complexes are necessary to satisfactory bread structure. Extracted polar lipids can be added to flour to restore its functionality. Some synthetic compounds act similarly. Finney (1971) suggests that polar lipids may be bound to gliadin by hydrophilic bonds and to gultenin by hydrophobic bonds, and that the presence of these bonds may cement these proteins and contribute structurally to gas-retaining complexes. The role of hydrophobic bonds is also stressed by Ponte and Baldwin (1972) who found that added hydrocarbons can affect bread properties by promoting a rigid lipid-protein complex.

Lipoproteins in food fabrication

A trend toward "textured" and "fabricated" food tailored toward specific uses introduces many synthetic foods which undoubtedly have properties and problems associated with lipoproteins. The subject is too broad to discuss here. Suffice it to say that texture, binding properties, wettability and other so-called "functional" properties of the polymers (proteins and polysaccharides) which constitute the backbone of the "fabricated" material will often depend on their interaction with lipids. This is likely to be true of filaments and films prepared from soy protein, or from single-cell proteins, as well as of micelles, granules and particles of fish protein concentrate or of isolated milk proteins. Some examples of such effects are available in the literature. A recent paper by Wu and Bates (1972) for instance reports a study of thermally initiated lipoprotein film formation at air-"soymilk" interfaces, and also in model systems containing phospholipids and safflower oil.

Another example is provided by the work of Leo and Betscher (1970) who found that stearoyl-2-lactylate improved the functionality of coffee whiteners. They suggest that it forms a complex with sodium caseinate and improves the characteristics of lipoprotein membranes. Note that this compound is claimed to improve bread properties which also depend on lipoprotein structures.

REACTIONS OF PEROXIDIZING LIPIDS WITH PROTEINS

A GREAT DEAL of evidence indicates

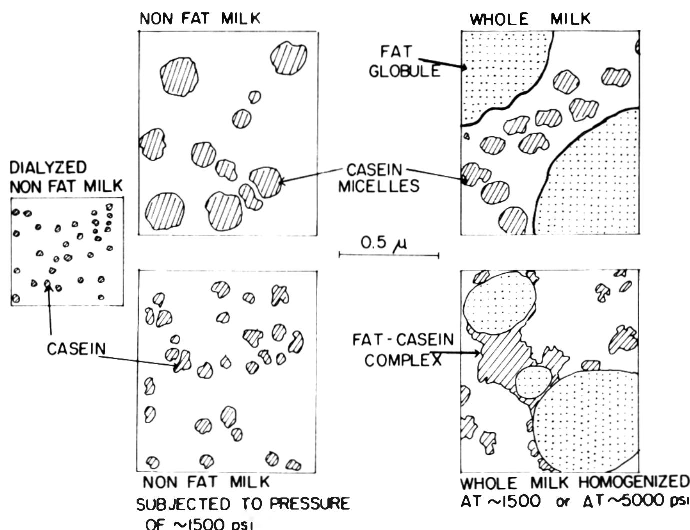


Fig. 10—Changes in casein micelles due to exposure of nonfat milk to pressure, and fat-casein complex formation in homogenized whole milk. For comparison, dissociation of casein micelles as a result of dialysis of nonfat milk is also shown. Schematic diagrams based on electron micrographs published by Henstra and Schmidt (1970) and Schmidt and Buchheim (1970).

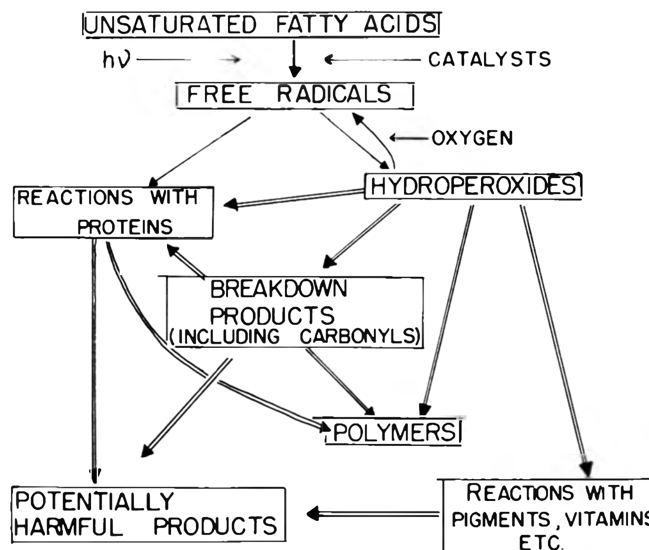


Fig. 11—Schematic representation of reactions initiated by lipid oxidation.

that peroxidation of lipids in lipid-protein systems results in reactions with the proteins. These reactions often lead to formation of colored, insoluble or sparsely soluble complexes.

Peroxidation of unsaturated fatty acids is a free radical reaction, the course of which is shown schematically in Figure 11. The distribution of absorbed oxygen is shown schematically in Figure 12. As can be seen, the major initial products consist largely of hydroperoxides. As oxidation progresses, breakdown products of hydroperoxides accumulate and eventually the hydroperoxide concentration decreases. The reaction is very sensitive to the environment, especially to temperature, electromagnetic radiation, state of dispersion as well as to the presence of catalysts and inhibitors.

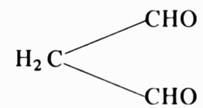
The reactions of peroxidizing lipids may be important biologically, either because they result in toxic or otherwise biologically active compounds in foods; or because there is actual peroxidation in vivo. In vivo peroxidation may be involved in:

(a) Damage to lipoprotein membranes of subcellular particles;

(b) Formation of the so-called "aging pigment;"

(c) Crosslinking of various polymers in aging animals. The possibility of in vivo peroxidation is still a constroversial subject, but is supported by: (1) Extracted lipids show signs of peroxidation in some tissues even when they are extracted under conditions minimizing danger of oxidation; (2) Free radical signals in tissues are increased in some disease states including some malignancies; (3) Sporadic studies have shown life-prolonging effects of antioxidants, and some diseases and toxic effects (for instance, carbon tetrachloride poisoning) are alleviated by antioxidant intake; (4) Studies on isolated subcellular particles including liver and brain microsomes, and liver mitochondria show that peroxidation of membrane lipids can occur and cause severe functional damage; and (5) Studies on isolated proteins show that reactions with peroxides damage the biological functions of such proteins.

Reaction products of lipid peroxidation react with proteins in a number of ways, Crawford et al., 1967. Malon-



aldehyde (MA), is a product of hydroperoxides of linolenic, arachidonic and other oxidized fatty acids, and forms the basis for the use of the TBA test. Chio and Tappel (1969) have shown that reactions between MA and amino acids lead to the formation of fluorescent complexes which are similar to the so-called "aging pigment," a lipoprotein isolated from tissues of aging animals, and attributed to peroxidative reactions. Buttkus (1967) showed that MA reacts readily with myosin in solutions at 20, 0 and −20°C, and that this reaction is accelerated in the frozen state. At room temperature lysine, histidine, tyrosine, arginine and methionine were the reaction sites. In the frozen state, histidine was not attacked but (MA) reacted irreversibly with lysine. Kummerow and coworkers (Kummerow, 1966; Nishida and Kummerow, 1960; Narayan et al., 1964) studied formation of lipid protein complexes in model systems. They found that the complex formation occurred only when lipids were peroxidized, that different proteins responded differently and that the reactions depend on the environment. They attributed the complex formation to secondary bonds, presumably including H-bonds. Pokorny (1963) and Pokorny and Janicek (1968) studied casein reactions with peroxidized lipids. They also found strong dependence of complex formation on environment, especially the polarity of the solution. They attributed the complex formation to hydroperoxides. Tappel's group in California (Roubal and Tappel, 1966) found distinct similarity between effects of reactions with oxidizing lipids on proteins, and the effects of ionizing radiations. They found damage to several proteins including cytochrome C, hemoglobin and ovalbumin. Cytochrome C damage in reaction with hydroperoxides was also reported by

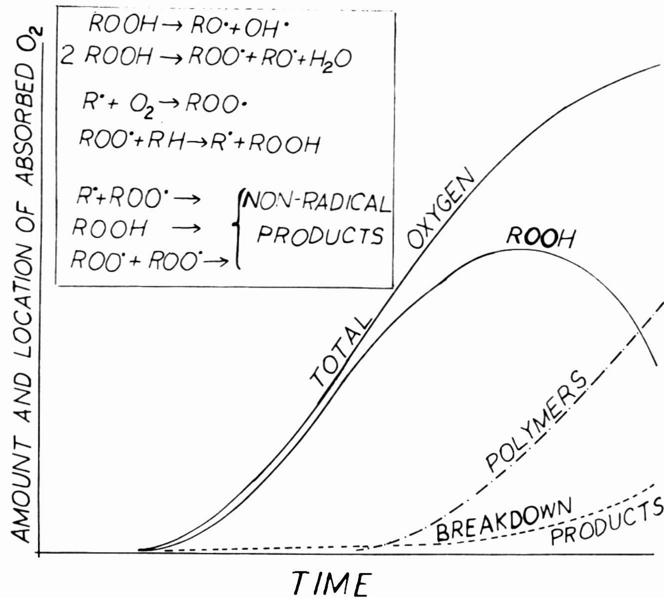


Fig. 12–Distribution of oxygen absorbed by peroxidizing fatty acids.

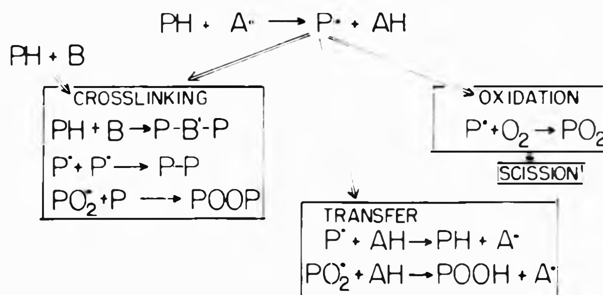


Fig. 13–Potential interactions of proteins with lipid oxidation.

O'Brien and Frazer (1966). The work of Roubal and Tappel led to their suggestion that protein insolubilization can result from protein-protein interactions initiated by lipid-free radicals as shown in Figure 13.

We studied gelatin-linoleate interactions in the dry state (Zirlin and Karel, 1969). From our results, we concluded that reactions of gelatin with lipids can lead to scission of the protein as well as to crosslinking as shown in Figure 13.

In subsequent work we have shown a strong similarity between radiation effects and reactions of proteins with peroxides, and that apparently in both, free radicals may form on α-carbons of the proteins and cysteyl radicals may form in proteins containing cysteine or cystine. The sulfur-containing proteins are more likely to crosslink than gelatin or collagen which contain no sulfur.

We are presently attempting to obtain defininte proof that protein free radicals are actually formed during reactions with lipid peroxides. This is a difficult task, and some authors including Roubal (1970) in his most recent work consider that the ESR signal in proteins after exposure to oxidized lipids is caused by lipid peroxides complexed with proteins.

The role of free radical in lipoprotein formation, reactions and properties is a most challenging subject currently being studied at several institutions. Our group is concentrating on the following aspects of reactions of fatty ester peroxides with proteins in solutions and in the dry state:

(a) Nature, origin and fate of free radicals;
(b) Similarities between these reactions and sensitized photooxidation and radiation effects;
(c) Fate of individual amino acid residues;
(d) Potential biological significance of these reactions. This is an interdisciplinary effort, and in particular the biological aspects are studied in collaboration with our toxicology group under G. Wogan, our animal pathology laboratories under P. Newberne and with R. Wurtman, who, with his colleagues, has made great strides in the effects of light on hormones.

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JFS BASIC SCIENCE

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A STANDARDIZED REVERSED PASSIVE HEMAGGLUTINATION TECHNIQUE FOR THE DETERMINATION OF BOTULINUM TOXIN

INTRODUCTION

RAPID DETECTION and identification of the toxins of *Clostridium botulinum* are important for the proper treatment of those afflicted and the determination of the implicated food so that it may be removed from distribution as quickly as possible. The currently accepted method for detection and identification of these toxins in foods, stomach contents and blood is to inject the suspect material into mice, some of which have been protected with specific antitoxins (Crisley, 1964). Quantitation of the toxin is also done by mouse assay, involving the injection of 0.5 ml of a serial dilution of the suspect material intraperitoneally into white mice weighing approximately 20g. The fraction dead in each dilution is recorded over a 96 hr period and the dilution that will kill 50% of the mice (LD_{50}) determined. A variation of this technique is to inject 0.5 ml of the undiluted extract intraperitoneally or 0.1 ml intravenously and measure the time from challenge to death. This latter technique is often referred to as the rapid assay because if high amounts of toxin are present, death will occur in about 0.5 hr if the dose is 10^6 LD_{50}, 1 hr if the dose is 10^5 LD_{50} and 2 hr if the dose is 10^4 LD_{50}. In our experience and in the experience of others (Dack, 1956; Segner and Schmidt, 1968) it is important that the mice die with typical symptoms of botulism or false interpretations may result. This necessitates that mice must be observed at frequent intervals throughout the test period, which could be as long as 96 hr if the toxin level is low. Another factor detracting from the use of the mouse test is that mice of the right age and weight must be made available continually.

Numerous techniques have been developed in recent years attempting to overcome many of the disadvantages of the mouse assay. Any replacement technique, however, should approach the mouse test in sensitivity to be acceptable and should also possess the advantage of being more rapid. Few of the techniques offered as a replacement fulfill these requirements, and many have other disadvantages as well. The use of goldfish in place of mice has been suggested by Crisley (1960) but has never gained acceptance. The fluorescent antibody (FA) technique has been investigated by Kalitina (1960), Bulatova and Kabanova (1960), Boothroyd and Georgala (1964), Walker and Batty (1964), Midura et al. (1967), Lynt et al. (1971) and others with conflicting results and varying degrees of success. A distinct disadvantage of the FA technique is that it does not detect soluble toxin and therefore the mouse test is still required to demonstrate toxicity. Other techniques, such as immunodiffusion (Vermilyea et al., 1968; Anderson and Niedermeyer, 1970; Miller and Anderson, 1971) have limited value because they either lack the required sensitivity, are not extremely rapid or require elaborate procedures.

Rycaj (1956) demonstrated that the hemagglutination (HA) technique could be used to detect minute amounts of type A toxin in foods with greater sensitivity than that achieved with guinea pigs. Yafaev and Chepelev (1961), using indirect hemagglutination, claimed sensitivity equal to that achieved with animal assay but were unable to differentiate between types A and B toxin. Johnson et al. (1966) demonstrated cross-reactions between antisera and toxins of types A and B but were able to eliminate those cross-reactions by titration of antitoxins with the heterologous antigens. Uemura and Sakaguchi (1971) using reversed passive hemagglutination (RPHA) for the detection of type E toxin showed a toxin detection level of less than 10 LD_{50}. They experienced cross-reaction between type E antitoxin and 500 LD_{50} of type A toxin but not with 2,300 LD_{50} of type B toxin or with 7,500 LD_{50} of type F toxin.

The above RPHA techniques have formed a basis for the development of a practical test for the qualitative and quantitative determination of the toxins in foods. This paper introduces a means for the standardization of the test with toxoid prepared from crystalline toxin so that results from different laboratories can be made comparable.

EXPERIMENTAL

Sheep erythrocytes

Formalin-preserved sheep erythrocytes (SRBC) (Difco) were washed once with 0.038M $NaHSO_3$ in 0.85% saline (Silverman et al., 1968) and then three times with saline. The washed cells were diluted to a concentration of 2.5% with saline and refrigerated until needed.

Antisera

Antiserum against partially purified (alcohol precipitated) botulinum type A toxin was prepared by injection of rabbits with alum adsorbed toxoid (supplied by the Michigan State Department of Health). Antiserum against crystalline type A toxin was prepared by injection of rabbits with toxoid in Freund's complete adjuvant.

Antitoxin globulin was prepared from rabbit antiserum by saturation with $(NH_4)_2SO_4$ to 50%. The precipitate was dissolved in saline to the original volume and reprecipitated with

$(NH_4)_2SO_4$ to 50% saturation. This procedure was repeated a third time. The final precipitate was dissolved in saline to half the original volume of serum, dialyzed in the refrigerator against 0.15M borate-buffered saline (pH 8.4) until free of SO_4^{2-} ions, dispensed in 1 ml quantities in screw-cap tubes and stored frozen until needed.

Procedure for tanning SRBC

An equal volume of 1:20,000 tannic acid (Baker) in phosphate-buffered saline (PBS), pH 7.3, was added to washed SRBC, and incubated at 37°C for 10 min with gentle mixing. The tanned cells were centrifuged and washed twice with PBS, pH 7.3, and resuspended in saline to a concentration of 2.5%.

Procedure for sensitizing SRBC

The concentration of globulin needed for optimal sensitization of tanned SRBC was determined by titrating known amounts of toxin with SRBC treated with varying concentrations of globulin. The dilution selected varied slightly from one lot of antiserum to another.

Sensitization of tanned SRBC was as follows: one volume of 2.5% tanned SRBC, one volume of appropriately diluted antitoxin globulin (diluted with PBS, pH 6.4), and four volumes of PBS, pH 6.4 were mixed and allowed to react at 24–27°C for 10 min with gentle swirling. The sensitized cells were centrifuged and washed twice in PBS (either pH 6.4 of pH 7.3) containing 0.5% normal rabbit serum (NRS) and resuspended in the same to a concentration of 2.5% for use in the tube test or 1% for use in the microtiter test. Tanned cells were washed twice and resuspended as above and used as controls.

Chromic chloride method of coating cells

A slight modification of the method of Gold and Fudenberg (1967) was used to sensitize cells. Appropriately diluted antitoxin globulin was mixed with an equal volume of a solution of 0.1% $CrCl_3$ in 0.85% NaCl and 0.1 ml of a 25% solution of washed SRBC was added immediately. The mixture was incubated at 24–27°C for 5 min, centrifuged and washed three times with 0.85% saline. The washed cells were resuspended in PBS, pH 6.4, containing 0.5% NRS to a concentration of 1% for use in the microtiter test.

Hemagglutination procedures

Serial 1:2 dilutions of crystalline toxin, toxoid or toxin-containing samples were prepared in 0.5 ml amounts in tubes (the diluent used was 0.5% NRS in PBS, pH 6.4 or 7.3) and 0.05 ml of 2.5% SRBC of the same pH was added. Hemagglutination patterns were read after incubation for 2 hr at 24–27°C. In the microtiter test, serial dilutions of crystalline toxin, toxoid or toxin-containing samples were prepared in the wells of plastic plates with microtiter loops; 0.025 ml of 1.0% SRBC was added to 0.025 ml of dilution and the tests read after incubation for 1 hr at 24–27°C.

Sample preparation

Cultures of *C. botulinum* types A-F, grown in Cooked Meat Medium (Difco) for 48–72 hr were centrifuged to remove cells. The cell-free culture filtrate was used in the hemagglutination and mouse assay procedures. Known quantities of crystalline botulinum type A toxin were added to food products. Cans of commercially sterile food products were also inoculated with spores of *C. botulinum* type 62A and type B Lamanna and spores of *C. sporogenes* PA 3679. The can lids were sterilized by alcohol flaming, punctured with a sterile ice pick, inoculated by means of a needle and syringe and resealed with solder. The cans were allowed to incubate at 35°C until swollen and were examined for toxicity. Toxin was extracted from the food products by crushing the food in a mortar and pestal using a volume of gelatin-phosphate buffer, pH 6.8, equal to the volume of sample, followed by centrifugation to remove particulate matter. The clear supernatant was collected for assay.

Elimination of nonspecific reactions

With some products it was necessary to add washed SRBC to the extract to remove substances causing nonspecific agglutination. This was done by adding 0.1 ml of packed red cells which had been centrifuged and washed five times, to 1.0 ml of extract. After reacting for 10 min at 24–27°C, the mixture was centrifuged to remove the cells.

Trypsin or pepsin (2 × crystalline, salt free) at a final concentration of 1 mg per ml also was used to treat food extracts in which nonspecific hemagglutination reactions were encountered. The pH of the extract was adjusted to 6.5, if necessary. The enzyme-extract mixture was incubated at 35°C for 30 to 60 min prior to analysis.

Preparation of toxin and toxoid

Crystalline type A toxin was prepared by the method of Duff et al. (1957) with slight modifications which included washing the original acid precipitate at pH 4.6 instead of 5.0 and centrifugation instead of filtration when separating the dissolved toxin from the washed precipitate. When dissolving the toxin in buffer for alcohol precipitation, it is important that the protein concentration be between 10–20 mg/ml. The solution for crystallization should have a protein concentration of 10–15 mg/ml. The crystallization was allowed to proceed for several days at 4°C. Crystals were removed from the mother liquor ammonium sulfate solution by centrifugation at 10,000 × G for 15 min at 4°C in a refrigerated centrifuge. These crystals were dissolved in 0.05M sodium phosphate buffer at pH 6.8 to give a concentration of about 10 mg per ml, as determined by the absorbance at 278 nm ($E_{1cm}^{1\%}$=16.5), and dialyzed against the same buffer to remove the ammonium sulfate. The yield of crystalline toxin based on the toxin content of the culture was about 15%. Toxoid for injection of rabbits was prepared by adding formaldehyde to the crystalline toxin solution to a final concentration of 0.35%, and allowing the mixture to react at 24–27°C for several weeks. Loss of toxicity was determined by injecting 0.5 ml of the solution into mice. After all toxicity was lost, the solution was diluted to contain the equivalent of 1 mg of toxin per ml based on the absorbance of the toxin solution after dialysis. The toxoid was stored at 4°C.

Standard toxin and toxoid solutions

A standard toxoid solution for use in the hemagglutination test was prepared by diluting the above toxoid solution with 0.05M phosphate buffer, pH 6.8, to yield a solution containing 0.2 μg of toxoid per ml. A standard toxin solution was prepared by diluting the dialyzed crystalline toxin solution (untoxoided) with buffer to a final toxin concentration of 0.2 μg per ml.

Mouse assay

White mice about 3-wk old and weighing between 18 and 25g were used. The number of mouse LD_{50} per ml was determined by injecting 0.5 ml of the diluted solution of toxin or clarified food extract intraperitoneally into each of six mice and observing the number dead over a period of 96 hr. Two-fold serial dilutions were made with sterile 0.05M sodium phosphate buffer, pH 6.8. The percent kill in each dilution was plotted against the dose on probit-log paper. The best straight line was fitted by inspection and the dose corresponding to probit 5 was taken as the LD_{50}. Sterile procedures were adhered to in order to reduce the possibility of infection in the mice.

RESULTS

BOTH THE TUBE and microtiter RPHA technique can be used successfully for toxin detection. At pH 7.3, titers obtained by the microtiter technique were twofold (one dilution) less than those obtained with the tube test. At pH 6.4, the sensitivity of the two techniques was identical (per ml of sample). Nonspecific hemagglutination reactions were encountered in the first two tubes of the dilution series (1:4) using uninoculated culture filtrates and sensitized SRBC and diluent at pH 7.3. These nonspecific reactions were eliminated using sensitized SRBC and diluent at pH 6.4. No HA reactions were encountered at pH 6.4 when toxic

Table 1—Comparison of results obtained from the assay of crystalline botulinum type A toxin by mouse assay and reversed passive hemagglutination

		Toxin concentration as determined by	
Sample	Toxin (μg/ml)[a]	Mouse assay (μg/ml)[b]	Hemagglutination (μg/ml)
1	0.01	0.014	0.0125
2	0.1	0.107	0.1
3	1.0	0.8	1.0

[a] Amount of toxin per ml based on absorbance at 278 nm ($E_{1cm}^{1\%}$=16.5) of a solution of crystalline toxin with a concentration of 300 μg per ml and diluted to these concentrations with gel-phosphate buffer.

[b] 1 μg of crystalline type A toxin as determined from a large number of assays is equivalent to 3.3 × 10^4 mouse LD_{50} using our strain of white mice at a weight of about 20g and 3–4 wk of age.

culture filtrates were incubated with tanned unsensitized cells, but at pH 7.3, nonspecific reactions were encountered in the first tube of the dilution series (1:2).

Toxoided crystalline type A toxin and crystalline type A toxin react identically in the HA test. The end-points of HA reactions were the same on a mg/ml basis using either toxoid or toxin. Toxin titers obtained using sensitized SRBC prepared by the chromic chloride method were fourfold (two dilutions) higher than titers obtained using cells prepared by the tannic acid method. The mean end-point for 20 HA tests with crystalline type A toxin or toxoid was 0.0008 μg per ml using chromic chloride sensitized SRBC. This would represent a toxin level of about 27 mouse LD_{50} per ml assuming one mouse LD_{50} equal to 0.00003 μg.

The crystalline toxin prepared for this work assayed 3.5×10^7 mouse LD_{50} per mg. This value is consistent with other preparations of crystalline type A toxin. Good correlation of results was obtained between the mouse test and reversed passive hemagglutination as presented in Table 1.

Hemagglutination results obtained using antiserum against crystalline type A toxin showed no significant difference when compared to results obtained using antiserum against the partially purified toxin. No HA reactions were obtained when crystalline toxin in phosphate buffer, toxic culture filtrate or toxic food extract were heated in boiling water for 15 min. Culture filtrates containing toxins of types B, C, D, E or F showed no cross-reactions with type A antiglobulin sensitized cells. Likewise, no hemagglutination reactions were encountered with culture filtrates or food extracts inoculated with strains of *C. sporogenes.*

Assays for crystalline type A botulinum toxin added to various food products met with limited success initially. Nonspecific hemagglutination reactions were encountered with some products. These were eliminated by treatment of the extract with washed SRBC prior to adding the sensitized cells. Treatment of the extract with trypsin or pepsin in some cases also aided in the elimination of nonspecific reactions without any adverse effect on the toxin titer. Pepsin was slightly better than trypsin for eliminating these nonspecific reactions with some products.

Nonspecific hemagglutination reactions were not encountered with all lots of SRBC when analyzing food extracts for toxin content. Some lots of cells could not be used at all for hemagglutination reactions and were discarded. Several lots did not nonspecifically agglutinate in the presence of food extracts and gave excellent hemagglutination patterns.

Cans of product which had been inoculated with either *C. botulinum* types A or B spores or *C. sporogenes* spores were examined in a blind study. These cans had been inoculated and coded in such a way as to make their contents unknown to the technician examining them for toxicity. All cans containing type A toxin were correctly detected by hemagglutination. No HA reactions were encountered with extracts from cans inoculated with either *C. botulinum* type B or *C. sporogenes* spores.

Table 2—Content of C. botulinum type A toxin in a food extract based on the end-point in the hemagglutination test

End-point tube number	Toxin concentration of extract (μg/ml)	(LD_{50})
1	0.0016	53
2	0.0031	103
3	0.00625	208
4	0.0125	417
5	0.025	833
6	0.05	1,667
7	0.1	3,333
8	0.2	6,667
9	0.4	13,333
10	0.8	26,667
11	1.6	53,333
12	3.2	106,667

The toxin content of a food extract can be read directly from Table 2 if the end-point for a particular lot of sensitized SRBC using the standard toxoid solution is at tube number 8. For example, a toxic food extract with an end-point at tube number 5 would contain 0.025 μg/ml of toxin. However, using the standard toxoid solution the end-point obtained with various lots of sensitized SRBC may vary and will not always be at tube number 8. When this occurs, the toxin content of an extract cannot be read from the table and the following formula must be used:

$$\text{Toxin content of an extract} = 0.2\ \mu g/ml \times 2^x$$

where x represents the difference in the number of tubes between the end-point of the toxoid standard and the end-point of the unknown extract, 0.2 μg/ml is the equivalent amount of toxin in the toxoid standard and the value 2 represents the twofold dilutions used in the test. The value of x is negative if the end-point tube number of the unknown extract is less than the end-point tube number of the toxoid standard, and positive if the end-point tube number of the unknown extract is greater than the end-point tube number of the toxoid standard. For example, if the end-point of the standard fell at tube number 7 and the end-point of the extract was at tube number 5, the value of x would be −2. If the end-point of the standard fell at tube number 7 and the end-point of the extract was at tube number 8, the value of x would be 1. In calculating the amount of toxin per ml or g of food, any dilution of the food made in preparing the extract must also enter into the calculation.

The following example may help to illustrate how the toxin content in a food extract is determined. With a particular lot of sensitized SRBC, the end-point using the standard toxoid solution was at tube number 6. Using the same lot of cells, the end-point of a toxic food extract was at tube number 4. The value of x to be used in the equation is −2. Therefore,

$$\text{Toxin content of the extract} = 0.2\ \mu g/ml \times 2^{-2} = 0.2\ \mu g/ml \times \tfrac{1}{4} = 0.05\ \mu g/ml$$

The amount of toxin present per ml of food extract would be 0.05 μg.

DISCUSSION

THE EXCELLENT correlation between the reversed passive hemagglutination technique and the mouse assay for detecting botulinum type A toxin should make the RPHA technique valuable in the food industry as a screening test for detecting the presence of toxin in foods. Detection and quantitation of toxin in a food extract can be accomplished within 1.5 to 3 hr, with the type of toxin determined simultaneously. The reagents and equipment are relatively inexpensive and the test can be performed easily by a technician. The test, at present, is not as sensitive as the mouse assay, but it is sufficiently sensitive for practical purposes. With the reagents used in this work, 0.0008 μg/ml or 27 mouse LD_{50}/ml of type A toxin was detected. Only type A has been investigated, but there is good indication that the technique will work equally well for the other types.

The variation among lots of SRBC and the variation in preparing sensitized cells from one time to another and from laboratory to laboratory necessitate the use of some standard to make results comparable. It has been demonstrated that the use of toxoid against crystalline type A toxin serves as an excellent standard for this purpose. Toxoid is indistinguishable from toxin in the hemagglutination reaction and has the added advantage of being nontoxic and stable for long periods of time. The toxoid used in these studies has remained stable under refrigeration for l yr.

No cross-reactions were encountered between the type A antiglobulin sensitized cells and toxins of the other types as reported by some investigators. The reason for this is not clearly understood, but undoubtedly the purity of the immunizing toxoid and the quality and titer of the antiserum would greatly influence the occurrence or lack of cross-reactions.

The method used for the sensitization of cells appears to influence the sensitivity of the hemagglutination technique. When using cells sensitized by the chromic chloride method, the sensitivity of the RPHA technique was invariably greater than when using cells sensitized by the tannic acid method. The chromic chloride method also has the advantage of being a one-step method and being considerably more rapid.

Differences in cell patterns and endpoints in the hemagglutination reaction were noted between various lots of commercially available SRBC. Some cells formed tight, compact buttons to indicate a negative reaction whereas others formed more diffuse buttons. Some lots were not acceptable for use in hemagglutination tests because of the spontaneous agglutination encountered with some food extracts. The time required for hemagglutination patterns to develop also varied from lot to lot. It is advisable that when a good lot of cells is encountered, a large quantity of these cells be acquired. Formalinized, unsensitized cells remain acceptable for over a year and when sensitized and stored at 4°C are satisfactory for at least 4 to 5 months.

Nonspecific hemagglutination reactions were not encountered with all products tested, but when encountered, were easily eliminated by one of several treatments. The method of choice would be influenced by the product and it is suggested that an assay technique be determined for each type of product to be examined.

Reversed passive hemagglutination is a rapid screening technique applicable to the detection of type A botulinum toxin in culture filtrates or food extracts. With the reagents used in this work, 0.0008 μg/ml of type A toxin was detected. Nonspecific reactions encountered with some food extracts were easily eliminated. The technique presented minimizes differences encountered between different laboratories, using different antisera and different lots of cells by using a standardized toxoid solution. This standardized toxoid solution will be available upon request for research purposes and can be obtained through Dr. E.J. Schantz at the Food Research Institute, Madison, Wisc.

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CONTRIBUTION OF ADSORPTION TO VOLATILE RETENTION IN A FREEZE-DRIED FOOD MODEL CONTAINING PVP

INTRODUCTION

IN A PREVIOUS paper we reported that in a freeze-dried system containing PVP (polyvinylpyrrolidone) and n-propanol the retention of most of the alcohol could be explained by entrapment of the alcohol in microregions formed by PVP (Chirife et al., 1973). There appeared to be a competition for water sorbing sites by the entrapped propanol, resulting in reduction of water sorbing capacity at levels below the BET monolayer value.

The ability of various polar sites in hydrophilic polymers to adsorb either water or alcohol has also been observed by Le Maguer (1972) and Fogiel and Heller (1966).

In the present study we show the extent to which adsorption is important in the retention of n-propanol in freeze-dried PVP solutions.

MATERIALS & METHODS

Model system preparation

The model system consisted of a water-soluble polymer (PVP), ^{14}C-labeled n-propanol and water.

The system was prepared by dissolving the desired amount of PVP in water and adding n-propanol. 5-ml aliquots of the solution were pipetted into 50-ml Erlenmeyer flasks, frozen as specified below and then freeze dried at room temperature and at a chamber pressure of less than 100μ for 48 hr in a Virtis freeze drier (Model 10–MRTR). Temperatures were not measured during freeze drying. The drying took place with limited heat transfer and unheated platens at low chamber pressures. No melting or collapse was observed in any of the samples during freeze drying. The conditions of freeze drying were identical to those previously found by us to give high volatile retentions in carbohydrate-volatile systems.

The volume of solution per flask and resultant sample thickness varied in some experiments, as noted under Results & Discussion.

The composition of the system was fixed as the following initial concentration expressed in weight percent: PVP 20%, n-propanol 1%, water 79%.

Samples were frozen by one of two methods: rapid freezing was accomplished by immersion of flasks in liquid nitrogen; slow freezing by placing the stoppered flasks in still air at −40°C.

PVP

Polyvinylpyrrolidone K-30 (molecular weight 40,000) was obtained from Matheson, Coleman and Bell (East Rutherford, N. J.).

N-propanol

Reagent grade n-propanol was mixed with ^{14}C-labeled n-propanol to give the desired specific radioactivity. The radioactive propanol was obtained from International & Nuclear Corp., Irving, Calif.

Humidification experiments

In several experiments freeze-dried PVP-n-propanol systems were humidified by placing tared and weighed flasks in vacuum desiccators containing saturated salt solutions, which maintained the desired constant relative humidities.

N-propanol analysis

The n-propanol content was determined by measuring the radioactivity of the samples with a liquid scintillation counter.

The dried samples of PVP were dissolved in water (to 10% solution); 1 ml of this solution was added to 10 ml of water-miscible scintillator (2,5-diphenyloxazole, 1g; naphthalene, 100g; dioxane to 1,000 ml volume) in the counting vial, and the resulting solution was counted with a liquid scintillation counter (Nuclear Chicago Corp., 720 series).

N-propanol adsorption

Some experiments on sorption of n-propa-

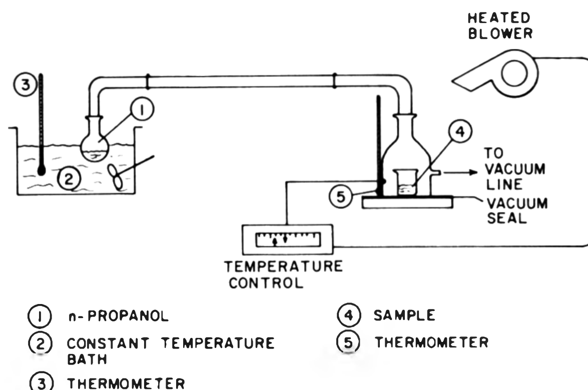

Fig. 1—Apparatus for study of n-propanol adsorption on PVP.

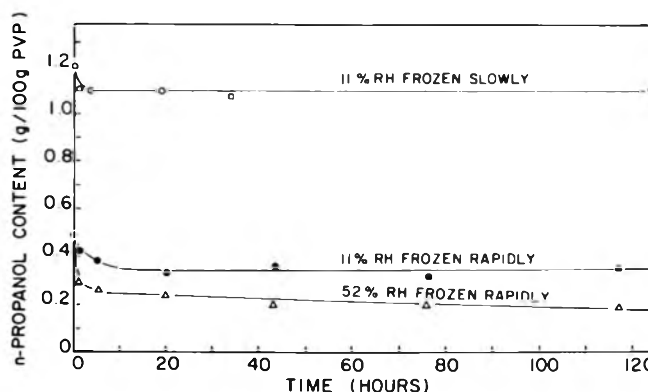

Fig. 2—Retention of n-propanol in freeze-dried PVP humidified to 11% and 52% RH.

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nol in freeze-dried PVP were carried out as shown in Figure 1. The sample was maintained at 24°C by circulating heated air from a blower operated by a temperature control. The activity of the n-propanol vapor in the evacuated system shown in Figure 1 was regulated by controlling the temperature in the cold bath.

RESULTS & DISCUSSION

RETENTION of n-propanol after freeze drying solutions of PVP and n-propanol depends on freezing rate, sample thickness, drying conditions and the concentration of PVP and propanol (Chirife et al., 1973). Under typical drying conditions for 25% PVP, 1% n-propanol solution, the retention was 0.49g n-propanol/100g PVP in rapidly frozen samples and 1.2g/100g in slowly frozen systems. Most of the retained alcohol was entrapped internally in microregions, as evidenced by lack of desorption in the absence of structural disruption by water (Chirife et al., 1973).

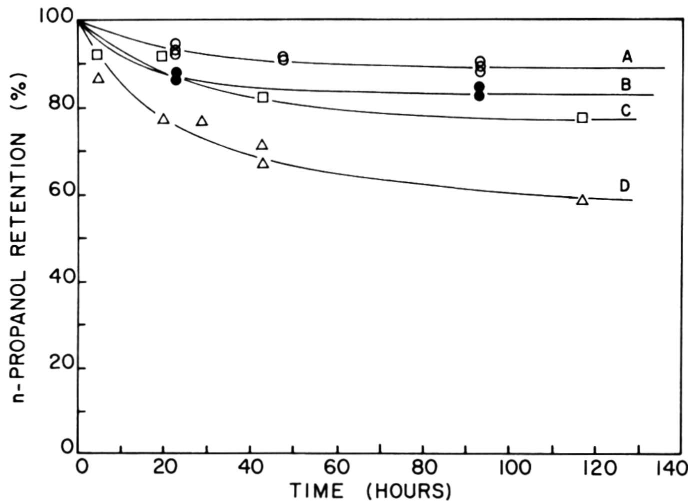

Fig. 3—Retention of n-propanol in freeze-dried PVP during desorption at 37°C and 50°C: (A) Slowly frozen, desorption at 37°C; (B) Slowly frozen, desorption at 50°C; (C) Rapidly frozen, desorption at 37°C; (D) Rapidly frozen, desorption at 50°C. N-propanol content after freeze drying: (A) and (B): 1.2g/100g PVP; (C) and (D): 0.49g/100g PVP.

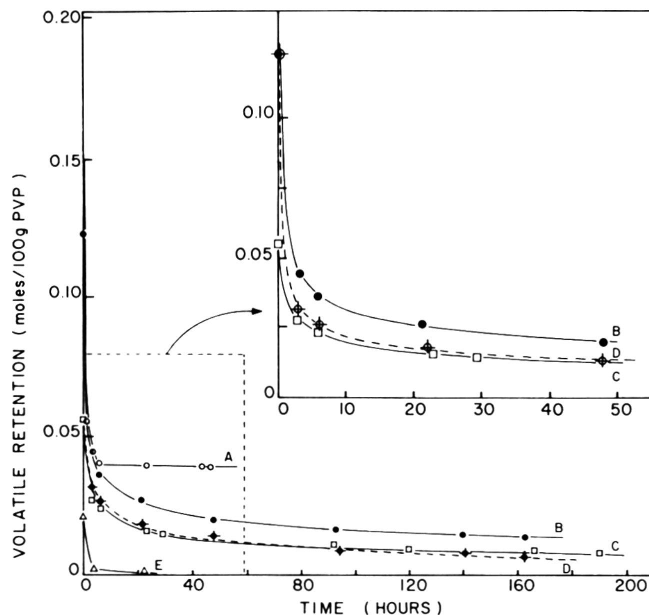

Fig. 4—Desorption of water and of alcohols after adsorption on freeze-dried PVP: (A) Water, desorption at 25°C; (B) N-propanol (adsorption at propanol activity of 0.2) desorption at 25°C; (C) N-propanol (adsorption at propanol activity of 0.11) desorption at 25°C; (D) N-propanol (adsorption at propanol activity of 0.11) desorption at 37°C; (E) N-heptanol, desorption at 25°C.

There appeared to be indications, however, that part of the alcohol may be retained after freeze drying by strong adsorption. Relative contribution of adsorption is likely to be most pronounced in rapidly frozen systems in which the microregion development is less complete than in slow frozen systems, especially in the case of polymers which have a low mobility, and in which entrapment is more difficult.

Figure 2 shows the n-propanol content in freeze-dried PVP after humidificaiton to 11% and 52% RH. In the PVP system the BET monolayer value for water, at which microregion disruption begins to occur, corresponds to 30% RH (Chirife et al., 1972). The loss at 11% RH therefore may be due to desorption of the adsorbed fraction; relative contribution is greater in rapidly-frozen PVP than in slowly-frozen PVP, even though the absolute losses at 11% RH are comparable (0.11g/100g PVP for slow frozen and 0.15g/110g for rapidly frozen).

Fast and slow frozen freeze-dried PVP-n-propanol systems were kept at very low humidity by placing samples in evacuated desiccators containing activated charcoal and calcium sulfate at 37°C and 50°C with the loss of n-propanol determined. Results are shown in Figure 3. In all cases there is a loss asymptotically approaching a value dependent on temperature. These losses are likely to correspond to the portion of total alcohol which is held by "adsorption irreversible by freeze drying" but reversible by desorption at a higher temperature. The remaining n-propanol is held in microregions which are impermeable until disrupted by treatment with water vapor or possibly other structure-disrupting treatments (polar solvents, very high temperature). The fractional contribution of adsorption is larger for rapidly frozen than for slowly frozen samples. However, the absolute losses do not differ much: at 50°C and 93 hr 0.20g n-propanol/100g PVP are lost in slowly frozen samples, and 0.19g/100g PVP in rapidly frozen samples.

In order to confirm that a portion of the total propanol retained after freeze drying of PVP is capable of being held by adsorption, we conducted adsorption-desorption experiments. Freeze-dried samples of PVP containing no propanol were placed in a vacuum oven at 95–100°C for 72 hr to remove the last traces of water. Adsorption of n-propanol was then carried out at room temperature (24°C) by exposing the samples to the alcohol vapor at propanol activities of 0.20 or 0.11. The samples were then transferred to a vacuum desiccator con-

taining activated charcoal and calcium sulfate; desorption of n-propanol was followed with time at either 25°C or 37°C.

Figure 4 shows the decrease of adsorbed amount with time. It can be seen that, following an initial rapid rate of desorption, the removal of n-propanol continues very slowly and appears to approach a constant retention level; this level decreases with increase in temperature (curves B and D).

The interpretation of this phenomenon could be simply made on the basis of the energies of activation of desorption (Bliznakov and Polikarova, 1966). The removal of each successive portion of alcohol is more difficult due to the increase of the energy barrier, until a value is reached of the energy of desorption at which the rate of the process becomes practically zero. This will determine the irreversible amount of adsorbed n-propanol.

However, that the situation is not so simple is clearly illustrated by curve C in Figure 4, which corresponds to a sample adsorbed initially at a lower vapor activity (0.11) than that for curve B (0.20) and even more dramatically in Figure 5 which shows desorption of n-propanol after exposure to saturated vapor of the alcohol up to the adsorption of about 12%. The amount of retained n-propanol depends on the initial amount adsorbed and on the activity of the sorbate. Even at the relatively low vapor activities in Figure 4 n-propanol is capable of penetration into the PVP structure and subsequent entrapment.

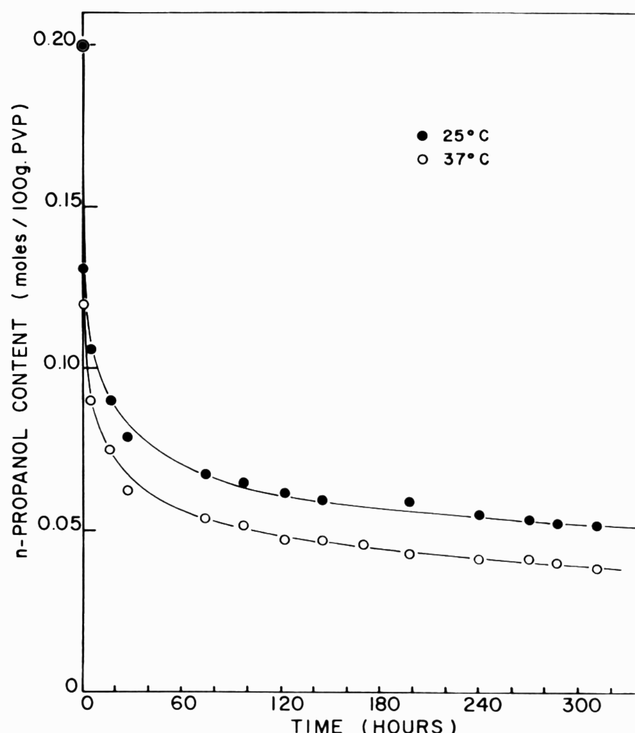

Fig. 5–Desorption of n-propanol adsorbed by dry PVP by exposure to saturated vapor.

This fact makes difficult to assign the total retention observed in adsorption experiments to a true adsorption process. This is also confirmed by the results showed by curve E (Fig. 4) corresponding to the desorption of adsorbed n-heptanol: the retained amount is practically zero. Heptanol does not readily dissolve PVP, in contrast to n-propanol.

The retention of strongly adsorbed alcohol in the polar PVP in preference to water would be difficult to accept in view of the relative polarities of water and propanol. However, our experiments in the PVP system showed that there is water retention in addition to n-propanol retention: 20 samples of 20% PVP solutions (3.5 mm thickness) without volatile were freeze dried in standard conditions for 48 hr. The samples were analyzed gravimetrically (24 hr, vacuum oven at 95–100°C) for residual moisture content. No significant difference was found between slow and fast frozen samples. The "water retention" was found to be 0.66 ± 0.11g water/100g PVP.

For fast frozen samples the retention of n-propanol was 0.49g n-propanol/100g PVP. On molar basis the retention of water alone is much higher than the total retention of the n-propanol, which also included the alcohol entrapped in microregions. The values for water are: 14.7 moles/mole PVP; for n-propanol they are 3.27 moles propanol/mole PVP.

Desorption of previously adsorbed water was studied in the following experiment: Samples of PVP freeze dried without n-propanol were placed in a vacuum oven at 95–100°C for a period of 24 hr. The dry weight was recorded. The samples were placed in a vacuum desiccator over a constant humidity solution until they reached an equilibrium moisture content of 13.5g water/100g PVP. The samples were then removed and desorbed by evacuation over a bed of calcium sulfate. Figure 4, curve A shows the decrease of the adsorbed amount (on molar basis) of water with time. It can be seen that below about 0.039 moles water/100g PVP (~0.71g water/100g PVP) there is almost no decrease with time. This amount of adsorbed water is close to the water content retained after freeze drying (0.66 ± 0.11g water/100g PVP). This strong semipermanent adsorption responsible for part of the retention is referred to as irreversible adsorption, which is defined as the situation occuring when there is adsorbed material which cannot be removed by evacuation at the temperature at which the adsorption was carried out (Clark-Monks et al., 1970).

Additional evidence for adsorption as a mechanism for part of retention in the PVP systems was obtained from experiments with layered systems. Samples were prepared by rapidly freezing alternate layers of a solution containing no volatile and layers of a solution containing the volatile. Each layer was completely frozen before the next layer was added. After the standard cycle of freeze drying (48 hr) the layers were separated for individual analysis. In some samples the layers were separated by thin brass mesh to avoid any "contamination" between layers. In other samples, an already freeze-dried layer of PVP with no n-propanol was placed on the frozen layer containing the volatile.

Good agreement was found among all the samples: it was observed that the amount of n-propanol adsorbed in the dry layers was about 10% of the total retention found in the layers originally containing the volatile. The absolute amounts were 0.047–0.061g n-propanol/100g PVP.

We can now explain the observed results by the hypothesis that, in addition to the bulk of the retained alcohol which is entrapped in impermeable microregions, a portion of the retained alcohol and a portion of the water remaining after freeze drying are held by adsorption outside the impermeable microregions.

The situation in which two vapors are in competition for sites capable of adsorbing either vapor can be approximated by equations (1) and (2) derived from the Langmuir isotherm, provided that the as-

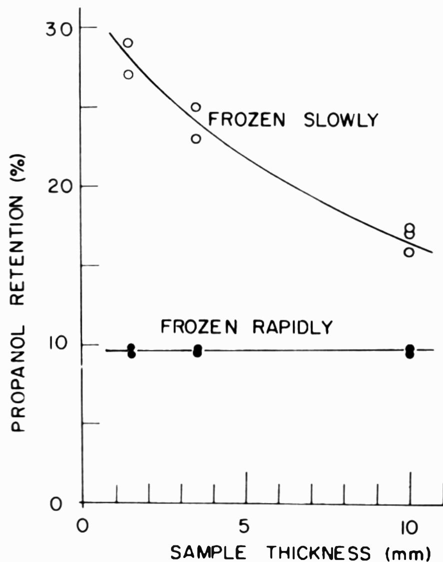

Fig. 6—Effects of freezing rate and of sample thickness on retention of n-propanol in freeze-dried PVP.

sumption is made that there is no heterogeneity of the solid surface, and no opening of additional sites or adsorbate interactions during adsorption:

$$n_w = \frac{N_w b_w p_w}{1 + b_w p_w + b_a p_a} \quad (1)$$

$$n_a = \frac{N_a b_a p_a}{1 + b_a p_a + b_w p_w} \quad (2)$$

where N_w = number of sites available for water sorption;
N_a = number of sites available for alcohol sorption;
p_a = partial pressure of alcohol;
p_w = partial pressure of water;
n_a = number of sites occupied by alcohol;
n_w = number of sites occupied by water; and
b_a, b_w = constants

The above equations show that only when $b_w p_w + b_a p_a << 1$ is the adsorption of the two vapors independent of one another.

The experimental observations of competition by sorption sites in the PVP between water and n-propanol (Chirife et al., 1973) and the observed losses of alcohol at low relative humidities (Fig. 2) can be explained qualitatively through the set of equations (1) and (2). However, these equations would have to be modified to give quantitative information, since adsorption of polar vapors on polar polymers does involve surface heterogeneity, interactions between vapors, as well as changes in number of available sites during sorption (Le Maguer, 1972). Perhaps this could be partially accounted for through the study of Ross and Oliver (1964) who devised a means of allowing quantitatively both for intermolecular attraction and for surface heterogeneity at the same time.

Our results confirm that the total amount of alcohol remaining in the freeze-dried PVP solutions is composed of a fraction entrapped in impermeable microregions, and a fraction adsorbed, in competition with water, in locations from which it can be desorbed, albeit slowly.

In slow frozen samples the entrapped fraction accounts for most of the retained n-propanol and the effect of processing variables are explainable entirely by the microregions theory (Flink and Karel, 1970b).

In the case of fast-frozen PVP, however, the adsorbed fraction is sufficiently large to cause some deviations from behavior attributable entirely to entrapment.

According to the microregions theory (Flink and Karel, 1970b), retention decreases with increasing thickness primarily because the more rapid drying and steep moisture gradients in thin samples decrease the time during which the moisture content in the microregions is high enough to permit volatile escape. Figure 6 presents the retention of n-propanol in fast- and slow-frozen PVP as a function of thickness of sample. In fast-frozen PVP the retention is independent of the thickness of the sample. This observation may be due to a combination of adsorption and entrapment, since at the end of the drying period desorption will be less complete in thicker samples than in the thinner samples.

Adsorption and desorption of organic volatiles on food components has been studied by other investigators, including Rey and Bastien (1962), Issenberg et al. (1968) and Le Maguer (1972).

Maier (1969; 1970; 1971; 1972) and Gray and Roberts (1970) studied the binding of various volatile organic compounds to foods and food components and found strong interactions between specific substrates and some adsorbates.

Maier (1972) observed that sorption of ketones usually required either the presence of water to allow ketone penetration into polymeric aggregates, or presence of fat in which the ketones apparently dissolve. In absence of water there was some sorption of acetone on zein, starch and pectin. The adsorption was irreversible. Infrared measurements confirmed that absorption was due to (ketone)–C=O··· HO–(polymer) hydrogen bonds.

Binding of alcohols to the peptide CO groups of poly-L-proline was demonstrated by the shift of the OH stretching vibration of the alcohols (Strassmair et al., 1971). (PVP)–C=O···HO–(alcohol) bonds are probably involved in the PVP-n-propanol interactions.

In conclusion, we showed that in rapidly frozed, freeze-dried polymer solutions, adsorption may contribute significantly to the total amount of volatile retained after freeze drying. Our results do not allow us to determine the exact amount held by adsorption rather than entrapment, but in most of the cases we studied, the major fraction is that held by entrapment in microregions.

Further studies on more complex systems are underway.

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PEA LIPIDS AND THEIR OXIDATION ON CARBOHYDRATE AND PROTEIN MATRICES

INTRODUCTION

DETERIORATION in pea quality may be a result of microbiological, enzymatic and nonenzymatic changes. The latter two are of particular importance in the preservation of fresh and processed peas. In both cases deterioration occurs mainly via lipid oxidation resulting in off-flavor development. Whitfield and Shipton (1966) revealed in off-flavored frozen green peas as many as 12 lipid oxidation products consisting of the carbonyl compounds n-anals, 2-en-als, 2,4-diene-als and 2-ones. Bengtsson and Bosund (1964) suggested that many of these volatiles are formed either by lipoxidase action in fresh peas or by autoxidation in the processed peas. Eriksson (1967) demonstrated that for peas 5–8% of the total lipoxidase content is located in the skin, 80% in the outer and 12% in the inner tissue of the cotyledon.

An earlier study on fresh pea lipoxidase conducted in this laboratory revealed the presence of lipoxidase isoenzymes and their association with subcellular particles and cytoplasm. The activity of the enzyme was the highest in presence of a free fatty acid pool, lower in the presence of neutral lipids and absent in the presence of polar lipids. Furthermore, the study indicated that mitochondrial volume changes induced by soaking of pea seeds in the presence of iron and copper ions resulted in the nonenzymatic oxidation of mitochondrial lipids (Haydar and Hadziyev, 1973a, b).

Pea seeds used in the present investigation contained about 6% protein consisting mainly of albumins and globulins. The albumins represented 8–15% of the total protein and probably serve a structural rather than a storage function in the embryo. They contain many enzymes and electrophoresis and ultracentrifugation studies revealed them to be polydisperse moving with low molecular weight compounds. Their isoelectric point was found to be at pH 6.0. Globulins represented on the average 70% of the total protein. They are rich in acidic amino acids and are composed of two distinct proteins: legumin and vicilin. Their molecular weights are much higher than the albumins and their isoelectric points are at pH 5.5 for vicilin and pH 4.8 for legumin (Danielsson and Lis, 1952). Klimenko and Pinegina (1964) have shown that independent of pea variety legumin to vicilin ratio is 1:1.13. The globulins are considered to be the major storage proteins and as revealed by Varner and Schidlovski (1963) are present in peas as large roughly spherical bodies, embedded in amorphous matrix protein.

The pea seeds used in this study as reported by Schoch and Maywald, (1968) contain starch granules with a highly restricted swelling characteristic and a high iodine-binding capacity. The starch content was 33% on a dry weight basis and had a 70% amylose content. These figures are consistent with those reported by McCready et al. (1950).

The data for localization of starch and globular proteins and those for intercellular localization of pectin in peas meristematic cell walls, suggest a spatial segregation of starch pectin and storage proteins from lipids. However, such a segregation would be altered in processed peas. As reported by Haisman (1962) cooked peas should be considered as a conglomerate of swollen tissue cells in a solubilized pectin gel. In purees the starch and globular bodies would in part lose their identity. There is thus a possiblity that globulins, pectin and starches may assume the role of a matrix in subsequent oxidation of the available lipids. Therefore, this study was undertaken to investigate the extent of lipid oxidation occuring in various lipid-matrix combinations. The envisaged approach for our previous and this study is illustrated on Figure 1.

This report presents the results of the individual lipid components of neutral and polar fractions and the results of the oxidation of these fractions on carbohydrate matrices consisting of amylose, amylopectin, cellulose and pectin, and pea seed protein matrices consisting of albumins and globulins.

Abbreviations

Abbreviations used throughout the paper include: DG, diglycerides; DGDG, digalactosyl diglyceride; DPG, diphosphatidyl glycerol; FA, fatty acid; FFA, free-FA; MGDG, monogalactosyl diglyceride; NL, neutral lipids; PA, phosphatidic acid; PC, phosphatidyl choline; PE, phosphatidyl ethanolamine; PG, phosphatidyl

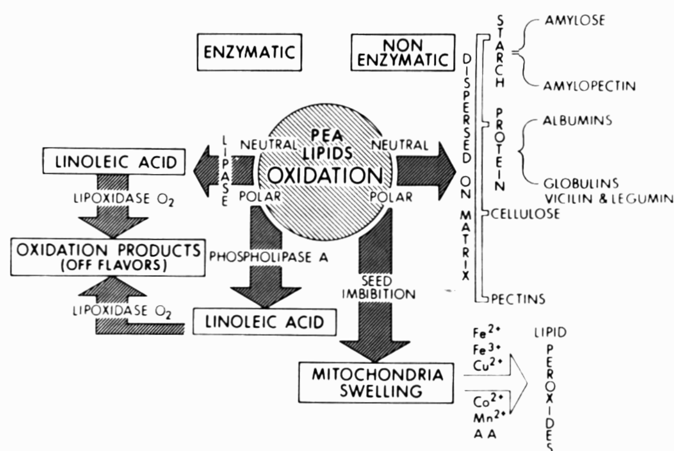

Fig. 1—Lipid oxidation pathways in pea seeds. The pathway via mitochondria swelling occurs during soaking of seed. The oxidation via enzymatic pathway is readily stopped by blanching of peas. The oxidation of lipids dispersed on solid matrices occurs in blanched whole peas or purees.

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glycerol; PI, phosphatidyl inositol; PL, polar lipids; PS, phosphatidyl serine; SG, sterol glycoside; TG, tryglycerides; and TL, total lipids.

EXPERIMENTAL

Pea lipids

Isolation and purification of total pea lipids. Three varieties of peas, Homesteader, Laxton and Stratagem were analyzed. Crude lipids were extracted from 100-g samples by refluxing the ground seeds with 4–6 vol of chloroform-methanol (2:1, v/v) for 5 min. After filtration, the residue was reextracted with hot fresh solvent mixture and the procedure repeated until a colorless residue was obtained. The extracts were combined and the solvent evaporated at room temperature using a vacuum flash rotary evaporator.

Nonlipid contaminants were removed by either the technique of Folch et al. (1957) or that of Williams and Merrilees (1970). In the first, the crude lipid extract (≃ 1 liter) was mixed thoroughly in a separatory funnel with 0.2 its volume of an aqueous solution of 0.58% sodium chloride. The mixture was kept at 4°C until two phases separated. The upper phase was then removed and the remaining interface was rinsed twice with small amounts of upper phase pure solvent. The lower phase was evaporated to dryness and the resulting residue was then dissolved in anhydrous ethanol-free chloroform prepared by refluxing water washed chloroform over calcium chloride for 2–3 hr. The lipid solution was then flushed with nitrogen and stored in darkness at –20°C.

In the second purification procedure, to the crude lipid extract about 15g of Sephadex G 25 was added and the solvents evaporated until no liquid remained in the flask. Then, the Sephadex was resuspended in 300 ml of anhydrous chloroform and dried down once more, after which it was again suspended in chloroform and poured into a chromatographic column (4.5 cm ID). The lipids were eluted with 200 ml anhydrous chloroform, concentrated and stored as mentioned earlier.

Lipid fractionation

Neutral and polar lipids. Total lipids (TL) were separated into neutral (NL) and polar lipids (PL) on a 2.2 × 40 cm silicic acid column. Silicic acid 100 mesh for chromatography from Mallinckrodt (New York, N.Y.) was washed with hot methanol followed with hot acetone and then dried at 105°C for 2 hr. Batches of Hyflo Super-Cel (Fisher Scientific Co., Fair Lawn, N.J.) were also treated in the same way. A mixture of 15g silicic acid and 5g of Hyflo Super-Cel was suspended in anhydrous chloroform and poured into the column. When the chloroform was drained, the TL were applied in the ratio of 30 mg/g silicic acid. The NL were eluted with 250 ml chloroform and the PL with 250 ml chloroform methanol (1:1, v/v). Solvents were evaporated and the remaining lipids were dissolved in anhydrous chloroform and stored until further use.

Composition of neutral and polar liquids. The individual lipid components of NL and PL fractions were identified and isolated by using the method of thin-layer chromatography (TLC). For thin-layer plates Silica gel G according to Stahl (Merck, Darmstadt, Germany) was used as an adsorbent. A slurry of 30g gel and 60 ml water was spread on 20 × 20 cm plates. The thickness of the layer was 200μ or 500μ in the preparative plates. The plates were dried at room temperature, activated at 110°C for 1 or 2 hr depending on thickness of the layer, and stored in a desiccator until used. After applying samples, the chromatograms were developed in chambers well lined with filter paper saturated with the solvent. The following solvent systems were used: ethyl ether-petroleum ether b.p. 40–60°C (20:100, v/v) for NL and (10:100, v/v) for sterols. One of two solvent systems was used for two-dimensional development of PL. The first system was that of Nichols and James (1964) using in the first dimension chloroform-methanol-7N ammonia (65:25:4, v/v) and chloroform-methanol-acetic acid-water (170: 25:24:4, v/v) in the second dimension, The second system was that of Rouser et al. (1970) in which the first solvent is chloroform-methanol-28% aqueous ammonia (65:25:5, v/v) and the second chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, v/v). In two-dimensional TLC the first solvent was evaporated usually under a vacuum of 28 in at room temperature for 20 min.

Spots were visualized by spraying the plates with 50% sulfuric acid and charring at 100°C for 10–15 min. Rhodamine 6 G or 2',7'-dichlorofluorescein were used for nondestructive detection of the spots. The first was prepared on the day of use by mixing 1 ml of stock solution of 0.1% (w/v) dye in methanol with 25 ml of 2N ammonia. Plates were sprayed with this solution or with 2',7'-dichlorofluorescein (0.5% in 95% ethanol) and viewed while still damp under UV light.

The identity of the spots was confirmed by comparison with authentic samples obtained from Hormel Institute (Austin, Minn.) and by specific detection methods. Glycolipids appeared as violet or green spots after spraying with 0.2% (w/v) anthrone in concentrated sulfuric acid followed by heating at 70°C for 20 min (Rosenberg et al., 1966). Spraying the plates with 0.2% ninhydrin in water saturated butanol followed by heating for several minutes at 105°C in an oven saturated with moisture, resulted in red-violet spots for lipids containing free amino groups.

The reagent prepared as described by Vaskovsky and Kostetsky (1968) was used for detection of phospholipids. Ammonium molybdate (16g) was dissolved in 120 ml of water to give solution I. Concentrated sulfuric acid (40 ml) and 10 ml of mercury were shaken with 80 ml of solution I for 30 min to give, after filtration, solution II. Concentrated sulfuric acid 200 ml followed by solution II was added carefully to the rest of solution I. The cooled mixture was diluted with water to 1 liter. Phospholipids were detected immediately after spraying at room temperature as blue spots on a white background.

For detection of PC, the plate was dried at 110°C for 15 min to remove traces of water and was sprayed while hot with 0.25% solution of cis-aconite anhydride in acetic anhydride. After the developments of red spots, the background was removed by spraying the plate with distilled water. The background disappeared and PC showed as a bright red violet spot (Vaskovsky and Suppes, 1971). Cis-aconite anhydride was prepared by heating the acid at 140°C under a vacuum of 5–10μ for 15 min. The residue was treated with hot benzene, cooled and the crystallized anhydride recovered by filtration using a Buchner funnel.

The spots were recovered from the plates as usual and extracted with chloroform methanol 1:1. Silicic acid was then removed by low-speed centrifugation and the individual lipids obtained were used for further studies.

Fatty acid analysis. For preparation of fatty acid methyl esters (FAME) a transesterification procedure was applied. An amount of 10–20 mg NL was gently refluxed in 2 ml of 1% (w/v) sulfuric acid in methanol for 2 hr while for PL, 10–20 mg was esterified under nitrogen with 2 ml of 2% (w/v) sulfuric acid in methanol in sealed ampules at 65°C overnight. After esterification the contents were diluted with water and the FAME were extracted with hexane, washed with 0.2N potassium carbonate and finally with water. The residual water was removed by anhydrous sodium sulfate and the methyl esters were then concentrated in a stream of nitrogen and used directly for GLC. For GLC analysis a Bendix Model 2500 Gas Chromatograph equipped with FID detector was used with glass columns 6 ft × 1/8 in., packed with 15% EGS coated on AW, 100/120 mesh Chromosorb P. Isothermal separation at 185°C was achieved using N_2 flow rate of 60 ml min. The fatty acids were identified by means of a calibration curve of retention times vs. equivalent chain length and by comparing with known methyl ester standards obtained from Hormel Institute.

For IR analysis of FA, the peas TL were used. After the saponification of lipids in ethanolic KOH at 65°C and in an atmosphere of nitrogen, the unsaponifiables were removed by ether extraction, while the FFA were recovered and their aliquots methylated by cold and by hot esterification procedures. The latter involved heating FFA at 60°C for 2 min in a closed vial with 14% methanolic BF_3. The cold esterification was achieved by allowing the FFA to stand at room temperature for 45 min with 5% methanolic sulfuric acid. The IR recordings of isolated FAME was done in the neat state and involved a wave-length range between 2.5 and 15μ using a Perkin Elmer Spectrometer Model 21, while for resolution in the region of 8.25–11.1μ a Beckman Model 12 was used.

Matrices

Ash free Whatman No. 1 paper for chromatography was used as cellulose matrix. Pectin, geno-pectin slow set from Food Products Ltd. (Montreal, Quebec). Amylose was from Sigma Chem. Co. (St. Louis, Mo.) and amylopectin from Nutritional Biochem Corp. (Cleveland, Ohio). The proteins used as matrices were albumins and globulins which were isolated before their use from pea seeds. Wrinkled-type pea seeds variety Homesteader (100g) were ground and extracted overnight by stirring with 350 ml of 0.05M phosphate buffer pH 7.0 containing 0.2M NaCl. The slurry was filtered through cheese-cloth and the debris-free homogenate was then centrifuged at 10,000 × G for 10 min. Proteins in the supernatant were precipitated overnight by 70% ammonium sulfate saturation and centrifuged at 10,000 × G for 10 min. The pellet was dissolved in 40 ml of the extraction buffer and dialyzed against running tap water overnight, followed by distilled water for 5 hr and finally against double distilled and deionized water until the absence of traces of chloride ions. The dialysate with protein suspension was centrifuged at 10,000 × G for 10 min to yield the globulin pellet (vicilin and legumin) and the supernatant with albumins. The latter was dialyzed for an additional 5 hr against deionized water and centrifuged at 10,000 × G for 10 min. The supernatant yielded the pure albumins. The pellet was com-

bined with the previous pellet and dialyzed against deionized water for 5 hr. The dialysate was centrifuged at 10,000 × G for 10 min and the pellet consisting of pure globulins was dissolved in a small volume of 0.2M buffered NaCl solution. Albumin and globulin fractions were then lyophilized by freezing in a dry ice-acetone bath and drying under a vacuum of 10–20μ for 24 hr. All isolation steps were performed at 2–4°C.

Preparation of the model systems and oxidation assay

The filter paper technique of Karel (1960) was adopted for studying the matrix effect on lipid oxidation. Filter paper discs 3.5 cm in diameter were dipped for 10 sec in 1% aqueous solution of the matrix except for globulin when water contained 0.2M sodium chloride. The discs were dried in air on a stainless steel wire tray for 10 min and then dried at room temperature for 24 hr in a vacuum of 10–20μ. The dehydrated filter papers were weighed, dipped for 10 sec in chloroform solutions containing 10–20% NL or PL, and the dehydration procedure repeated. Four filter paper discs were placed in the reaction flask of a Gilson differential respirometer. The oxidation was conducted in air at 50°C for 20–50 hr. To eliminate the effect of oxygen depletion, the flasks were flushed with dry air at suitable intervals, 10–15 hr at the beginning, and 2–5 hr near the end of the run. All experiments were done at least in duplicate.

RESULTS

Pea lipids

Separation and identification. Pea seeds contained 3–4% TL extractable by a hot chloroform methanol mixture (2:1, v/v). Laxton had the highest TL lipid content while Homesteader had the lowest, and Stratagem was in between (Table 1). The NL/PL ratio varied considerably between varieties and was highest for Laxton while that of Homesteader and Stratagem was low and comparable. The IR analysis of FA methyl ethers of the peas TL revealed the characteristic hydrocarbon chain absorptions at 3.4, 6.85, 7.25 and 13.85μ. No superimposed bands of functional groups such as –OH or C=O were detected. The ratio of absorbance at 3.3μ to that at 3.5μ (=C–H stretching) (Arnold and Hartung, 1971) being for Homesteader 0.285, Laxton 0.319 and 0.311 for Stratagem, revealed a degree of unsaturation equivalent to iodine numbers of 118, 124 and 121, respectively. The absence of a peak at 10.36μ and its presence at 4μ region indicated the ab-

Table 1–The TL percentage and the ratio of NL/PL in the investigated pea seed varieties[a]

Variety	% Moisture	% TL	NL/PL Ratio
Homesteader	15.4	3.0	1.8
Laxton	17.5	3.9	2.3
Stratagem	16.0	3.4	1.7

[a] Percentages are calculated on dry basis

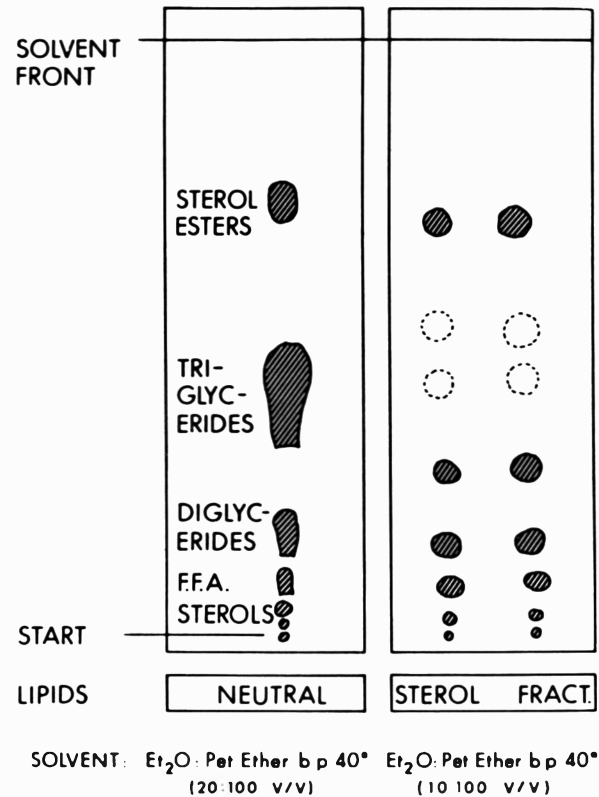

Fig. 2–One-dimensional TL chromatograms of neutral and nonsaponifiable fractions of pea seed lipids, using as adsorbent silica gel G (Merck).

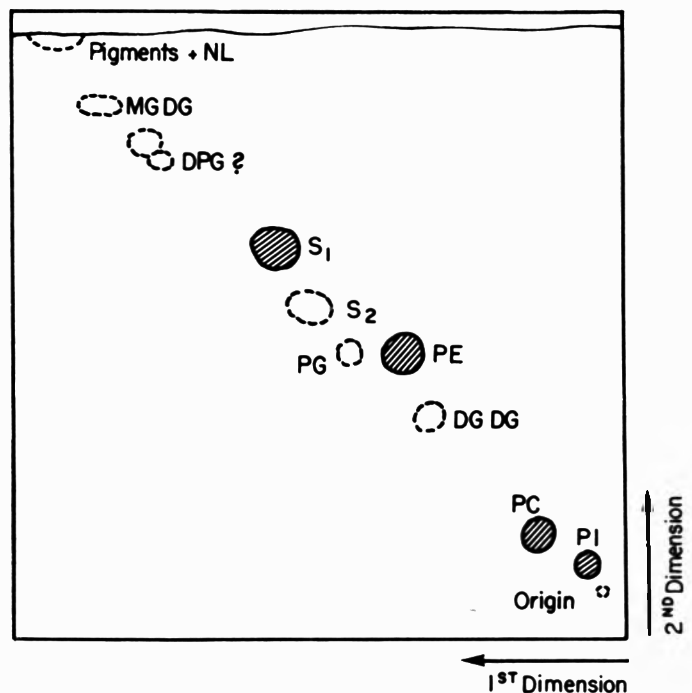

Fig. 3–Two dimensional TL chromatograms of pea seeds' PL. The adsorbent layer was 200μ of silica gel G (Merck). Solvent system was chloroform-methanol-7N ammonia (65:25:4, v/v) in the first dimension, and chloroform-methanol-acetic acid-water (170:25:24:4, v/v) in the second dimension (Nichols and James, 1964). P-chloromercuribenzoate (PCMB) added to the extraction solvent to inhibit phospholipase D and other enzyme activities during extraction did not bring about a change of pattern of the lipids on the chromatograms.

sence of trans and the presence of only cis unsaturation. Furthermore, a high resolution recording in the region of 8.25–11.1μ revealed the absence of any absorption peak due to conjugated dienes. All these findings were the same for FA samples of the three varieties regardless of the esterification procedure used.

The NL of pea seeds as revealed by one-dimensional TLC (Fig. 2) consist mainly of TG, small amounts of DG, FFA and free and esterified sterols. Pea sterols recovered from the unsaponifiable fraction after alkaline saponification of TL were also complex in composition. One dimensional chromatograms obtained by a slightly less polar solvent than that used for developing NL revealed seven well defined spots within this class of compounds (see Fig. 2).

The PL of pea seeds were separated by two-dimensional TLC using two different solvent systems. Both systems revealed the presence of 9–10 clear spots (Fig. 3 and 4) of which PI, PC, PE and SG (S_1) were the major lipids. Among these, PE appeared as the most intensive spot upon charring the plate with sulfuric acid. Present to a lesser extent were MGDG, DGDG, PG, traces of NL and pigments, a stationary faint spot at the origin, a sterol spot (S_2) and possibly DPG. No spots representing PA or PS were apparent in either solvent system. Using the first solvent system (Fig. 3), two overlapping spots appeared below the spot containing MGDG, but appeared as one spot when the second solvent system was used (Fig. 4).

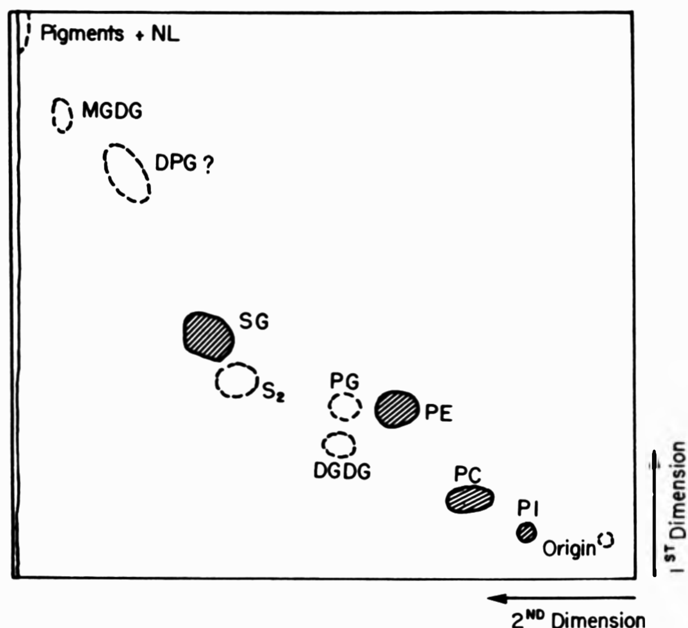

Fig. 4–Two-dimensional TL chromatograms of pea seeds' PL. The adsorbent layer was 200μ of silica gel G (Merck). Solvent system used was chloroform-methanol-28% ammonia (65:25:5, v/v) in the first dimension, and chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5, v/v) in the second dimension (Rouser et al. 1970).

Table 2– The FA composition of total, neutral and polar pea lipids

FA[a]	Homesteader			Laxton			Stratagem		
	TL	NL	PL	TL	NL	PL	TL	NL	PL
12	tr[b]	tr	tr	tr	tr	tr	tr	tr	0.7
14	tr	tr	tr	tr	tr	tr	tr	tr	0.5
15	tr	tr	tr	tr	tr	tr	tr	tr	0.7
16	11.4	10.1	17.6	11.5	10.9	18.0	11.2	9.9	12.5
16:1	tr	tr	0.7	tr	tr	0.9	tr	tr	3.2
16:2(or 17)	tr	tr	0.7	tr	tr	0.6	tr	tr	1.8
18	4.0	3.8	5.6	3.4	2.9	3.4	4.5	4.0	4.7
18:1	27.6	26.7	24.8	34.9	29.3	30.6	31.5	29.8	30.9
18:2	48.1	46.4	46.1	41.5	43.4	42.1	45.2	45.5	41.8
18:3	8.3	12.4	3.6	8.3	12.4	3.2	7.4	10.3	2.8
Iodine value (by IR)	118.0			124.0			121.0		
DBI[c]	1.49	1.57	1.29	1.43	1.53	1.25	1.44	1.52	1.26

[a] Expressed as % of total FA content
[b] tr, less than 0.5%
[c] DBI, double bond index, an indication of unsaturation degree is as useful as iodine value, when the lipid samples are available in microscale amounts (Lyons et al., 1964). The values are expressed as the summation of the weight percent of fatty acid multiplied by the number of double bonds it contains per molecule and divided by 100.

Among PL, all individual phospholipids gave positive reactions with the phosphorus detecting reagent; the intensity of the blue-colored spots being strongest for PE followed by PC and then PI. PE spot gave a distinct red color with ninhydrin, and PC gave a positive reaction with the choline detecting reagent. The spots containing galactolipids, MGDG and DGDG, gave a violet color when sprayed with α-naphthol in sulfuric acid before heating. Sterols were revealed by their distinct purple color developed at the beginning of charring with sulfuric acid. Besides the use of specific spraying reagents the identity of the major lipids was also confirmed by the migration pattern of identical commercial samples or individual lipid samples prepared in this laboratory (MGDG and DGDG). In addition, IR spectra were used for further confirmation of some lipid compounds.

Spectra of IR absorption for MGDG and DGDG in the solid state (potassium bromide pellet) revealed major absorption bands at 2.9, 3.4, 5.75 and 9.4μ, the first major peak corresponding to –OH stretching from sugar moiety, the second as a double peak to the symmetrical and nonsymmetrical stretching of hydrocarbon chain, the third to stretching of C=O in ester group, and the fourth as a wide band resulting from C-O stretching of ester group. The small bands representing unconjugated cis double bond appeared at 3.3 and 6.1μ reflecting the bending vibration of olefinic hydrogen atoms. Cis double bonds, in addition, were easily detected by a rather broad band at the region of 14–15μ. From the spectra recorded, it appears that none of these bands even that of –OH could be used to distinguish MGDG from DGDG. Nevertheless, the –OH absorption band was reliable in distinguishing galactolipids from some phospholipids such as PE and PC.

Identification of PE and PC in ethanol free chloroform solutions has been carried out by recording their spectra in the region of 8.5–11μ. Absorption patterns related to nitrogenous moieties were reflected by a single peak at 9.3μ for PE

and by a double absorption peak between 9–9.7μ for PC.

FA composition. The FA composition of total, polar and neutral pea seed lipids is presented in Table 2. In all cases the presence of about 10 FA was apparent. Linoleic acid was the major FA up to 40–50% in TL, PL and NL of pea seed, followed in decreasing order by oleic, palmitic, linolenic and stearic acids. The other FA including the odd carbon number acids were present only in traces. Minor differences in FA ratios were observed in TL among the three varieties. Thus, Homesteader had a slightly higher content of linoleic but a lower content of oleic acid. The ratios of the other FA were comparable. The FA composition of PL revealed, with no exception, a higher content of saturated acids such as palmitic and stearic, but a lower content of linolenic acid, when compared to those of NL. The ratios of linoleic and linolenic acids were comparable in both NL and PL. Among the three varieties, Homesteader had the highest content of linoleic and the lowest content of stearic acid in both lipid fractions, i.e., NL and PL. For PL fraction, the lowest amount of palmitic acid was found in Stratagem. Nevertheless, differences in FA composition of the three varieties investigated were not sufficiently high for varietal characterization.

As seen from Table 3 all pea seeds' PL components analyzed contain high amounts of palmitic and oleic acids but only traces of linolenic acid. Among the PL, PC had the highest content of palmitic and the lowest content of stearic acid, while both SG and MGDG had the highest content of oleic acid. When FA composition of the two galactolipids were compared, MGDG had considerably higher levels of saturated acids such as myristic and stearic, and a higher level of oleic acid but a much lower level of linoleic acid, than those in DGDG. From the FA composition found for the six major constituents of the seed PL analyzed, no FA specificity for a given lipid compound could be ascertained.

The low level of unsaturated FA's and the corresponding low values for Double Bond Index (DBI) for glycolipids were unexpected (Table 2). Therefore, to verify the identity of these lipids their isolation was repeated but this time from pea leaves and purified pea chloroplasts. The lipids thus obtained cochromatographed with those of seeds, though their FA composition differed substantially. The unsaturated acids present in MGDG and DGDG were oleic 2.8 and 2.4, Linoleic 11.7 and 5.4 and linolenic 82 and 83.8%, respectively. The corresponding DBI values were thus high being 2.73 for MGDG and 2.65 for DGDG which values agreed with unsaturation degrees for green plant tissue (O'Brien and Benson, 1964).

Table 3–The FA composition of some major PL of pea seeds var. Homesteader

FA[a]	PI	PC	PE	SG	MGDG	DGDG
10	tr	tr	tr	1.1	tr	0.9
12	1.0	tr	tr	3.6	3.2	1.4
14	1.7	0.8	tr	4.8	4.2	1.0
15	0.6	0.6	tr	tr	tr	0.7
16	35.5	41.9	22.4	18.6	21.4	20.5
16:1	1.6	0.9	tr	3.2	tr	tr
16:2(or 17)	1.6	1.4	tr	tr	tr	1.2
18	13.4	3.3	6.9	10.4	12.4	6.7
18:1	34.1	26.1	46.9	47.8	40.4	33.2
18:2	10.0	24.3	23.5	10.5	18.4	34.2
18:3	tr	tr	tr	tr	tr	tr
DBI	0.55	0.76	0.94	0.72	0.77	1.02

[a] Expressed as % of total FA content

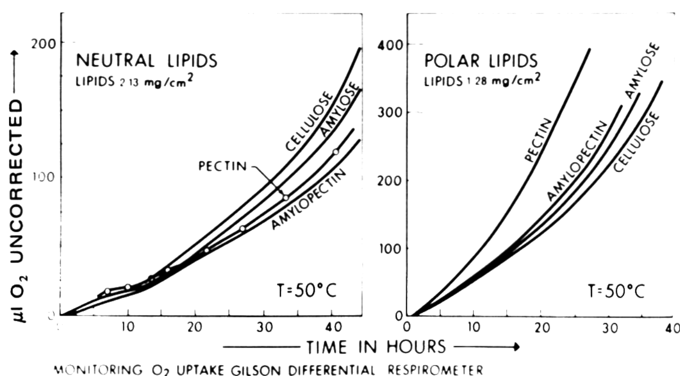

Fig. 5–Oxygen uptake of neutral and polar pea lipids as influenced by carbohydrates used as matrices. The filter paper disc procedure (Karel, 1960) and a Gilson Differential Respirometer were applied.

Matrix effect on lipid oxidation

Preliminary experiments with 30 mg of NL and 18 mg of PL, coated on one filter paper disc, and oxidized at 25°C and 35°C resulted in an uptake of 0–2 μl O_2 in the first 48 hr, and less than 2 μl for NL, and less than 7 μl for PL in the initial 24 hr, respectively. Therefore, oxidation experiments were conducted at 50°C. At this elevated temperature about 120 mg of neutral and 70 mg of polar pea lipids were found satisfactory for obtaining reliable readings of oxygen uptake when proteins and carbohydrates were applied as matrices. Furthermore, when these amounts of lipids were coated on one filter paper disc, having a surface area of 14 cm^2, by dipping the disc in a 50% chloroform solution of lipids, the amounts of lipids adsorbed were poorly reproducible. Satisfactory results were obtained when 10–15% solution of lipids were used and instead of one, four filter paper discs per 15 ml flask of the respirometer. Due to the high gelling property of pectin and to the high retrogradation of pure amylose solutions, these matrices along with those of proteins were prepared in 1% aqueous solutions. The amount of solid support adsorbed per disc averaged 3.0 mg, i.e., 0.215 mg/cm^2. On such matrices the amounts of NL and PL adsorbed reflected a ratio of 1.67 which approximately simulated that found in pea seeds.

The effects of carbohydrates such as amylose, amylopectin and pectin on the oxidation rate of polar and neutral pea lipids are shown in Figure 5. The rate of oxidation, being expressed in accumulated actual oxygen consumption vs. time, was for NL slow at the first 12 hr, after which time it increased and after 40 hr reached a value of 1–1.5 μl O_2/mg of lipid applied. These results have shown that lipids coated on cellulose disc itself which was not precoated and thus had a role of a cellulose matrix, were oxidized at a higher rate than lipids present on cellulose precoated with other carbohydrates.

Among carbohydrates, the oxidation rate with amylopectin after 40 hr was 26% less than that found for amylose,

while with pectin as matrix, the oxidation was highest at the initial 12 hr, but decreased by time and after 22 hr ranked between the rates for amylose and amylopectin. In comparison to cellulose, the matrix of pectin induced a 26% inhibition in oxygen uptake. When this inhibition is compared to that of amylopectin which had the highest retardation degree of oxidation, the inhibition was still about 11% less effective.

Contrary to peas' NL, the oxidation rate for PL when expressed per mg of adsorbed lipid was more than four times higher. In these experiments pectin as matrix exerted the highest promoting effect thus giving the highest oxidation rate, while cellulose gave the lowest. The effects of amylose and amylopectin were similar, but in comparison to NL their effects on lipid oxidation were reversed; amylose being the lower. Finally, with the exception of pectin all matrices exerted an equal effect during the initial stage of oxidation, and with all matrices the oxidation proceeded with no apparent induction period.

When recorded oxidation rates and previously given FA composition for peas' NL and PL are compared, it appears that the unsaturation degree is not of primary importance but the polarity of the lipid which is being oxidized.

The effect of proteins such as pea albumins and globulins on the oxidation rate of lipids is shown in Figure 6. Again there was a small effect in promoting oxidation of NL. After 40 hr the cumulative oxygen uptake for albumin matrix was about 16% less than that of globulins. When compared to cellulose matrix, used in these experiments as control, both proteins were slightly retarding the oxidation rate. After 40 hr of oxidation their retardation degree averaged around 13%, being slightly higher for albumin.

Oxidation rates for peas' PL were very high. This was particularly the case with globulins as matrix, which after 20 hr was 2.8 times higher than that with albumin, while after 40 hr of oxidation the results were not comparable by direct manometric readings. The albumin matrix effect was similar to that of cellulose but again slightly retarding the oxidation degree. With albumin as matrix, the oxidation rate of PL when expressed per mg lipid and compared to that of NL was 3.4 times higher after 40 hr, while with globulin, it was 13 times higher after only 20 hr of oxidation. This again suggests that polarity rather than unsaturation degree has the primary influence in lipid oxidation. Furthermore, the results showed clearly that globulin and not albumins was the main oxidation promoting matrix.

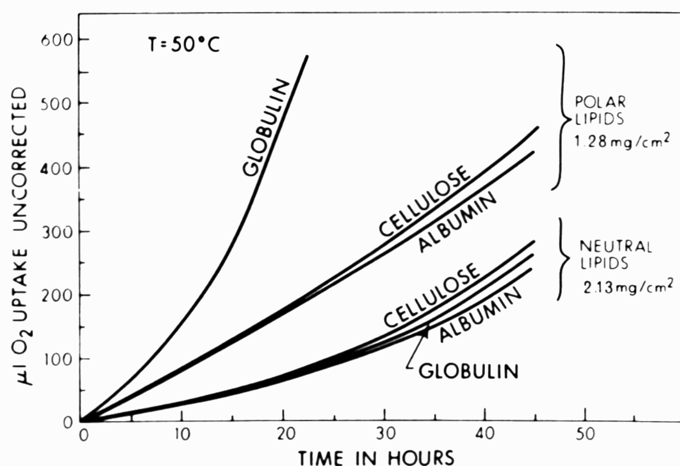

Fig. 6–Oxygen uptake of neutral and polar pea lipids as influenced by pea proteins used as matrices. Readings were corrected only for oxygen uptake by proteins themselves. Other details as in Fig. 5.

DISCUSSION

PEA SEEDS' PL investigated are more complex than NL. The major seeds' PL found were PE, PC and PI in decreasing order. The results are not in agreement with those reported in the literature which also are inconsistent. Wagenknecht et al. (1959) reported the absence of PI in dry pea seeds, while Quarles and Dawson (1969) reported PI as a major PL second only to PC. The latter authors reported the presence of PG, DPG, PA and PS in minute amounts, and N-acyl-PE in a relatively large amount; however, the last three lipids were not detectable in our study. But, while Adhikari et al. (1961) suggested the presence of galactolipids in pea seeds merely by galactose determination, the present results confirmed the presence of MGDG and DGDG and established the presence of two other sterol glycolipids that were not reported earlier. The absence of lyso derivatives of PC and PE on the other hand indicated the absence of phospholipase action in the seeds investigated.

The fatty acid composition of pea seeds' TL indicated only slight variations between varieties, although large differences from those reported in the literature were apparent. In the present study the percent composition of the FA content was as follows: $C_{18:2}$, 45; $C_{18:1}$, 32; C_{16}, 11; $C_{18:3}$, 8; and $C_{18:0}$, 4. The comparative data reported by Bengtsson and Bosund (1966) were 55, 12, 23, 9 and 2, respectively. On the other hand the FA composition of PL revealed a lower degree of unsaturation than that of NL, which agrees with the early generalizations about seed lipids made by Aylward (1956), and with the results reported by other authors (Lee and Mattick, 1961; Bengtsson and Bosund, 1966).

The subsequent oxidation study with neutral and polar pea lipids, as influenced by matrix revealed distinct differences among matrices as well as lipid fractions. Thus the amylopectin NL system consistently performed a retarding action in lipid oxidation. This result was unexpected since it has been well established (Senti and Erlander, 1964) that amylose and not amylopectin forms nonstoichiometric inclusion compounds with fatty acids. Such compounds are stable both in a solid state and solution. More recently Wren and Merryfield (1970) have shown that in wheat, starch lysolecithin, not free fatty acids, is the lipid constituent of the natural inclusion compound. In the present study the dry amylose matrix was soaked in chloroform solutions of the lipids under investigation. Such a procedure should place the suitable lipid components within the amylose helix. In the case of neutral lipids the absence of any retarding effect of the amylose on oxidation suggested that such an interaction did not occur. On the other hand the presence of amylose brought about a consistent decrease in the rate of oxidation of PL. This observation suggested that PL formed an inclusion compound.

Glycolipids, mono- and digalactosyldiglycerides were also present in the PL fraction. Wehrli and Pomeranz (1970) demonstrated the interaction of these with wheat flour starch. They concluded that the polar galactosyl moiety was bound to the starch, the highly unsaturated fatty acid chains being free in an extended trans conformation. In the present study the oxidation rate of PL on carbohydrate matrices even though reduced by the presence of the carbohydrate was still four times higher than that of NL under the same conditions. To ascribe the

oxidation rate difference solely to the exposed glycolipid fatty acids would not be justified. Firstly, these are minor constituents of the PL fraction; secondly, they were found to be less unsaturated than the glycolipids of cereals reported by Graveland (1968) and even less so than those of pea leaves.

The pectin matrix effect on lipid oxidation appears to be two-fold. In oxidation of NL it retards whereas for PL it enhances the oxidation. Bishov and Henick (1961) indicated that pectin generally exhibits a protective action, which action correlates with the charge on the pectin molecules. The results of the present study have shown that retardation is exclusively confined to NL but not to PL. In the latter case an interaction between the negatively charged matrix and the zwitter-ion components of PL has to be assumed. If this assumption is valid then the basic electrophylic nitrogen of PC and PE would interact, while the nucleophylic centers released would orient the fatty acid chains to a sterically more exposed conformation, thus providing an increased rate of oxidation.

The protein matrix effect on lipid oxidation was relatively small with the NL and substantial with the PL fraction. Togashi et al. (1961) demonstrated that the higher the charge on the gelatin surface the lesser the rate of oxidation. If the measure of the charge is reflected by a numerical difference of the protein isoelectric points and pH, as suggested by these authors, then in the present study in which proteins were isolated in a buffer of pH 7.0, globulins should bring about a higher retardation than albumins. However, the results were consistently opposite: globulins promoted while albumins retarded the rate of lipid oxidation.

From the results discussed above it must be concluded that lipid polarity rather than its degree of unsaturation is the predominant factor controlling the rate of oxidation. In addition, it is evident that physical and chemical interactions of lipids and matrices do influence the rate of oxidation. The role and mechanisms of these interactions are the subject of our current investigation.

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CALCIUM ACTIVATION OF SOYBEAN LIPOXYGENASE

INTRODUCTION

SOYBEAN LIPOXYGENASE is unique among dioxygenases in not requiring a metal activator or prosthetic group. Compounds such as cyanide, fluoride, azide and diethyl dithio carbamate do not inhibit lipoxygenase (Holman and Bergstrom, 1951; Tappel, 1961). An activator, thought to be protein, was described (Balls et al., 1943; Kies, 1947) but never fully characterized. Smith and Sumner (1948) disputed the existence of such an activator.

Franke and Frehse (1953) reported that barley lipoxygenase gained activity when dialyzed against tap water but lost activity when dialyzed against distilled water. They found that Ca^{2+} in the tap water was responsible for the increased activity but could not demonstrate a similar Ca^{2+} activation with soybean lipoxygenase. Koch (1968) found that navy bean lipoxygenase and soybean lipoxygenase were activated by Ca^{2+}.

Yamamoto et al. (1970) were unable to confirm that purified soybean lipoxygenase was activated by Ca^{2+}, but they isolated a Ca^{2+}-activated isozyme of the usual soybean lipoxygenase. The Ca^{2+} was effective in overcoming a substrate inhibition of the isozyme. Holman et al. (1969) reported that as little as 1×10^{-7}M Ca^{++} activated the soybean lipoxygenase that Holman isolated and studied in 1947.

Recently, Koch et al. (1971) have extended their studies of Ca^{2+}-activated lipoxygenases to the effects of Ca^{2+} concentration, of the order of Ca^{2+} addition, of buffer effects and of response to Tween 20 and a cold water insoluble material from navy beans. These studies confirm Ca^{2+} activation of lipoxygenase but raise questions about how Ca^{2+} is acting. For example, Ca^{2+} activates lipoxygenase when added to the reaction mixture before the enzyme but does not activate when added after the enzyme.

Our interest in soybean lipoxygenase derives from the importance of the enzyme in causing off flavors in soy products. We chose to examine the Ca^{2+} activation because of conflicting reports in the literature as to the existence of Ca^{2+} activation and because of the questions about the mechanism of Ca^{2+} activation.

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EXPERIMENTAL

Materials

We purchased partially purified soybean lipoxygenase from Sigma Chemical Co., St. Louis, Mo., and did no further purification. Concentration was 1 mg ml^{-1} in deionized water.

Crude soybean extract was prepared according to Dillard et al. (1960). We prepared navy bean extracts the same way except that the beans were not defatted.

Lipoxygenase 1 and lipoxygenase 2 were prepared as described under Methods.

The linoleic acid was Sigma Chemical Company's Grade III, approximately 99% pure, in 1-g ampules. Open ampules were stored in a freezer under N_2. We prepared stock solutions of linoleic acid in ethanol at 10 mg ml^{-1}. This stock solution was diluted in buffer as needed for individual assays.

Trilinolein came from Supelco, Inc., Bellefonte, Pa., and was stored in a 60% ethanol–40% acetone mixture. The ethanol-acetone mixture contained 20 mg ml^{-1} trilinolein, which was added directly to buffer for assays.

Distilled, deionized water and reagent grade chemicals were used.

Methods

All lipoxygenase assays measured O_2 disappearance with a polarographic O_2 electrode (Beckman Instruments or Yellow Spring Instruments) and model SRG recorder (Sargent Welch). The reaction vessel used with the Beckman electrode contained 47 ml, with the electrode inserted in the side just above a magnetic stirrer, while the YSI electrode was inserted in a 3-ml reaction vessel (Model 53 YSI oxygen monitor).

Reaction mixtures with the Beckman electrode contained 45 ml of buffer (0.2M Tris, pH 8 for most experiments), 1 ml of the stock solution of either linoleic acid or trilinolein as substrate (7.9×10^{-4}M linoleic acid or 5×10^{-4}M trilinolein) and 1 ml of enzyme. Reaction mixtures used with the YSI electrode contained 7×10^{-4}M linoleate dispersed with 0.004% Tween 20 in 0.2M Tris at pH 8.

At the start of an assay, the O_2 electrode was adjusted to 100% after equilibration with buffer and substrate. Addition of enzyme started the reaction. Enzyme activity is reported in units of μmoles O_2 min^{-1} ml^{-1} of enzyme. All assays were at 25°C, and oxygen concentration was assumed the same as oxygen dissolved in water at 1 atm and 25°C.

We removed phytates by using the alkali precipitation method of Smith and Rackis (1957) as modified by Wang (1971).

Lipoxygenase 1 and 2 were prepared by extracting 100g defatted soy flour with 1 liter of 0.05M acetate pH 4.5 containing 0.1 mM Ca^{2+}. The extract was clarified by centrifugation and taken to 40% saturation with $(NH_4)_2SO_4$. The precipitate was rehydrated with 0.01M phosphate pH 6.8 and dialyzed overnight against the same buffer. The supernate was taken to 70% saturation, and the precipitate was rehydrated with 0.01M phosphate pH 6.8 and dialyzed overnight against the same buffer.

Both the 40% and 70% ammonium sulfate precipitates were chromatographed on DEAE Sephadex A-50 (Sigma Chemical Co.) using 0.01M phosphate pH 6.8 and a NaCl gradient for elution. The 40% precipitate gave two peaks with lipoxygenase activity. The first peak corresponded to lipoxygenase 2 and the second peak corresponded to lipoxygenase 1 as isolated by Christopher et al. (1970). The 70% ammonium sulfate precipitate yielded only one peak on chromatography and that was lipoxygenase 1.

RESULTS & DISCUSSION

WE FOUND that rates of O_2 uptake are proportional to amounts of lipoxygenase,

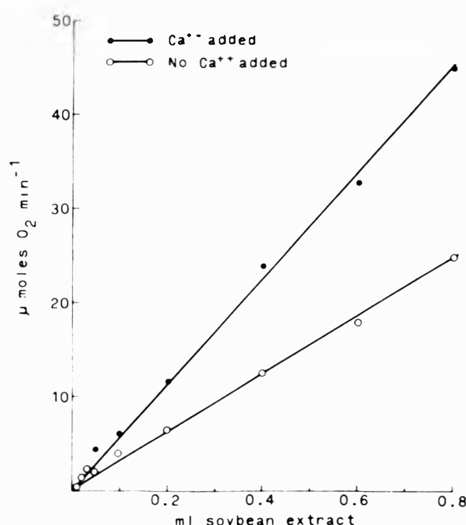

Fig. 1—The linear response of oxygen uptake to amount of lipoxygenase and the activation of the reaction by Ca^{2+}. The reaction mixture contained 7.9×10^{-4}M linoleic acid, the indicated amount of soybean extract as enzyme, and 1×10^{-3}M Ca^{2+} when added concurrently with enzyme. The buffer was 0.2M tris at pH 8.0, and the temperature was 25°C.

at least up to 10 μmoles O_2 min^{-1} for each reaction mixture. Figure 1 shows, for crude soybean extracts, that O_2 uptake is proportional to the amount of lipoxygenase for rates as high as 45 μmoles O_2 min^{-1} at pH 8, while Figure 2 shows that with Ca^{2+} added at pH 7.5, the proportionality between enzyme added and O_2 uptake only exists to about 10 μmoles min^{-1}. Mitsuda et al. (1967) found that amounts of crystalline lipoxygenase are proportional to O_2 disappearance up to 0.7 μmoles min^{-1} in a 3-ml reaction mixture. This corresponds to 10 μmoles min^{-1} in a 47 ml reaction mixture, since the amount of O_2 change that can be measured polarographically depends on the volume of the reaction mixture.

One of the limiting factors in polarographic measurements is the response time of the Clark type electrode. A rate of 10 μmoles O_2 min^{-1} in a 47 ml reaction volume corresponds to a change of approximately 210 μM min^{-1}, or about 90% of the saturation value of 240 μM O_2 in water at 25°C. The response time of the Beckman electrode we used is 90% of the actual change in 10 sec, and the rate of 45 μmoles min^{-1} shown in Figure 1 is roughly a 100% change in O_2 in 15 sec or close to the absolute limits of the electrode. Thus, the response time of the electrode is not responsible when 10 μmoles min^{-1} is the upper limit of linearity between O_2 uptake and amounts of lipoxygenase, but the limiting factor is not known.

Figures 1 and 2 also show the definite Ca^{2+} activation found when soybean extract was used as enzyme and linoleic acid as substrate. We explored several variables for their effect on Ca^{2+} activation, with the results shown in Table 1. If trilinolein is used a substrate, no Ca^{2+} activation can be detected. Dialysis of a soybean extract decreased activity and decreased the response to Ca^{2+}. If Ca^{2+} is used in the extraction of lipoxygenase, then added Ca^{2+} has no effect, and the activity on trilinolein is decreased. Fractionating a soybean extract with $(NH_4)_2SO_4$ (50% saturation gave a preparation that responded to Ca^{2+} approximately the same as soybean extract.

Further studies of the effect of Ca^{2+} on three different lipoxygenase preparations are summarized in Table 2. The water extracts from soybeans and navy beans are activated by Ca^{2+}, but a purified commercial lipoxygenase is not.

The lack of response to Ca^{2+} by purified lipoxygenase can be explained by the results of Yamamoto et al. (1970). They found that one isozyme (lipoxygenase a), which corresponds to lipoxygenase 1 as described by Christopher et al. (1970) and is the most studied lipoxygenase from soybeans, was inhibited by Ca^{2+}, and that another isozyme, lipoxygenase b according to Yamamoto et al. (1970) or lipoxygenase 2 according to Christopher et al. (1970), is activated by Ca^{2+}. The activation was characterized as overcoming substrate inhibition. Holman et al. (1969) found Ca^{2+} activation of lipoxygenase at 10^{-7}M Ca^{2+}, a concentration several orders of magnitude less than the Ca^{2+} concentration used by others. Since Holman et al. (1969) worked with lipoxygenase a or 1, their results conflict with those of Yamamoto et al. (1970).

We isolated lipoxygenase 1 and 2 from a soybean extract and tested both isozymes for their response to added Ca^{2+}. Figure 3 shows that lipoxygenase 1 is not activated by Ca^{2+} but slightly inhibited whereas Figure 4 shows lipoxygenase 2 is activated by Ca^{2+}. We also extracted the purified commercial lipoxygenase and attempted to isolate lipoxygenase 1 and 2

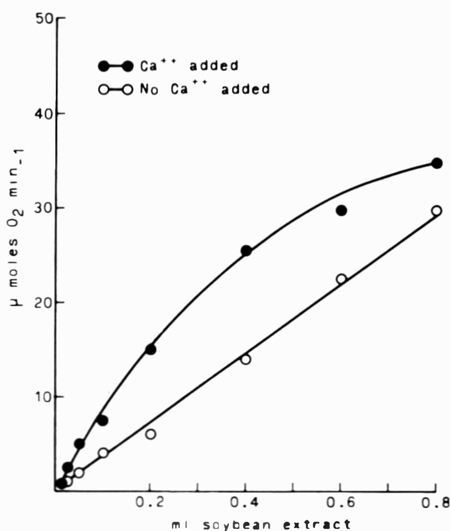

Fig. 2–The response of oxygen uptake to lipoxygenase and the activation of the reaction by Ca^{2+}. Reaction conditions were the same as for Figure 1 except the pH was 7.5.

Table 1–Ca^{2+} activation after several lipoxygenase treatments as measured on linoleic acid and on trilinolein[a]

	Activity in μmoles O_2 min^{-1} ml^{-1} on:			
	Linoleic acid		Trilinolein	
	No Ca^{2+}	Ca^{2+}	No Ca^{2+}	Ca^{2+}
Soybean extract	23.5	39.5	17.4	13.5
Dialyzed soybean extract[b]	19.8	25.0	14.1	14.0
$CaCl_2$ treated extract[c]	16.9	15.4	8.5	7.2
Dialyzed $CaCl_2$ treated extract	18.8	21.1	9.0	8.7
Ammonium sulfate precipitate[d]	31.5	42.8	8.8	6.2
Ammonium sulfate supernate	0.4	1.4	–	–

[a] Reaction mixture contained 0.2M tris pH 8, 7.9 × 10^{-4}M linoleic acid or 5 × 10^{-4}M trilinolein, and 1 × 10^{-3}M Ca^{2+} when added. Total volume was 47 ml; 25°C.
[b] Dialysis at room temperature for 24 hr using frequent changes of deionized H_2O.
[c] The extraction was done in the presence of 10^{-2}M Ca^{2+}.
[d] Precipitate from 50% saturation with $(NH_4)_2SO_4$ rehydrated in 0.2M tris pH 8.

Table 2–Ca^{++} activation of three different lipoxygenase preparations and dependence of the Ca^{++} activation on the sequence of addition

	Activity: μmoles O_2 min^{-1} ml^{-1}		
Sequence of addition to the reaction mixture[a]	Partially purified enzyme[b]	Navy bean extract	Soybean extract
Buffer + linoleic acid + enzyme	5.4	0.05	23.5
Buffer + Linoleic acid + enzyme + Ca^{++}	4.8	0.04	23.0
Buffer + linoleic acid + Ca^{++} + enzyme	5.1	4.8	28.5
Buffer + linoleic acid + (Ca^{++} + enzyme)[c]	5.3	6.7	39.5

[a] The reaction mixture contained 0.2M tris pH 8, 7.9 × 10^{-4}M linoleic acid and 1 × 10^{-3}M Ca^{2+} when added. Total volume was 47 ml; 25°C.
[b] This lipoxygenase preparation was Sigma Type I containing 1 mg ml^{-1}.
[c] Ca^{2+} and lipoxygenase were mixed and held for approximately 1 min before adding to the reaction mixture.

from it. We could only find lipoxygenase 1 and conclude that this is the reason for lack of Ca^{2+} activation with the commercial lipoxygenase.

Koch et al. (1971) found that Ca^{2+} activation of crude lipoxygenase occurs only when Ca^{2+} is added to the reaction mixture before or concurrently with enzyme, and we have confirmed this (Table 2). Such a finding precludes a simple combination of Ca^{2+} with lipoxygenase to form an active complex and is an important piece of information about the mechanism of activation by Ca^{2+}. Consequently, we investigated some possible explainations for the importance of sequence of addition of Ca^{2+} and lipoxygenase.

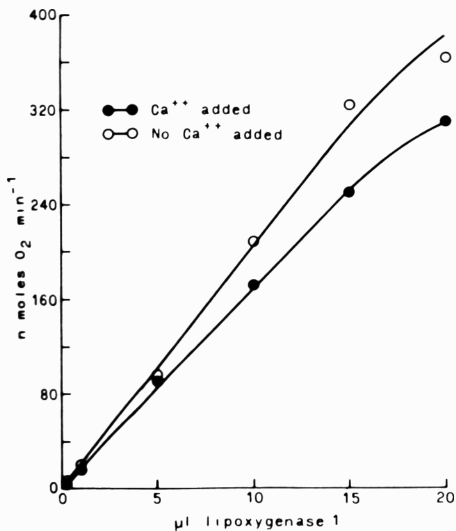

Fig. 3—Response of lipoxygenase 1 to Ca^{2+} addition. Assay was done with YSI electrode and 3 ml reaction mixture. Substrate was 7 X 10^{-4}M linoleate dispersed with 0.004% Tween 20. Buffer was 0.2M tris pH 8 at 25°C. Ca^{2+} was added before enzyme and was 7 X 10^{-4}M when used.

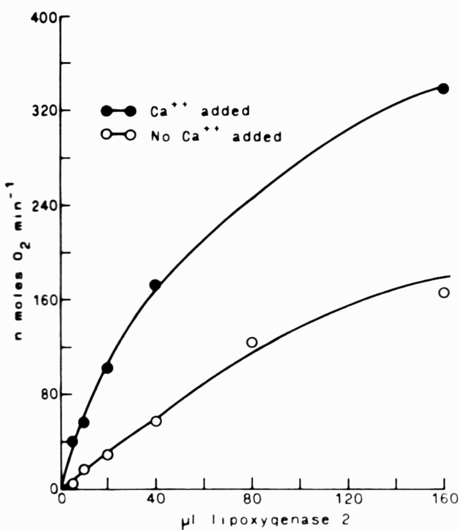

Fig. 4—Response of lipoxygenase 2 to Ca^{2+} addition. Assay conditions the same as for Figure 3.

Allen (1968) proposed that lipoxygenase activity was lost on addition to reaction mixtures due to adsorption on glass surfaces. We reasoned that the glass adsorption might explain the peculiar sequence dependence of Ca^{2+} activation if Ca^{2+} could prevent glass adsorption but could not free the lipoxygenase after adsorption. To test this possibility we passed lipoxygenase (soybean extract) through 3m of glass tubing, 4 mm bore. No adsorption occurred as measured by a decrease in lipoxygenase activity, and the crude extract still was activated by Ca^{2+}; thus glass adsorption did not provide an explaination for the mechanism of Ca^{2+} activation.

We investigated the possiblity that phytates may be involved in Ca^{2+} activation of lipoxygenase. Koch et al. (1971) reported that cold insoluble material recovered from soybeans or navy beans would enhance Ca^{2+} activation, and they speculated that this enhancement may be due to a protein-phytate complex with Ca^{2+} binding ability. We prepared lipoxygenase free of phytates by using the alkali precipitation of Smith and Rackis (1957) as modified by Wang (1971). Figure 5 shows the response of soybean extract to several steps in the procedure to remove phytates: whey production from precipitation of protein at pH 4.5; adjustment to pH 8 with NaOH and dialysis. At each step the soybean extract was assayed at several different pH values with and without Ca^{2+} added. The results indicate that the procedure to remove phytates does remove something that causes Ca^{2+} to activate lipoxygenase.

To test the effect of phytate added back to the phytate-free lipoxygenase, we used a commercial preparation of sodium phytate at a concentration of 2 mg phytate per ml of enzyme (this is calculated to be approximately 2X the phytate concentration in the original soybean extract). The lipoxygenase with added phytate had the same activity as phytate-free lipoxygenase and still did not respond to Ca^{2+}. Hence, although a procedure for removal of phytates caused Ca^{2+} activation of lipoxygenase to be lost, addition of phytate to the lipoxygenase did not return Ca^{2+} activation.

To learn whether Ca^{2+} was interacting mainly with lipoxygenase or with linoleic acid, we varied the concentration of Ca^{2+} and lipoxygenase with constant linoleic acid and then varied the concentration of Ca^{2+} and linoleic acid, keeping lipoxygenase constant. Figure 6 shows the results of varying Ca^{2+} and lipoxygenase concentrations. These results indicate that, regardless of the concentration of lipoxygenase, the Ca^{2+} concentration that gives maximum activation stays practically constant. Figure 7 shows what happens when Ca^{2+} and linoleic acid concentrations are varied and lipoxygenase is held constant. The Ca^{2+} concentration at which maximum activation occurs varies in relation to the linoleic acid concentration. The maximum Ca^{2+} activation occurs at Ca^{2+} concentration that is approximately equimolar to the linoleic acid concentration.

Since soybean extract contains two lipoxygenases, one activated by Ca^{2+} and the other inhibited by Ca^{2+}, the peak of activity, as Ca^{2+} concentration changes, may result from a combination of activities of the two isozymes of lipoxygenase.

The confirmation of the finding of Yamamoto et al. (1970) that two lipoxy-

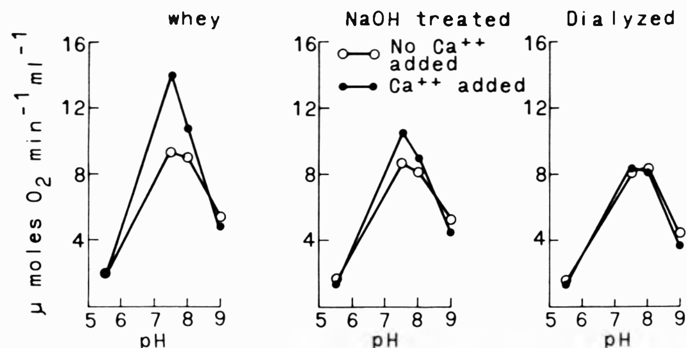

Fig. 5—The response of the lipoxygenase to treatment for removal of phytates. The enzyme was soybean extract treated as described in the text. Temperature 25°C, linoleic acid was 7.9 X 10^{-4}M, and Ca^{2+} was 1 X 10^{-3}M when added concurrently with enzyme. Buffer was 0.2M tris for pH 8 and 9; 0.2M phosphate for pH 5.5 and 7.5.

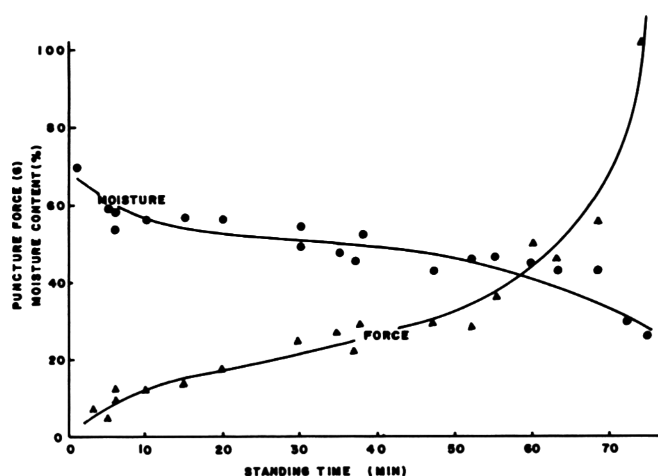

Fig. 1–Effect of ambient dehydration time upon soymilk film strength (penetration force) and moisture content.

Table 2–Dispersions employed for film formation

System	Composition (%) Total solids	Crude Protein	Lipid	P/L ratio
Soymilk	5.2	2.8	1.6	1.8
SPI	3.0	2.8	–	–
Peanut milk	4.8[a]	1.3	2.7	0.5
	5.6	1.6	3.0	0.5
Cottonseed milk:				
Glanded:				
Whole seed	4.8	2.0	1.7	1.2
Whole defatted	4.4	2.2	0.7	3.1
Kernel	5.8	3.0	1.8	1.7
Kernel defatted	6.9	3.5	1.4	2.5
Cyclone flour	3.0	2.2	0.04	57.9
Glandless: Kernel	5.1	2.2	1.6	1.4
NFDM	5.0	1.8	–	–
Whey protein conc (WPC)				
Na	5.0	2.8	0.4	7.0
Ca	5.0	2.8	0.4	7.0
Acid	5.0	2.9	0.4	7.2
Na Caseinate	3.0	2.8	–	–
Cottage cheese whey (Liquid)	5.0	0.8	–	–

[a] Water extracted, pH 6.8

Table 3–Film penetration force (by Instron) and subjective film strength score of NFDM and soy films as affected by forming time intervals (moisture content: 55.0 ± 2.3%)

Film formation time (min)	NFDM films Instron (g)	NFDM films Subjective score	Soy films Instron (g)	Soy films Subjective score
10	134 ± 19	5.0	30 ± 10	3.0
15	506 ± 54	5.0	70 ± 0	3.5
25	430 ± 53	5.0	80 ± 10	4.0
35	513 ± 31	5.0	74 ± 17	4.0
55–70	–	–	100 ± 10	4.5

tained subjectively by noting the mechanical characteristics of the film during formation, withdrawal and handling as follows:

0 = no surface film formed
1 = film formed but too weak to be removed as a discrete sheet
2 = film may be removed from liquid surface but falls from rod immediately
3 = film may be removed and drained, but portions tear from rod while hanging
4 = films dry as smooth sheets without tears
5 = film may be handled and easily manipulated while withdrawing or drying

These subjective scores were used to describe the film strength of each film formed during the duplicated runs. Mean subjective scores were averaged and reported for each experiment.

Analytical

Samples were finely ground for: moisture determinations (air oven 5 hr at 100°C); crude protein (micro Kjeldahl) and total lipid (2g sample extracted with 60 ml petroleum ether for 6 hr) (AOAC, 1970).

Protein and mass incorporation efficiencies (PIE and MIE) were obtained by dividing the protein and dry mass of total films recovered by the corresponding quantity in the original aqueous system. Rate of film formation (g/min-m^2) was calculated from the average dry weight of film, formation time and pan surface area.

RESULTS & DISCUSSION

Film strength

Measurements by the balance and Instron methods were quite erratic and tedious to perform. However, some useful data in Figure 1 indicate the influence of standing time (ambient dehydration) upon film penetration force determined on the balance. As films dried, their resistance to puncture increased accordingly. Below 40% moisture, soy films toughened markedly. Similarly, as films formed and thickened their resistance to shear also increased, as measured by the Instron. Nonfat milk films were very strong and soymilk films were weaker, but still quite acceptable (Table 3). The film-strength and forming-time relationship showed that both NFDM and soybean films reached maximum strength within 15 min. The 5-point subjective scale of film strength proved quite useful as a routine measurement of film recovery characteristics, although a rapid, accurate and sensitive instrumental method relating film strength to fundamental mechanical parameters is still needed.

Soy protein isolate system

Figure 2 indicates the film formation characteristics of model SPI systems at five concentrations and three pH's. With this high protein ingredient (96.4% protein, dry basis) the protein content of all SPI films averaged about 83%, independent of the initial concentration. Dispersed total solids below 4.3% adversely influenced MIE, PIE, film strength and

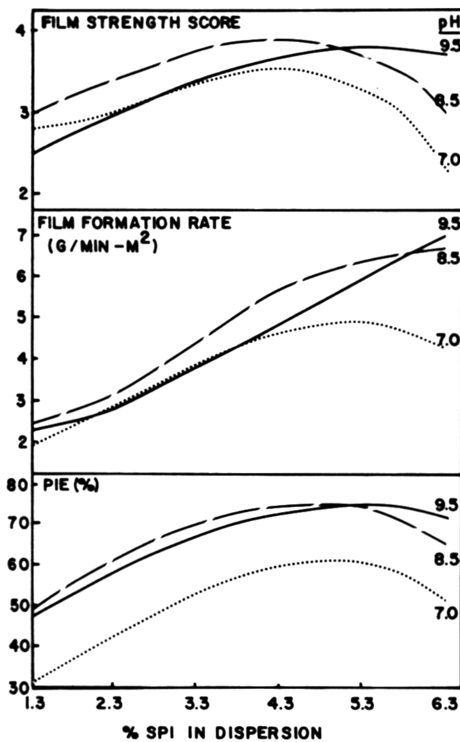

Fig. 2–Film formation characteristics as affected by concentration and pH of SPI aqueous dispersion.

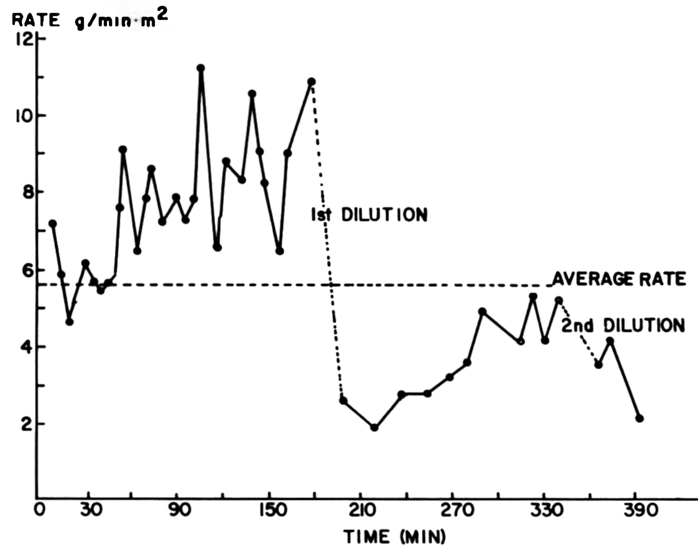

Fig. 3–Rate of film formation and withdrawal over a typical soymilk run.

particularly the formation rate. At concentrations higher than 4.3%, film formation rate increased but PIE and film strength decreased, especially at lower pH's, 7.0 and 8.5. Conditions for obtaining the best compromise between PIE and film strength without appreciably reducing film formation rate were 4.3% solids at pH 8.5 or 5.3% solids at pH 9.5. However, these higher pH's generally result in darker, but not objectionable films as noted previously. Soymilk systems also showed similar pH and concentration effects (Wu and Bates, 1972b).

When total solids were maintained at 6.3% by replacing 1% of the SPI with lipid or carbohydrate at pH 8.5, there was a marked enhancement in film PIE and strength without decreasing formation rate (Table 4). An additional 1% replacement was less effective. The films from systems with added lipid or carbohydrate also appeared to be smoother and of more even texture than those from SPI alone, which tended to form cracked films. This indicates that the presence of appropriate amounts of secondary components is essential for good film formation indices and better film quality (Wu and Bates, 1972b). However, a preliminary survey of the film formation potential of many protein systems, only a few of which are reported in Table 2, revealed that poor performance frequently occurred in systems having a low protein-lipid (P/L) ratio. Thus excessive lipid is more detrimental to film formation than the complete absence of lipid.

While the concentration and composition of a film-forming dispersion is continually changing due to film removal and water evaporation, film strength and composition are relatively constant as formation rate decreases. Figure 3 shows a typical 390 min soymilk run with two dilutions. In general, film formation should be allowed to proceed until signs of gelation are evident and dilution is required. Residues from several second dilutions have been pooled and diluted but additional films were formed slowly. Thus a compromise must be adopted between PIE and formation rate based upon values of the starting ingredients, films, residues and cost of operation.

Peanutmilk systems

The local availability of peanuts and the relatively high protein and lipid content made this oilseed a useful ingredient for film formation. Thoroughly deskinned, raw peanuts produced a light cream-colored peanutmilk with 5.6% total solids, 1.6% protein and 3.0% lipid. Figure 4 summarizes the effect of added SPI, sodium caseinate or 1:1 mixtures of the two to peanutmilk systems. Peanut-

Table 4–Film formation characteristics of SPI dispersions as affected by adding components (pH 8.5, temp 90 ± 5°C)

SPI solids (%)	Dispersion: CH_2O corn syrup solids	Dispersion: Lipid (%)	Dispersion: WPC Na	MIE (%)	PIE (%)	Film strength mean subjective	Film formation rate g/min·m²	Film protein (%)
6.3	0	0	0	71.4	65.0	3.0	6.7	83.5
5.3	1.0	0	0	73.3	68.1	4.0	5.6	71.8
5.3	0	1.0	0	79.7	73.6	4.0	6.6	71.4
5.3	–	–	0.8	80.7	73.4	3.3	5.2	79.2
4.3	2.0	0	0	66.5	62.3	3.7	5.1	58.7
4.3	0	2.0	0	79.7	71.4	3.8	7.1	56.2

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FORMATION OF A POTATO CHIP-LIKE FLAVOR FROM METHIONINE UNDER DEEP-FAT FRYING CONDITIONS

INTRODUCTION

THE CHEMICAL COMPOSITION of the flavor of potato chips has been extensively studied. Dornseifer and Powers (1963, 1965) reported the presence of several carbonyl compounds in potato chips. Later, Deck et al. (1973) and Chang (1967) conducted a systematic isolation, fractionation and identification of volatile flavor compounds from potato chips with a good desirable flavor. They identified a total of 53 compounds; these included eight nitrogen compounds, two sulfur compounds, 14 hydrocarbons, 13 aldehydes, two ketones, one alcohol, one phenol, three esters, one ether and eight acids. This group of investigators (Deck and Chang, 1965) were also the first to have found a series of seven pyrazine compounds as components of potato chip flavor. Recently, Buttery et al. (1971) added 18 pyrazine and pyridine compounds to the list.

The precursors of these compounds and the chemical mechanisms of their formation, however, have not been established. Nevertheless, these flavor compounds were evidently produced by the components of the potato when they were treated under deep-fat frying conditions as those used in the manufacture of potato chips. Since amino acids and sugars were two of the major constituents of potatoes (Fitzpatrick et al., 1965), an attempt was made to determine the flavor characteristics and the chemical composition of the volatile compounds produced by various amino acids, particularly methionine, when they were deep-fat fried.

EXPERIMENTAL

Model system for treating components of potatoes under deep-fat frying conditions

A cotton ball was moistened with three times its weight of water. The moistened cotton ball, containing 75% water, was similar to an inert piece of potato. The active components in potatoes, either amino acids or sugars, or their various combinations, were added into the water before it was used to moisten the cotton ball. The moistened cotton ball was then deep-fat fried to study the flavor characteristics produced by any component or combination of components of the potato.

The optimum conditions for producing an oil with a potato chip-like flavor were as follows. Three cotton balls, each weighing 0.5g and containing 1.5 ml of a solution of 2.78g of methionine in 100 ml of water, were deep-fat fried in 2,500 ml of a refined, bleached and freshly deodorized cottonseed oil at 180–185°C for 1-1/2 min. It was important that the peroxide value of the oil before using did not exceed 1.0 meq/kg. Immediately after the frying, the cotton balls were removed from the oil. After the frying operation was repeated with another three cotton balls, an oil with the desired potato chip-like flavor was obtained. Other oils and fats, such as corn oil and hydrogenated cottonseed oil, had been used successfully to replace the cottonseed oil.

In order to avoid excessive oxidative degradation of the oil, the fryings were carried out under an atmosphere of nitrogen. A 1/4 in. diam aluminum tube, was inserted into the bottom of a Sunbeam household deep-fat fryer (A) as shown in Figure 1. The section of the tubing on the bottom of the fryer (B) was bent into a loop and perforated with pinholes at equidistance with the end closed. Nitrogen was bubbled through the oil with the use of the aluminum tubing at a predetermined rate measured by a flowmeter (C) before the heating was started until the oil was cooled down to room temperature.

The fryer was snugly fitted into a stainless steel cone (D). The cone had an alembic edge and was wrapped with aluminum tubing cooled with running water. Thus the condensate collected inside the cone could not drip back into the heated oil, avoiding secondary decomposition reactions. Nitrogen was also blown into the cone in order to remove air from the surface of the oil. It was important to immerse the moistened cotton balls into the oil during frying. This was accomplished by inserting an aluminum stick (E) through the cotton balls (F) and then hooking the aluminum stick onto the bottom of the frying basket (G), as shown in Figure 1.

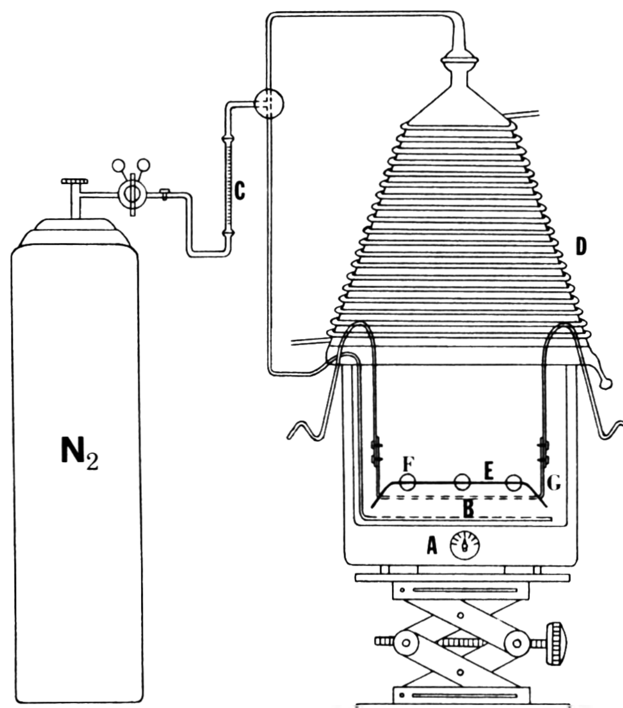

Fig. 1—Apparatus used for deep-fat frying in the model system

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Methionine used

DL-Methionine, M.A. (Mann Research Laboratories, New York, N.Y.) was used. It produced only one spot by two-dimensional paper chromatography.

RESULTS & DISCUSSION

Aroma generated by amino acid under deep-fat frying conditions

Five different amino acids were selected according to their chemical structures (Table 1). Each of them was treated under deep-fat frying conditions with the use of the model system. The aroma thus generated by each of the amino acids as judged by an experienced panel of six persons is shown in Table 1. The strong pleasant potato chip-like aroma could be produced by either the D- or L-isomers of methionine or their mixtures. After the cottonseed oil was used for the deep-fat frying of moistened cotton balls containing methionine, it retained a strong potato chip-like flavor. The flavor of the oil was evaluated by an experienced panel of six persons. The volatile flavor compounds in this oil can be isolated by subjecting the oil to 90°C under a vacuum of less than 0.01 mm Hg for 1-1/2 hr. The isolated volatile compounds had a strong potato chip-like aroma. When they were dropped on a perfumers' paper stick, the aroma lingered on the stick for many hours, thus indicating that the compounds responsible for the potato chip-like aroma were of relatively high boiling points.

Addition of glucose to methionine in the model system did not improve the characteristic of the flavor produced. At weight ratios (methionine:glucose) of 1:1 to 7.7:1, the cotton balls were dark brown in color after deep-fat frying and the odor was reminiscent of burnt brown potato chips. At weight ratios (methionine:glucose) of 16.6:1 to 33:1, the cotton balls were light brown to yellow in color after deep fat frying and the odor was reminiscent of that of ordinary potato chips. Since a potato chip-like flavor could be produced by methionine alone without glucose and without the development of a dark brown color, it appeared that potato chip flavor might not entirely be due to browning reactions. It probably could also be produced by simple thermal decomposition of some amino acids, such as methionine.

Since it was noted that the compounds responsible for the potato chip-like aroma were of relatively high boiling points, they probably could not be formed by direct degradation of methionine which had a rather low molecular weight itself. It was suspected that the potato chip-like aroma was produced by interactions between the thermal oxidative decomposition products of the oil and the thermal degradation products of methionine. For example, Rizzi (1972) reported that a variety of pyrazine compounds could be formed by the reaction of alpha-dicarbonyls with alpha-amino acids.

The production of a potato aroma by methionine has been reported previously by Herz and Shallenberger (1960). They claimed that heating methionine and glucose in water at 100°C and heating the said reactants with mineral oil to 180°C could both yield a potato aroma. In the present investigation, only a weak aroma was detected when either methionine or methionine and glucose were heated to 100°C in water. When methionine or a mixture of methionine and glucose were heated in an oil, a pleasant potato and tomato-like aroma was generated, starting at 50°C and reaching a maximum of 110°C. When heated over 130°C, the characteristic of the aroma began to deteriorate. At 180°C, the aroma was obnoxious (Chang and Reddy, 1971). Elodé et al. (1966) also claimed that heating an aqueous methionine solution with sugars to 100° and 180°C yielded a cabbage-like odor. The potato chip-like aroma was noticed only when methionine was treated under deep-fat frying conditions.

It was known that methional was a Strecker degradation product of methionine (Ballance, 1961). However, the flavor produced by treating methionine under deep-fat frying conditions was found to be more pleasant and more reminiscent of that of potato chips than a solution of methional in oil. Furthermore, deep-fat frying of moistened cotton balls containing methional did not yield the same flavor as heating methionine under the same conditions.

Quantitative estimation of volatile flavor compounds

In the model system, a total of approximately 125 ppm of methionine by weight of oil was deep fat fried in moistened cotton balls to produce an oil with a potato chip-like flavor. However, the amount of methionine actually reacted to produce the desired flavor was much less than the total amount used. The amount of methionine in the moistened cotton balls before and after frying was analyzed by paper chromatography. The methionine spot was developed with the use of ninhydrin and its quantity was measured by absorption at 570 mμ. It was found that only 24 ppm of methionine by weight of the oil was reacted to produce the desired flavor.

The amount of volatile flavor compounds in the oil with the potato chip-like flavor was extremely small. By maintaining the flavored oil at 90°C for 1-1/2 hr under 0.01 mm Hg, only 3 ppm of volatile compounds by weight of the oil could be isolated. However, these 3 ppm of volatile compounds must be responsible for the potato chip-like flavor, because after they were removed, the oil was practically bland in odor and flavor.

Effect of the chemical structure of methionine upon its ability to produce a potato chip-like flavor

The chemical structure required for the generation of the potato chip-like fla-

Table 1—Aroma generated by different amino acids

Amino acid	Aroma generated under deep-fat frying conditions
Threonine	Wet hair, earthy
Proline	Stale popcorn, bitter
Histidine	Stale popcorn
Cystine	Slightly meaty
Methionine	Strong potato chip-like

Table 2—Effect of chemical structure upon the production of a potato chip-like flavor by methionine under deep-fat frying conditions

Compound	Structures	Characteristic of flavor produced under deep-fat frying conditions
D-Methionine L-Methionine DL-Methionine	CH_3-S-CH_2-CH_2-CH(NH_2) COOH	Good potato chip-like
S-Methyl-L-cysteine	CH_3-S-CH_2-CH(NH_2) COOH	Good potato chip-like
DL-Ethionine	CH_3-CH_2-S-CH_2-CH_2-CH(NH_2) COOH	Good potato chip-like
S-Ethyl-L-cysteine	CH_3-CH_2-S-CH_2-CH(NH_2) COOH	Obnoxious (cooked turnip)
Methionine hydroxy analog	CH_3-S-CH_2-CH_2-CH(OH) COOH	Obnoxious (cooked turnip)
S-Carboxymethyl-L-cysteine	HOOC-CH_2-S-CH_2-CH(NH_2) COOH	Obnoxious (cooked turnip)

vor during the deep-fat frying of methionine was quite specific (Table 2). Methionine, either D- or L- or DL-, generated a good potato chip-like flavor. Elimination of a carbon in the carbon chain of methionine did not affect its ability to produce a good flavor. Lengthening the methyl group at the end by one carbon atom could still produce good potato chip-like flavor. However, when both happened at the same time, that was, when the terminal methyl group was lengthened, and the carbon chain was shortened, an obnoxious flavor, instead of a potato chip-like flavor, was produced. The amino group was also important to the production of potato chip-like flavor. Replacement of this group with a hydroxyl group resulted in the production of an obnoxious flavor. The terminal methyl group was also important to the potato chip-like flavor production. Replacement of one hydrogen atom by a carboxyl group also resulted in the generation of an obnoxious flavor.

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DISTRIBUTION OF DIELDRIN IN MILK FRACTIONS

INTRODUCTION

SINCE MOST of the chlorinated hydrocarbon pesticides are lipid soluble, they have been more frequently found in the fatty portions of foodstuffs. Mann et al. (1950) reported that residue levels of DDT were much higher in the cream and butter than in the skim milk and whole milk from which the various products were made. Langlois et al. (1964, 1965) also found that either the intentionally added DDT and dieldrin or the physiologically incorporated lindane and endrin were recovered almost entirely in the cream and butter fractions after separation. The amounts of residues in each fraction were generally proportional to the total lipid content in that fraction. Residue levels on a fat basis varied somewhat with fractions and pesticides.

Beroza and Bowman (1966) demonstrated that both organochlorine and organophosphate pesticides added to milk were almost entirely found in the cream phase. They suggested that these chemicals were absorbed onto the fat globules in milk. Hugunin and Bradley (1969) reported that skim milk, buttermilk and a high density pellet of buttermilk contained greater amounts of some organochlorine pesticides and proposed that this was due to the association of residues with the phospholipid in milk fat. The same researchers (Hugunin and Bradley, 1971) later suggested that pesticides were binding to the fat globule membrane proteins or whey proteins. The stabilities and distributions of several chlorinated pesticides during processing varied with the nature of the chemicals and the processing techniques (Li et al., 1970).

The present study was planned to investigate the specific relationship between dieldrin and the different lipid classes in the milk system. Comparisons were made of the distribution of dieldrin in naturally contaminated milk and milk to which the pesticides had been added. Physical methods were used to separate fresh raw milk into various fractions and the total lipid, phospholipid and dieldrin contents of the fractions were determined.

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MATERIALS & METHODS

Milk

Two kinds of milk samples were used in this research. One was collected from cows which had ingested dieldrin and another was from uncontaminated cows. Both milk samples were obtained from the Michigan State University Dairy herd. Appropriate amounts of dieldrin in acetone solution (the ratio of acetone solution to milk was 10 ml to 10 gal) were added dropwise with stirring to the uncontaminated milk at a 0.5 ppm level on a weight basis and the mixture was allowed to equilibrate for 45 min at 37°C with intermittent stirring.

Chemicals

Dieldrin containing 99% HEOD (1, 2, 3, 4, 10, 10-hexachloro-6, 7-epoxy-1, 4, 4a, 5, 6, 7, 8, 8a-octahydro-1, 4-endo-exo-5, 8-dimethanonaphthalene) was purchased from Applied Science Laboratories, Ann Arbor, Mich. Organic solvents used were nanograde (Mallinckrodt) or reagent grade.

Separation of major milk fractions

The method employed was essentially that of Brunner (1965) and Swope (1968). Uncooled fresh raw milk was separated into cream and skim milk with a Delaval disc-type separator. Cream was washed three times with deionized distilled water and reseparated. The washed cream was cooled overnight and churned to form butter and buttermilk. Butter was then melted at 45°C to yield butteroil and butter serum. The latter was further centrifuged to remove excess butteroil.

The buttermilk fractions collected each day were preserved with penicillin G and streptomycin sulfate (Quirk, 1965) and 0.01% BHT and were stored at 4°C until a sufficient amount of this fraction was obtained. Thus, up to 6 days collections of buttermilk fractions were pooled for the preparation of fat globule membrane fractions. Preliminary tests showed that these preservatives did not interfere with the GLC determination of dieldrin.

Throughout the entire separation process, each fraction was weighed and a sample of each fraction was taken before that fraction was further separated into other fractions. All samples were stored at −20°C until used for lipid and dieldrin analyses.

Preparation of fat globule membrane fractions

The procedures of Swope (1968) with slight modifications were used. Pooled buttermilk fractions were filtered and centrifuged at 2,000 × G for 20 min to eliminate excess butterfat. The aqueous solution was designated as refined buttermilk. Differential centrifugation was employed to separate the membrane materials from buttermilk into three pellets, i.e., 7,500S, 230S and 35S. The crude 7,500S and 230S fractions were washed twice with deionized water and recentrifuged. All of the membrane fractions were freeze dried prior to extraction of lipid and pesticide. A preliminary study showed that freeze drying at 100μ Hg for 24 hr had no significant effect on the residue levels in several fractions.

Table 1—Lipid and dieldrin content of major fractions of milk containing physiologically incorporated dieldrin

Fraction	Lipid[a] (%)	Phospholipid[a] in total lipid (%)	Dieldrin concentration, ppm[b], in Lipid	Dieldrin concentration, ppm[b], in Fraction
Whole milk	4.05	0.71	0.83 ± 0.09	0.034 ± 0.004
Skim milk	0.58	1.94	0.89 ± 0.10	0.005 ± 0.001
Cream (unwashed)	53.63	0.37	0.83 ± 0.02	0.445 ± 0.011
Butter	83.00	0.31	0.86 ± 0.15	0.714 ± 0.125
Butteroil	99.20	0.01	0.87 ± 0.09	0.863 ± 0.089
Buttermilk (refined)	1.67	11.84	0.58 ± 0.04	0.010 ± 0.001
Butter serum (refined)	1.92	23.96	0.50 ± 0.07	0.010 ± 0.001

[a] Average of duplicate determinations. Pooled fractions from six individual milk collections and separations were used for each determination.
[b] Average and range of duplicate determinations

Extraction and determination

Lipid and dieldrin in the major fractions were extracted with ethanol, ethyl ether and petroleum ether, and the extracted lipid was subjected to acetonitrile-petroleum ether clean-up using the procedures described by the Food and Drug Administration (1968). Further clean-up with a Florisil-celite column was accomplished according to recommendations by the Shell Chemical Company (1964).

Slightly modified extraction procedures were adopted for buttermilk and butter serum to facilitate a better separation between the solvent phase and the aqueous phase and to recover the lipids completely. 3.5 vol of ethanol, 1 vol of ethyl ether and 2.5 vol of petroleum ether were used in the first extraction, whereas, 1 vol of petroleum ether was used in each of the second and third extractions. The residual phase was diluted with 2.5 vol of distilled water and then extracted with ethyl ether and petroleum ether following the original procedure.

Free lipids of the membrane material were extracted with petroleum ether (Cerbulis, 1967). The residue portion was rehydrated with 10 vol of water and then the bound lipids were extracted with ethanol, petroleum ether and ethyl ether following the same procedures as used for buttermilk.

Quantitative determinations of dieldrin were carried out on a F and M Hewlett-Packard Model 5750B research gas chromatograph equipped with a tritium electron capture detector. The operating conditions were 4' × 1/4" OD coiled glass column prepacked with 3.8% SE-30 on 80/100 mesh Diatoport S; injection port temperature 240°C; column oven 200°C; detector 210°C; flow rate of carrier gas (helium) 45–50 ml/min; and purge gas (90% argon-10% methane) 50 ml/min. Sample size was 1-4 μl. The peak heights of dieldrin in the samples were compared with those provided by standard concentrations to give quantitative data.

A portion of the extracted lipid was used for phosphorus determination employing the method of Morrison (1964). Phospholipid content was estimated by multiplying the phosphorus values by 25.

RESULTS & DISCUSSION

Physiologically incorporated dieldrin

The total lipid, phospholipid and dieldrin content of various major fractions of milk containing physiologically incorporated pesticide are shown in Table 1. Lipid content ranged from 0.01 to 23.06% with the butter serum containing the greater amount of phospholipid. The dieldrin concentration of each fraction on a weight basis was closely related to the lipid content of that fraction. On a lipid basis, whole milk, cream, skim milk, butter and butteroil contained comparable concentrations of dieldrin, whereas refined buttermilk and butter serum containing higher levels of phospholipid contained lower levels of dieldrin. The distribution of phospholipid among these fractions is similar to that reported by Privett et al. (1968) except that the skim milk obtained in the present study contained lesser amounts of phospholipid.

The percentage yield of major fractions from whole milk and the relative distribution of dieldrin and lipid in each fraction are shown in Table 2. Although the skim milk contained very little dieldrin on a weight basis (Table 1), it comprised more than 90% of the total weight of milk. Therefore, it accounted for a significant quantity of dieldrin and lipid in milk. On the other hand, unwashed cream accounted for about 6% of the total weight of milk, but contributed almost 85% of both the total lipid and dieldrin in the milk system. Similarly, butter and butteroil accounted for only a small fraction of the milk, but contributed a relatively large amount of lipid and dieldrin. From these data, it is again observed that the distribution of dieldrin is proportional to the lipid distribution in milk.

In order to learn more about the nature of the relationship between dieldrin and various types of lipids, the free lipid and bound lipid phases were extracted from the membrane pellets. Phospholipid content of these two lipid phases was also determined. Since the fat soluble pesticides tend to be extracted with the nonpolar solvent along with the free lipid phase even if these residues are originally associated with the bound lipid, any individual analysis of residue in either of these two types of lipids may not reflect completely the actual original pesticide distribution. Correlations of residue with different types of lipids, however, still can be made by comparing the free and bound lipid levels with the residue levels on a lipid basis in different pellet fractions. The analytical data of this phase of the study are shown in Table 3.

The 230S and 35S pellets were the major fractions of membrane materials, and the total lipids extracted from these two pellets were used for residue analysis. The amount of 7,500S pellet was too small for accurate residue analysis, especially at the low residue levels encountered. Results in Table 3 show that although 230S and 35S pellets contained different levels of phospholipid, and free and bound lipid, the concentrations of dieldrin in the total lipid were comparable with each other. It appears that at the membrane levels of the milk system and/or at these low levels of residue, the deposition of pesticide is not affected by the various types of lipids.

As comparisons were made between Table 1 and 3, it was observed that the phospholipid content was much higher

Table 2—Relative distribution of lipids and dieldrin in major fractions of milk containing physiologically incorporated dieldrin

Fraction	Fraction wt[a] (%)	% Distribution in fraction: Lipid[b]	Dieldrin[b]
Whole milk	100.00	100.00	100.00
Skim milk	93.60[c]	13.40	14.49
Cream (unwashed)	6.40	84.74	84.78
Cream (washed)	3.74	–	–
Butter	2.43	49.80	51.60
Butteroil	2.00	48.98	50.76
Buttermilk (refined)	1.31	0.54	0.40
Butter serum (refined)	0.27	0.13	0.08

[a] Average of duplicate preparations. Each represents the average of six individual milk collections and separations
[b] Average of duplicate determinations. Pooled fractions of six individual milk collections and separations were used for each determination.
[c] Estimated by the difference between whole milk and unwashed cream

Table 3—Lipid and dieldrin content of freeze-dried fat globule membrane pellets prepared from milk containing physiologically incorporated dieldrin

Pellet[a]	Fraction wt (%)	% Lipid in Pellet[b] FL	BL	TL	% Phospholipid in FL	BL	TL	Dieldrin concentration, ppm[c], in TL	Pellet
7500S	2.92	9.56	8.62	18.18	–	–	–	–	–
230S	43.05	26.00	7.65	33.65	12.22	57.33	22.96	0.28 ± 0.02	0.088 ± 0.0006
35S	54.03	39.77	8.38	48.15	19.34	37.70	71.87	0.27 ± 0.03	0.129 ± 0.016

[a] Each value is the average of duplicate preparations of pellets from pooled buttermilk. Each pooled buttermilk results from six individual milk collections and separations
[b] FL = Free lipids, extracted with petroleum ether; BL = Bound lipids, extracted with ethanol, ethyl ether and petroleum ether; TL = Total lipids = FL + BL.
[c] Average and range of duplicate determinations

but the dieldrin level was much lower in the membrane fractions than in the refined buttermilk. This finding supports the concept that an inverse relationship exists between the residue level on a fat basis and the phospholipid content. This relationship has already been observed among the major milk fractions.

Milk containing added dieldrin

In this phase of the study, both crude and refined buttermilk and unwashed and washed cream were examined. Additionally, a portion of skim milk was also refined by centrifugation at 2,000 × G for 15 min to remove excess milkfat. The results of analysis of the major fractions of this intentionally contaminated milk are shown in Tables 4 and 5. Data indicated that the distribution patterns of lipids and dieldrin were similar to those found in the milk containing physiologically incorporated pesticide. Concentrations of residue in various fractions were closely related to the total lipid content.

On a fat basis, similar levels of dieldrin were found in whole milk, crude skim milk, butter, cream and butteroil fractions. Lower levels were found in buttermilk and butter serum and the greatest amount of dieldrin was detected in the refined skim milk fraction. The lower concentrations of dieldrin in buttermilk and butter serum were probably due to the higher phospholipid content of these two fractions as was observed above with the naturally contaminated milk. The same correlation was not applicable to the refined skim milk which contained high levels of phospholipid and also contained high concentrations of dieldrin. One factor possibly involved in such an "irregular" relationship is the relatively large quantity of aqueous skim milk fraction in the whole milk (90% or more of the milk is skim milk). The aqueous phase of skim milk may contain some of the residues. Reports have indicated that dieldrin may be soluble in water at 0.186 ppm (Park and Bruce, 1968) and at much higher levels in blood serum (Moss and Hathaway, 1964). Although only a small amount of the pesticide dissolved would be in the serum phase of milk, it will contribute significantly to the residue concentrations expressed on a fat basis for the refined skim milk fraction since it has a very low fat content. Earlier reports indicating that skim milk contained higher levels of residues on a fat basis (Hugunin and Bradley, 1969; Li et al., 1970) were probably due to the same reasons, i.e., the low fat content of skim milk and solubility of residues in the aqueous phase; rather than that higher amounts of residues were associated with the phospholipid. Hugunin and Bradley (1971) reported that casein and whey proteins increased the solubility of DDT and dieldrin in an aqeous model system.

The percentage recovery of various fractions from milk and the distribution of lipid and dieldrin among various fractions are shown in Table 5. Comparable to the results obtained with the naturally contaminated milk, the added pesticide was also largely recovered in the cream phase after separation. Washing of cream caused some losses of lipid and dieldrin, but the washed cream still contained more than 70% of the total lipid and dieldrin of milk. Butter and butteroil contained 69.64 and 68.82% of the added pesticide, respectively, and buttermilk and butter serum fractions accounted for only a small portion of the lipid and residue in milk. The original skim milk fraction accounted for about 12% of the total lipid and dieldrin in milk, but the refined skim milk contributed only approximately 2% of the lipid and dieldrin to the milk system. Different values of the percentage distribution of dieldrin in milk fractions were observed between the naturally contaminated milk and milk with added pesticide (compare Tables 2 and 5). However, this variation was mainly due to the different lipid distributions in these milk samples and was also affected by the separation techniques used. It is readily apparent that the absolute quantity of pesticide recovered from any frac-

Table 4—Lipids and dieldrin content of major fractions of milk containing added dieldrin[a]

Fraction[b]	Lipid (%)	Phospholipid In total lipid[c]	Dieldrin concentration[c] ppm, in Lipid	Fraction
Whole milk	3.87	0.55	13.07	0.505
Skim milk	0.51	—	13.19	0.067
Skim milk (refined)	0.09	10.32	14.60	0.013
Cream (unwashed)	34.47	0.33	12.77	4.413
Cream (washed)	45.62	0.22	12.70	5.782
Butter	83.86	0.26	12.79	10.729
Butteroil	99.00	0.06	12.98	12.850
Buttermilk	3.83	0.82	8.42	0.315
Buttermilk (refined)	1.92	6.32	8.01	0.166
Butter serum (refined)	1.18	22.62	6.89	0.082

[a] Dieldrin was added to milk at 0.5 ppm level on a fresh weight basis.
[b] Milk fractions were prepared from two independent milk collections and separations.
[c] Average of duplicate determinations of individual preparations

Table 5—Relative distribution of lipids and dieldrin in major fractions of milk containing added dieldrin[a]

Fraction[b]	Fraction wt[c] (%)	% Distribution in fraction[d] Lipid	Dieldrin
Whole milk	100.00	100.00	100.00
Skim milk	90.00[e]	11.89	12.17
Skim milk (refined)	90.00[e]	2.03	2.23
Cream (unwashed)	10.00	88.42	86.30
Cream (washed)	6.28	74.48	71.93
Butter	3.30	71.38	69.64
Butteroil	2.72	69.51	68.82
Buttermilk	2.95	2.93	1.84
Buttermilk (refined)	2.95	1.45	0.95
Butter serum (refined)	0.38	0.12	0.06

[a] Dieldrin was added to milk at 0.5 ppm level on a fresh weight basis.
[b] Milk fractions were prepared from two independent milk collections and separations.
[c] Average of duplicate preparations
[d] Average of duplicate determinations of individual preparations
[e] Estimated by the difference between whole milk and unwashed cream

tion of milk varies with the total amount of lipid in that fraction.

The total lipid content of the membrane pellets as shown in Table 6 is similar to the earlier findings with the naturally contaminated samples. Free and bound lipids were not determined in this phase of the study. The final clear center layer of the supernatant and the final top layer after centrifugations were collected and analyzed. Concentrations of dieldrin in 7,500S, 230S pellets and the final top layer were similar on a fat basis, while the 35S pellet and the center layer contained slightly lower levels of this pesticide. Overall results showed that lower values of dieldrin concentrations were again found in these membrane fractions when compared to other major fractions.

Control milk was separated and analyzed in the same manner as the intentionally contaminated milk. Dieldrin content in these control fractions ranged from 0.053 to 0.22 ppm on a fat basis; or from 0.001 to 0.083 ppm on a weight basis. These values were much smaller than the values obtained from the milk containing added pesticide, therefore no adjustment was made on the residue concentrations of fractions from milk with added pesticide.

Factors relating to the distribution of dieldrin

Because of the lipophilic property of chlorinated hydrocarbon pesticides, it has long been conceded that most of the physiologically incorporated residues are within the fat globules of milk. By physical separations, Langlois et al. (1964, 1965) and Li et al. (1970) proved that these pesticides are essentially recovered in the cream and butter fractions. The present study confirms these findings and further indicates that major amounts of residues are found in the butteroil fractions.

Added chlorinated pesticides have also been found mostly in the cream and butter phase (Langlois et al., 1964, 1965; Beroza and Bowman, 1966) suggesting that pesticides were absorbed onto the fat globules rather than incorporated within the fat body. The present research showed that the added dieldrin was primarily recovered in the butteroil fraction, similar to the findings with the physiologically incorporated pesticide. This study implies that dieldrin is preferentially distributed into the neutral or free lipid fractions of milk regardless of whether it is the consequence of original deposition, the penetration of pesticide through the membrane or is the results of shifting to an altered orientation during separation.

Hugunin and Bradley (1969) and Li et al. (1970) reported that in some instances the concentrations of pesticides were higher in the skim milk, buttermilk and high density membrane pellets, in some other instances these higher levels were not observed for buttermilk, and in other instances, no difference at all was detected. They proposed that the higher residue concentrations found in skim milk and buttermilk on fat basis were due to the associations of pesticides with the phospholipid or the lipoproteins in these fractions. However, they did not report the total lipid content of their milk products or fractions. It has been observed in the present study that the amount of lipid present in a fraction varies significantly with the separation techniques involved, especially for the low-fat containing fractions such as skim milk and buttermilk. In addition, the residue levels expressed either on a weight basis or on a fat basis varies with the lipid content in that fraction. It is very important that all of the lipids should be extracted if the subsequent residue analysis is to be based on the isolated lipid. Lawrence and Burke (1969) also reported that the residue levels tended to be higher on a fat basis if only a portion of the total fat was extracted from milk and analyzed.

The variations between the data obtained by the earlier workers and the present study are probably due to the variance in the lipid content, the techniques used in fraction separations and in lipid extractions. One such difference in separation techniques was that Langlois et al. (1964, 1965) and Li et al. (1970) used pasteurized unwashed cream to make buttermilk while raw and thrice-washed cream was used in the present investigation. The composition of these buttermilks would be different and this difference influenced the distribution of residues.

In addition to the factors discussed above such as the relatively higher phospholipid content in buttermilk, butter serum and membrane fractions tended to decrease the residue concentrations on a fat basis, and the slight solubilities of pesticide in the aqueous phase tended to raise the residue levels on a fat basis for the skim milk fractions, other factors may also be involved. The variations in glyceride species, phospholipid composition, fatty acid moieties and lipoprotein structures may also influence the distribution patterns of pesticide residues in a milk system.

Table 6–Lipid and dieldrin content of freeze-dried fat globule membrane materials prepared from milk containing added dieldrin[a]

Pellet[b]	Fraction wt (%)	Lipid[c] (%)	Phospholipid in total lipid[c] (%)	Dieldrin concentration, ppm[c], in: Lipid	Pellet
7500S	4.20	17.50	36.79	4.71 ± 0.23	0.824 ± 0.040
230S	60.72	29.76	33.47	4.93 ± 0.27	1.467 ± 0.080
35S	35.08	51.35	30.20	3.24 ± 0.16	1.663 ± 0.082

Supernatant[b]	Dry matter (%)	Lipid in dry matter[d] (%)	Phospholipid in total lipid[d] (%)	Dieldrin concentration, ppm[d], in: Lipid	Dry matter	Solution
Final center layer	0.107	7.22	14.71	3.37	0.243	0.0003
Final top layer	0.163	8.07	14.96	5.12	0.413	0.0007

[a] Dieldrin was added to milk at 0.5 ppm level on a fresh weight basis.
[b] Obtained from pooled buttermilk of two independent preparations
[c] Average and/or range of duplicate determinations
[d] Single determination

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ACID PRODUCTION BY Streptococcus lactis IN LOW-LACTOSE SKIM MILK

INTRODUCTION

THE LACTOSE CONTENT of normal cow's milk is never a limiting factor in dairy fermentations. However, it may be desirable to limit fermentation in certain cultured dairy products by removing some of the lactose from milk (Mocquot and Hurel, 1970). An example would be in the production of sour cream where it is desirable to produce a very thick cream with a low acid content. The maximum viscosity occurs around pH 5.2 to 5.3, but it is difficult to stop fermentation at this level. Fermentation could be limited by reducing the lactose in the cream. The same principle could be used to control fermentation in other dairy products such as buttermilk or yogurt. In addition, the removal of lactose and supplementation of milk with glucose would allow the consumption of this nutritious food by persons with limited lactose tolerance. The latter problem is becoming increasingly important to the dairy industry (McCracken, 1971). Studies have shown that 10–15% of adult Caucasians and 70% of adult Negroes in the United States are lactose intolerant (Bayless and Rosensweig, 1966; Cuatrecasas et al., 1965). Other studies have shown that American Indians (Welsh et al., 1967), African Negroes (Cook and Kayubi, 1966), Greek Cypriots (McMichael et al., 1966) and Asians (Chung and McGill, 1968; Davis and Bolin, 1967; Huang and Bayless, 1968) also have a high incidence of lactose intolerance (95% of Asians). The consumption of fermented dairy products (e.g., buttermilk, acidophilus milk, and yogurt) by lactose intolerant people has been advocated by some authors because they believe that these products are lactose free (Rosensweig, 1969). Analysis of these products for lactose shows that there is only slightly less lactose present after fermentation than before (Reineccius et al., 1970). The value of surplus milk as a protein source for peoples of underdeveloped countries is greatly reduced because of the lactose intolerance.

The objective of this study was to determine if acid production during dairy fermentations could be controlled by limiting the lactose content of the milk.

EXPERIMENTAL

LACTOSE LIMITED skim milk was prepared from 11% reconstituted nonfat milk (Matrix Mother Culture Medium, Galloway-West, Fond du Lac, Wisconsin) as described by Morr et al. (1967). This method involves cycling milk several times through Sephadex G-25 in a basket centrifuge. Skim milk (1 liter) was distributed evenly over the inside surface of a slowly rotating (60 × G) bed of Sephadex G-25. After a short equilibration period of about 5 min the rotor was accelerated to 1,000 × G and held for 10 min. The eluant collected from the centrifuge was recycled through the Sephadex bed after the bed was washed three times with deionized water.

The treated milk was steamed for 60 min, cooled to 32°C, and inoculated with a 1% inoculum of *Streptococcus lactis* C_2F previously grown in 11% reconstituted nonfat milk at 21°C for 18 hr. Acid production was measured by changes in titratable acidity (TA) as determined by titrating 9g of milk with 0.1N NaOH to a phenolphthalein end point. The Sephadex treated milk was supplemented with increasing concentrations of lactose or glucose, with yeast extract, pancreas extract, or metal solution or with a combination of these growth stimulants as indicated in the text. The stock metal solution had the following composition: K_2HPO_4, 3g; NH_4Cl, 3g; $MgCl_2 \cdot 6H_2O$, 20g; $CaCl_2$, 2g; $FeCl_3 \cdot 6H_2O$, 0.5g; $ZnSO_4 \cdot 7H_2O$, 0.5g; $CoCl_2 \cdot 6H_2O$, 0.25g; $CuSO_4 \cdot 5H_2O$, 0.25g; and $NaMoO_4$, 0.25 g in 1,000 ml water. 10 ml of the stock metal solution were added per liter of Sephadex treated milk. Lactose concentration in the treated milk was determined by the phenol sulfuric method (Dubois et al., 1956) and by gas chromatography (Reineccius et al., 1970). Both methods gave comparable results.

RESULTS & DISCUSSION

FIGURE 1 compares acid production of *S. lactis* C_2F in Matrix milk to treated milk which was cycled two (2×) and three (3×) times through Sephadex G-25. The treated milk did not support maximum acid production by *S. lactis* C_2F. In the control there was a total change of 0.54% TA as compared to only 0.2 and 0.1% in milk cycled two and three times, respectively. The treated milk contained less than 0.2% lactose as contrasted with approximately 6% lactose in the control culture. These results suggested that limited lactose content may have contributed to the small change in TA. However, Figure 1 also shows that when the treated milk was supplemented with 4% lactose, acid production did not increase. The

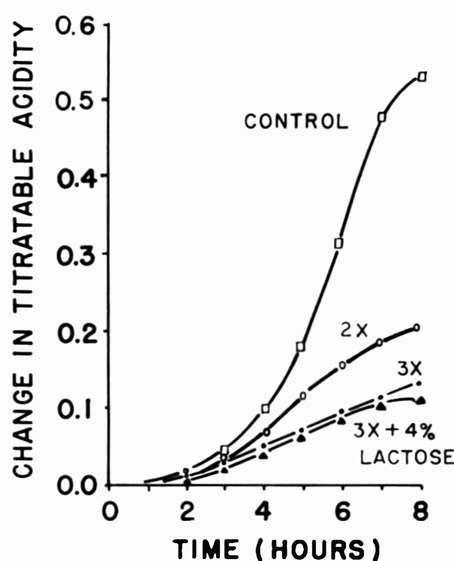

Fig. 1—Acid production by S. lactis C_2F in low-lactose milk (2× and 3× milks were cycled two and three times through Sephadex G-25)

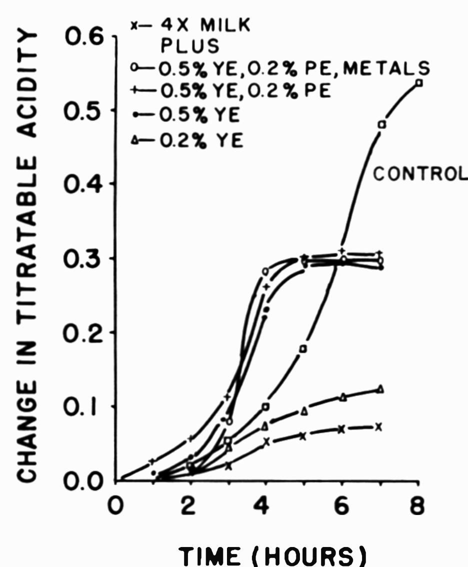

Fig. 2—Acid production by S. lactis C_2F in low-lactose milk (4×) supplemented with yeast extract (YE), pancreas extract (PE) and a metal solution.

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change in TA was only 0.1%, indicating that other essential nutrients were also removed by the Sephadex treatment. The difference in TA between 2× and 3× milks was apparently due to lower levels of nutrients other than lactose.

Figure 2 illustrates efforts to stimulate acid production in the treated milk (4×) by adding yeast extract, pancreas extract, or metal solution. The addition of 0.2 or 0.5% yeast extract stimulated acid production with 0.5% yeast extract giving the highest stimulation. Increasing the concentration of yeast extract above 0.5% did not further stimulate acid production. The addition of pancreas extract or the metal solution to the yeast extract supplemented milk did not result in greater acid production but a shorter lag period was noted when these growth supplements were added. The above data suggest that lactose may now be the factor responsible for limiting acid development. The influence of adding the same ingredients to treated milk supplemented with 4% lactose is shown in Figure 3. Acid production nearly equivalent to the control was attained by the addition of yeast extract, pancreas extract and metals. Reiter and Oram (1968) have shown that iron, magnesium and postassium are required by certain lactic streptococci for growth, and that these organisms failed to grow in milk treated with ion exchange resin. Olson and Qutab (1970) also recommended the addition of iron, magnesium and selenium to milk to significantly improve the rate of acid production by streptococci. The Sephadex treatment used in our experiments would have removed most of the metals required for growth (Morr et al., 1967).

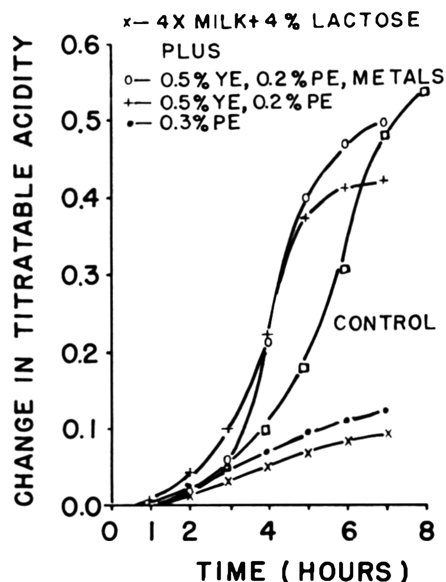

Fig. 3–Acid production by S. lactis C_2F in low-lactose milk (4×) supplemented with 4% lactose, yeast extract (YE), pancreas extract (PE) and a metal solution.

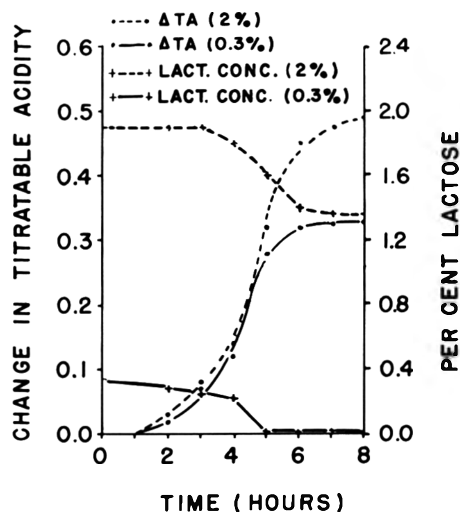

Fig. 4–Correlation between acid production by S. lactis C_2F and lactose content in low-lactose milk supplemented with 0.5% yeast extract, metals and lactose (0.2% and 1.8%).

The results shown in Figure 4 suggest that lactose was the substrate limiting acid production in Sephadex treated milk supplemented with yeast extract and metals. In the milk with 0.3% lactose, acid was produced by *S. lactis* C_2F until the lactose was exhausted from the medium. In the milk containing 2.0% lactose, a change of 0.5% TA was observed and the lactose concentration decreased from 2.0 to 1.4%. Acid production probably stopped due to the accumulation of toxic lactic acid or to limitation of some other required nutrient.

The results suggested, however, that the lactic fermentation can be controlled by limiting the availability of carbohydrate. To test this hypothesis, increasing amounts of lactose were added to treated milk supplemented with yeast extract and metals (Fig. 5). The fermentation stopped after a 0.2% change in TA in the treated milk containing 0.2% lactose. By increasing the lactose concentration to 0.3, 0.4, 0.5, and 0.6%, the acid production also increased and stopped upon the exhaustion of lactose. Increasing the concentration of lactose above 0.7% did not stimulate further acid production. Thus, under these conditions, *S. lactis* C_2F was utilizing 0.5–0.6% lactose in its fermentation. Figure 6 shows that the fermentation could also be controlled by varying the glucose concentration. Supplementation of Sephadex treated milk with increasing concentrations of glucose, resulted in an equivalent increase in acid production comparable to that observed for lactose addition. McKay et al. (1972) have recently reported that lactic streptococci are not stable with respect to lactose fermentation. Therefore, the use of glucose would prevent or eliminate any variation which might occur in the starter's ability to ferment lactose.

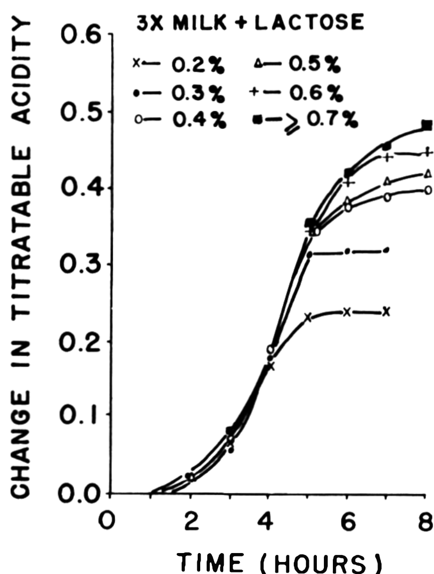

Fig. 5–Acid production by S. lactis C_2F in low-lactose milk supplemented with 0.5% yeast extract, metals and increasing amounts of lactose.

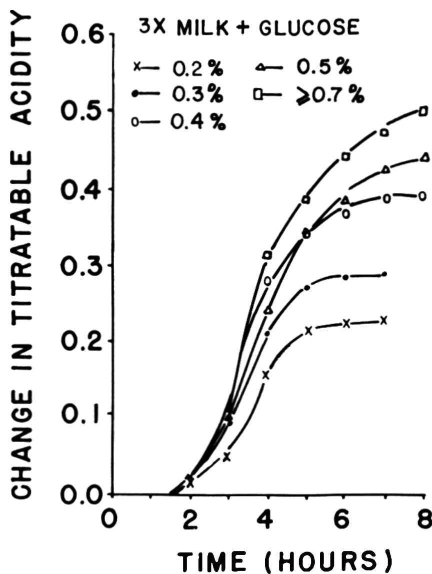

Fig. 6–Acid production of S. lactis C_2F in low-lactose milk supplemented with 0.5% yeast extract, metals and increasing amounts of glucose.

In conclusion, results indicate that acid production by *S. lactis* C_2F can be controlled by adding lactose or glucose to milk that has been passed through Sephadex G-25 and supplemented with yeast extract, pancreas extract and metals. The method of Morr et al. (1967) used for removing lactose from milk is being used commercially and should present few problems in manufacture. Fermented dairy products produced by the method proposed could be consumed by lactose intolerant people.

Future publications will deal with texture and flavor aspects of this research.

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POLYPHENOL OXIDASE OF ROYAL ANN CHERRIES: PURIFICATION AND CHARACTERIZATION

INTRODUCTION

PHYSIOLOGICAL INJURY to Royal Ann cherries results in a rapid formation of dark brown and reddish pigments in the injured area. This color change has been attributed to the action of polyphenol oxidase (*o*-diphenol: O_2-oxidoreductase, EC 1.10.3.1) on the natural phenolic substances of plant tissues (Corse, 1964). The phenolic substances are oxidized to quinones, which either react with themselves or with other phenolics to form the brown pigment called melanin (Eskin et al., 1971).

Polyphenol oxidases (PPO) have been purified and studied from peaches (Wong et al., 1971), cranberries (Chan and Yang, 1971), mushrooms (Kertesz and Zito, 1962; Nakamura et al., 1966), carrots (Khandobina and Geraskina, 1969), apples (Walker and Hulme, 1966; Shannon and Pratt, 1967), potato (Patil and Zucker, 1965) and bananas (Palmer, 1963). The purpose of this work was to purify and study the properties of the PPO of Royal Ann cherries.

EXPERIMENTAL

Cherry samples

Royal Ann cherries (24–26° Brix) were hand picked, placed in polyethylene bags, iced and brought to the laboratory where the stems and pits were removed. After freezing at −30°C, the cherries were freeze dried. During freeze drying, the temperature of the heating plates did not exceed 32°C. Approximately 2 hr elapsed between picking and freezing of the cherries. The freeze-dried cherries were placed in enameled cans, flushed with N_2 and sealed under a vacuum of 27 in. of Hg. The canned, freeze-dried cherries were stored at −23°C until used.

Preparation of PPO

Freeze-dried cherries were powdered in a stainless steel Waring Blendor for 2 min in the presence of liquid nitrogen. The powder was placed in a brown bottle, sealed and stored in a desiccator at 5°C until used. The extraction procedure was as follows: 6 ml of freshly prepared 20% aqueous polyethylene glycol (PEG 20,000) was added to a centrifuge tube and chilled in an ice bath. 4g of cherry powder were added to make a thick paste. This paste was allowed to stand for 3 min, mixed with 9 ml of cold acetone and gently stirred for 1 min. After centrifuging, at 31,000 × G for 5 min, the supernatant was discarded and the pellet was rinsed with 1 volume of cold acetone. The precipitate was extracted with 100 ml 0.05M acetate buffer (pH 5.6) by stirring for 12 min in an ice bath. After filtering through a nylon cloth, centrifugation of the filtrate at 27,000 × G for 10 min resulted in a clear supernatant. The active enzyme was precipitated from the supernatant by the addition of 3 volumes of cold acetone to 2 volumes of extract. This mixture was stirred for 2 min and allowed to stand for 90 min in an ice bath. The supernatant was decanted and discarded while the cloudy suspension was centrifuged at 20,000 × G for 10 min. After decanting the supernatant, the precipitate was dissolved in a minimum volume of distilled water. All operations were carried out either in a cold room or in an ice bath and all solutions were cooled before use.

Protein content of the enzyme preparations was determined by the method of Kalckar (1947). The phenolic content was determined by the spectrophotometric method described by AOAC (1960) and expressed as μg tannic acid per ml of enzyme preparation.

Polyphenol oxidase assay procedure

Enzyme activity was determined by measuring the increase in absorbance at 400 nm with a Beckman DB recording spectrophotometer. The reference cuvette contained 2 ml of 0.2M phosphate buffer (pH 7.0) and 1 ml 10 mM catechol. Sample cuvette contained 1 ml 0.2M phosphate buffer, 1 ml 10 mM catechol and 1 ml of enzyme solution. Temperature of the reaction mixture was 30°C. Under these conditions, linearity was maintained for 2 min. One unit of enzyme activity was defined as the amount of enzyme that caused a change in absorbance of 0.001/min. The initial rate of the enzyme catalyzed reaction was proportional to the concentration of the enzyme solution.

Sephadex chromatography

Sephadex G-100 (Pharmacia), particle size 40–120μ, was prepared as described by the manufacturer (Technical Data Sheet No. 6). A 2.5 × 95 cm column was prepared and equilibrated with 0.050M acetate buffer (pH 4.5). 10 ml of the enzyme extract was dialyzed against this buffer overnight and applied to the column. Chromatography was performed with the same buffer. The eluent was continuously monitored at 280 nm and collected in 10 ml fractions.

Ion-exchange chromatography

Pre-swollen microgranular diethylaminoethyl (DEAE)-cellulose with a capacity of 1.0 meq/g was obtained from Reeve Angel, Inc. and prepared according to Whatman Advanced Ion-exchange Cellulose Laboratory Manual. A 2.5 × 45 cm column of DEAE-cellulose was packed at room temperature. The packed column was equilibrated overnight with starting buffer (1 mM phosphate buffer, pH 6.2) at 4°C. After the sample was applied to the column, one void

Table 1—Extraction of cherry PPO by various methods[a]

Extraction method	Activity (units/ml)	Absorbance 280 (nm)	Absorbance 260 (nm)	Protein (mg/ml)	Specific activity (units/mg)	Phenolic content (μg tannic acid/ml)
1. Buffer[b]	200	6.6	6.8	4.5	44	165
2. PVP + buffer	373	5.5	5.8	3.7	101	95
3. Acetone powder + buffer	420	6.0	6.5	3.9	108	90
4. Acetone powder + PVP + buffer	425	5.5	5.9	3.6	118	92
5. PEG + buffer	540	12.8	13.0	8.9	61	75
6. PEG – acetone + buffer	450	5.5	6.5	3.2	141	65
7. Dowex 1 – X8 + buffer	210	2.5	2.3	1.9	111	52

[a] The results are the average of three trials on different crude powder preparations.
[b] 0.05M acetate, pH 5.6

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volume of starting buffer was allowed to flow through the ion exchange resin. This was followed by a concave elution gradient generated by using six chambers of a Varigrad. The six chambers contained 0, 30, 60, 50, 40 and 100 ml of the final buffer (1M phosphate, pH 6.2), respectively. Total volume of each chamber was made to 100 ml with the starting buffer. At the end of the chromatographic run, the DEAE-cellulose was re-equilibrated by eluting with 100 ml of 0.5M KH_2PO_4 and with starting buffer until the pH had returned to 6.2.

Polyacrylamide-gel electrophoresis

Discontinuous vertical gel electrophoresis was performed similar to that described by Petropakis et al. (1969) with the exception that the electrode buffer was 0.0165M Tris-0.039M glycine, pH 8.75. Model EC-470 vertical gel electrophoresis apparatus (EC Apparatus Corporation) was used. Gel concentrations were 4%, 10% and 20% for the spacer, running and plug gels, respectively. Spacer and plug gels were approximately 2.5 cm in length. The temperature was maintained at 0°C throughout the run by circulating cold water through the cooling channels of the electrophoretic cell. A sample of 150 µl of the enzyme solution containing 10% sucrose and a small amount of bromophenol blue was placed in the sample slot of the gel. Polyphenol oxidase activity in the gel strips was detected by immersing the strip for 10 min in 100 ml of 10 mM catechol-0.2M potassium phosphate (pH 7.0) containing 0.05% p-phenylenediamine. The strip was then treated with 100 ml of 1 mM ascorbic acid for 5 min. After 48 hr in distilled water, the strips were placed in 30% ethyl alcohol. Bands were stable for more than 2 months at room temperature in this solution. In studies to determine the effect of inhibitors on the PPO isozymes, the gel strips were incubated in 100 ml of 10 mM inhibitor solution for 1 hr at room temperature. After this incubation period, the inhibitor solutions were poured off and replaced with 100 ml of substrate solution.

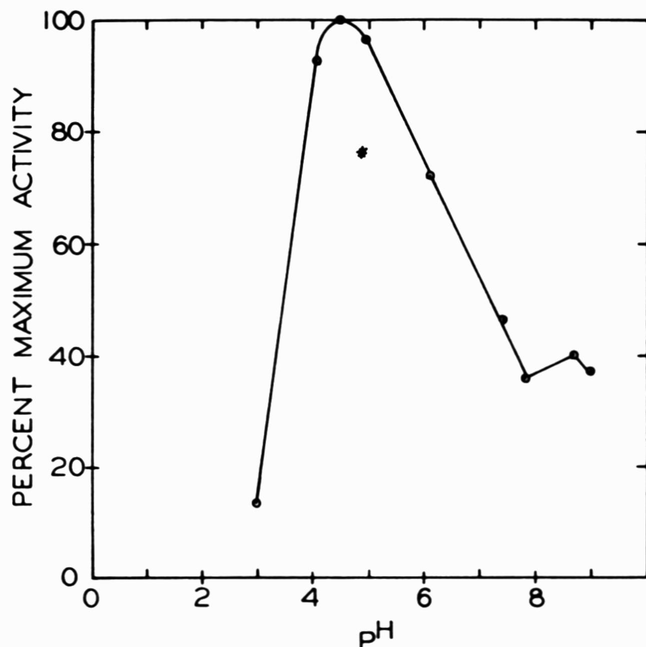

Fig. 1—pH stability of Royal Ann cherry PPO. Results are the average of four different trials of crude powder preparation. (*) control.

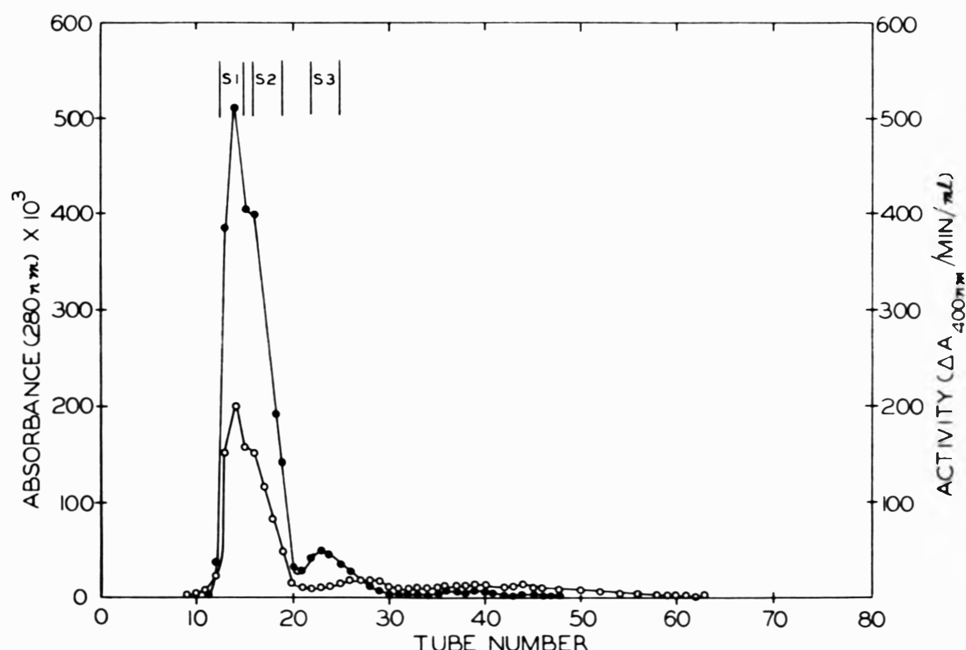

Fig. 2—Elution profile of PPO on Sephadex G-100. Equilibrated and eluted with 0.05M acetate, pH 4.5, flow rate 12 ml per hr at 4°C. -•-•-•-, PPO activity (units/ml); -○-○-○-, absorbance at 280 nm.

RESULTS & DISCUSSION

Enzyme extraction

The data in Table 1 reveal that the extraction of the crude powder with buffer (0.05M acetate, pH 5.6) resulted in low total and specific activities. The presence of polyvinlypyrrolidone (PVP) (Loomis and Battaile, 1966) in the buffer (0.5g PVP/g crude powder) reduced the phenolic compounds substantially and increased the activity of the PPO. When the crude powder was extracted with acetone prior to buffer extraction (method No. 3) the phenolic content was also decreased and activity was increased. Addition of PVP to the buffer (No. 4) did not affect either the phenolic content or the activity of the extract. Use of PEG (1.5 ml of 20%/g crude powder) in the buffer (No. 5) yielded the highest activity of any of the methods used with a low phenolic content. However, a considerable amount of PEG remained in the extract as revealed by the high absorbance at 280 and 260 nm.

Highest specific activity was obtained when PEG was mixed with the crude cherry powder and acetone and the resulting powder was extracted with buffer (No. 6). This method is described in the experimental section. The use of Dow 1-X8 (Lam and Shaw, 1970) in the extracting buffer did not appear to increase the activity, although this resin resulted in the lowest phenolic content. Therefore, the best extraction method was the PEG-acetone treatment followed by extraction with buffer. This preparation was used in the following work and is referred to as the PEG-acetone extract.

Protamine sulfate has been used in crude enzyme extracts to precipitate nucleic acids and their anionic biopolymers (Felix, 1960). Protamine sulfate has also been used in purification of esterases

from plant sources (Montgomery et al., 1969; Veerabhadrappa and Montgomery, 1971). Addition of protamine sulfate solution to the PEG-acetone extract resulted in a reduction of PPO activity and specific activity. Therefore, protamine sulfate was not used in the purification of cherry PPO.

75% of the PPO activity from the PEG-acetone extract was precipitated at 10% saturation with ammonium sulfate. The purity of this precipitate had increased approximately 9-fold. Difficulty was encountered in dissolving the precipitate; therefore, this method of purification was abandoned. However, precipitation of PPO from the PEG-acetone extract with acetone was a convenient method to concentrate the activity and to remove the excess PEG, which interfered with protein determinations.

pH Stability

To determine the pH of buffers to use in column chromatographic studies, the pH stability of PPO was determined. pH of the PPO solutions was adjusted by the careful addition of 1 mM NaOH or HCl. No change in the pH was noted during the 48 hr storage period at 4°C. The results of these studies are presented in Figure 1. Maximal stability for 48 hr appeared to be at pH 4.5. A striking observation was that samples adjusted with either acid or base between pH 3.9 and 6.0 had higher activity than the control at pH 4.8. Swain et al. (1966) found a similar increase in the activity of broad bean PPO activity when the pH was altered by acid or base. In their work, the optimum stability was at pH 4.5–4.7. These authors (Swain et al., 1966) concluded that at neutrality the prosthetic groups of the enzyme were masked by the tertiary structure of the protein and alteration of this structure by a change of pH or an anionic detergent resulted in a more active enzyme.

Gel filtration

The elution profile of PPO from Sephadex G-100 revealed three fractions (Fig. 2). Tubes 12–15, 16–19 and 22–25 were pooled and called fractions S1, S2 and S3, respectively. These fractions were concentrated by lyophilization and stored at −20°C for further studies.

The data in Table 2 show that the specific activities of fractions S1 and S2 were not increased by gel filtration while fraction S3 showed a slight increase in purification. However, fraction S3 contained only 4% of the total activity of the acetone precipitate. Chromatography on Sephadex G-150 and G-200 did not improve the separation of these fractions. Therefore, since Sephadex gel-filtration did not appear to fractionate and purify PPO, the method was abandoned as a means of purifying cherry PPO.

Ion-exchange chromatography

Attempts to fractionate PPO by ion-exchange chromatography on carboxymethyl (CM)-cellulose were not successful. With DEAE-cellulose, two fractions designated DE1 (tubes 32–35) and DE2 (tubes 36–39) were obtained (Fig. 3). DE1 and DE2 contained 45% and 33% of the total activity of the acetone precipitate, respectively (Table 3). The most active tube in DE1 resulted in a 200-fold purification while the most active tube in DE2 was purified 180-fold. These fractions were dialyzed for 24 hr against three changes of 1 mM potassium phosphate (pH 6.2), concentrated by lyophilization and stored at −20°C for further studies.

pH Optimum for polyphenol oxidase activity

Results of this study (Fig. 4) show a bell-shaped curve for the PEG-acetone extract with a maximum of 7.0. On the other hand, both fractions DE1 and DE2 demonstrate a broad plateau at a pH 7.3–7.8. A pH optimum of near neutrality has been observed for the PPO of apricot (El-Tabey and Cruess, 1949), eggplant (Rhoades and Chen, 1968), cranberry (Chan and Yang, 1971), bean leaf (Racusen, 1970) and peach (Wong et al., 1971). Apple PPO was found to have two pH optima, 5.2 and 7.3, with the activity at pH 5.2 several times greater than that at pH 7.3 (Shannon and Pratt, 1967).

Table 2—Purification of PPO from Royal Ann cherry with Sephadex G-100

Purification step	Volume (ml)	Total activity (units × 10⁻³)	Total protein (mg)	Specific activity[a] (units/mg × 10⁻³)	Yield (%)	Purification (fold)
1. PEG-acetone extract	100	25.5	240.0	0.1		
2. Acetone precipitate[b]	10	48.5	12.0	4.0	190	40
3. Sephadex G-100						
fraction S1	35	17.9	4.9	3.7	70	37
fraction S2	40	16.0	4.0	4.0	62	40
fraction S3	40	1.9	0.3	6.3	7	63

[a] Most active tube of each fraction
[b] Acetone precipitate dialyzed against 0.05M acetate, pH 4.5

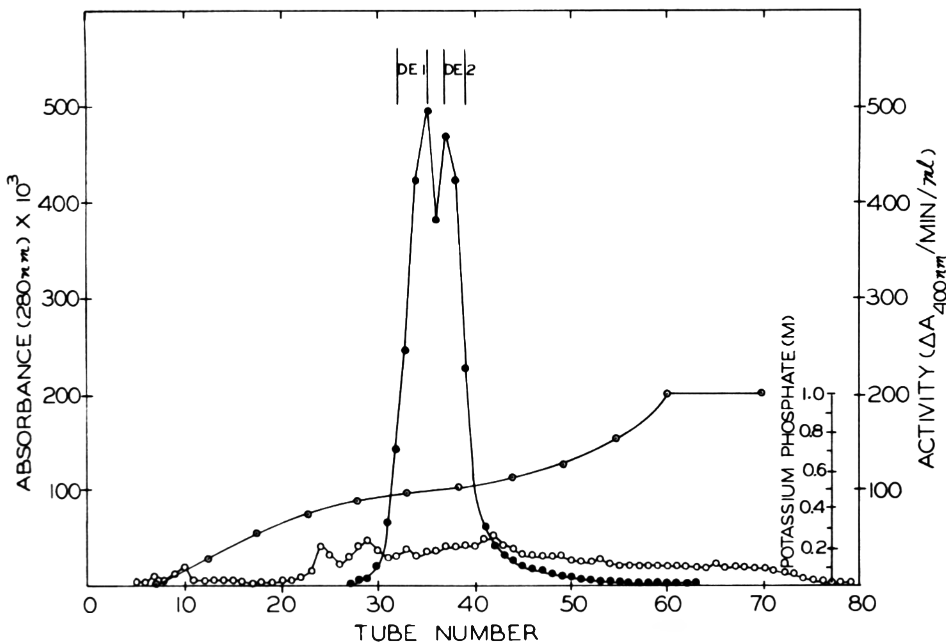

Fig. 3—Chromatography of PPO on DEAE-cellulose. Eluted with a concentration gradient of potassium phosphate, pH 6.2, flow rate 50 ml per hr at 4°C. -●-●-●-, PPO activity (units/ml); -○-○-○-, absorbance at 280 nm; -⊙-⊙-⊙- potassium phosphate concentration.

Table 3—Purification of Royal Ann cherry PPO by DEAE-cellulose chromatography

Purification step	Volume (ml)	Total activity (units × 10^{-3})	Total protein (mg)	Specific activity[a] (units/mg × 10^{-3})	Yield (%)	Purification (fold)
1. PEG-acetone extract	80	20.4	192	0.1		
2. Acetone precipitate[b]	8	43.2	8.5	5.0	211	50
3. DEAE-cellulose						
fraction DE1	40	19.8	1.0	20.0	97	200
fraction DE2	30	14.4	0.8	18.0	70	180

[a] Most active tube of each fraction
[b] Acetone precipitate dialyzed against 1 mM phosphate, pH 6.2

Table 4—Michaelis constants and maximum velocities of fractions of Royal Ann cherry PPO

PPO fraction	K_M (mM)	V_{max} (Abs)
Acetone precipitate	46	766
DE1	43	1000
DE2	53	1000

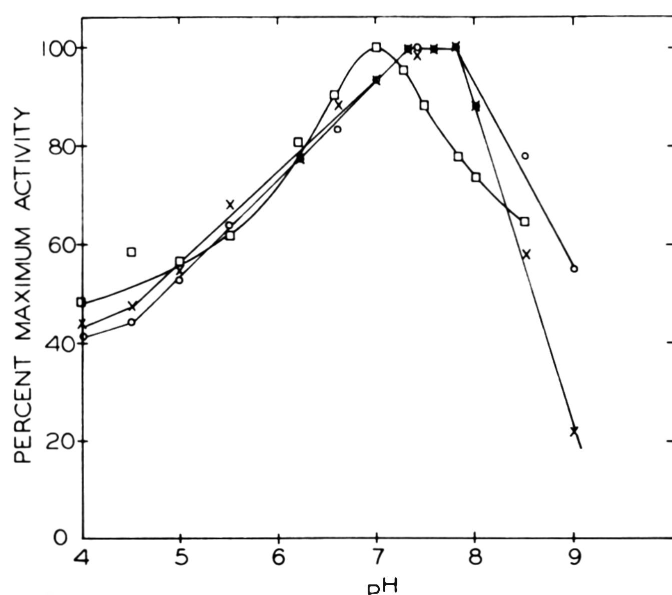

Fig. 4—Effect of pH on PPO activity. -□-□-□-, PEG-acetone extract; -x-x-x-, DE1; -o-o-o-, DE2. Acetate buffer was used between pH 4.0–6.0; phosphate buffer, pH 6.0–8.0; and Tris-HCl, 8.0–9.0.

Effect of substrate concentration

The effect of substrate concentration on the initial reaction rate of cherry PPO was determined for each fraction. Michaelis constants (K_M) and maximal velocities (V_{max}) are shown in Table 4. These were calculated from S/v vs. S plots (not shown). Polyphenol oxidase from the acetone precipitate, DE1 and DE2 fractions had similar K_M values which differed only slightly from the K_M reported for the Bartlett pear PPO (Tate et al., 1964), but were about 80-fold different from the PPO of tobacco root (Sisler and Evans, 1958). The V_{max} of fractions DE1 and DE2 was the same, while that of the acetone precipitate was about 25% less.

Substrate specificity

Various monophenolic and *o*-diphenolic compounds were used to determine the substrate specificity of the acetone precipitate and fractions DE1 and DE2. Protein concentration of all three fractions was adjusted to approximately 0.1 mg/ml. Rate of the reaction for the different substrates was measured at maximal wavelengths after a 5 min reaction time. Data presented in Table 5 reveal that all fractions were active towards the *o*-diphenols but not with monophenols. These results indicate that cherry PPO cannot catalyze the oxidation of monophenol to *o*-diphenol. This compares favorably with observations on the substrate specificity of other plant PPO with the exception of mushroom (Krueger, 1950) and potato (Alberghina, 1964) PPO. Mason (1956) reported that monophenolase is less stable than *o*-diphenolase. Since the acetone precipitate did not show monophenolase activity, if any monophenolase activity were present in the Royal Ann cherry, it was not lost during chromatography. When *p*-cresol was used as substrate, the three fractions did not show activity for 20 min; but when a mixture of 4-methyl catechol and *p*-cresol was used, activity was observed almost immediately. These results are similar to those of Long and Alben (1968). Of the substrates used in this study, pyrogallol was oxidized most rapidly by the acetone precipitate and fraction DE1, while fraction DE2 oxidized 4-methyl catechol most rapidly. This indicates that the substrate specificity of DE1 and DE2 was different.

Inhibitor studies

Several compounds known to reduce the enzymic browning of fruits were used as inhibitors of PPO. Sodium diethyldithiocarbamate (DEDTC), dithiothreitol, and potassium metabisulfite delayed the start of the reaction from 2 to 5 min. Inhibition by the various compounds was calculated from the rate of the change in absorbance at 400 nm immediately following this induction period. With catechol as substrate, the three enzyme preparations showed similar behavior towards the inhibitors (Table 6). All of the compounds used in this study inhibited all the preparations of cherry PPO. Sodium chloride was the least potent inhibitor of those used. DEDTC, dithiothreitol, potassium metabisulfite and potassium cyanide appear to inhibit PPO at very low concentrations. DEDTC has been shown to complex the copper prosthetic group of PPO (Swain et al., 1966; Vaughan and Butt, 1970; and Grncarevic and Hawker, 1971) and to inhibit the PPO of broad bean (Swain et al., 1966), grapes (Segal and Segal, 1969), *Microbacterium leprae* (Prabhakaran et al., 1969), grape berries (Grncarevic and Hawker, 1971) and peaches (Wong et al., 1971).

Heat inactivation

5 ml of enzyme solution were placed in a prewarmed test tube at 75°C and portions were withdrawn at various intervals, cooled and assayed for residual activity. The rate of decrease of PPO activity at 75°C appears to follow first order kinetics for 7 min (Fig. 5). Differences in

Table 5–Substrate specificity of Royal Ann cherry PPO

Substrate	Wavelength (nm)	Activity (units/ml) Acetone ppt	DE1	DE2	Ratio of activity (DE1/DE2)
Catechol	400	355	220	150	1.5
Pyrogallol	334	715	490	175	2.8
4-Methyl catechol	400	650	305	390	0.8
Dopa	460	205	80	60	1.3
Chlorogenic acid	400	225	165	110	1.5
1-Epinephrine	470	205	140	100	1.4
Tyramine	472	0	0	0	0
1 (–) Tyrosine	472	0	0	0	0
p-Cresol	400	0	0	0	0

Table 6–Effect of different inhibitors on PPO fractions

Inhibitor	Concentration	Acetone ppt inhibition (%)	DE1 inhibition (%)	DE2 inhibition (%)
NaCl	100 mM	0	0	0
	333	14	26	16
	400	18	32	19
Diethyl-dithiocarbamate	83 μM	61	77	68
	166	93	97	99
	333	100	100	100
Potassium metabisulfite	83 μM	54	43	48
	166	100	100	100
Dithiothreitol	67 μM	50	43	45
	83	100	100	100
	166	100	100	100
Thiourea	33 μM	36	30	32
	166	48	66	71
	333	57	86	87
KCN	33 μM	40	39	45
	166	100	100	100

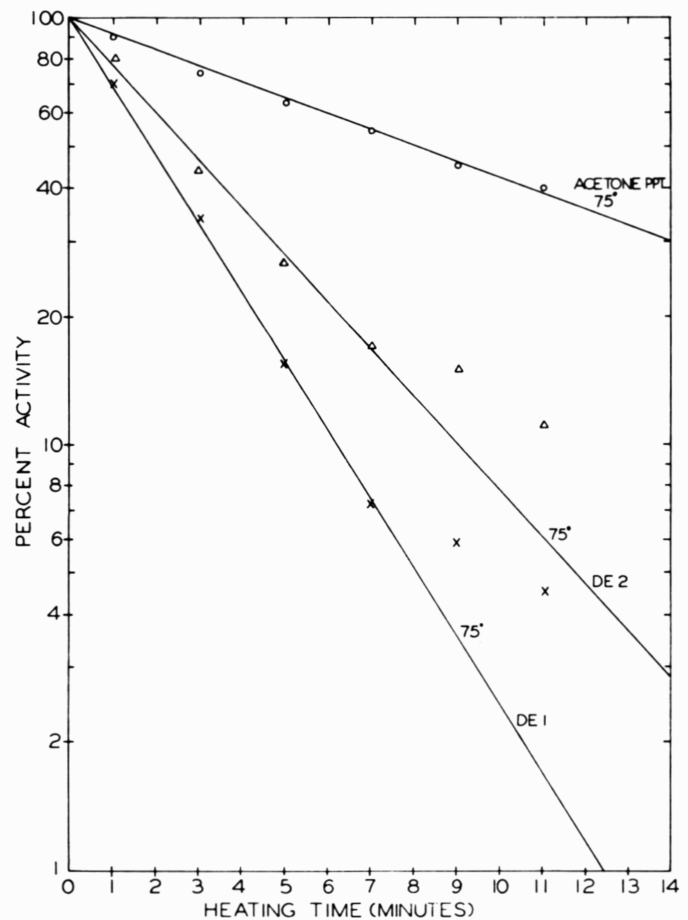

Fig. 5–Heat inactivation of Royal Ann cherry PPO.

heat stabilities were apparent between fractions DE1 and DE2 and the acetone precipitate. Half-lives of DE1, DE2 and acetone precipitate at 75°C were 1.9, 2.7 and 8 min, respectively.

Changes in the absorption spectrum of the reaction mixture

The absorption spectrum of the enzyme solution from the acetone precipitate shows a typical maximum of 280 nm (Fig. 6). The spectrum for catechol showed a sharp peak maximizing at 275 nm. The absorption spectrum of the reaction mixture of pH 7.0 was determined at various times after initiation of the reaction (Fig. 7). Maximal absorption at 400 nm occurred after 5 min. This is in agreement with the data for eggplant (Rhoades and Chen, 1968) and cranberry (Chan and Yang, 1971) PPO. the spectra in Figure 7 reveal that as the reaction proceeded there was a decrease in absorbance at 400 and 230 nm and an increase at 290 nm. This indicates that the initial reaction product, which absorbed at 400 nm, reacted further to form substances that absorbed at 290 nm. The decrease in absorbance at 400 nm could account for the observed decrease in the rate of the PPO reaction at 400 nm after 3 or 4 min. The identity of the compound absorbing at 290 nm was not investigated.

Polyacrylamide-gel electrophoresis

Electrophoretic pattern of the PEG-acetone extract and the acetone precipitate prepared from Royal Ann cherries is presented in Figure 8. In addition, patterns obtained with only bromophenol blue and no sample are shown in Figure 8. From strip 3 of Figure 8 band D was identified as bromophenol blue, while strip 4 indicates that band E was due to an artifact which does not require the presence of the sample. Mason (1956) reported that the nonenzymatic deamination of glycine with catechol forms a deep brown color and the most probable product to deamination was glyoxylic acid. Therefore, band E appears to be due to the glycine ions which were present in the electrode buffer and formed the trailing ions of the discontinuous buffer system.

In the PEG-acetone extract, group A consists of four bands, while groups B and C had only one band each. In contrast to this, group A of the acetone precipitate revealed a dark band that migrated at a slower rate than those of the PEG-acetone extract. Also, group B of the acetone precipitate has three bands. From these observations, it is apparent that the precipitation with acetone causes changes in the isozymic pattern of cherry PPO.

Fractions from the chromatography of the acetone precipitate on Sephadex G-100 were separated by gel electrophoresis. These bands appeared too light to publish, however, on the original gels it

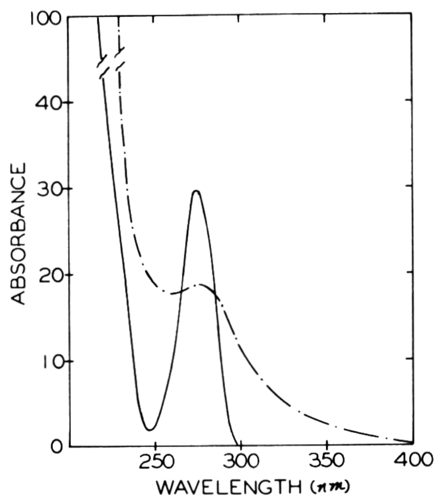

Fig. 6–Absorption spectra of catechol and PPO preparation. ——, catechol; –·–·–, PPO.

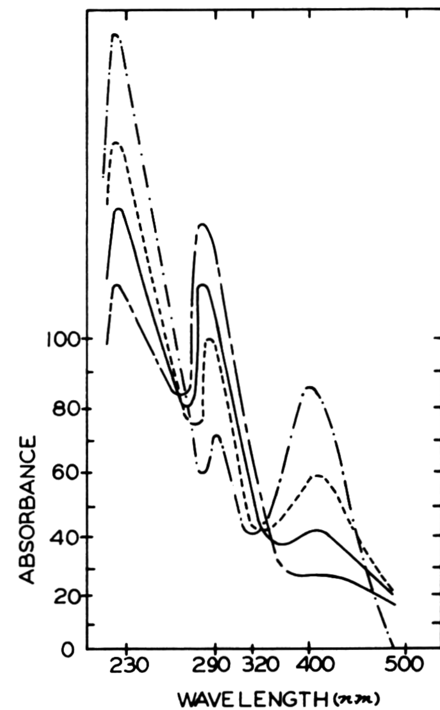

Fig. 7–Absorption spectra of PPO assay mixture at various times after addition of the enzyme. –·–·–·, 1 min; ----, 10 min; ——, 20 min; —— – ——, 40 min.

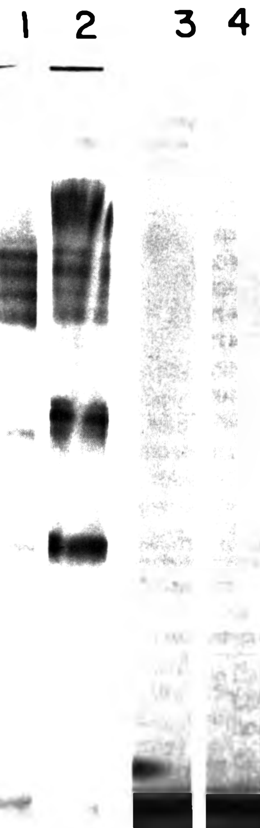

Fig. 8–Electrophoretic patterns of (1) PEG-acetone extract; (2) acetone ppt; (3) bromophenol blue; and (4) no sample. Catechol was used as substrate.

was evident that group A was concentrated in the first portion of the PPO peak while group C was more dominant in the last portion of the peak. Group B was found between groups A and C. This indicates that the PPO differ in molecular size. Similar observations were made from the electrophoretic patterns of the fractions from DEAE-cellulose (not shown). There was a predominance of groups A and B in the first fraction of PPO activity eluted from DEAE-cellulose, while group C was concentrated in the later fractions. Since polyacrylamide-gel electrophoresis separates proteins on the basis of size and net charge, the size of the PPO would decrease and the net charge would increase from group A to group B to group C.

Data presented in Figure 9 show that catechol, chlorogenic acid, dopa and *p*-cresol served as substrates for groups A, B and C with the chromogenic patterns of dark brown, light blue, deep red and red, respectively. With 10 mM pyrogallol no activity was evident (strip 6); however, at 100 mM activity was detectable. In the first band of group A, the oxidation of pyrogallol produced a yellow chromogen. Group C was the only group of cherry PPO to show activity toward 4-methyl catechol. This was revealed as a red color. *p*-Cresol was oxidized by the enzyme in the gel but did not serve as a substrate in the spectrophotometric assay. Pomerantz and Warner (1967) demonstrated an activator function for dopa in tyrosine hydroxylation catalyzed by hamster melanoma PPO. In agreement with this, Long and Alben (1968) reported that with a spectrophotometric assay the presence of an activator (4-methyl catechol) was necessary for the oxidation of *p*-cresol by mushroom PPO, while in the absence of this activator, the enzyme was inactive toward *p*-cresol. When 4-methyl catechol was added to the spectrophotometric assay, cherry PPO also oxidized *p*-cresol. This activity was greater than with 4-methyl catechol alone. Whether 4-methyl catechol serves as an activator for the *p*-cresol or vice versa is not known. In the gel, *p*-phenylenediamine may have also acted as an activator for the oxidation of *p*-cresol.

To determine if the isozymes of cherry PPO could be dissociated, the acetone precipitate preparation was dialyzed against 0.25% sodium dioctylsulfosuccinate for 24 hr and 10 mM $CaCl_2$ for 16 hr. Spectrophotometric assay showed no differences in the activity of these treated portions from that of untreated samples. Strips 2 and 3 of Figure 9 demonstrate that with catechol as substrate, no differences were noted in the gel electrophoretic patterns of these two treatments. This study indicates that no dissociation occurred.

In Figure 9, strips 9–15, are the re-

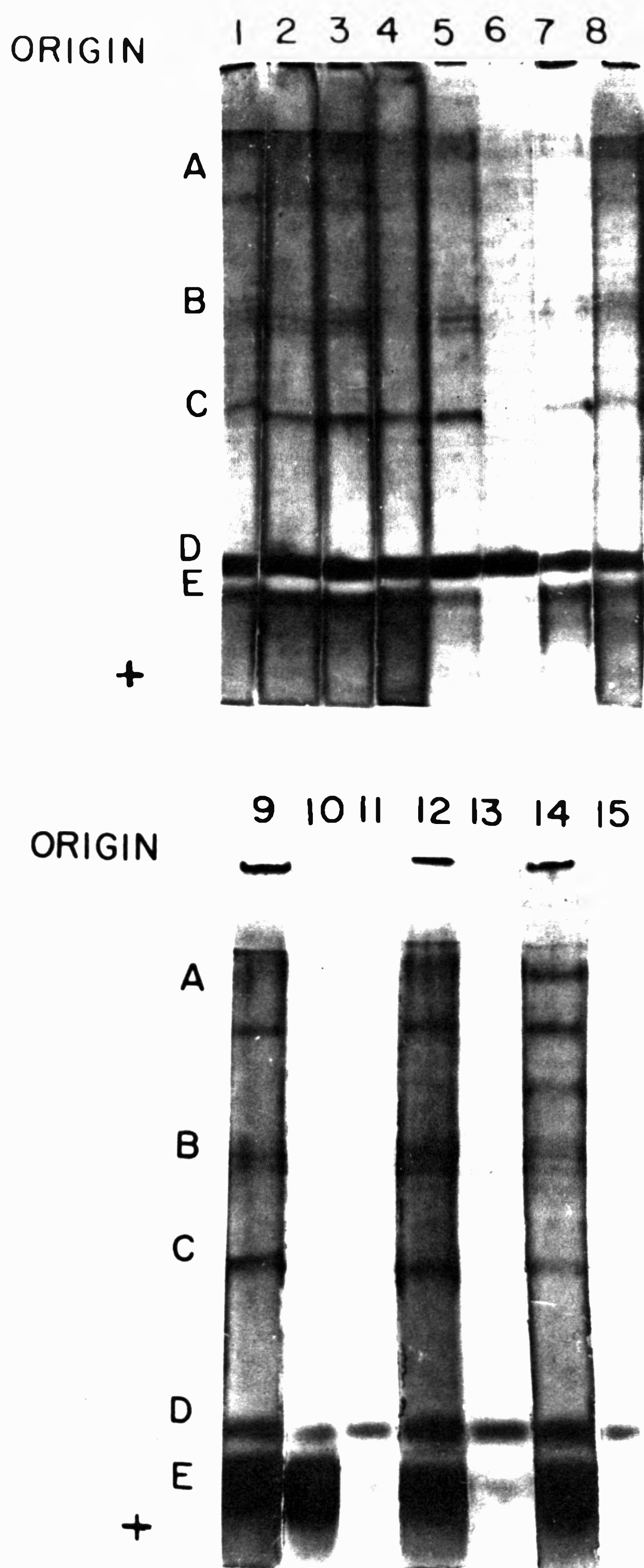

Fig. 9—Electrophoretic patterns of PPO with different substrates (1–8) and inhibitors (9–15): (1) control (catechol); (2) dialyzed against sodium dioctylsulfosuccinate–catechol as substrate; (3) dialyzed against $CaCl_2$–catechol as substrate; (4) 4-methyl catechol; (5) chlorogenic acid; (6) pyrogallol; (7) p-cresol; (8) dopa; (9) control–no inhibitor; (10) potassium cyanide; (11) potassium metabisulfite; (12) thiourea; (13) dithiothreitol; (14) 2, 3-dihydroxynaphthalene; and (15) diethyldithiocarbamate.

sults of the inhibitor study on the activity of PPO. These patterns reveal that diethyldithiocarbamate (DEDTC), potassium metabisulfite, dithiothreitol, and KCN inhibited all three groups of the PPO, while thiourea and 2,3-dihydroxynaphthalene did not inhibit any of bands of PPO activity. All of the bands within a given group appeared to behave similarly towards the inhibitors. Since some of these inhibitors showed an induction period in the spectrophotometric assay treatment with inhibitor, the gels were immersed in distilled water for 12 hr, before being placed in the substrate solution. PPO activity was partially restored by this treatment. Therefore, the inhibition appears to be reversible.

Discussion

Although PVP has been used for the binding of phenolic compounds in plant enzyme extracts, in the present investigations PEG protected cherry PPO from inactivation better than PVP. Walker and Hulme (1965) have reported that PVP in high concentrations will bind protein as well as phenolic compounds. Badran and Jones (1965) and Arakji and Yang (1969) reported that PEG was a more effective pheonlic binding agent than PVP for extracting PPO from green banana and cranberry, respectively. In the present study, PEG was effectively removed from the enzyme preparation by acetone precipitation.

Fractionation of cherry PPO by chromatography on Sephadex G-100 indicates that these enzymes have different molecular sizes. The two fractions obtained from chromatography on DEAE-cellulose appeared to differ in their substrate specificity and heat stability, but exhibited similar sensitivities toward the inhibitors used in this investigation. Since each fraction contained a portion of the other, the properties of the fractions would be changed as higher purification was obtained.

Smith and Krueger (1962) separated five active components of mushroom PPO by chromatography on hydroxyapatite. Using polyacrylamide-gel electrophoresis, Constantinides and Bedford (1967) showed that mushroom PPO was composed of nine distinct isozymes. Three of these isozymes were active towards l-tyrosine, while the others oxidized dl-dopamine. These authors also found that potatoes contained 11 bands and apples three bands of PPO active towards dopa. With bean leaf PPO, Racusen (1970) obtained three isozymes with gradient electrophoresis but only one fraction was observed on chromatography on DEAE-cellulose.

Previous workers (Bouchilloux et al., 1963 and Jolley et al., 1969) reported that under certain conditions the association and dissociation of PPO isozymes

could take place. However, those isozyme patterns presented in Figure 9, strips 2 and 3 show that sodium dioctylsulfosuccinate did not cause dissociation of cherry PPO. This may have been due to lack of the reagents in the electrode and gel buffers. However, similarities between the samples treated with the reagents and those not treated would indicate that if dissociation had occurred, reassociation had taken place to form the same bands as were present before the treatment. Wong et al. (1971) also noted that the bands of peach PPO were not dissociated into subunits by urea. Therefore, it is possible that the PPO of cherry may exist in isozymic forms that are not composed of subunits.

Chromatographic evidence suggests that cherry PPO are separable into two or three fractions of different molecular size and charge density. Polyacrylamide-gel electrophoresis also demonstrated three groups which varied in either molecular size, charge density or both. Therefore, the PPO system of Royal Ann cherries appears to be composed of three enzymes with similar properties. Electrophoresis indicated two of these enzymes are composed of isozymes.

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NONVOLATILE ACIDS OF STRAWBERRIES

INTRODUCTION

ORGANIC ACIDS are important constituents of food products, not only as chelating agents for iron and copper, but for control of pH and inhibition of enzymes. The importance of pH and acidity to color of strawberries has been demonstrated (Sistrunk and Cash, 1968; Wrolstad et al., 1970). While the pH of different varieties of strawberries varied from 3.2–3.7 under conditions of processing, generally varieties with lower pH were more stable in color (Sistrunk and Cash, 1968). Wrolstad et al. (1970) found that color quality as rated by a taste panel was higher on varieties with a pH below 3.4.

The predominant acid in strawberries was found to be citric acid with lesser quantities of malic (Whiting, 1958; Hulme and Wooltorton, 1958). In Royal Sovereign strawberries, malic acid was approximately 10% and quinic acid 2% of the total acidity. Trace amounts of other acids also were separated on a silica gel column (Hulme and Wooltorton, 1958).

In strawberries that were fed radioactive fructose the radioactive peaks corresponded closely with organic acid titration peaks (Markakis and Embs, 1964). About 2.5% of the labeled fructose was incorporated into citric acid which represented 75% of the total acidity. Malic acid was second and phosphoric the third highest in concentration.

Maturity had a significant influence on organic acid patterns in fruits (Whiting, 1958; Amerine, 1950; Hulme and Wooltorton, 1957; Markakis et al., 1963). In Royal Sovereign strawberries, there was a large decrease in malic acid and an increase in quinic acid during maturation (Hulme and Wooltorton, 1958). Citric acid decreased slightly between the green and red-ripe stages of maturity.

The present study was initiated to determine nonvolatile acids in different maturities and varieties of strawberries.

MATERIALS & METHODS

THE VARIETIES Earlibelle, Sunrise and selection SIUS 253 were harvested and separated into two maturities. Fruit that was half-red to full-red (firm-ripe) and from full-red to overripe (full-ripe) were capped and frozen in polyethylene bags. Fruit of Surecrop variety were stripped to simulate mechanical harvest and separated into five groups: hard-green, mature-green, pink, firm-ripe and full-ripe. Approximately 5-lb samples of firm-ripe and full-ripe fruit were capped and packed in polyethylene bags for freezing while hard green, mature green and pink fruit were ripened to a red color at 21°C before freezing at −18°C.

For organic acid analyses, 200g of partially thawed fruit were blended in a blender for 1 min. A 20-g aliquot was taken and blended for 5 min with 80 ml acetone. The slurry was filtered and washed by suction filter. The extracts were evaporated at room temperature in large petri dishes in a fume hood. The residue was taken up in 10 ml distilled water and passed through a 10 × 200 mm cation exchange col-

Table 1—Total acidity and titration values of seven fractionated acids of strawberries

Variety	Number of Peak 1	2	3	4	5	6	7	% total acids as citric[a]
Surecrop	(ml of 0.01N NaOH)							
Hard-green	1.8	1.6	3.5	38.6	148.1	0.7	4.9	1.30
Mature-green	2.4	2.7	2.7	41.2	120.6	0.7	4.2	1.11
Pink	1.8	2.2	2.5	40.4	116.8	0.4	3.9	1.13
Firm-ripe	1.7	1.4	2.3	36.8	105.4	0.5	3.4	0.97
Full-ripe	1.5	1.3	1.3	30.2	92.3	0.4	3.1	0.85
Earlibelle								
Firm-ripe	1.4	0.9	2.4	40.2	203.2	0.4	4.6	1.65
Full-ripe	1.8	0.5	2.1	50.0	174.1	0.4	4.4	1.50
Sunrise								
Firm-ripe	3.4	1.9	5.5	39.0	154.2	0.5	8.3	1.37
Full-ripe	2.7	1.2	4.1	37.1	149.8	0.5	8.1	1.37
SUIS 253								
Firm-ripe	2.6	1.6	3.0	38.0	136.6	0.3	3.2	1.19
Full-ripe	2.1	1.5	2.5	36.4	131.9	0.3	2.7	1.14

[a] Total acids after cation exchange step

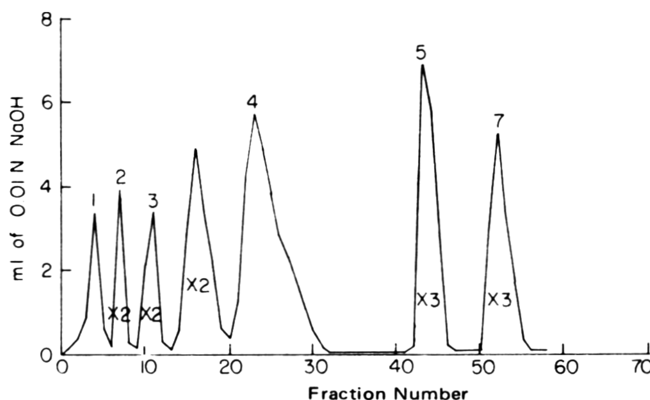

Fig. 1—Titration peaks of column chromatographic fractions of known acids. The following acids and quantities were chromatographed: (1) L-aspartic, 10 mg; (2) L-glutamic, 10 mg; (3) quinic, 20 mg; succinic, 15 mg; (4) malic 20 mg; (5) citric, 30 mg; (7) phosphoric, 20 mg.

umn packed with Dowex 50W-X4 (50-100 mesh) in the H+ form to remove pigments. After collecting 60 ml through the column essentially 100% of acids were removed. Samples were divided into two lots, one being titrated with 0.1N NaOH to obtain total acidity.

Rexyn 201 (Mfd. by Fisher Scientific Co.) in the acetate form (200–400 mesh) was used for fractionating the organic acids. The strong anion exchange resin, Rexyn 201 chloride was converted to the acetate form by adding 1N Na acetate to 10g of resin in a beaker. After standing for 1 hr the acetate was decanted off and the resin rinsed several times with distilled water and finally with 0.1N acetic acid. An 8 × 130 mm column was packed with the slurry by applying 2 lb pressure from a small pump. Glass wool was placed on the bottom and tops of column. 30 ml (10g) of sample was passed onto the column and six fractions were collected during adsorption. The column was filled with 2N acetic acid and attached to an automatic fraction collector with a volumetric attachment set to deliver 5 ml. The eluting solutions were in the following order: 50 ml 2N acetic acid, 50 ml 4N acetic acid, 80 ml 6N acetic acid and 150 ml of 6N formic acid. Approximately 72–75 fractions, including the original six, were sufficient to elute the organic acids. The determinations were replicated three times and only means of the data are shown. This method is similar to that used by Hulme and Wooltorton (1957). Fractions were transferred to petri dishes and dried in a ventilated fume hood at room temperature before titrating with 0.01N NaOH. Also effluent volumes for known acids were established by the same procedure. The resin was forced from the column after each sample and reconditioned by rinsing with distilled water and adding 1N Na acetate. By adding a small amount of resin each cycle, the resin was used repeatedly with excellent results. Dowex 50W-X4 was reconditioned by adding a small amount of nitric acid to the wet resin in a beaker to hydrolyze the pigments. Then the resin was washed several times with H_2O before returning to the column.

The paper chromatographic technique was adapted from the method of Williams and Patterson (1964). Samples were extracted by the same procedure as stated previously. After passing through the cation exchange column to remove pigments, samples were concentrated to dryness, taken up in 5 ml aliquots of 50% ethanol, and spotted on Whatman 3MM paper. Known acids were also spotted at the rate of 1–2 mg per spot from a solution of the acid containing 50 mg/5 ml. The R_f values of the known acids were measured and compared with R_f values of the unknowns in the samples.

Table 2–Strawberry acids and standards separated on Whatman 3 MM paper[a]

Acid	Strawberry acid R_f	Standard acid R_f	Fraction on Rexyn 201 column
	$R_f \times 100$		
Galacturonic	12(tr)	12	14
Glutamic	18	18	7
Quinic	38	37	11
Phosphoric	42	42	52
Citric	58	59	43
Malic	65	64	23
Chlorogenic	69	70	48
Succinic	–	84	16

[a] Developed by ascending technique at 22°C in n-Butanol-formic acid-water (10:3:10 v/v)

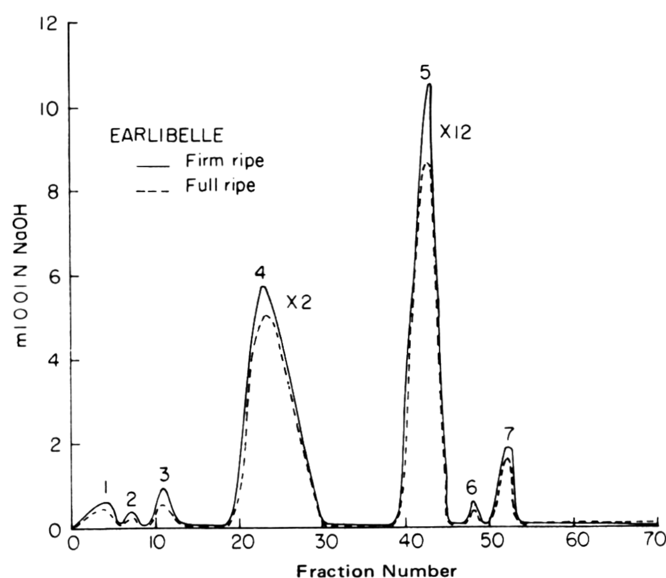

Fig. 2–Titration peaks of firm-ripe and full-ripe Earlibelle strawberries. Tentative identification of the acids: (1) aspartic; (2) glutamic; (3) quinic; (4) malic; (5) citric; (6) not identified; (7) phosphoric.

RESULTS & DISCUSSION

TOTAL ACIDITY was lower in fruit that was riper in most varieties, especially in Surecrop where part of the fruit was ripened after harvest (Table 1). The total acidity was the same for the two maturities in Sunrise and not greatly different in Earlibelle and SIUS 253. The most important organic acids were identified by comparison of the R_f values (Table 1) and the titration peaks of known acids (Fig. 1). Differences in organic acids were more apparent in peak 4 (malic) and peak 5 (citric) as a result of maturity and variety (Table 1). Malic acid increased until the mature-green stage in Surecrop and then decreased through the full-ripe stage. Peaks 1 and 2 were identified as aspartic and glutamic acids since these peaks came off consistently at fraction 4 and 7 respectively. These acids were higher in mature-green fruit of Surecrop. Peak 3 (quinic) was fairly stable in green Surecrop fruit that was ripened after harvest although this peak decreased sharply in the full-ripe fruit. This was different from the results of Hulme and Wooltorton (1958) in which quinic acid increased with maturity although they did not analyze full-ripe fruit. Peak 5 (citric) was much higher in the hard-green stage and decreased rapidly with maturity in Surecrop. Peak 6 was not identified in this study but according to the data of Markakis et al. (1963) peak 6 was probably chlorogenic acid. This acid was not greatly different in any of the samples. Phosphoric acid (peak 7) was higher in greener fruit, although the difference was small between firm-ripe and full-ripe fruit.

Titration curves for the firm-ripe and full-ripe fruit of Earlibelle variety reveal that there were no qualitative differences in the organic acids between the two maturities (Fig. 2). The results for the other varieties were similar in that no other peaks were present. In most instances, the fractionated acids were higher in firm-ripe as compared to full-ripe fruit (Table 1). Malic acid (peak 4) was higher in firm-ripe as compared to full ripe fruit except in Earlibelle. Citric acid

(peak 5) was slightly higher in firm-ripe fruit of Sunrise and SUIS 253 but in Earlibelle and Surecrop the differences were much greater between the two maturities. Succinic acid did not appear on either the chromatograms or anion exchange peaks although other workers have reported this acid in strawberries (Hulme and Wooltorton, 1958; Markakis and Embs, 1964).

Since earlier studies (Wrolstad et al., 1970; Sistrunk and Cash, 1968) have shown that pH and acidity were important for color of strawberries, it was felt that nonvolatile acid differences might provide information that would be helpful in developing varieties with better color. Firm-ripe fruit of many varieties was found to be more stable in color than full-ripe fruit in accelerated temeprature studies in a water bath (data not shown). The only major difference between the two maturities of the four varieties reported here was the higher total acidity in greener fruit. Malic and citric acids were responsible for most of the difference (Table 1).

In conclusion, the nonvolatile acids in these varieties did not vary qualitatively but there were quantitative differences between varieties and maturities. It is possible that the ratio of citric to malic acid in different genetic lines and maturities could affect color.

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ANTHOCYANINS OF ROSELLE (Hibiscus sabdariffa, L.)

INTRODUCTION

THE CALYCES of Roselle (Hibiscus *sabdariffa* L.) have been used to produce a red, pleasant flavored extract for use as a food and beverage in a number of tropical countries. Esselen and Sammy (1973) reviewed the chemistry and food uses of Roselle and cited a number of applications.

Yamamoto and Oshima (1932) obtained a crystalline anthocyanin from Roselle which they called "hibiscin" and assigned the structure, cyanidin-3-glucoside. They changed it later (1936) to delphinidin-pentoside-glucoside. Forsyth and Simmonds (1954) also reported the presence of delphinidin and cyanidin in Trinidad Roselle. Seshadri and Thakur (1961) reported the presence of gossytrin, a yellow pigment and hivisein which they claimed to be identical with daphniphyllum. The pigments of Roselle imported from Formosa were recently reexamined by Shibata and Furukawa (1969) who reported the presence of Cn-3-G and Dp-3-GX. The Dp-3-GX, which was obtained in crystalline form, was reported to be identical with daphniphyllin from Daphniphyllum *macropodum* Miq.

This report describes the isolation and identification of anthocyanins from Trinidad Roselle using a solvent system of higher resolution – BFW developed by Fuleki (1969). BFW is a particularly useful solvent for anthocyanin separation. Its use has revealed a number of other pigments not usually noted in a number of fruit products.

EXPERIMENTAL

A 1 LITER sealed jar containing fresh Roselle calyces (Early Dwarf variety) in 1.5N HCl/ethanol was shipped airmail from Trinidad, West Indies and used in this work. The calyces were macerated in a blender with methanol, filtered and the pulverized calyces re-extracted with 1% HCl in methanol. The filtrate was combined and concentrated in vacuo under 30°C.

The solvent systems used are listed in Table 1.

Authentic pigments

The preparation of authentic pigments from the sources listed in Table 2 followed the standard chromatographic procedure.

Isolation of pure Roselle pigments

The pigment extract was streaked on 46 × 57 cm sheets of Whatman No. 3 MM paper and developed by decending chromatography with BFW. Extended development was continued until the fastest moving band reached to about 3–4 cm from the bottom edge of the paper to achieve maximum separation. Numbering of pigment bands was begun with the fastest moving pigment. The developed chromatograms were air dried, cut, the pigment bands eluted with MAW and the solvent evaporated in vacuo under 30°C. Each pigment was further purified with 15% HAc, BFW, 15% HAc in that order.

Identification of Roselle anthocyanins

The identification of pigments followed in general the spectroscopic and chromatographic methods described by Harborne (1967).

Spectral measurement. UV and visible spectra were obtained with a Perkin-Elmer UV-visible-NIR recording spectrophotometer (#450) on the purified pigments dissolved in 0.01% HCl in spectrograde methanol.

Rf, R_{Cy3G} and R_G determination. These values were determined by spotting the pigments with authentic markers on Whatman No. 1 paper in specified solvent systems. R_{Cy3G} is chromatographic mobility relative to cyanidin-3-glucoside and is used with BFW. Most pigments have slow mobility in this solvent and prolonged development (with the solvent front off the paper) up to 2 or 3 days is required for meaningful R_{Cy3G} measurement. In R_G determination (chromatographic mobility relative to glucose), glucose is allowed to travel to 1/3 distance from the bottom edge of the paper to resolve the dissacharides.

Acid hydrolysis. The anthocyanin was hydrolyzed by heating approximately 2 ml pigment solution with 2 ml of 2N HCl in a water bath for 1 hr. After cooling, the anthocyanidin was extracted with amyl alcohol. The mineral acid was removed from the aqueous sugar solu-

Table 1–Solvent systems for chromatography

Abbreviation	Composition
BFW	n-butanol-formic acid-water (100:25:60)
15% HAc	glacial acetic acid-water (15:85)
MAW	methanol-glacial acetic acid-water (90:5:5)
1% HCl	concentrated HCl-water (3:97)
HAc-HCl	water-glacial acetic acid-HCl (82:15:3)
BAW	n-butanol-glacial acetic acid-water (4:1:5) upper phase
BuHCl	n-butanol-2N HCl, 1:1, upper phase
Formic	formic acid-concentrated HCl-water (5:2:3)
Forestal	glacial acetic acid-concentrated HCl-water (30:3:10)
Phenol	phenol-water (4:1)
BBPW	n-butanol-benzene-pyridine-water (5:1:3:3)
BEW	n-butanol-ethanol-water (4:2:2.2)

Table 2–Rf values of Roselle anthocyanins

Pigments	Source	Solvent systems					R_{Cy3G} × 100
		1% HCL	HAc-HCl	15% HAc	BuHCl	BAW	BFW
		Rf(× 100)					
2		7	24	62	23	36	99
3		28	52	70	22	31	81
4-1		3	16	48	11	23	54
4-2		26	54	70	17	25	60
Authentic markers:							
Cn-3-G from V. dentatum (Du and Francis, 1973a)		6	25	63	23	36	100
Cn-3-Gal from C. mas (Du and Francis, 1973b)		7	25	62	22	33	89
Dp-3-G from pomegranates (Harborne, 1967)		3	17	49	12	23	53
Cn-3-GX from V. dentatum (Du and Francis, 1973b)		28	53	71	22	33	80

tion remaining after extracting the anthocyanidin by repeatedly extracting it with 10% di-n-octylmethylamine in chloroform (Smith and Page, 1948). The sugar solution was washed twice with chloroform to remove residual amine. Both the aglycones and sugar solutions were concentrated by evaporation. The aglycones were then identified by comparison of Rf values with authentic aglycones on the same paper in BAW, Formic and Forestal (delphinidin and cyanidin were obtained from grapes and cranberries respectively). The sugars were developed with known sugar markers in BBPW, BAW and Phenol, and detected by anilin hydrogen phthalate (Partridge, 1945).

Controlled acid hydrolysis. The hydrolysis was carried out as described above except that small aliquots were withdrawn after 1, 2, 3, 4, 5, 6, 8, 10, 15, 20 and 60 min of heating. The samples were applied along with authentic anthocyanins directly on Whatman No. 1 papers and developed in two typical solvents: BFW and 1% HCl.

Peroxide hydrolysis. Sugars attached to the 3-position of anthocyanin were removed by the method of Chandler and Harper (1961) with minor modification. About 2 drops of 30% hydrogen peroxide was added to the solution (ca 1 ml of concentrated pigment) and let stand for 4 hr. A trace amount of Pd catalyst was then added to decompose excess hydrogen peroxide and let stand overnight. The decolorized solution was then washed with di-n-octylmethylamine and chloroform successively. One drop of ammonia was added and the solution evaporated to dryness. The sample was then spotted on the paper along with glucose and authentic disaccharides in BAW, BEW and BBPW. The reaction mixture could also be streaked on Whatman No. 1 paper and purified in BAW. Location of the sugar band could be detected by spraying just the end of the paper with Partridge's reagent. The sugar strip was cut out, eluted with methanol, concentrated and analyzed for partial or complete hydrolysis products with 2N HCl.

Total anthocyanin determination

The total anthocyanin content of air-dry calyces (13.5% H_2O) was estimated by the method of Fuleki and Francis (1968) and expressed as mg Dp-3-G per 100g dry calyces. The molar extinction coefficient of Dp-3-G established by Asen et al. (1959) was used in recalculating the $E^{1\%}_{1cm}$ at 543 nm. Triplicate determinations were made to give an average figure. Extracting solvent was changed to 1% HCl/methanol instead of 0.1N HCl in enthanol in the original method. Absorbance was determined in Hitachi Perkin-Elmer 139 UV-Vis. spectrophotometer.

Table 4—Products of acid hydrolysis

Pigments	Aglycone[a]	Sugars[b]	Hydrolyzed intermediate[c]
2	Cy	G	–
3	Cy	G,X	Cy-3-G
4-1	Dp	G	–
4-2	Dp	G,X	Dp-3-G

[a] Compared with authentic aglycones (Dp from grape, Cy from cranberry) in Formic, Forestal and BAW
[b] Compared with standard sugars, in BBPW, Phenol and BAW
[c] Developed in 1% HCl and BFW with authentic pigments

The calculation is as follows: Molar extinction coefficient of Dp-3-G = 2.9 X 10^4 using a M.W. of 518.5. On a $E^{1\%}_{1cm}$ basis, this is 559. A sample weighing 4.12g made up to 500 ml and diluted 1 to 20, gave an absorbance of 0.28. This is 1.5g pigment per 100g dry sample.

RESULTS & DISCUSSIONS

QUANTITATIVE DETERMINATION of total anthocyanin in dry Roselle calyces indicated that there were about 1.5g anthocyanin/100g on a dry weight basis when expressed in terms of Dp-3-glucoside.

Prolonged chromatography of Roselle pigment extract in BFW (3 days) showed the presence of six anthocyanin bands. Numbering of bands starts from the fastest moving one upward. Bands 1, 5 and 6 were trace pigments and present at a very low concentration. Unless special efforts were made to collect sufficient materials, their identification is impractical. Band number 2 represented a minor pigment in Roselle and bands 3 and 4 the major predominant pigments.

Band number 2 pigment

This minor anthocyanin yielded no intermediary product on controlled acid hydrolysis. The end products of hydrolysis were identified as cyanidin and glucose. The Rf values of this pigment were identical to authentic Cy-3-G in all solvent systems compared on the same chromatograms (Table 2). Spectroscopic data also substantiated that the compound in question was a 3-substituted cyanidin derivative (Table 3). The pigment is thus identified as cyanidin-3-glucoside.

Band number 3 pigment

This anthocyanin represents the second major pigment in Roselle. Spectral data suggested that it is a 3-substituted glycoside of cyanidin. Controlled acid hydrolysis of this anthocyanin showed the presence of Cy-3-G as a hydrolyzed intermediate (Table 4). Complete acid hydrolysis gave cyanidin, glucose and xylose as the end products. Hydrogen peroxide hydrolysis removed a disaccharide from the pigment which showed chromatographic mobility similar to that of sambubiose (Table 5). The purified disaccharide also gave glucose and xylose on partial and complete acid hydrolysis. The pigment is thus identified as cyanidin-3-sambubioside. The similarity of Rf values of this compound to the authentic Cy-3-sambubioside isolated from *V. dentatum* further confirms its identity (Du and Francis, 1973a, b).

The occurrence of Cy-3-sambubioside as the second major pigment in roselle was not reported by previous workers. This is probably due to the types of solvent systems used and avoidance of prolonged chromatography during the separation process, since this pigment has Rf values close to No. 4-2 (see below) in aqueous solvents and merged as one band. Chromatography for 2 days in BAW or BFW would effectively separate the two.

Band number 4 pigments

This pigment band separated into two components in 15% HAc. A minor slow moving band (4-1) and a predominant major faster moving band (4-2).

No. 4-1 yielded delphinidin and glucose as the end products of acid hydrolysis. Spectral evidence indicated that it is a delphinidin-3-glucoside. Rf comparison with authentic delphinidin-3-glucoside suggested that it is Dp-3-G. This pigment is also a minor component in roselle anthocyanins. It is present in quantity more than Cy-3-G, but lesser than Cy-3-sambubioside.

No. 4-2 pigment is the most abundant

Table 3—Spectral[a] data of Roselle anthocyanins

Pigment	Evis.max	Euv.max	Euv.max/ Evis.max	440/ Evis.max	310/ Evis.max	Al shift
2	528	281	68	25	15	+
3	528	280	67	24	14	+
4-1	541	276	59	22	16	+
4-2	542	277	56	20	13	+

[a] Spectral data obtained on Perkin-Elmer 450 UV-Visible-NIR spectrophotometer

Table 5—Chromatographic mobility of disaccharides from Roselle anthocyanins

Disaccharides	R_G[a] BAW	BEW	BBPW	Acid hydrolysis of disaccharides
3	75	78	68	G,X
4-2	75	79	67	G,X
Authentic marker:				
GX (*V. dentatum*)	76	79	70	

[a] Chromatographic mobility relative to glucose

anthocyanin in roselle and is the major pigment responsible for its reddish-violet color. It also crystallizes more easily than most pigments. Controlled acid hydrolysis showed the presence of delphinidin-3-glucoside as an intermediate. Complete acid hydrolysis gave delphinidin, glucose and xylose as end products. Spectral data suggested that the sugars are attached to the 3-position of delphinidin. This is further indicated by the removal of a disaccharide from the 3-position by hydrogen peroxide hydrolysis. Acid hydrolysis of this disaccharide gave glucose and xylose. Thus the pigment is identified as delphinidin-3-sambubioside.

CONCLUSION

WITH THE EXCEPTION of detecting Cy-3-sambubioside as the second major anthocyanin in Roselle, the identification of other pigments; Cy-3-G, Dp-3-G and Dp-3-GX, are in agreement with those reported by Shibata and Furukawa (1969). Dp-3-sambubioside and Cy-3-sambubioside are the major pigments, Dp-3-G and Cy-3-G are present in minor concentration.

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OXIDATIVE CHANGES IN OXYMYOGLOBIN DURING INTERACTION WITH ARGININE LINOLEATE

INTRODUCTION

AUTOXIDATION of MbO_2 (oxymyoglobin) is known to be influenced by various factors such as pH (George and Stratmann, 1954; Matsuura et al., 1962; Brown and Mebine, 1969), temperature (Matsuura et al., 1962; Brown and Mebine, 1969; Brown and Dolev, 1963a, b), partial pressure of oxygen (George and Stratmann, 1952; Matsuura et al., 1962; Brown and Mebine, 1969), metallic ions (Snyder and Skrdlant, 1966) and bacteria (Robach and Costilow, 1961). Besides, it has been shown that the rates of heme-catalyzed oxidation of unsaturated lipid are similar with ferrous and ferric hemoproteins (Robinson, 1924; Brown et al., 1963; Hirano and Olcott, 1971) and that in the oxidation of unsaturated lipid, ferrous hemoprotein is converted into its ferric form (Brown et al., 1963). Thus, it is presumed that the interaction of ferrous hemoproteins such as MbO_2 with unsaturated lipids may participate to some extent in the color deterioration of meat during storage. However, little information is available in this regard.

The interaction of MbO_2, the major pigment responsible for the desirable red color of fresh meat, with linoleate was studied primarily with regard to the oxidative changes of MbO_2.

EXPERIMENTAL

Materials

Crystalline sperm whale Mb was obtained from Mann Research Laboratory. Crystalline tuna Mb was prepared from the dark muscle of big eye tuna, *Parathunnus sibi*, by the method of Schmid (1949). Crystalline bovine serum albumin was obtained from Nutritional Biochemical Co. MbO_2 was prepared by reduction of MetMb with $Na_2S_2O_2$ and subsequent passage over a column of mixed bed ion exchange resin (Bio-Rad AC 501 × 8) to remove breakdown products of $Na_2S_2O_4$.

Arginine linoleate was used in this study because it is easily dispersed in acid buffer solution. Linoleic acid (Hormel Institute product) was used without purification. Arginine linoleate was prepared by the method of Chang et al. (1960): a methanolic solution of arginine was mixed with an equimolar methanolic solution of linoleic acid and the methanol was evaporated under reduced pressure to obtain the white solid, arginine linoleate. The solid was dispersed in nitrogen saturated distilled water just before use.

Method

The reaction system was prepared by adding the arginine linoleate in nitrogen saturated water and the MbO_2 to phosphate buffer in an Erlenmeyer flask, which was kept at room temperature, 20 ± 0.5°C or placed in a water bath at 5 ± 0.5°C. Concentration of MbO_2 in the reaction system was 0.07 mM, corresponding to that in the muscle of tuna fish (Brown, 1962). Concentrations of arginine linoleate were adjusted to 0.44–2.2mM. The interaction of MbO_2 with linoleate should be examined in the acid region, since the pH of the fish and mammal muscles is known once to fall to around 6 or lower after their death and then rises during their autolysis. Therefore, the final pH of the reaction system was adjusted to 6.28 with phosphate buffer. Final phosphate concentration was 0.2M.

To follow the oxidation of MbO_2, the absorption spectrum in the region between 450 and 700 nm was recorded at appropriate intervals, using a portion of the reaction mixture, which was subsequently returned to the flask. For convenience, the concentration of unoxidized MbO_2 in the system was calculated from the difference between absorbances at 580 (max) and 563 (min) nm: the difference at the initiation of reaction was taken to be that corresponding to 100% MbO_2.

The oxidation of linoleate was followed by measuring the absorbance at 233 and 280 nm (Lundberg and Chipault, 1947) after Mb was removed by adding ethanol to the reaction mixture.

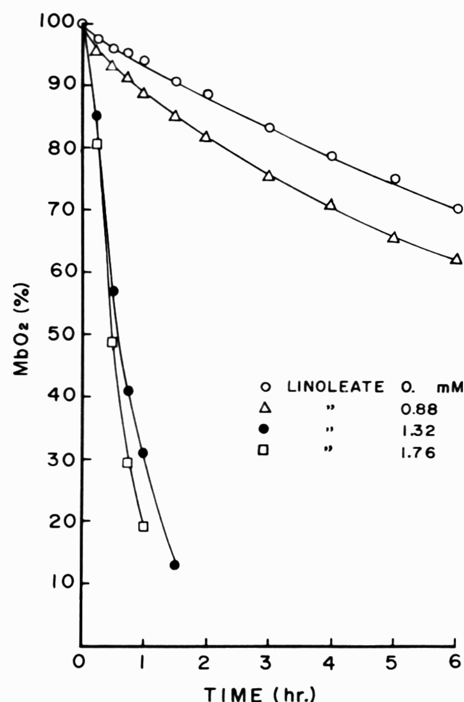

Fig. 1—Effect of linoleate on the oxidation rate of MbO_2. Each system contained sperm whale MbO_2 at concentration of 0.07 mM in 0.2M phosphate buffer, pH 6.28. The reactions were carried out at 20 ± 0.5°C.

RESULTS & DISCUSSION

Effect of concentration of arginine linoleate on the oxidation rate of MbO_2

The oxidation rate of MbO_2 was determined at 20°C in the presence of various concentrations of linoleate. The results obtained are shown in Figure 1. At pH 6.28 the linoleate at concentrations lower than 0.88 mM (Mb:linoleate molar ratio, 1:13) was almost ineffective. The slightly accelerated oxidation of MbO_2 observed in the early stage of the reaction may be due to a trace amount of oxidation products contained in the added linoleate. With addition of linoleate at concentrations higher than 1.32 mM (Mb:linoleate molar ratio, 1:19), the oxidation of MbO_2 was markedly accelerated. However, at pH 7.40 the linoleate at concentrations as high as 2.2 mM had little accelerative effect on the oxidation of MbO_2 in the early stage of the reaction.

Oxidation of linoleate

To determine the oxidation of linoleate, absorption spectra of the reaction system with 1.76 mM of added linoleate were recorded in the ultraviolet region after the system was treated with alcohol. The spectrum recorded immediately after the initiation of the reaction did not show any distinct absorption peak. After 15 min of the reaction, however, two dis-

tinct peaks due to oxidation products of linoleate appeared at about 233 and 280 nm in the spectrum (Tappel, 1955). The absorbances of these peaks increased with elapsed time, showing the heme-catalyzed oxidation of linoleate to occur. Figure 2 shows the relationship between the heme-catalyzed oxidation of linoleate and the rapid oxidation of MbO_2.

In the case of the reaction at concentrations of linoleate lower then 0.88 mM which did not accelerate the oxidation of MbO_2, no change was observed in the absorption spectrum in the ultraviolet region throughout the period of the reaction.

From these results, it is obvious that the accelerated oxidation of MbO_2 is caused by the action of some oxidation products of linoleate.

Changes in absorption spectrum of MbO_2

Figure 3 shows changes in the absorption spectrum of MbO_2 during the course of interaction with linoleate at pH 6.28, 20°C. Under these conditions, the oxidation of MbO_2 was markedly accelerated by linoleate as shown in Figure 1. The spectra shown in Figure 3 were corrected for turbidity in the sample solution by the method of Goldbloom and Brown (1966). The spectrum recorded immediately after the initiation of reaction showed two peaks at 580 and 543 nm characteristic of MbO_2. After 15 min, the intensity of the peaks at 580 and 543 nm decreased and absorbances at about 505 and 635 nm increased slightly. With elapsed time, the absorbances of the former two peaks further decreased with the increase in absorbances at about 505 and 635 nm which resulted in two distinct peaks, showing formation of MetMb. These spectrophotometric observations show that MbO_2 is rapidly converted into MetMb during the course of the interaction with linoleate and that the heme moiety of Mb is degradated in the reaction to some extent because the oxidation of MbO_2 was accompanied by slight reduction in absorbance of the Soret peak.

As a result of studies of the catalysis of unsaturated lipid oxidation by iron protoporphyrin derivatives, Brown et al. (1963) suggested that the ferrous form of the catalyst is inactive until its oxidation. On the basis of the proposed mechanism of the heme-catalyzed oxidation of unsaturated lipids, it is assumed that a small amount of MetMb which is converted from MbO_2 in the early stage of the reaction reacts with linoleate to initiate the chain reaction of linoleate oxidation; following this the oxidation products from the chain reaction accelerate the oxidation of MbO_2 to the ferric form. However, there seems to be no conclusive evidence showing that ferrous hemoprotein, especially MbO_2, is inactive as a catalyst. Further studies seem to be necessary in this regard.

Comparison of whale and tuna MbO_2 in regard to rate of oxidation

In the presence of 1.32 mM linoleate, the oxidation rate of whale MbO_2 was compared with that of tuna MbO_2 at pH 6.28, 5°C. The results obtained are shown in Figure 4. The whale MbO_2 oxidized slightly slower than that of tuna. Similarly, the linoleate was oxidized slowly in the system containing whale Mb compared with that containing tuna Mb. Brown et al. (1961) found that cysteine residue was contained in the globin molecule of tuna Mb but not in that of whale Mb, though it is not clear whether the structural difference between them has any reflection on the catalytic activity.

Effect of albumin on the oxidation rate of MbO_2

Wills (1965) has shown that the hemoglobin-catalyzed oxidation of linoleic acid is inhibited to some extent by addition of serum albumin. To examine the effect of protein on the interaction of MbO_2 with linoleate, serum albumin was added to the buffer solution of linoleate at pH 6.28 to give final concentrations of 0.1, 0.2 and 0.3% and the oxidation rate of MbO_2 was determined at 20°C. The results obtained are shown in Figure 5. Al-

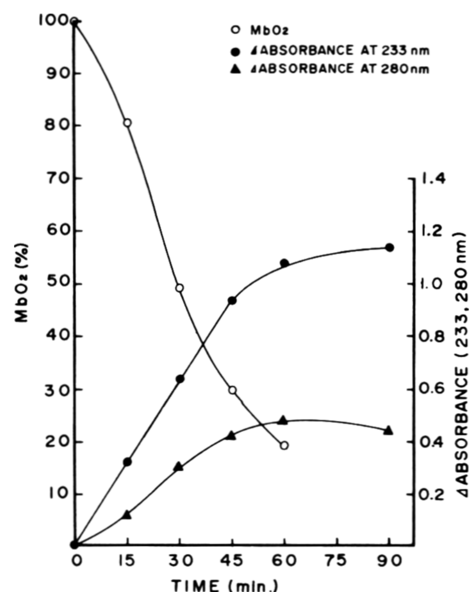

Fig. 2–Relationship between catalyzed oxidation of linoleate and of MbO_2. The system contained sperm whale MbO_2 (0.07 mM) and linoleate (1.76 mM) in 0.2M phosphate buffer, pH 6.28. The reaction was carred out at 20 ± 0.5°C.

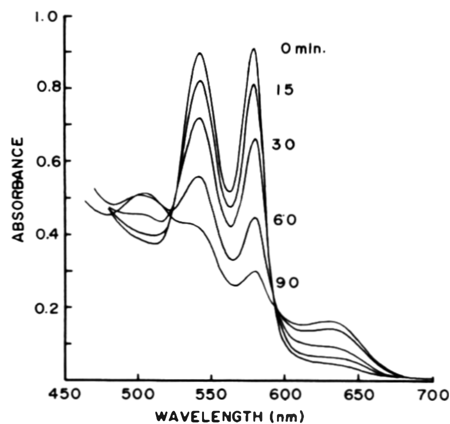

Fig. 3–Changes in absorption spectrum of MbO_2 in the course of interaction with linoleate. The system contained sperm whale MbO_2 (0.07 mM) and linoleate (1.32 mM) in 0.2M phosphate buffer, pH 6.28. The reaction was carried out at 20 ± 0.5°C. The numbers in the figure indicate time of the reaction in minutes.

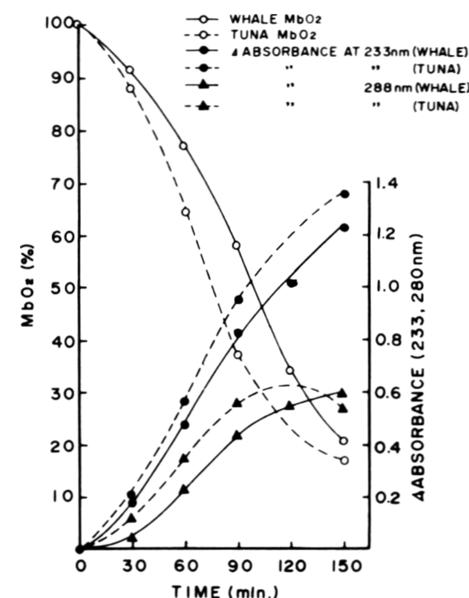

Fig. 4–Comparison of whale and tuna MbO_2 in regard to the rate of oxidation during interaction with linoleate. Each system contained MbO_2 (0.07 mM) and linoleate (1.32 mM) in 0.2M phosphate buffer, pH 6.28. The reactions were carried out at 5 ± 0.5°C.

bumin at a concentration of 0.1% had no effect on the accelerated oxidation of MbO_2 in its interaction with linoleate, while increased concentrations of added albumin had an inhibitory effect on the accelerated oxidation of MbO_2. Following addition of albumin at levels higher than 0.2%, no heme-catalyzed oxidation of linoleate was detected by spectrophotometric examination in the ultraviolet region.

In the course of this experiment, it was observed that the absorbances at 700 nm of the reaction systems containing albumin were usually lower than those of systems without added albumin. This indicates that the turbidity of the reaction system is reduced by the addition of the protein. The reduction of turbidity of the system seems to be ascribed to the combination of albumin with the linoleate dispersed in the buffer solution. The observed inhibitory effect of added albumin on the heme-catalyzed oxidation of linoleate may be associated with changes in micelle structure of linoleate in the system. However Wills (1965), on the basis of his results, has mentioned that the binding of linoleic acid to albumin does not significantly affect the rate of heme-catalyzed oxidation of linoleic acid.

Reduction of turbidity was also observed in the reaction system without added albumin at pH 7.40: when MbO_2 was added to the linoleate dispersed in buffer solution, the turbidity of the resulting mixture was markedly reduced, showing changes in the micelle structure of linoleate in this system. Under these conditions, neither accelerated oxidation of MbO_2 nor of linoleate were observed.

It is interesting to recall, in connection with the effect of a homogeneous system on the heme-catalyzed oxidation of linoleate, Lovern's suggestion (Lovern, 1946) that if the linoleic acid is present in a solution, instead of a suspension, the hematin-catalyzed oxidation of linoleic acid does not occur.

As is shown in the present study, the mode of interaction between MbO_2 and unsaturated lipids is very much influenced by the concentration of MbO_2, unsaturated lipids, and protein in the reaction system.

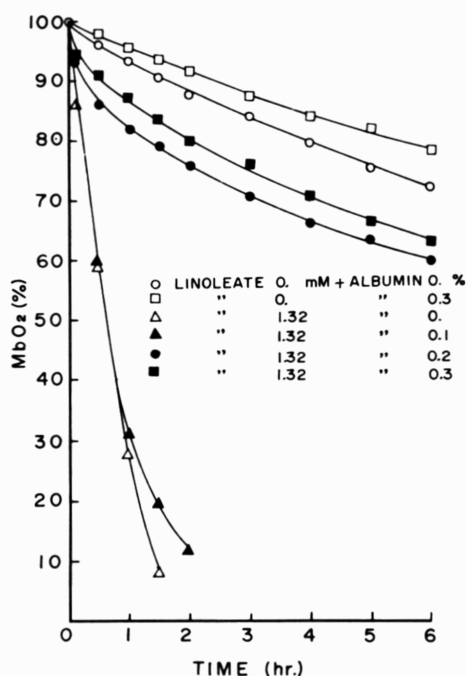

Fig. 5—Effect of serum albumin on the oxidation rate of MbO_2 during interaction with linoleate. Each system contained sperm whale MbO_2 (0.07 mM) in 0.2M phosphate buffer, pH 6.28. Linoleate and serum albumin were added to the system at concentrations shown in the figure. The reactions were carried out at 20 ± 0.5°C.

It is obvious that there are many problems to be solved before a conclusion is drawn that the likely interaction occurs in meat systems, although the occurrence of the oxidation of MbO_2 to MetMb by the above presented interaction seems to be probable in meat.

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EFFECT OF ALTERING ULTIMATE pH ON BOVINE MUSCLE TENDERNESS

INTRODUCTION

IN A REVIEW of the previous work relating pH to meat tenderness Bouton et al. (1971) concluded that it was difficult to determine the precise relationship between pH and meat properties. Often only a limited pH range had been studied or samples were unevenly distributed within the range and sometimes comparisons were made only between meats of high and low pH.

Bouton et al. (1957) reported a curvilinear relationship between taste panel scores and the ultimate pH of beef muscles (range 5.4–6.4) with a maximum toughness at pH 5.9. However, their data included only a few samples with pH values above 5.8. Miles and Lawrie (1970) reported that shear force values decreased linearly as the pH of rabbit muscles increased from 5.4 to 7.2.

To elucidate the relationship between ultimate pH and tenderness Bouton et al. (1971; 1972a, b) investigated changes in the ability of muscle proteins to retain water and in mechanical properties as the pH of sheep muscles was varied from 5.4 to 7.0. They considered that although ultimate pH influenced both myofibrillar strength and adhesion between muscle fibers, those measurements reflecting myofibrillar toughness were most affected. Water retention by muscles increased with increasing ultimate pH but the increase, in the range between pH 5.4–6.0, was small and unlikely to have much effect on tenderness. Differences in fiber contraction state were considered likely to be the predominant factor affecting tenderness in muscle at between pH 5.4 and 6.0.

In the present experiment beef muscles, in which the ultimate pH values were varied from 5.4 to 7.0 by pre-slaughter injections of adrenaline, were used to confirm the earlier results obtained for mutton (Bouton et al., 1972a, b). Additional information was gained by:

(a) assessing the effects of pH on taste panel scores for tenderness and juiciness;
(b) determining how the extent of fiber shortening (thermal contraction) during cooking influenced the relationship between ultimate pH and between-fiber adhesion measurements;
(c) determining whether the large variability in shear force values in the pH range 5.4–6.0 could be due to pre-rigor shortening; and
(d) investigating whether the relationships between pH and mechanical properties were influenced by reducing myofibrillar strength either by preventing muscles from shortening or by aging at 0–1°C for 3 wk.

MATERIALS & METHODS

Animals and pre-slaughter treatments

A group of 20 Hereford steers aged about 2 yr and with a mean liveweight of 408 ± 9 kg were used. They were penned individually and fed alfalfa hay plus commercial cattle cubes for periods of 3-10 wk until slaughter.

Subcutaneous injections of 1:200 (w:v) adrenaline chloride in 0.3% aqueous ascorbic acid solution (or equivalent concentrations of an aqueous solution of adrenaline bitartrate) were administered to 17 of the 20 animals in single or multiple doses given at selected times pre-slaughter. The total amount administered over the entire pre-slaughter period ranged from 7.7–82.5 mg/100 kg liveweight. By systematically varying the dose, number of injections and times of injections before slaughter it was possible to obtain pH values close (± 0.1 in 15 of 17 animals) to pre-selected values. To obtain low ultimate pH values care was taken to reduce pre-slaughter stress to a minimum when two of the three untreated animals were killed. The third animal was chased about in a yard prior to slaughter to produce an intermediate ultimate pH value. Using all of these techniques it was possible to achieve an even distribution of pH values over the range 5.4–7.0 (see Figs. 1 and 2).

Post-slaughter treatments

As soon as possible after slaughter, and immediately after splitting, one side of each carcass was hung from the Achilles tendon (also described later as normal hanging) while the other side was hung from the aitch bone using the method of Hostetler et al. (1970; 1972). It has been shown, (Hostetler et al., 1972; Bouton et al., 1973) that aitch bone hanging of carcasses causes stretching (or prevents pre-rigor shortening) in all of the muscles selected for use in the present experiments. For convenience muscles from Achilles tendon hung sides are called 'normal' muscles and muscles from aitch bone hung sides are referred to in this paper as stretched muscles. It is realized that, strictly, only muscles with sarcomere lengths greater

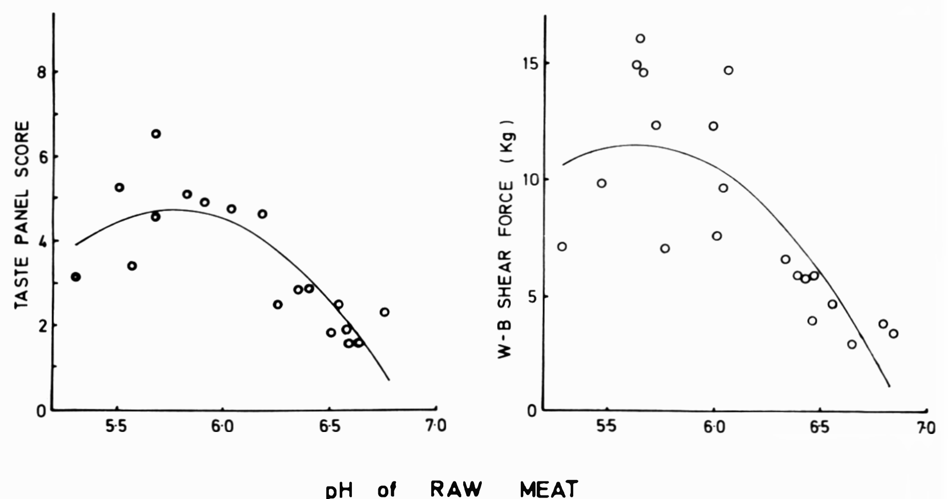

Fig. 1—Warner-Bratzler (WB) shear force values (kg) and taste panel tenderness scores for unaged, normal longissimus dorsi muscles showing the effect of ultimate pH. The lines shown are the lines of best fit.

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than the rest length (about 2.3 μm according to Voyle, 1969) can be termed stretched. However, when sides are hung from the aitch bone, it has been demonstrated (Hostetler et al., 1972; Bouton et al., 1973) that, with the exception of the longissimus dorsi (LD) muscle, all the muscles used in the experiments reported here were stretched. The 'stretched' LD muscles although they had sarcomere lengths of less than 2.3 μm had significantly longer sarcomeres than those obtained for the LD muscles from normally hung sides.

Selection and treatment of muscles

Table 1 summarizes the muscles used, the treatments applied and the objective or subjective assessments carried out. In all cases muscles from both the aitch bone and normally hung sides of the same carcass were used. The selected muscles were all removed after the sides had been hung at 0–1°C for 2 days post-slaughter. The LD (from the lumbar region, posterior from the last rib) and semimembranosus (SM) muscles were each cut into two parts, one of which was cooked that day (2 days post-slaughter) while the other part was vacuum sealed in cryovac and aged at 0–1°C for a further 21 days before cooking. There is little further increase in tenderness after an aging period of 21 days at 0–1°C (Bouton and Harris, 1972c). Samples were weighed before and after aging to determine weep or moisture losses. The two within-muscle locations were assigned alternately so that each was equally represented in the 2-day and 23-day groups. The muscles cooked 2 days after slaughtering are, for convenience, referred to as 'unaged' while those cooked 21 days later are termed 'aged' samples.

The effects of pH on subjectively determined tenderness were assessed using the LD (thoracic region between the 5th and last rib), adductor (A) and rectus femoris (RF) muscles. Objective measurements of between-fiber adhesion were made on both the unaged and aged SM muscles and on the unaged vastus lateralis (VL), biceps femoris (BF) and gluteus medius (GM) muscles using the method of Bouton and Harris (1972a, b, c). Moisture retention measurements were made on raw samples of both unaged and aged LD and SM muscles and also on both unaged and aged LD muscle samples cooked at 60, 70 and 90°C.

Table 1—Treatment carried out on selected normal and stretched muscles from beef sides which were hung either from the Achilles tendon or from the aitch bone

Muscle	Cooking temp °C	Time after death the muscle was used (days)		Measurement made					
		2	23[a]	T.P.[b]	W-B[c]	Ch[d]	F[e]	A[f]	WHC[g]
Semi-membranosus SM	60	+	+	–	–	–	–	+	+
	80	+	+	–	+	+	+	–	+
Longissimus dorsi LD	60	+	+	–	–	–	–	–	+
	70	+	+	–	–	–	–	–	+
	80	+	+	–	+	+	+	–	–
	90	+	+	–	–	–	–	–	+
	Oven	+	–	+	–	–	–	–	–
Vastus lateralis VL	60	+	–	–	–	–	–	+	–
	80	+	–	–	–	–	–	+	–
Biceps femoris BF	60	+	–	–	–	–	–	+	–
	80	+	–	–	–	–	–	+	–
Gluteus medius GM	60	+	–	–	–	–	–	+	–
	80	+	–	–	–	–	–	+	–
Rectus femoris RF	Oven	+	–	+	–	–	–	–	–
Adductor A	Oven	+	–	+	–	–	–	–	–

[a] Samples weighed before and after aging to determine weep losses
[b] T.P. = Subjective assessment
[c] W-B = Warner-Bratzler shear force
[d] Ch = Instron compression
[e] F = Fiber tensile strength
[f] A = Adhesion
[g] WHC = Moisture retention properties
+ means treatment or measurement carried out
– means no treatment or measurement made.

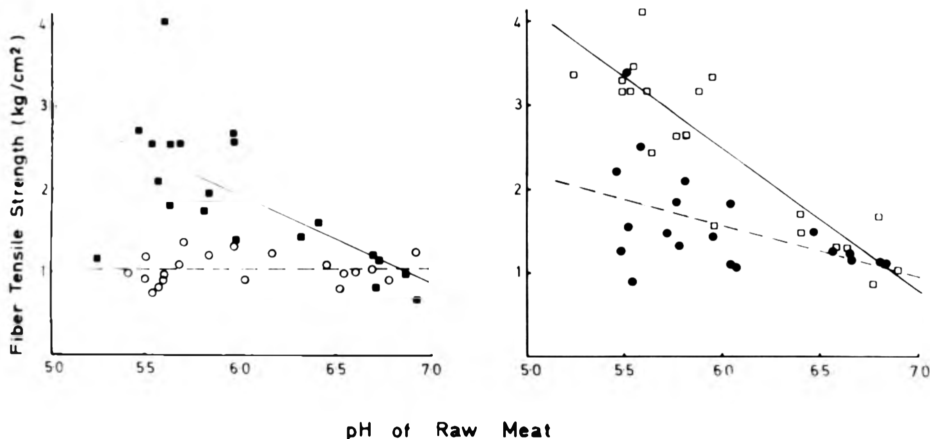

Fig. 2—Fiber tensile strength (kg/cm²) for aged (-o--o-) and unaged (—■——■—) normal SM muscles; aged (-●--●-) and unaged (—□——□—) stretched SM muscles, cooked at 80°C for 1½ hr, as a function of ultimate pH. The lines shown are the lines of best fit.

Cooking methods

Muscle samples used for objective assessments were cooked, after weighing, in polyethylene bags which were tightly clipped round the samples and totally immersed in thermostatically controlled water baths at the selected cooking temperature for the prescribed times. Since Giles (1969) has shown that the decrease in the length of meat fibers was small at a cooking temperature of 60°C and comparatively large at 80°C, the samples for mechanical measurements, cut into rectangular blocks weighing 180-200g, were cooked at either 60 or 80°C for 90 min. Samples of the VL, BF and GM muscles were cooked at both temperatures so that adhesion measurements could be made. Adhesion measurements on the SM were made only on samples cooked at 60°C. LD samples for moisture retention measurements weighed 100-120g and were cooked for 1 hr at 60, 70 or 90°C. Samples of this size cooked under these conditions have moisture retention properties identical to the larger samples which were necessary for the mechanical measurements.

After cooking, the bags and contents were removed from the baths and cooled in cold running water. If samples were required for subsequent water-holding measurements, the cooking juices were retained and their solids content determined by evaporating a weighed aliquot to dryness at 105°C for 18 hr. Cooked meat samples were carefully dried with paper tissues and reweighed to determine cooking losses after which they were stored overnight at 0–1°C before apportioning sub-samples for the various objective and water-holding measurements.

Samples for the taste panel were cut into steaks 18–20 mm thick and cooked in an air circulating oven at 175°C to an internal temperature of 77 ± 2°C. This oven-cooking method was used because the taste panel members were more familiar with samples prepared in this way than with samples cooked in polyethylene bags.

Mechanical measurements

Shear force measurements were made with a modified Warner-Bratzler shear device to assess myofibrillar strength (Bouton and Harris, 1972b). An Instron Universal Testing Machine (type TM-M) was used for compression and tensile tests. The compression values reported were those for "chewiness," defined by Bouton et al. (1971). Tensile measurements on samples held with fibers at right angles to the strain were considered a measure of the strength of the connective tissue holding fibers together (Bouton and Harris, 1972a) and are referred to in the text as adhesion measurements. Tensile measurements on samples with the fibers parallel to the applied strain were regarded primarily as a sensitive measure of changes in fiber tensile strength (Bouton and Harris, 1972b). However it is appreciated that, since each fiber is surrounded by a connective tissue sheath, there is a contribution from the connective tissue. This contribution is likely to vary with the number of fibers per unit cross sectional area. Details of all these tensile measurements have been described by Bouton and Harris (1972a, b, c).

Subjective assessment

Cooked samples were cut into 12–15 mm cubes so that individual tasters received samples which could be bitten either along or across the muscle fibers.

Twelve panel members were selected from the laboratory staff on their ability to detect differences in meat toughness. At each panel sitting duplicate pieces of meat, from each of four samples, were evaluated. Panelists tasted the samples in predetermined random orders. The effects of hanging treatments were assessed by tasting samples from any two muscles from normal and aitch bone hung sides of each carcass at one panel sitting. Tenderness was recorded on a 9-point, structured scale: very tough (8), tough (6), slightly tough (4), tender (2) and very tender (0). Juiciness was recorded on a similar 9 point scale, structured very dry (8), dry (6), slightly dry (4), juicy (2) and very juicy (0).

Measurement of moisture retention properties

The method used for the measurement of moisture retention properties was a development of the centrifugal technique of Akroyd as described by Bouton et al. (1971). The parameters measured were water-holding capacity (WHC), water retained after cooking (WRC) and expressed juice (EJ) and these have all been defined by Bouton et al. (1972a). Solids content of the juice lost in cooking and in the expressed juices were also determined.

Measurement of pH

The pH values, of both raw and cooked meat, were measured directly using a Phillips Digital pH meter PW9408 with a Phillips probe-type combined electrode (C64/1). The pH values of the raw meat were measured, at room temperature, 22°C, just prior to cooking. Ten pH readings were made on each sample of each muscle and the mean used as the pH of the sample. The pH values measured 2 days after slaughter were regarded as ultimate pH values, i.e., the lowest pH values reached postmortem. A similar number of readings were taken on cooked samples after they had cooled to room temperature.

Statistical treatment of results

Animals were selected at random for adrenaline treatments. Regression analysis was carried out on the data obtained for each particular muscle from sides of animals subjected to the same hanging treatment to show the dependence of the selected measurements on pH, to estimate the regression coefficients and to establish the lines of best fit. Regression coefficients were tested for homogeneity and analysis of covariance used to test the significance of differences between adjusted treatment effects.

RESULTS & DISCUSSION

THE RESULTS obtained are considered in terms of the effects of pH on tenderness as assessed by subjective and objective measurements and also of changes in adhesion values and moisture retention properties.

Effects of pH on subjective tenderness

The tenderness scores recorded by the taste panel are linearly and significantly related to pH for both the normal and stretched samples from all three muscles (LD, A and RF) studied and tenderness increases with increasing ultimate pH. The tenderness of the normal LD muscles is significantly ($P < 0.001$) and linearly related to pH but the regression is significantly ($P < 0.01$) improved by fitting a quadratic component. This result indicates a maximum toughness at pH's of about 5.8–6.0 (see Fig. 1). Correlation coefficients between pH and tenderness scores of 0.83, 0.81 and 0.90 are found for the normal LD, A and RF respectively and of 0.59, 0.79 and 0.85 for the stretched LD, A and RF muscles. The overall effect of stretching is to significantly ($P < 0.05$) improve tenderness for all three muscles at low pH values although at high pH values (near 7.0) stretching has no effect.

Panel scores for juiciness show no significant relationship with pH. This is an interesting result since, even with the large decrease in cooking loss which occurs as pH increases, there is apparently no significant difference in the amount of moisture which is detected as it is squeezed out in the chewing process.

Effect of pH on objective measurements

The measurements used were intended to assess the effects of pH on the myofibrillar and connective tissue components of toughness. Bouton and Harris (1972a, b, c) concluded that shear force values are a good measure of myofibrillar strength, little influenced by variations in connective tissue strength. They considered that connective tissue strength influences Instron compression values more than shear force values and that tensile measurements of fiber strength are influenced by both myofibrillar and connective tissue components. SM and LD muscles, both normal and stretched, gave similar results so that only detailed data for the LD muscles are given. Table 2 shows the dependence on pH of the compression, fiber tensile strength and shear force measurements of normal and stretched, aged and unaged LD muscle samples. The compression results are significantly ($P < 0.001$) and linearly related to pH. The shear force measurements are also significantly and linearly related to pH but in most cases the regressions are significantly improved by including a quadratic component, except in the case of the aged, normal LD samples. The shear force results for the normal, unaged LD muscles are shown, as a function of pH, in Figure 1, for comparison with the taste panel results on the same muscle.

Table 2–Regression analyses for compression, fiber tensile and W-B shear measurements on the LD muscle, cooked at 80°C for 1½ hr, to show effects of pH

Source of variance			dF	Mean square: Compression	Fiber tensile	W-B
LD	pH	Linear	1	2.134***	1.293**	3536.51***
Normal		Quad	1	0.131	0.693*	696.20*
		Error	17	0.058	0.085	154.31
LD	pH	Linear	1	1.532***	1.794**	184.26*
Stretched		Quad	1	0.069	0.593*	155.53*
		Error	17	0.025	0.119	24.4
LD	pH	Linear	1	2.220***	0.0004	1192.37**
Normal		Quad	1	0.019	0.0001	299.01
Aged		Error	17	0.030	0.015	75.68
LD	pH	Linear	1	1.286***	0.0003	28.57
Stretched		Quad	1	0.014	0.011	25.24**
Aged		Error	17	0.028	0.009	2.94

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Compression and shear force values obtained for both the SM and LD muscles show that the pH dependence of these measurements are different for the two hanging treatments and for the unaged and aged muscle samples. The slopes of the regression lines obtained for the aged and stretched samples are significantly ($P < 0.01$) decreased relative to the regression lines obtained for the normal, unaged samples. The regression lines for all four treatments converge at high pH. At high pH, therefore, no reduction in shear force or compression values is produced either by stretching or by aging.

Fiber tensile strength measurements for the unaged LD and SM muscles are highly dependent on pH (see Fig. 2 for SM results and Table 1 for LD results) but when fiber tensile strength is reduced as a result of aging there is no significant relationship between pH and fiber tensile strength. At high pH values there is no difference between the fiber tensile strength values obtained for either muscle for any of the four treatments. At low pH values the stretched SM and LD muscles have greater fiber tensile strength values than those obtained for the 'normal' muscles. However only for the SM muscles is this effect due to stretching significant ($P < 0.001$). This effective increase in tensile strength due to stretching is believed to be a function of fiber packing density which is greater in stretched SM than in stretched LD or normal SM and LD muscles (Bouton and Harris, 1972c).

Table 3—Regression analyses and analyses of variance for the WHC measurements on raw LD and SM muscles to show the effects of pH, hanging (or stretching) treatment and aging

Source of variance			dF	Mean square WHC – LD	Mean square WHC - SM
Normal, unaged	pH	Regression	1	1.6782***	1.4549***
		Error	19	0.0206	0.0216
Normal and aged	pH	Regression	1	1.0227***	2.1322***
		Error	19	0.0082	0.0282
Stretched, unaged	pH	Regression	1	1.4640***	1.0244***
		Error	19	0.0280	0.0189
Stretched and aged	pH	Regression	1	0.7843***	1.3066***
		Error	19	0.0213	0.0136
Animal (A)			19	0.0417**	0.0448**
Hanging treatment (S)			1	0.1163***	0.0757**
A x S			19	0.0074	0.0063
Aging (AG)			1	0.2431***	0.0186
AG x S			1	0.0228	0.0245
Error			38	0.0122	0.0121

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 4—Regression analyses and analyses of variance on WRC and EJ measurements on cooked LD muscles to show effects of pH, hanging treatment, and cooking temperature on the WRC results

Source of variance			dF	Mean square WRC	Mean square EJ
LD normal 60°C	pH	Linear	1	0.9033***	0.0695*
		Error	18	0.0144	0.0127
LD normal 90°C	pH	Linear	1	0.7142***	0.1042*
		Quadratic	1	0.0591*	–
		Error	17	0.0121	0.0166
LD stretched 60°C	pH	Linear	1	0.6822***	0.0273
		Error	18	0.0166	0.0165
LD stretched 90°C	pH	Linear	1	1.0425***	0.0631
		Quadratic	1	0.0845*	–
		Error	17	0.0103	0.0152
Animal (A)			19	0.0724***	
Hanging treatment (S)			1	0.1480***	
A x S			19	0.0081	
Aging (AG)			1	0.6657***	
Temperature (TE)			2	20.2640***	
S x AG			1	0.0528*	
S x TE			2	0.0012	
AG x TE			2	0.0320	
S x AG x TE			2	0.0034	
Error			190	0.0081	

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Effect of pH on adhesion values

Tensile measurements of adhesion between the muscle fibers were considered a measure of connective tissue strength by Bouton and Harris (1972a, b, c) who also showed that myofibrillar contraction state and length changes during cooking affected adhesion values. Adhesion values were greater in older than in younger animals (although shear values were similar) and adhesion values were unaffected by aging treatments which decreased shear values markedly (Bouton and Harris, 1972a, c).

Results obtained in the present experiment for SM muscles cooked at 60°C (where thermal contraction of the muscle fibers was minimal) also show that no significant change is produced by aging and that stretching significantly ($P < 0.001$) decreases adhesion values. The adhesion values obtained for the SM, VL, BF and GM muscles cooked at 60°C show no significant effect due to pH. At 80°C, when there is large thermal fiber contraction (Giles, 1969), adhesion values obtained for the VL and BF muscles decrease significantly ($P < 0.01$) with increased pH. The GM muscle shows a trend towards decreased adhesion values at high pH but the relationship only approaches significance at $P < 0.05$. These results suggest that thermal contraction could be a factor influencing pH-dependence of the adhesion between muscle fibers.

Since adhesion values for the VL, BF, GM and SM muscles cooked at 60°C are not significantly affected by pH, analysis of variance could be used to test for muscle and treatment differences. Mean values for normal and stretched muscles are significantly different, 0.59 and 0.48 kg/cm^2 respectively (standard error ± 0.02). The mean values for the BF, VL, SM and GM muscles cooked at 60°C are 0.87, 0.50, 0.45 and 0.31 kg/cm^2 respectively (standard error ± 0.03).

Effect of pH on moisture retention properties of raw meat

Table 3 shows the regression analyses for the water-holding capacity (WHC) measurements on the raw SM and LD muscles. These WHC results are significantly ($P < 0.001$) dependent on pH. Stretching significantly increases WHC as does aging. This increase in WHC with aging is significant only in the case of the LD muscle. An increase in WHC due to stretching was previously found in sheep muscle (Bouton et al., 1972a). Weep losses from the normal and stretched LD and SM muscles during aging decrease ($P < 0.05$) with increasing ultimate pH. Stretched muscles lose significantly ($P < 0.001$) less weep than the normal muscles (mean values 1.85 and 3.81% respectively with a standard error of 0.04). Howard and Lawrie (1956) showed that the psoas major (sarcomere length about 3.3 μm), a stretched muscle, lost less in weep than the LD (sarcomere length about 1.7 μm), a mildly contracted muscle.

Effect of pH on moisture retention properties of cooked meat

Table 4 shows regression analyses of results for the WRC and expressible juice (EJ) for LD muscles cooked at 60 and 90°C. The WRC results obtained for samples cooked at 70°C have a similar significant ($P < 0.001$) linear relationship with pH to those obtained for the 60 and 90°C samples. The results for all three cooking temperatures are included in the analysis of co-variance on the WRC values to show the effects of stretching, aging and cooking temperature. A quadratic component improves the WRC/pH relationship for both the unaged and aged muscles. Stretching improves the ability of the meat to retain its moisture during cooking while aging has the reverse effect. Stretching tends to increase and aging to decrease EJ values.

The effects of cooking temperature on WRC and EJ are similar to those reported previously (Bouton et al., 1972a) for mutton. WRC and EJ both decrease with increasing temperature. WRC values increase markedly with increasing pH but the increase in EJ values is small and not always significantly related to pH. Taste panel scores for juiciness are not significantly related to pH. It would appear that juiciness is more closely related to EJ values, which changes little with increasing pH, than to WRC values when there is wide variation in pH values.

CONCLUSIONS

THE RESULTS presented in this paper show that increasing ultimate pH generally increases tenderness whether tenderness is assessed by objective or by subjective methods. This increase in tenderness is concomitant with increasing water-holding capacity which is also highly correlated with pH. Large variations in shear force values are found for muscles such as the SM and LD (see Fig. 1) with pH values less than 6. This agrees with the mutton results of Bouton et al. (1972b). This variation or scatter in shear force values is much less marked for muscles which have been stretched or aged to reduce myofibrillar toughness. Myofibrillar contraction state and hence toughness can be affected by conditioning temperature, the temperature at which the muscle goes into rigor (which is also related to the rate of onset of rigor) and by the degree of slack allowed the muscle by its skeletal attachment (Newbold and Harris, 1972). With so many conflicting factors liable to affect tenderness in normal animals, with ultimate pH values of less than 6.0, it would be surprising if there were always a direct relationship between pH and tenderness.

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EFFECT OF FEEDING A PROTECTED SAFFLOWER OIL SUPPLEMENT ON THE COMPOSITION AND PROPERTIES OF THE SARCOPLASMIC RETICULUM AND ON POSTMORTEM CHANGES IN BOVINE SKELETAL MUSCLE

INTRODUCTION

RECENTLY it has been shown (Cook et al., 1970, 1972; Scott et al., 1970, 1971) that the fatty acid composition of the milk and body fats of ruminant animals is markedly altered when the diet is supplemented with a polyunsaturated oil embedded in a matrix of formaldehyde-treated casein to protect it from hydrogenation in the rumen. Such a supplement could also be expected to alter the fatty acid composition of the membrane phospholipids. It has been shown with rats that changes induced in the fatty acid composition of the phospholipids of the sarcoplasmic reticulum (SR) by certain treatments are accompanied by changes in the activity of the sarcoplasmic calcium pump (Seiler and Hasselbach, 1971; Seiler et al., 1970). It was of some interest, therefore, to determine whether supplementing the diet of ruminants with a protected polyunsaturated oil for a period immediately before slaughter affected the physical or chemical changes that take place in muscle during the period between slaughter and the development of rigor mortis.

Ox sternomandibularis muscle, the muscle used in the present work, when excised and stored at 1°C soon after slaughter shortens during the development of rigor mortis by up to about 50% of its freshly excised length (Locker and Hagyard, 1963). Similarly, when frozen soon after slaughter and then thawed this muscle may shorten by more than 70% of its freshly excised length (Marsh and Leet, 1966; Scopes and Newbold, 1968). These phenomena, known as cold shortening and thaw shortening respectively, are thought to result from the release of ca^{2+} from the sarcoplasmic reticulum (Bendall, 1960; Cassens and Newbold, 1967a). Stimulation of postmortem glycolysis can also be attributed to the release of Ca^{2+} from the SR (Scopes and Newbold, 1968).

The present paper compares (1) the fatty acid composition of the phospholipids of the SR, (2) the Ca^{2+}-accumulating and ATPase activities of the SR; and (3) postmortem physical and chemical changes believed to depend on the release of Ca^{2+} from the SR, in muscles from cattle fed a supplement of protected safflower oil and from unsupplemented cattle.

MATERIALS & METHODS

Animals and rations

Sternomandibularis muscles from four weaner steers 9–10 months old and weighing about 200 kg and four 21-month-old steers weighing about 250 kg were used. Two animals from each age group (the control animals) were fed a diet made up of 60% grass hay and 40% sorghum grain. The other two steers from each group (the treated animals) were fed the same diet as the controls except that it was supplemented with 22.5% by weight of protected safflower oil made up of two parts by weight of safflower oil and one part by weight of casein and prepared as described by Scott et al. (1971). After 6 wk on these diets the animals were slaughtered at the local abattoir and the sternomandibularis muscles removed. All postmortem treatments and SR preparations were commenced within 2 hr after slaughter.

Measurement of cold shortening and thaw shortening

Strips about 0.5 cm^2 in cross section and 5–6 cm long were excised from the muscles longitudinally to the direction of the fibers. Cold shortening at 1°C was measured in 2–3 strips of each muscle as described by Cassens and Newbold (1967a) and thaw shortening in 2–3 strips frozen in air at −22°C and thawed at room temperature (23°C) 24 hr later as described by Scopes and Newbold (1968).

Chemical determinations

Pieces of muscle stored under N_2 at room temperature were sampled at 2, 4, 6, 8, 10 and 26 hr postmortem for pH determination and chemical analysis. Measurements of pH were also made at 26 hr and 72–96 hr on samples of muscle placed at 1°C 2 hr postmortem. Methods of measuring pH, of preparing perchloric acid extracts and of analyzing these extracts for glycolytic products and intermediates and adenine nucleotides were as previously described (Newbold and Scopes, 1967, 1971).

Preparation and properties of SR

The muscle fraction centrifuging down between 8000 and 28000 × G was prepared by the method of Martonosi et al. (1968) except that it was washed only once with 0.6M KCl-buffer and 1 mM dithiothreitol was present throughout the preparation. The final suspending medium contained 5 mM dithiothreitol. Protein was determined by the method of Lowry et al. (1951).

The uptake and release of Ca^{2+} were measured at 23°C and pH 6.3 in a medium containing 100 mM KCl, 20 mM histidine, 1 mM ATP, 1mM $MgCl_2$, 2.5mM phosphoenolpyruvate, 8 units of pyruvate kinase/ml, 20 μM $^{45}CaCl_2$ and 0.06–0.10 mg SR protein/ml by the Millipore filtration method of Martonosi and Feretos (1964). Filters with an average pore diameter of 0.22 μm and siliconized glassware (Petroff et al., 1964) were used. The reaction was started by the addition of SR.

Basal and total ATPase activities were measured at 37°C and pH 7.2 in 50 mM Tris buffer containing 5 mM ATP, 5 mM $MgCl_2$ and 5 mM sodium azide. When measuring basal ATPase activity the medium also contained 1 mM ethylene glycol bis-(β-aminoethyl ether)N,N'-tetraacetic acid (EGTA). Measurements of ATPase activity were also made in the presence of EGTA and absence of azide. The concentration of SR used in determining basal ATPase activity was 0.14–0.25 mg/ml and in determining total ATPase activity 0.06–0.10 mg/ml. The reaction was started by the addition of ATP and stopped 10 min later by the addition of one volume of ice-cold 10% trichloracetic acid. Inorganic orthophosphate production was

Table 1—Fatty acid composition of the phospholipids of SR preparations from muscles of control and treated animals[a]

Fatty acid	Animals: Control (3)	Animals: Treated (4)
14:0	0.9 ± 0.2	1.3 ± 0.2
16:0	14.5 ± 1.4	14.4 ± 1.0
16:1	3.0 ± 0.6	2.2 ± 0.4
18:0	15.1 ± 2.0	14.5 ± 1.3
18:1	22.6 ± 3.3	12.1 ± 1.1*
18:2	13.3 ± 0.3	34.9 ± 1.2*
18:3	2.7 ± 0.1	1.7 ± 0.5
20:4	9.6 ± 0.9	8.3 ± 0.9
22:6	3.7 ± 0.5	1.6 ± 0.7
Polyenoic / Saturated + monoenoic	0.53 ± 0.05	1.05 ± 0.04*

[a] Values are mean values ± S.E. and are expressed as percentages of the total fatty acid content. Numbers of animals are given in parenthesis.
* Significantly different ($P < 0.05$) from values for control animals

measured by the method of Fiske and Subbarow (1925).

Isolation and analysis of lipids

Lipids were extracted from the SR preparations by the method of Folch et al. (1957). Methods used in separating the phospholipid from the other lipid fractions and in determining its fatty acid composition were as described by Scott et al. (1967).

RESULTS & DISCUSSION

Fatty acid composition of the SR phospholipids

Phospholipids account for more than 80% of the total lipid content of the sarcoplasmic reticulum, the remainder being made up by triglycerides, cholesterol and cholesterol esters (Fiehn et al., 1971; Meissner and Fleischer, 1971; Waku et al., 1971).

Because of the loss of one phospholipid extract the number of age and treatment comparisons that could be made was limited. In Table 1 the fatty acid composition of the SR phospholipids is given as the mean for all animals in the same treatment group regardless of age. Supplementing the diet with protected safflower oil significantly affected ($P < 0.05$) only the linoleic (18:2) and oleic acid (18:1) contents of the SR phospholipids. The linoleic acid content increased 2.6-fold, the oleic acid content decreased substantially and the ratio of the polyenoic to saturated + monoenoic fatty acids doubled. These results are in keeping with those of Cook et al. (1972) who found substantial increases in the proportions of linoleic acid and decreases in the proportions of oleic acid in the plasma, liver and skeletal muscle phospholipids of steers fed a similar protected-safflower oil supplement.

Properties of the SR

The rate of Ca^{2+} uptake by the SR, the total amount taken up at 23°C, the rate of Ca^{2+} release when the reaction mixture was placed at 0°C after 5 min at 23°C, and the total amount of Ca^{2+} released in 7 min at 0°C were not affected by the age or diet of the animal (Fig. 1). Further, when the reaction mixture was subsequently returned from 0°C to 23°C, Ca^{2+} was again taken up rapidly by all preparations and after about 2 min the total uptake was the same as in reaction mixtures that had not been cooled (Fig. 1).

The rate of uptake of Ca^{2+} based on the 1 min value was 31.5 ± 6.3 nmoles/mg protein/min and the mean total uptake about 40 nmoles/mg protein. (The total uptake did not exceed 75% of the added Ca^{2+} except in one preparation from the control animals and one from the treated where it was about 90%).

Neither the basal nor extra ATPase activity was affected by diet. The basal ATPase activity amounted to 0.37 ± 0.02 and the extra ATPase activity to 0.49 ± 0.02 μmoles/mg protein/min. In the presence of EGTA, azide had the same effect on the ATPase activity of SR preparations from both control and treated animals, the basal ATPase activity as measured here (i.e., the activity in the presence of azide) being about 80% of the activity in the absence of azide.

These findings indicate that substantial changes can be made in the fatty acid composition of the SR lipids without affecting the Ca^{2+} uptake or ATPase activities of the SR.

Postmortem shortening

Neither age nor diet had a significant effect on the rate or extent of cold shortening or thaw shortening. Cold shortening amounted to 36.6 ± 2.4% and thaw shortening to 76.9 ± 0.5% of the freshly excised length.

Postmortem metabolism

Initial pH values of the sternomandibularis muscle (i.e., values 2.0 hr after slaughter) and pH values after storage of the muscle at 1°C for 26 and 72–96 hr were not significantly affected by age or diet, mean values ± S.E. for all muscles being 6.91 ± 0.02, 6.30 ± 0.04 and 5.89 ± 0.04, respectively. The last of these values, the ultimate pH at 1°C, was higher than the ultimate pH at 23°C (i.e., the pH after 26 hr under N_2 at 23°C) for both age groups. Muscles from both control and treated animals within each age group reached the same ultimate pH at 23°C but the value for the four younger animals (5.75 ± 0.02) was significantly higher than that for the four older animals (5.65 ± 0.01). The effect of temperature on the ultimate pH is in keeping with the results of Cassens and Newbold (1967b).

The rate of pH fall at 23°C was unaffected by age or diet. Similarly, the total acid soluble phosphorus content of the muscle was the same for all control and treated animals as also were the rates and patterns of change in the concentrations of glucose-1-phosphate + glucose-6-phosphate + fructose-6-phosphate, fructose-1,6-diphosphate + 1/2 triose phosphate, 2- and 3-phosphoglycerates + phosphoenolpyruvate, pyruvate, lactate, glucose, α-glycerophosphate, ATP, ADP, AMP and NAD.

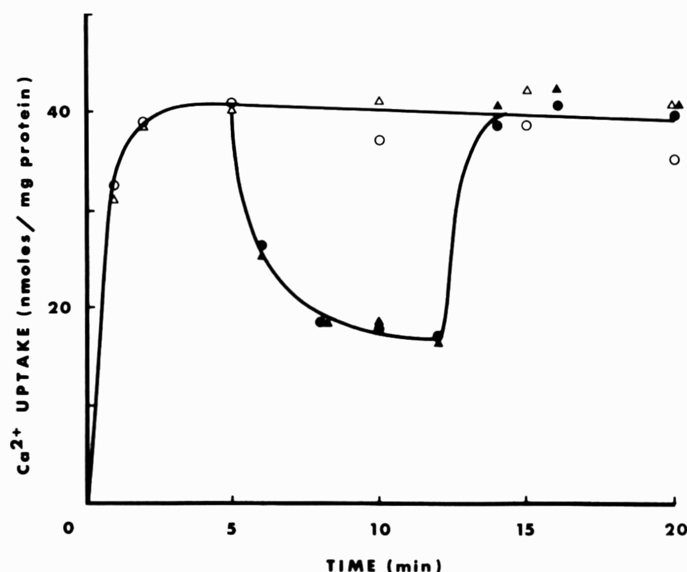

Fig. 1—Ca^{2+} uptake at 23°C and release at 0°C. Open symbols: uptake at 23°C. Closed symbols: release when the reaction medium was placed in ice for 7 min after 5 min at 23°C, and subsequent uptake when returned to 23°C. Circles and triangles are means for the preparations from the four control and four treated animals respectively.

CONCLUSION

IT IS APPARENT that none of the properties of the SR or pre-rigor changes in muscle examined in the present work was influenced by substantial changes in the fatty acid composition of the SR lipids. Thus there is no reason to suppose that meat from ruminants fed a protected safflower oil supplement should be handled differently pre-rigor to meat from unsupplemented animals.

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MYOFIBRIL FRAGMENTATION IN BOVINE LONGISSIMUS DORSI AS AN INDEX OF TENDERNESS

INTRODUCTION

THE DEGREE of myofibril fragmentation caused by mechanical treatments has been associated with chemical and physical changes during rigor mortis development and postmortem aging of meat (Takahashi et al., 1967, 1970; Davey and Gilbert, 1969; Fukazawa et al., 1969; Sayre, 1970).

In electron microscopic studies of the fine structure in myofibrils, Fukazawa et al. (1969) suggested that the increased tendency for fragmentation postmortem might be due to the decay of the zig-zag configuration on the z-line and pointed out the importance of breaks at the z/I junctions for the increased fragmentation. Phase contrast micrographs (Davey and Gilbert, 1967) of beef muscle aged 4 days at 15°C showed that the z-line disappeared as the myofibrils disintegrated into individual A-bands after brief mechanical treatment.

Although the tendency for myofibril fragmentation following mechanical treatment to increase during postmortem aging may have a direct relationship to tenderness, very little information has been presented to evaluate the importance of myofibril fragmentation as a factor in tenderness variations among animals. Sayre (1970) found that fragmentation generally corresponded to tenderness in chicken pectoralis muscles, when tenderness changes were produced by different treatments affecting postmortem glycolyses. The purpose of this study was to investigate the relationship between the extent of myofibril fragmentation and tenderness in bovine longissimus dorsi. The samples were from young bulls on identical feeding and husbandry, leaving genetic background as the main source of variation between animals. Myofibril fragmentation was measured directly by phase contrast microscope as well as indirectly by spectrophotometric measurements of myofibril suspensions.

EXPERIMENTAL

Meat sampling

Samples of longissimus dorsi were taken from the carcass between the eighth and tenth rib of Danish Black Pied breed. The bulls were reared at the Egtved progeny testing station and slaughtered at 450 kg live weight. Samples were excised 24 hr postmortem and aged for 9 days at 2–4°C in Cryovac bags. The experimental group consisted of 20 young bulls chosen at random from a group of 88 animals at the progeny testing station.

Shear force measurements

Tenderness of the meat was determined by the Warner-Bratzler shear device after deep-fat frying at 150°C until a sample center temperature of 70°C had been reached. Cores (1.27 cm diam) were drilled parallel to the muscle fibers from the center region of the roasts and were cut right angled to the fibers. The average was calculated on the basis of 10 measurements.

Homogenization procedure

A 1 cm thick cross section of raw meat was stored at −18°C until used for the experiments. Prior to use steaks were allowed to thaw at 4°C. 8-g samples were scraped from the surface of the meat, homogenized by a Sorvall omni mixer (a blade homogenizer) for 30 sec at 11,000 rpm in 50 ml KCl-phosphate buffer (0.06M KCl, 0.05M potassium phosphate, pH 7.0) and poured through a nylon mesh to remove connective tissue and gross material. After centrifugation of the suspension for 3 min at 400 × G, the sediment was washed by three cycles of 50 ml buffer solution followed by centrifugation for 30 min at 2000 × G.

Measurement of fragmentation

The myofibril sediment was examined by a phase contrast microscope at 1000× magnification; 100 fibrils were chosen at random. The length of fragments was determined by counting the amount of sarcomers per fibril and the average length of fragments calculated on the basis of 100 fibrils. Particle size of homogenized fiber piece suspensions was determined essentially as described by Davey and Gilbert (1969), but we used a spectrophotometer with emission photometry equipment to measure emission values instead of turbidity readings. Of the washed myofibril sediment, 0.4g were dispersed in the KCl-phosphate buffer solution under standard conditions using a small magnet stirring apparatus. In order to examine the effect of nitrogen concentration on emission measurements, four samples from the suspension of each animal were studied after dispersion in the buffer solution to give approximately 0.2, 0.4, 0.6 and 0.8 mg N per ml.

Nitrogen determinations

Total nitrogen concentration of the myofibril sediment was determined by the Kjeldahl method.

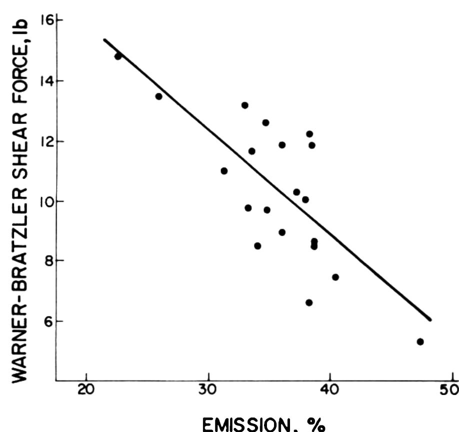

Fig. 1—Relationship between adjusted emission measurements on myofibril suspensions and Warner-Bratzler shear force (n = 20).

RESULTS & DISCUSSION

TENDERNESS AVERAGE and standard deviation measured on experimental group, were 10.28 lb and 2.49 lb respectively. For the total of animals on the Progeny Test Station, the figures this year were 9.36 lb and 2.43 lb. Consequently the selected group is applicable as representative of the typical level and variation of tenderness.

Effect of nitrogen concentration on emission measurements

Davey and Gilbert (1969) found that turbidity readings measured on suspensions of myofibril fragments increased as the particle size decreased as well as finding a positive linear relationship between turbidity readings and protein concentration. Therefore, in order to compare turbidity readings between animals it is essential to adjust the measurements to equivalent nitrogen concentration.

From dilutions of the washed fibrillar sediments corresponding to a nitrogen content varying from 0.2–0.8 mg N/ml, we found a positive linear relationship between nitrogen concentration and emission measurements. However, the hypothesis that all animals should have the same regression coefficient had to be rejected because a homogeneity test proved a significant difference between the regression

Table 1—Simple correlation coefficients between Warner-Bratzler shear force, % nitrogen and measurements of myofibril fragmentation (n = 20)

		1	2	3	4
1.	Warner-Bratzler	–	0.61**	0.37	–0.78***
2.	No. of sarcomers/fibril	–	–	0.37	–0.62**
3.	% N	–	–	–	–0.50*
4.	Emission	–	–	–	–

* $P < 0.05$
** $P < 0.01$
*** $P < 0.001$

Table 2—Analysis of variance on Warner-Bratzler shear force measurements estimated by a two-variable model of regression

Variable[a]	DF	R^2	F	P
No. of sarcomers/fibril + emission	17	0.64		
Emission after no. of sarcomers/fibril	1		12.30	< 0.005
No. of sarcomers/fibril after emission	1		1.20	ns

a Emission values are adusted to same content of nitrogen.

coefficients of the emission measurements on mg N/ml ($P < 0.001$). Consequently corrections in emission measurements were made to level the nitrogen concentration in the dilutions, based on the regression coefficients calculated on each animal. The nonhomogeneity of emission regression on the nitrogen concentration is attributed to the fact that different fragment sizes affect the influence of N-concentration on emission measurements.

Relationship between emission measurements and tenderness

Figure 1 shows the relationship between the Warner-Bratzler shear values of the heat-treated meat samples and the adjusted emission values measured on the myofibril suspensions from the raw meat.

Table 1 presents a number of correlation coefficients between the measured characteristics. A significant correlation between the number of sarcomers/fibril and the shear force was demonstrated ($r = 0.61$; $P < 0.01$). However, the shear force shows a higher correlation with the adjusted emission measurements ($r = -0.78$; $P < 0.001$).

The high correlation coefficients are attributable to the homogeneity of the material with regard to the quantity and quality of the connective tissue, as the animals were slaughtered at practically the same age and weight. Therefore, the variation of tenderness must be due substantially to the properties of the myofibrillar proteins, in this case measured as the degree of fragmentation after standard mechanical treatment.

The relationship between percentage of N in the myofibril sediment and tenderness of the meat ($r = 0.37$) may be due to the fact that with increasing shear force meat shows a tendency partly towards lower water binding capacity (Wismer-Pedersen, 1963) and partly towards larger fragment length after homogenization. This makes the sediment "pack" tighter (Davey and Gilbert, 1969).

The importance of adjusting the emission measurements to the same N-contents in the suspension can be seen from the fact that decreasing tenderness causes decreasing emission whereas the increase in N-contents has the opposite effect, thus concealing a part of the differences in emission values due to differences in tenderness.

Table 2 shows that the emission measurements contain all the information that the number of sarcomers/fibril gives about tenderness as the number of sarcomers/fibril contributes only slightly to further estimations of tenderness. On the contrary, the emission values contribute significantly to further estimations after considering the number of sarcomers/fibril as an explaining variable ($P < 0.005$).

The results of this study show that measurements of fragmentation after mechanical treatment reflect physical characteristics of the raw muscle tissue of high significance to meat tenderness in longissimus dorsi from young bulls after heat treatment. The close relationship may be due to a variable resistence in myofibril structure to physical disintegration which is unaffected by the denaturation and coagulation of the meat proteins as a result of heating.

Further studies have to be made to ascertain whether different degrees of myfibril fragmentation may be a function of (1) development if intra- and inter-myfibrillar linkage during rigor; (2) different courses of aging; or (3) whether genetic factors are involved in the resistence of myofibrils to mechanical treatment. In any event, measurement of fragmentation is a valuable micro method to predict tenderness in longissimus dorsi and the small sample size necessary for analyses makes spoilage of the carcass minimal.

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APPLIED SCIENCE and ENGINEERING

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AN EXPERIMENTAL STUDY OF THE OPTIMAL FEEDBACK CONTROL OF A FREEZE DRYER

INTRODUCTION

SEVERAL DRYING techniques and freeze-dryer designs which are intended to shorten drying times have been proposed in the literature. However, there has been no presentation of a thorough experimental and/or theoretical analysis of the optimal control strategy for the complete freeze drying cycle. Meo (1970) found that optimal feedback control, although practically desirable to implement, has not been achieved in freeze-drying. This should not be surprising since applications of optimal control theory to industrial processes have been few, in spite of the existence of well known theory. As discussed by Koppel (1968), optimal feedback control can only be realized for a restricted class of problems and is applicable to even fewer industrial problems.

A significant amount of research has been conducted to improve the efficiency of the typical radiant heat, batch freeze dryer. This work in the literature is in one sense an attempt to develop optimal operating conditions. Sandall et al. (1967) and Kan and de Winter (1968) have used nitrogen and helium atmospheres in the drying chamber to improve the thermal conductivity of the freeze-dried layer during freeze drying. Sandall et al. (1967) argue that the optimum pressure of nitrogen or helium in the drying chamber is that pressure which offers the best compromise between increasing heat transfer and decreasing mass transfer in the freeze-dried layer. This trade off point is said to occur when the drying rate passes from heat transfer limiting to mass transfer limiting. The transition from heat to mass transfer limiting drying is reported to occur at as high as 17 mm Hg for helium and as high as 9 mm Hg for nitrogen when freeze drying turkey breast. The experimental apparatus used by Sandall et al. (1967) presents very little resistance to mass transfer between the food product and the condenser surface due to the close proximity of the food product and the condenser. Such a situation did not exist in the experimental apparatus used in this work, and it may not exist in commercial freeze dryers, contributing to a lower pressure at which the transition from mass transfer to heat transfer limiting freeze drying takes place. Cox and Dyer (1972) investigated the influence of the pressure of a water vapor and air atmosphere on the drying time and predict that the pressure which produces the shortest drying time is that which offers the lowest total and water vapor pressures. Patents by de Buhr (1966), Kan (1966) and Mellor (1967) suggest that the total pressure should be cycled to achieve the shortest drying time. No mention is made of how or whether these policies change during the course of an entire freeze-drying run. Nor is mention made of radiator control, although it is perhaps clear that maximum radiator power consistent with product quality should be used.

This paper will present an experimen-

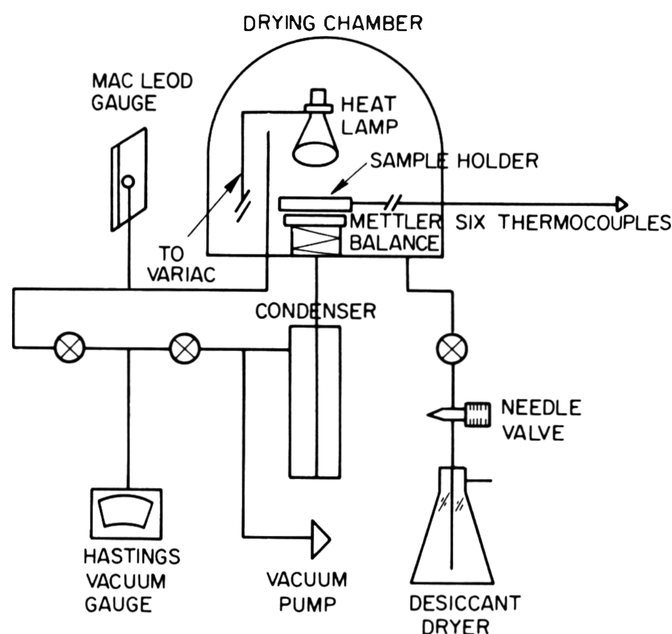

Fig. 1—Schematic of experimental freeze dryer.

[1] Present address: Artisan Industries Inc., Waltham, MA 02154

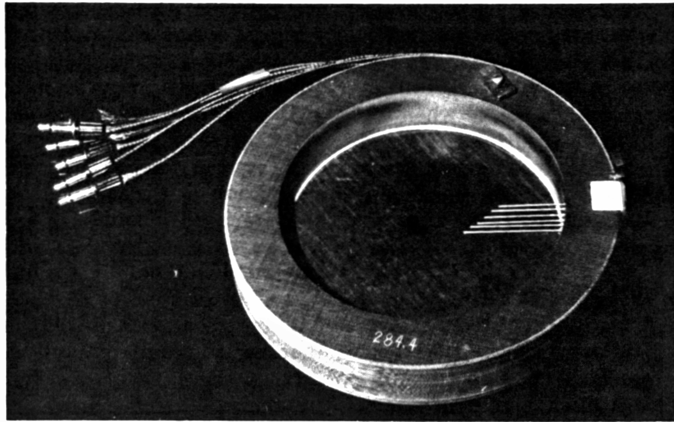

Fig. 2–Sample holder.

tal investigation of the radiator and total pressure control policies on the total drying time of a reconstituted instant milk product in a laboratory freeze dryer. An air-water vapor atmosphere in the drying chamber at pressures from 0.005–2.00 mm Hg will be considered. An experimentally derived near-optimal feedback control strategy for radiant heat freeze dryers which uses the temperatures of the heated surface(s) and frozen material (ice core) to determine the radiator output and the pressure in the drying chamber throughout the freeze-drying cycle will be presented. The control policies are subject to the constraints that the heated surface of the food product is not scorched, and the frozen material is not allowed to soften or melt. This paper focuses on the experimental and applied results of a larger work by Meo (1972). For the theoretical analysis of the optimal control strategy of a freeze dryer consult Meo and Friedly (1973).

EXPERIMENTAL

Apparatus

The apparatus is shown schematically in Figure 1. The sample is supported by a Mettler balance inside the glass bell jar, making possible continuous sample weight readings during drying. Sample temperatures are taken at three locations: just beneath the heated surface, at the middle and at the bottom of the 1/4-in. thick sample. The sample holder is the key piece of apparatus and is shown in Figure 2. Six thermocouple probes are permanently mounted in the side of the sample holder and make it possible to take temperature measurements at the same points in the sample from run to run. The heat of sublimation is provided by a heat lamp mounted over the sample. A variac controls the radiator power output. Using a heat lamp as a radiator makes it possible to rapidly change the temperature of the radiator, a strong advantage when the heated surface is near the scorch point or the frozen sample is in danger of melting. The pressure in the drying chamber is controlled by adjusting the atmosphere air bleed valve. Water vapor generated during drying is trapped in a dry ice/alcohol cooled cold trap located directly below the drying chamber. Proper use of the toggle valves permits either a measurement of the total pressure in the drying chamber by using a MacLeod gage or a measurement of the gas pressure down stream of the cold trap by using a Hastings gage. Because the Hastings gage is positively deflected by water vapor, it may be used to qualitatively measure the efficiency of the cold trap. Finally, the sample is made from a 14% solids solution of instant milk and water. Instant milk is experimentally convenient to use and has essentially uniform heat and mass transfer characteristics in both the frozen and freeze-dried states. 50g are poured into the sample holder and frozen in place, making a circular slab of frozen milk about 4 in. across and 1/4 in. thick. To insure a meaningful near-surface temperature reading, the thermocouple–even with the surface of the frozen sample–was covered with a thin layer of milk solution just before the sample holder was placed in the drying chamber.

Procedure

The sample was freeze dried under several sets of drying conditions. With the exception of one run, the reference run, the radiator control strategy was the same for all the runs. The heated surface of the sample was raised to 54.5°C (a temperature an arbitrary number of degrees below which actual scorching was visually observed on the surface of the freeze-dried sample) as rapidly as was possible without melting the frozen material in the sample. This was achieved with maximum power to the radiator, followed by automatic decreasing of power to maintain the surface temperature at 54.5°C.

Care was taken in each run to keep the frozen sample temperature below −7°C, the maximum permissible temperature which still gave an acceptable dried product.

Three sets of pressure conditions, however, were used. They were constant total pressure, pressure adjusted to maintain a constant frozen sample temperature, and cycled total pressure.

RESULTS & DISCUSSION

The reference run

The effectiveness of feedback control in freeze drying food products may be

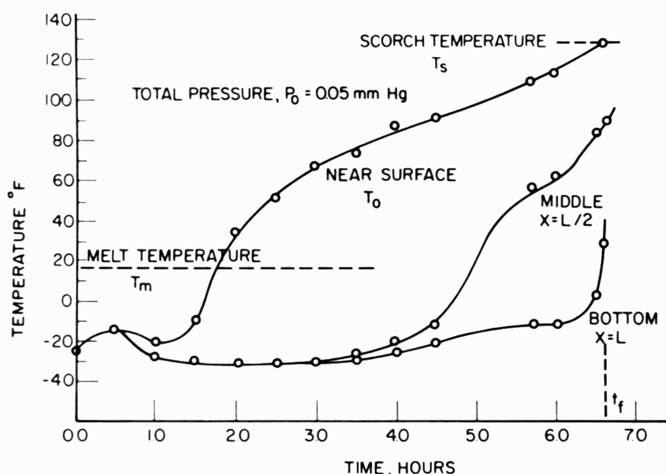

Fig. 3–Sample temperature histories in reference run, radiator power and chamber pressure held constant.

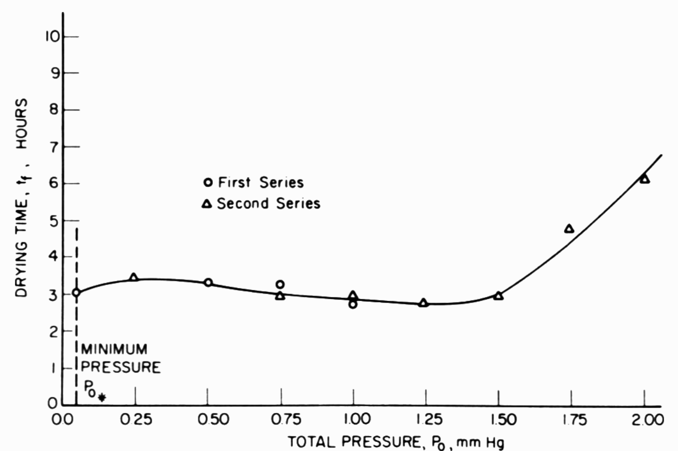

Fig. 4–Drying times obtained in constant pressure series of runs.

determined by comparing the drying times of a feedback control run and a reference run, a run conducted without any control. In the reference run, the radiator output and the total pressure in the drying chamber are kept fixed for the entire run. The best possible constant radiator power and pressure policies were used. The fixed radiator output was selected in order to raise the temperature of the heated surface of the food product to a point arbitrarily close to the onset of thermal damage to the freeze-dried layer just as the run is terminated. The fixed pressure level was taken as the minimum possible based on the results of Cox and Dyer (1972). The vacuum system was capable of holding a minimum pressure of 0.05 mm Hg. The temperature results of the reference run are shown in Figure 3. The surface temperature increases gradually until it approaches the chosen thermal damage temperature 54.5°C at the runs end. After an initial adjustment the ice temperature remains uniform until the ice front passes the measuring thermocouple. The drying time for the reference run was 6.6 hr. In this run and the ones to follow, the product was considered dry when the sample, made from 7g of instant milk and 43g of water, weighed 8g. This definition of run end point is based on considerations of the accuracy of the sample weighing system.

The constant total pressure series

An intuitive near-optimal feedback radiator control strategy can be based on maximizing the heat flux across the freeze-dried layer. Such a control policy would heat the free surface up to its maximum permissible temperature and hold it there for the duration of the run. The policy consists of a maximum power prescorch phase and a postscorch phase in which the power is changed proportional to the deviation in surface temperature away from the scorch point. An intuitive near-optimal feedback pressure control strategy is not as apparent. To study the influence of total pressure on the drying time, a series of runs, each using the radiator control strategy outlined above and a constant total pressure was made. Figure 4 shows the total drying time obtained as a function of the constant chamber pressure imposed. From 0.05 to 1.50 mm Hg, the curve is rather flat with a possible minimum at about 1.25 mm Hg. As suggested by Sandall et al. (1967) this is attributed to a trade off between increasing thermal conductivity and decreasing ΔT driving force for heat transfer in the freeze-dried layer. The optimum constant pressure run shows a nearly 60% reduction in drying time over that of the reference run in Figure 3.

It is convenient to divide the freeze-drying cycle of the constant total pressure runs into three phases. The time period before the heated surface reaches its maximum permissible temperature is

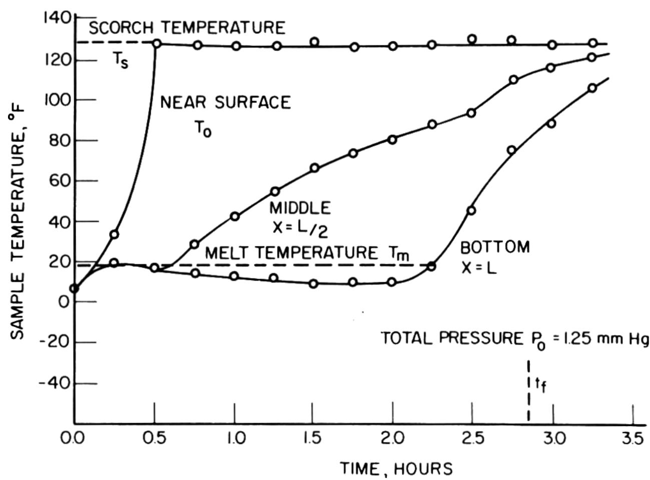

Fig. 5–Sample temperature histories during constant pressure series run at 1.25 mm Hg.

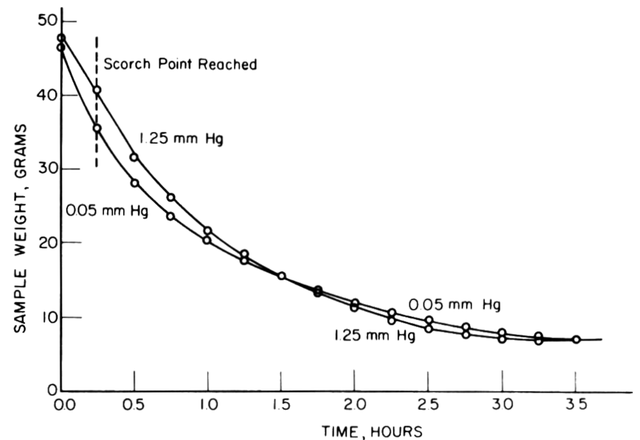

Fig. 6–Comparison of weight loss histories for low and near optimal constant pressure runs.

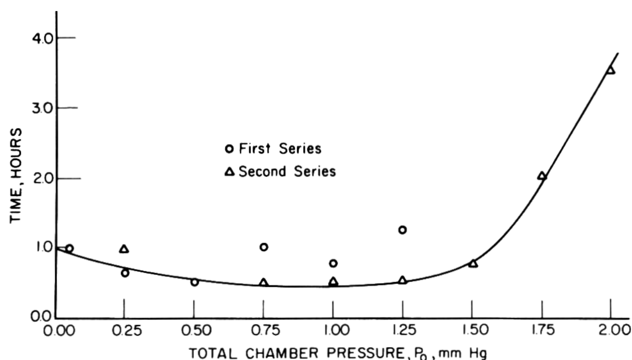

Fig. 7–Time required for ice interface to reach middle temperature probe in constant pressure series.

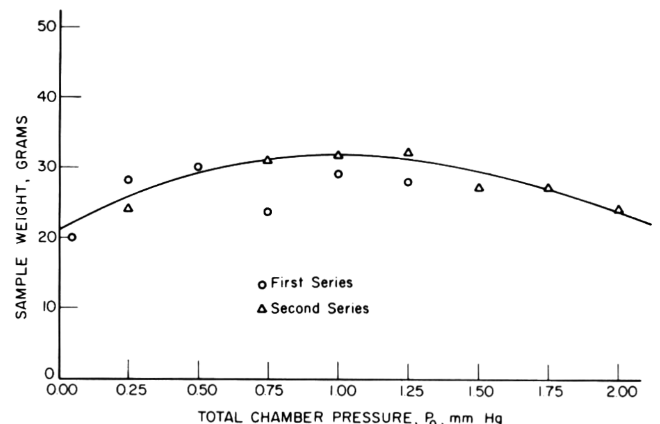

Fig. 8–Sample weight when ice interface reaches middle temperature probe in constant pressure series.

called the prescorch (actually thermal damage) phase. The time period between when the prescorch phase ends and the interface reaches the bottom (center, when drying from two sides) of the slab is called a postscorch phase. Finally, the time period between the end of the postscorch phase and the end of the run is called the terminal drying phase. These three phases are apparent in the typical temperature histories shown in Figure 5. This shows the run conducted at a constant total pressure of 1.25 mm Hg, the run with the shortest drying time. The prescorch phase is the first half hour of drying, the postscorch phase ends when the bottom probe temperature starts its rapid climb, and the terminal drying phase ends with the end of the run.

The influence of total pressure on the removal of water (ice) from the sample during two constant total pressure runs, one at 0.05 mm Hg and one at 1.25 mm Hg, is shown in Figure 6. Lower pressures produce better weight losses in the first half of the drying cycle, and near optimum pressures produce better weight losses in the second half of the drying cycle. This interesting phenomenon can be explained by an analysis of Figures 7 and 8 in which the time and sample weight at which the interface reaches the middle temperature probe are plotted. From Figure 7, one may see that the velocity of the interface during the first half of the constant total pressure drying cycles was fastest in a pressure zone around 1 mm Hg. From Figure 8, one may see that the weight of the freeze-dried layer is heaviest during the first half of the drying cycle in the same pressure range around 1 mm Hg, provided of course the frozen region density is unchanged from run to run. Combining these two observations, the key to rapid freeze-drying at a constant total pressure appears to be to move the interface as rapidly as possible during the early stages of drying because any moisture absorbed by the freeze-dried layer will be desorbed when the sublimation rate slows down, thereby improving the weight loss curve in the second half of the drying cycle. This statement assumes of course that the moisture absorbed by the freeze-dried layer is not detrimental to product quality.

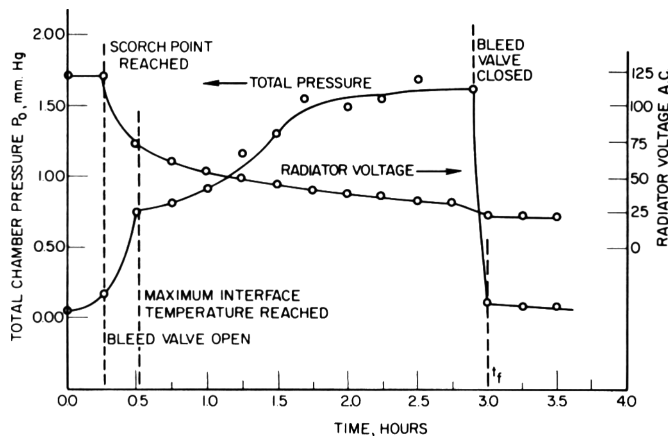

Fig. 9—Total pressure and radiator voltage policies holding surface and interface temperatures constant.

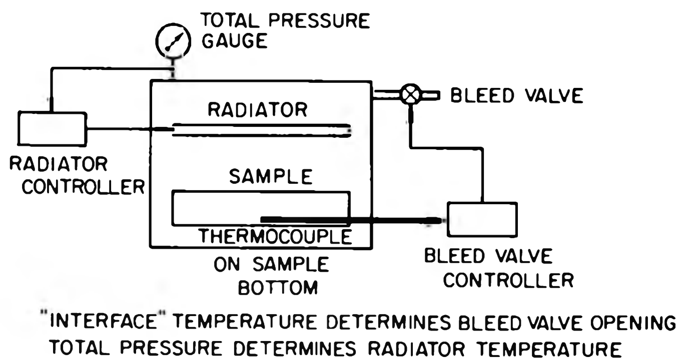

Fig. 10—Feedback control loops in prescorch phase.

Controlling the pressure

From Figure 5, it may be seen that the temperature of the frozen region remains essentially constant and very close to the maximum permissible ice interface temperature, −7°C. Meo and Friedly (1973) argue that theoretical considerations suggest that the true optimal behavior may involve segments when both the surface and ice temperatures are at their maximum values. A freeze-drying run using a chamber pressure feedback control strategy based on the above observation was made. The radiator voltage and total pressure policies are shown in Figure 9. The radiator control is typical of that in the constant pressure series of runs. However, here the pressure was controlled during the postscorch phase to hold the frozen region temperature constant at −7°C. This policy produced a drying time of 3.0 hr. It is believed that the drying time could be reduced somewhat further if the run were started with the frozen region at the constant postscorch temperature and held there during the prescorch phase. Nevertheless, a near-optimal drying time has been obtained using feedback controls on both the radiator power and the chamber pressure during most of the runs.

Additional experiments were performed to examine the effectivenees of cycling the chamber pressure in reducing the drying time, as described by Kan (1966), de Buhr (1966) and Mellor (1967). The chamber pressure was oscillated to produce interface oscillations between −32°C and −7°C with a period of the order of 10 min. The cycled pressure run gave a drying time of 10 min. The cycled pressure run gave a drying time of 3.3 hr, not as good as the best constant total pressure time of 2.7 hr. Comparing the drying curve of the cycled pressure run to that of the 1.25 mm Hg constant total pressure run, it was found that the efficiency of cycling the interface temperature appeared to improve with the thickness of the freeze-dried layer.

Feedback control loops

The information presented above may be used to formulate a feedback control policy for radiator power and total pressure during the entire freeze-drying run. The suggested policy treats each of the three phases of the drying cycle in separate ways. The feedback control loops operating during the prescorch phase are shown in Figure 10. The radiator temperature is linked to the total chamber pressure, and the bleed valve opening is linked to the frozen region temperature. The overall control strategy is to start the interface moving as rapidly as possible

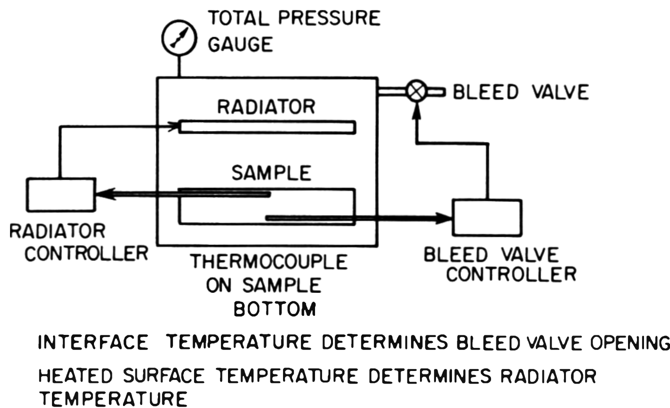

Fig. 11–Feedback control loops in postscorch phase.

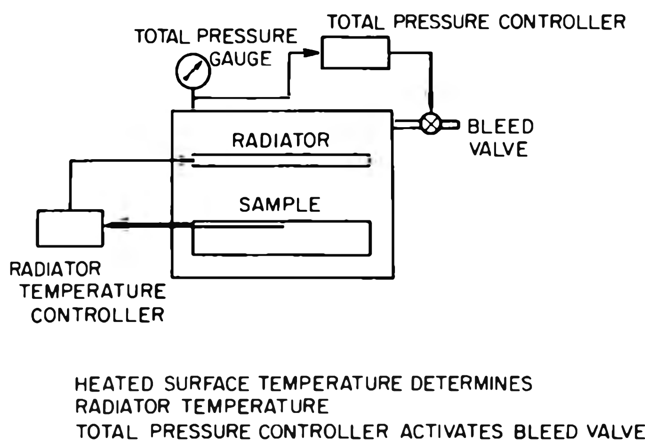

Fig. 12–Feedback control loops in terminal drying phase.

before the heated surface temperature reaches its maximum permissible temperature. The radiator feedback control strategy anticipates that the radiator may cause a sublimation rate so rapid that despite a fully closed bleed valve, the pressure in the drying chamber continues to rise. In such an event, the total pressure will rise to the radiator controller set point, and the radiator power will be forced to drop from its maximum value. The feedback control loops operating in the postscorch phase are shown in Figure 11. The radiator control policy maintains the heated surface at a constant temperature, and the bleed valve continues to maintain a constant frozen region temperature. A simple relay activated by the surface temperature reaching the scorch point can switch the radiator control into the postscorch phase. Similarly, when the interface reaches the bottom (center, when drying from two sides) temperature probe, the temperature of the probe will rise despite the closing of the bleed valve and a relay can switch the control loops to the terminal drying phase shown in Figure 12. The radiator feedback control loop is the same as in the postscorch phase but the bleed valve is used to regulate the total pressure.

It is believed that the feedback control strategy described above can be used to control commercial freeze dryers for a wide variety of food products. The experimental results suggest that the result will be near optimal. Only four pieces of information are required for each application. They are (1) the maximum total pressure in the prescorch phase; (2) the maximum frozen region temperature in the prescorch and postscorch phases; (3) the maximum heated surface temperature in the postscorch and the terminal drying phases; and (4) the desired total pressure in the terminal drying phase. When freeze drying coffee or other granulated food products, the heated surface temperature might be measured to a fair degree of accuracy by using a specially prepared thermocouple. A thin coating of liquid food product can be frozen onto a cylindrical thermocouple or thermistor temperature probe and this probe can be placed on the surface of a bed of frozen, granulated food product. After the layer of frozen food product on the temperature probe has been freeze dried, the probe temperature should offer a fair indication of the surface temperature of the surrounding granules of freeze-dried product. In this work, the heated surface temperature of the slab of instant milk was monitored by laying a thin thermocouple probe on the frozen sample surface before the sample was placed in a freeze dryer and laying a thin "bead" of liquid (instant milk) over the probe, thereby "welding" the probe to the frozen surface. Such a procedure ensured that the thermocouple was thermally shielded from the radiator by a layer of freeze-dried food product.

The interface temperature can be approximated in the case of granulated frozen food products by monitoring the water vapor pressure in the drying chamber. An allowance can be made for the water vapor pressure drop across the freeze-dried layer and by adding this value to the drying chamber water vapor pressure, the water vapor pressure at the interface can be estimated.

The freeze dryer control policies investigated in this work involve strategies not suggested before in the literature. They are applicable for the entire drying run and show as much as a 60% reduction in drying time for the apparatus used. The policy has been shown experimentally to be near optimal and the suggested feedback implementation is consistent with the most likely theoretical optimum discussed by Meo and Friedly (1973). The automatic feedback implementation of the control policy appears to be readily adapted for commercial use.

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ACCELERATED PORK PROCESSING. Fresh-Frozen Pork Chops

INTRODUCTION

PACKERS converting pork carcasses into wholesale cuts would profit by eliminating large holding coolers or using holding coolers more efficiently. Accelerated pork processing as described by Mandigo (1967, 1968) and Henrickson (1968) could result in a significant reduction in processing time while eliminating unnecessary processing steps.

Several laboratory studies demonstrated the feasibility of producing acceptable wholesale cuts of pork using the accelerated processing techniques (Moore et al., 1966; Weiner et al., 1966; Davidson et al., 1968; and Henrickson, 1968). However, Marsh and Legt (1966) and Weiner et al. (1966) noted that pork frozen within 35 min to 1 hr postmortem resulted in tough loins due to thaw rigor and cold shortening. Davidson et al. (1968) and Mandigo and Henrickson (1966) found no significant differences in percentage moisture and Warner-Bratzler shear values of hams due to accelerated vs. conventional processing methods. Weiner et al. (1966) and Moore et al. (1966) found fresh loins produced by the accelerated technique to be as acceptable as loins produced by conventional processing.

Mandigo (1968) indicated that accelerated processing of pork carcasses needed to be evaluated under commercial packing house conditions. Consequently, a study was implemented to determine the effect of accelerated and conventional processing of pork loins at the commercial packing house level on fresh-frozen pork chops.

MATERIALS & METHODS

THE LOINS from 60 pork carcasses used in this study were processed as wholesale fresh pork loins in the George A. Hormel and Company packing plant at Fremont, Nebr. Removal of the pork loin (NAMP No. 410, NAMP, 1970) from the carcass was done according to the standard methods of the packing plant.

Left and right sides were alternately assigned to the treatments of accelerated or conventional processing of pork loins. Accelerated processing is defined as the removal of the loin from the carcass within 1 hr postmortem, with fabrication to a finished wholesale cut prior to initial chilling of the cut. Conventional processing is a control, fabricated in the same manner, except fabricated after 24 hr of carcass chilling at 1.7°C.

The accelerated processed pork loins were divided into three groups according to the chill temperature used to chill the loins to an average temperature of 4.4°C after fabrication. Each group represented a differenct experiment. 20 loins were chilled at −45.5°C, −28.9°C and −17.8°C with an air flow velocity of 1.5m/sec. Paired loins from the same carcasses were conventionally processed and required 24 hr to reach a mass average temperature of 4.4°C at the thickest part. Accelerated processed pork loins required approximately 2 hr to reach a mass average temperature of 4.4°C.

The chilled loins were shipped to the University of Nebraska-Lincoln, and were frozen at −23.3°C. Five chops (1.27-mm thick) were removed anteriorly from the separation of the tenth and eleventh ribs while 15 chops were removed posteriorly, from the tenth and eleventh rib cut of the frozen loins. As soon as the pork chops were identified and trimmed of fat and bone, the boneless chops were placed in individual plastic bags and frozen. After all loin samples were processed, the bags were packed in dry ice and air-shipped to the U.S. Army Natick Laboratories, Natick, Mass.

The chops were stored at −28.9°C until they could be analyzed for tenderness. The frozen chops were weighed and a 5-g sample was removed from each chop to make a composite loin sample for proximate analysis, pH and water-holding capacity determination for each loin. The chops were reweighed and wrapped in aluminum foil to prevent moisture loss and facilitate identification. Each chop was identified as to loin number, processing method and chop location within the loin. The odd numbered chops from each loin were treated as fresh-frozen pork chops while the even number chops were freeze dried. This report will be concerned only with the fresh-frozen pork chops.

The proximate analysis (fat, protein, moisture and ash) was according to the procedures by AOAC (1965). The water-holding capacity test procedure was that of Wierbicki et al. (1957). The process was modified to use 10g of sample per centrifuge tube.

The fresh-frozen pork chops were equilibrated to 4.4°C and were penetrated with a Meat Penetrometer (Hinnergardt and Tuomy, 1970). The peak of the force curve was used in analyzing the data.

The penetrated raw pork chops were cooked to an internal temperature of 76.7°C using 6 psi steam pressure in a three compartment Steam Chef oven. The cooked chops were cooled for 2 hr at 4.4°C and then penetrated again. Following penetration, pork chops numbered seven and nine were paired and 17 and 19 were paired from each loin for evaluation by a 10-member taste panel.

Two samples were served at each taste panel session and evaluated for color, aroma, flavor, appearance and tenderness. The two samples represented a comparison of fresh-frozen pork chops from pork loins which were convention and accelerated processed.

The rating scales used by the taste panel for evaluation of color, aroma, flavor and appearance were based on a one to nine scale. A rating

Table 1—Tenderness of fresh-frozen pork chops equilibrated to 4.4°C before cooking as determined by the meat penetrometer[a]

Chill temp for accelerated processed loins[b]	Processing method for loins: Accelerated	Conventional	$S\bar{x}$
−45.5°C	3.31	3.03	0.06
−28.9°C	3.25	3.22	0.08
−17.8°C	4.30	4.42	0.10

[a] Shear force values reported as pounds force required to penetrate a 1.27-cm thick pork chop.
[b] Each chill temperature represents a separate experiment.

Table 2—Tenderness of fresh-frozen pork chops cooked to an internal temperature of 76.7°C at 6 psi steam pressure

Chill temp for accelerated processed loins	Processing method for loins: Accelerated	Conventional	$S\bar{x}$
	Meat penetrometer[c]		
−45.5°C	8.76[a]	8.67[a]	0.13
−28.9°C	8.27[a]	8.49[a]	0.14
−17.8°C	8.85[a]	9.06[a]	0.28
	10-member taste panel rating[d]		
−45.5°C	4.61[a]	4.83[b]	0.07
−28.9°C	3.81[a]	3.89[b]	0.04
−17.8°C	3.54[a]	3.45[a]	0.04

[a,b] Means with unlike superscripts are different ($P < 0.01$).
[c] Shear force values reported as pounds force required to penetrate a 1.27-cm thick pork chop.
[d] The acceptable tenderness range was from 3−7 using a rating scale of 1 = tough and 9 = tender.

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Table 3–Flavor, odor, color and acceptance of cooked pork chops evaluated by a 10 member taste panel[e]

Chill temp for accelerated processed loins	Processing method for loins: Accelerated	Conventional	$S\bar{x}$
	Flavor		
−45.5°C	5.55[a]	5.62[b]	0.04
−28.9°C	5.58[a]	5.53[a]	0.03
−17.8°C	4.96[a]	5.12[a]	0.04
	Odor		
−45.5°C	5.80	5.75	0.01
−28.9°C	5.97	5.96	0.01
−17.8°C	5.53	5.53	0.03
	Color		
−45.5°C	5.91	5.86	0.01
−28.9°C	6.05	6.10	0.01
−17.8°C	5.72	5.69	0.01
	Acceptance		
−45.5°C	5.71[c]	5.71[c]	0.03
−28.9°C	5.91[c]	5.95[c]	0.01
−17.8°C	5.56[c]	5.64[d]	0.03

a,b Means with unlike superscripts are different ($P < 0.05$).
c,d Means with unlike superscripts are different ($P < 0.01$).
e Rated on a 1–9 scale (1 = extremely poor; 9 = excellent)

of one was extremely poor while a rating of nine was excellent. The tenderness rating scale was also based on a one to nine rating. It differed from the hedonic scale used for color, aroma, flavor and appearance as only three of the nine points were given a word description relating to tenderness. The number one position was labeled tough, the number five position was designated as ideal tenderness, and the number nine position was labeled tender. Tough was defined as resembling leather and tender as offering no resistance to chewing, thus being mushy or lacking texture. Ideal tenderness was defined as being slightly chewy, firm and juicy. A pretest session was held to familiarize panel members in the use of the rating scales.

Data were analyzed using analysis of variance methods described by Bennett and Franklin (1954), Snedecor (1956) and Ralston and Wilf (1960) for differences due to processing methods, loins and chops.

Table 4–Percent cooking loss of pork chops from accelerated and conventionally processed pork loins

Chill temp for accelerated processed loins	Processing method for loins: Accelerated	Conventional	$S\bar{x}$
−45.5°C	34.42	35.05	0.54
−28.9°C	31.96	32.54	0.37
−17.8°C	38.95	36.90	0.82

Table 5–Proximate analysis of accelerated and conventional processed pork loins

Chill temp for accelerated processed loins	Processing method for loins: Accelerated	Conventional	$S\bar{x}$
	Moisture, %		
−45.5°C	70.38	70.49	.20
−28.9°C	69.93	70.07	.20
−17.8°C	70.31	70.67	.17
	Protein, %		
−45.5°C	22.55	22.46	.12
−28.9°C	22.48	22.32	.16
−17.8°C	22.06	22.23	.19
	Fat, %		
−45.5°C	6.52	6.11	.22
−28.9°C	6.26	6.22	.16
−17.8°C	5.75	5.65	.20
	Ash, %		
−45.5°C	1.11	1.13	.01
−28.9°C	1.13	1.13	.01
−17.8°C	1.13	1.13	.01

RESULTS & DISCUSSION

THE UNIFORM REMOVAL of pork chops from loins of accelerated and conventionally processed pork loins did not result in any differences between chop weights for the two processing methods. Chop size varied with the size of loins used. Since loins from the same carcass were used to compare processing methods for loins, the comparison was made using matched left and right loin pork chops of approximately the same size.

Objective measurement for tenderness, both prior to cooking and following cooking to an internal temperature of 76.7°C did not indicate a difference in tenderness of pork chops due to the processing method of pork loins within each experiment (Tables 1 and 2). In two of three experiments, the taste panels found significant ($P < 0.01$) differences between processing methods.

The meat penetrometer was used to objectively measure tenderness of 10 pork chops from each loin while the taste panel was allowed to test only two samples from each loin for each treatment. The fact that the penetrometer had a larger sampling of the chop population than did the taste panel may account for some of the variations in the taste panel findings. Within each experiment and tenderness evaluation technique, the actual difference in tenderness was very small. While the taste panel found only small tenderness differences in pork chops due to loin processing, the differences in tenderness were consistent. However, all of the taste panel tenderness values fell within an acceptable range.

Table 6–pH and water-holding capacity of accelerated and conventional processed pork loins

Chill temp for accelerated processed loins	Processing method for loins: Accelerated	Conventional	$S\bar{x}$
	pH		
−45.5°C	5.68[a]	5.75[b]	0.02
−28.9°C	5.63[a]	5.67[c]	0.01
−17.8°C	5.56[a]	5.59[a]	0.01
	Water-holding capacity[d]		
−45.5°C	22.88	22.38	0.83
−28.9°C	22.67	23.00	0.63
−17.8°C	24.87	24.77	0.74

a,b Means with unlike superscripts are different ($P < 0.01$).
a,c Means with unlike superscripts are different ($P < 0.05$).
d Water-holding capacity = percent juice lost.

The results of the taste panel for flavor, color, aroma and appearance are summarized in Table 3. The taste panel found no differences in the flavor, color, aroma or appearance of fresh-frozen pork chops from loins processed either by the accelerated or conventional method.

The percent cooking loss of pork chops (Table 4) from accelerated and conventionally processed pork loins was approximately the same within experiments. These results tend to confirm the work of Moore et al. (1966) who found no significant difference in cooking loss attributable to the hot (accelerated) or cold (conventional) cutting methods for loin samples.

No significant differences were found within experiments for moisture, protein, fat, water-holding capacity and ash due to loin processing method (Table 5 and 6). Moore et al. (1966) also found no significant differences in moisture loss of pork loin roasts because of the cutting method. However, differences in moisture, protein, fat and ash were found among loins ($P < 0.01$) due to animal variation.

The accelerated processed pork loins demonstrated a consistently lower final pH value than the conventionally processed pork loins even though this difference was not significant in experiment three (Table 6).

The data presented in Tables 1 through 6 tend to confirm the laboratory findings of Davidson et al. (1968), Mandigo and Henrickson (1966), Moore et al. (1966) and Weiner et al. (1966) which demonstrated the laboratory feasibility of producing acceptable wholesale cuts of pork using the accelerated pork processing method. From this study, one could conclude that acceptable fresh-frozen pork chops can be made using the accelerated processing technique for pork car-

casses under commercial processing conditions.

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ACCELERATED PORK PROCESSING. Freeze-Dried Pork Chops

INTRODUCTION

THE ECONOMIC potential of accelerated pork processing has been recognized by Mandigo (1968) and Henrickson (1968). Moore et al. (1966), Weiner et al. (1966), Hinnergardt (1972) and Hinnergardt et al. (1973) have shown that acceptable wholesale pork loins could be produced using accelerated pork processing techniques described by Mandigo (1967).

Fresh-frozen boneless pork chops from accelerated processed pork loins were not different in tenderness, color, aroma, flavor and appearance than were chops produced from conventionally processed loins (Hinnergardt et al., 1973).

Tuomy and Felder (1964) compared internal cooking temperatures of pork chops with plate temperatures for freeze-drying. They found that the freeze-dryer plate temperature was the only variable significantly affecting browning, tenderness and flavor of the final product. Aitken et al. (1962) found freeze-dried pork most acceptable when 60°C was not exceeded as the maximum freeze-dryer plate temperature. The actual temperature of the product being dried, rather than the plate temperature was the limiting factor (Tuomy and Felder, 1964). Tuomy (1970) indicated the quality of freeze-dried pork chops was lowered rapidly if the dry product temperature exceeds 54°C.

Tuomy and Helmer (1967) found that the freeze-drying process causes a toughening and a decrease in flavor acceptability in pork. They pointed out that freeze-drying resulted in tenderness and flavor variations that were greater for freeze-dried pork than comparable fresh samples.

Information has not been available on the effect of accelerated processing of pork loins with respect to the acceptability of freeze-dried pork chops. Consequently, a study was conducted to determine the effect of accelerated processing of pork loins on fresh-frozen and freeze-dried pork chops.

MATERIALS & METHODS

THE PORK CHOPS used in this study were obtained from 120 pork loins processed as wholesale fresh pork loins (NAMP No. 410, NAMP, 1970) in the George A. Hormel and Company packing plant at Fremont, Nebr. Removal of the pork loins from the carcass was done according to the standard methods practiced in the packing plant.

Left and right wholesale loins were alternately assigned to the treatments of accelerated or conventional processing of pork loins. The chilled loins were shipped to the University of Nebraska-Lincoln. At the University of Nebraska-Lincoln, the loins were frozen at −23.3°C. Once the loins were frozen, 1.27 cm thick chops were removed, packed in dry ice, and shipped to U.S. Army Natick Laboratories.

Ten chops from each loin were selected for freeze-drying. The fat trimmed, boneless pork chops were freeze-dried at 51.7°C plate temperature and a chamber pressure of 0.3–0.5 mm of mercury pressure. Identity of the pork chops during freeze-dehydration was maintained by use of dividers in the freeze-dehydrator trays. The dried chops were placed in plastic bags and labeled to maintain identity. The bags were then packed in beaded No. 10 cans. The oxygen in the headspace gas was reduced by evacuating the can with a vacuum of 736.6 mm on a mercury monometer for 1 min and flushing to atmospheric pressure with nitrogen gas. This procedure was repeated a second time before the can was sealed. The headspace oxygen content was reduced to approximately 0.75% as determined by the procedure outlined by Bishov and Henick (1966). The cans of freeze-dried chops were stored at 4.4°C until the chops could be analyzed.

Rehydration of the freeze-dried pork chops was accomplished by soaking the dry chops in 26.7°C water for 30 min. The freeze-dried pork chops were weighed before and after rehydration. These weights in addition to the fresh-frozen weight were used to calculate rehydration ratios and the percent of the original weight obtained upon reconstitution. The rehydrated chops were equilibrated to 4.4°C by holding 16 hr at 4.4°C.

The rehydrated, equilibrated pork chops were penetrated with a Meat Penetrometer described by Hinnergardt and Tuomy (1970). The peak of the force curve was used in analyzing the data. The penetrated freeze-dried raw pork chops were cooked to an internal temperature of 76.7°C using a 6 psi steam pressure in a three compartment Steam Chef oven. The cooked chops were cooled for 2 hr at 4.4°C and then penetrated again. Pork chops numbered six and eight were paired and 16 and 18 were paired from each loin for evaluation by a 10-member organoleptic panel.

Two samples were served at each taste panel session and evaluated for color, aroma, flavor, appearance and tenderness. The chops were cut into 10 pieces of equal size and served to the panelists. The two samples represented a comparison of freeze-dried pork chops and pork loins which were conventionally and accelerated processed. All chops submitted to the taste panel had been tested before hand with the Meat Penetrometer.

The rating scales used by the taste panel for evaluation of color, aroma, flavor and appearance were based on a one to nine scale. A rating of one was extremely poor while a rating of nine was excellent. The tenderness rating scale was based on a one to nine rating. It differed from the hedonic scale used for color, aroma, flavor and appearance as only three of the nine points were given a word description relating to tenderness. The number one position was labeled tough, the number five position designated as ideal tenderness and the number nine position was labeled tender. Tough was defined as resembling leather and tender as offering no resistance to chewing, thus being mushy or lacking texture. Ideal tenderness was defined as being slightly chewy, firm and juicy. A pretest session was held to familiarize the panel members in the use of the rating scales.

Data were analyzed using analysis of variance methods described by Bennett and Franklin (1954), Snedecor (1956) and Ralston and Wilf (1960).

RESULTS & DISCUSSION

THE MEAN WEIGHTS of the frozen, boneless pork chops prior to freeze-dehydration did not show a difference between pork chops from accelerated and conventionally processed pork loins (Table 1). A nonsignificant difference between pork chops from accelerated and conventionally processed pork loins was also noted in the dry weights (Table 1) and rehydrated weights (Table 2) of the freeze-dried pork chops. The rehydration ratio (Table 2) was not affected by accelerated processing.

The Meat Penetrometer (Hinnergardt and Tuomy, 1970) found no significant difference in tenderness of rehydrated, freeze-dried pork chops before cooking (Table 3). After cooking the rehydrated

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Table 1—Weight of fresh-frozen and freeze-dried boneless pork chops from conventionally processed pork loins[a]

Chill temp for accelerated processed loins	Processing method for loins: Accel-erated	Conven-tional	$S_{\bar{x}}$
	Fresh frozen		
−45.5°C	26.9	26.3	0.58
−28.9°C	25.3	25.7	0.68
−17.8°C	29.2	28.4	0.71
	Freeze dried		
−45.5°C	7.3	7.3	0.04
−28.9°C	7.1	7.2	0.01
−17.8°C	8.0	8.0	0.28

[a] Weights are in grams

Table 2—Rehydrated weights and rehydration ratio of freeze-dried pork chops from accelerated and conventional processed pork loins[a]

Chill temp for accelerated processed loins	Processing method for loins: Accel-erated	Conven-tional	$S_{\bar{x}}$
	Rehydration weight		
−45.5°C	21.8g	21.5g	0.12
−28.9°C	21.2g	21.5g	0.12
−17.8°C	24.0g	24.2g	0.13
	Rehydration ratio		
−45.5°C	2.990	2.993	0.005
−28.9°C	2.980	2.973	0.035
−17.8°C	3.018	3.024	0.006

[a] Rehydrated weight/dry weight

Table 3—Tenderness of freeze-dehydrated boneless pork chops equilibrated to 4.4°C before cooking as determined by the meat penetrometer[a]

Chill temp for accelerated processed loins	Processing method for loins: Accel-erated	Conven-tional	$S_{\bar{x}}$
−45.5°C	3.50	3.41	0.06
−28.9°C	3.65	3.64	0.08
−17.8°C	4.72	4.94	0.10

[a] Shear force values are reported as pounds force required to penetrate a 1.27-cm thick pork chop.

pork chops to 76.7°C internal temperature, the cooked chops were cooled to 4.4°C and penetrated again. The maximum force required to penetrate the 1.27-cm thick pork chops showed no difference in tenderness between freeze-dried pork chops from accelerated and conventional processed pork loins after cooking. However, the taste panel found freeze-dried pork chops from conventionally processed pork loins to be significantly ($P < 0.01$) more tender than those from accelerated processed pork loins in two out of three experiments. As can be seen from Table 4, all of the tenderness ratings were in the acceptable tenderness range. The apparent discrepancy between the two techniques for tenderness evaluation may be due to sample size. The Meat Penetrometer was used to evaluate 10 samples per loin as compared to only two samples per loin by the taste panel.

Table 5 reports the taste panel results for flavor, aroma, color and appearance. All of the pork chops freeze dried for the three experiments were freeze dried at the same time to avoid subtle variations that might have occurred due to freeze drying in separate batches. The organoleptic panel did not find any significant differences in flavor, aroma, color and appearance under these conditions due to accelerated processing of pork loins.

The percent cooking loss (Table 5) was based on rehydrated pork chop weights and cooked pork chop weights. Percent cooking loss was not affected by the processing method of the pork loins.

The data presented indicate the feasibility of producing acceptable freeze-dried pork chops by use of the accelerated processing technique for pork carcasses under commercial processing conditions.

Table 4—Tenderness of rehydrated pork chops cooked to an internal temperature of 76.7°C at 6 psi steam pressure[a]

Chill temp for accelerated processed loins[b]	Processing method for loins: Accel-erated	Conven-tional	$S_{\bar{x}}$
	Meat penetrometer		
−45.5°C	8.95[d]	8.73[d]	0.13
−28.9°C	8.01[d]	8.24[d]	0.14
−17.8°C	8.40[d]	8.61[d]	0.28
	10-Member taste panel rating[c]		
−45.5°C	4.21[d]	4.57[e]	0.07
−28.9°C	3.56[d]	3.82[e]	0.04
−17.8°C	3.36[d]	3.37[d]	0.04

[a] The chop was penetrated and then presented to the taste panel.
[b] Each chill temperature represents a separate experiment.
[c] The acceptable tenderness range was from 3–7 using a rating scale of 1 = tough and 9 = tender.
[d,e] Means with unlike superscripts are different ($P < 0.01$).

Table 5—Panel scores for taste attributes

Chill temp for accelerated processed loins	Processing method for loins: Accel-erated	Conven-tional	$S_{\bar{x}}$
	Flavor[a]		
−45.5°C	3.77	3.94	0.04
−28.9°C	3.73	3.82	0.03
−17.8°C	3.29	3.33	0.04
	Odor[a]		
−45.5°C	5.23	5.33	0.01
−28.9°C	5.39	5.41	0.01
−17.8°C	4.87	4.89	0.03
	Color[a]		
−45.5°C	5.59	5.58	0.01
−28.9°C	5.64	5.68	0.01
−17.8°C	5.33	5.34	0.01
	Appearance[a]		
−45.5°C	5.45	5.39	0.03
−28.9°C	5.52	5.58	0.01
−17.8°C	5.49	5.17	0.03
	Cooking loss (%)		
−45.5°C	24.63	25.05	0.54
−28.9°C	23.58	22.66	0.37
−17.8°C	26.90	27.00	0.82

[a] One to nine scale (1 = extremely poor; 9 = excellent).

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FEASIBILITY OF ADDING FREEZE-DRIED MEAT IN THE PREPARATION OF FERMENTED DRY SAUSAGE

INTRODUCTION

MANUFACTURE of fermented dry sausage is an important branch of the meat industry. In spite of the long history and ever-increasing demand, relatively little attention has been directed toward understanding the art of fermented sausage manufacture as practiced by relatively few specialists. During the preparation of dry sausage, a definite sequence for adding the meat materials and other ingredients is followed. The lean meat is either ground or cut, the fat meats added, followed by the addition of the seasoning-salt mixture (salt, nitrate, nitrite, glucose, sugar and spices), and further ground or cut until the desired texture is achieved.

Following preparation, the mix is stuffed into casings or placed in trays or sausage trucks and stored in controlled ripening rooms maintained at about 26–28°C with a relative humidity (RH) of 80–90% (Price and Schweigert, 1971) to permit the growth of selected fermentative bacteria. These bacteria ferment sugars to produce lactic acid which gives fermented sausages their characteristic tang and lowers the pH.

Following treatment in the ripening rooms, dry sausages are moved to drying rooms (maintained from 7.2–12.8°C, at 70–72% RH, and 15–20 air changes per hour) where they remain for several weeks to permit further development of the desired flavor, aroma and texture (MacKenzie, 1966). For economy, shortening the drying period would be desirable so that the product could be marketed in less time. This study was undertaken to assess the possibility of adding freeze-dried meat in the preparation of dry sausage to speed up drying and to evaluate physical and chemical changes during the 35-day drying period.

EXPERIMENTAL

Materials

Fresh hams, from a local meat packer, were deboned and separated into distinctly lean and fat portions. After the portions were thoroughly mixed, they were frozen to comply with C&MS regulations of pork to destroy trichinae (USDA, 1965). After the freezing treatment to destroy trichinae, a portion of the lean meat was removed from the freezer, allowed to partially thaw, ground once through a 3/16-in. plate, refrozen at −34.4°C and then dried in a Vacudyne Freeze Dryer, Model VPED-CX at 100 mμ of Hg until the dried product reached room temperature which usually took 2 days. The moisture content of the dried meat was 5.0% (AOAC, 1965). The dried meat was then vacuum packed and refrigerated until used.

Appropriate quantities of frozen fat and lean meat were removed from the freezer 2 days before the experiment and allowed to partially thaw.

Preparation of sausages

Five batches of sausage mixtures were prepared: four by mixing freeze-dried meat and partially thawed lean meat and pork tissue fat (ground once through a 1/2-in. plate) at the ratio of 1:4 (A), 1:5 (B), 1:6 (C) and 1:9 (D); and one without freeze-dried meat (E, control). Spices and curing ingredients were added which provided 71 mg $NaNO_2$, 575 mg $NaNO_3$, 240 mg sodium ascorbate, 9.0g sucrose, 4.5g glucose, 11.4g NaCl and 2.4g of commercial spices per pound of meat mixture. The lactic acid starter culture (Pediococcus cerevisiae) was a commercial product "Lactacel" which was used as recommended by the manufacturer (Merck Chemical Div., Merck & Co., Inc., Rahway, N.J.).

The individual batches were thoroughly mixed and re-ground through a 3/16-in. plate and stuffed into 52 mm D.S. fibrous casings. Each batch of sausage included 12–13 identical sausages, each weighing about 500g. Preparation of all sausage mixtures was carried out in a 1.7–3.3°C cooler. After stuffing, the sausages were placed in a 37.7°C, 80% RH room to ferment for up to 18 hr. After 7 and 18 hr of fermentation, pH values were determined. The sausages were then transferred to a drying room and further ripened for 35 days at 12.9°C and 70% RH with 5–10 fpm of air passing over the product.

Methods

Weight loss (moisture loss) was determined every day during the first 7 days and once per week thereafter. Two to three sausages were taken from each batch at the end of 7, 14, 21, 28 and 35 days of drying for chemical analysis, flavor evaluation and texture quality measurements.

The percentages of moisture, fat and protein in meat, raw materials and sausages were determined by AOAC methods (AOAC, 1965). Determinations of pH were made on a slurry of the sausage using a Fisher Model 320 pH meter. Free fatty acids and peroxide values were determined according to the method described by Cox and Pearson (1962).

Textural qualities of the sausages were measured using (a) the Allo-Kramer Shear Press and (b) the Warner Bratzler Shear. Allo-Kramer Shear values were determined on 1-cm thick slices of the product which included the peripheral layer of the slice. Warner-Bratzler Shear values were determined on 1-in. cores removed from the interior of the sausage.

Appearance, color and flavor were informally evaluated by several personnel in the Animal Products Laboratory.

The data were statistically analyzed using analysis of variance as outlined in Steel and Torrie (1960).

RESULTS & DISCUSSION

FERMENTED SAUSAGES were prepared by adding varying proportions of freeze-dried pork to the raw sausage mixture. The proximate compositions of the sausages prior to fermentation and drying, and after the 35-day drying period are shown in Table 1. Addition of the

Table 1—Proximate composition of each sausage group prior to fermentation and drying; and after the 35-day drying period

Sausage group[a]	Moisture Before %	Moisture After %	Fat Before %	Fat After %	Protein Before %	Protein After %
A	43.4	35.3	27.9	28.1	21.4	27.7
B	45.3	36.1	26.5	27.2	20.3	26.7
C	50.6	36.5	21.3	25.3	20.7	28.7
D	52.9	35.2	21.2	25.5	19.7	29.0
E	59.1	29.4	17.9	26.9	16.7	28.4

[a] Ratio of freeze-dried meat added to fresh meat: A = 1:4; B = 1:5; C = 1:6; D = 1:9; and E = 0.

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Table 2–Effect of adding varying ratios of freeze-dried meat to fresh meat on changes in pH during the fermentation and drying period

Time		Sausage group[a]				
		A	B	C	D	E
Fermentation (hr)	0	5.86	5.89	5.87	5.87	5.86
	7	5.77	5.88	5.78	5.78	5.70
	18	5.20	5.02	4.85	4.85	4.86
Drying (day)	7	5.01	4.89	4.89	4.86	4.76
	14	4.80	4.75	4.75	4.70	4.78
	21	4.87	4.79	4.79	4.72	4.77
	28	4.78	4.70	4.72	4.68	4.70
	35	4.70	4.70	4.68	4.68	4.65

[a] Ratio of freeze-dried meat added to fresh meat: A = 1:4; B = 1:5; C = 1:6; D = 1:9; and E = 0.

Table 3–Percent moisture content of sausages containing varying ratios of freeze-dried meat to fresh meat at various intervals of the drying period

Drying time (day)	Sausage group[a]				
	A	B	C	D	E
0	43.4	45.3	50.6	52.9	59.1
1	42.0	43.8	48.7	50.5	54.5
2	41.0	42.7	47.2	48.6	51.2
3	40.2	41.7	45.9	47.0	47.1
4	39.4	40.8	44.6	45.6	45.8
5	38.9	40.2	43.7	44.5	44.0
6	38.4	39.0	42.9	43.6	42.3
7	38.4	38.7	41.8	42.3	40.0
14	35.8	35.5	37.6	36.9	32.4
21	33.4	32.8	33.9	33.8	27.3
28	32.1	31.0	31.5	30.3	24.4
35	29.6	28.0	28.5	26.6	22.2

[a] Ratio of freeze-dried meat added to fresh meat: A = 1:4; B = 1:5; C = 1:6; D = 1:9; and E = 0.

freeze-dried product at the ratios of 1:4, 1:5, 1:6 and 1:9 decreased the initial moisture contents 15.7, 13.8, 8.5 and 6.2%, respectively. The addition of freeze-dried meat increased the percentages of protein and fat in the raw sausage mixture of sausage groups A, B, C and D.

However, after the 35-day drying period, the percentages of moisture, fat and protein were nearly the same in all sausage groups with the exception of the moisture content in sausage group E, which contained 6% less moisture than the experimental sausages.

Changes in pH during the fermentation and drying period are shown in Table 2. A pH of 5.3 or below is considered normal for ripened dry sausage (Price and Schweigert, 1971). In this study, the pH dropped to 5.2 or lower after 18 hr of fermentation. A desirable acidity or pH was obtained in fermented sausages prepared with the proportions of freeze-dried meat used in this study. In general, the decrease in pH was slower in the sausage groups containing the higher proportion of freeze-dried meat; however, this difference was not significant. There was a significant effect ($P < 0.05$) of fermentation and of drying time on the development of acidity in the product. As drying time increased, pH decreased.

Depending on the type of product, the time required for dry sausage to reach the dry stage (35–40% shrink) may range from 1 to 6 months. Environmental conditions (temperature, humidity and air changes) are important factors which influence the physical and chemical characteristics of the product. In general, the conditions under which dry sausage is most favorably processed in the drying room are from 7.2–12.8°C DB and 70–74% RH with 15-25 air changes per hour passing over the product (MacKenzie, 1966). These types of sausages are dried not less than 25 days and at not less than 7.2°C. The extent to which the addition of freeze-dried meat shortened the drying period in our study is shown in Table 3. On a equivalent moisture content basis, the addition of freeze-dried meat shortened the drying period by 5–6 days for sausage group A, 4 days for sausage group B, and 2 days for sausage groups C and D. Table 4 shows percent weight loss during the drying period. Proportion of freeze-dried meat, as well as drying time, had a significant effect ($P < 0.05$) on the amount of moisture lost during the drying period. The results clearly show that weight loss was greater for the sausage group containing no freeze-dried meat and decreased as the proportion of freeze-dried meat increased. At the end of the 35-day drying period, three times as much moisture was lost in the group containing no freeze-dried meat as in the group containing the highest proportion of freeze-dried meat. The sausages in Group E became wrinkled, showed some evidence of case-hardening and hollow spots were observed in some of the sausages after 28 days of drying. The appearance of the sausages in groups C and D was similar to the sausages in group E, but none of the defects, such as hollow spots and misshapes was found. Sausages in group A and B had less shrinkage, fewer wrinkles and retained their cylindrical shape throughout the drying process.

Table 4–Percent weight loss during the drying period of sausages containing varying ratios of freeze-dried meat to fresh meat

Sausage group[a]		Drying period (day)										
		1	2	3	4	5	6	7	14	21	28	35
A	No. of sausages	12	12	12	12	12	12	12	9	6	4	2
	Wt. loss	1.4	2.3	3.2	4.0	4.5	5.0	5.0	8.0	10.0	11.3	12.8
	S.E.	±0.0	±0.2	±0.0	±0.0	±0.1	±0.2	±0.2	±0.3	±0.3	±0.3	±0.6
B	No. of sausages	12	12	12	12	12	12	12	10	7	5	3
	Wt. loss	1.5	2.6	3.6	4.5	5.1	6.3	6.6	9.7	12.5	14.3	17.3
	S.E.	±0.0	±0.0	±0.1	±0.1	±0.1	±0.2	±0.2	±0.2	±0.3	±0.9	±0.6
C	No. of sausages	13	13	13	13	13	13	13	10	7	4	3
	Wt. loss	1.9	3.4	4.7	6.0	6.9	7.7	8.8	13.0	16.7	19.1	22.1
	S.E.	±0.1	±0.1	±0.1	±0.1	±0.1	±0.1	±0.1	±0.2	±0.2	±0.3	±0.1
D	No. of sausages	11	11	11	11	11	11	11	9	6	4	2
	Wt. loss	2.3	4.3	5.9	7.3	8.4	9.3	10.6	16.0	19.1	22.6	26.3
	S.E.	±0.1	±0.2	±0.2	±0.3	±0.3	±0.1	±0.3	±0.4	±0.5	±0.4	±0.4
E	No. of sausages	8	8	8	8	8	8	8	6	4	3	1
	Wt. loss	4.6	7.9	12.0	13.3	15.1	16.8	19.1	26.7	31.8	34.7	36.9
	S.E.	±0.1	±0.2	±0.2	±0.5	±0.3	±0.1	±0.3	±0.3	±0.2	±0.2	

[a] Ratio of freeze-dried meat added to fresh meat: A = 1:4; B = 1:5; C = 1:6; D = 1:9; and E = 0.

Table 5—Effect of adding varying ratios of freeze-dried meat to fresh meat on the amount of free fatty acids liberated during the drying period[a,b]

Drying time (day)	Sausage group[c]				
	A	B	C	D	E
0	0.54	0.55	0.55	0.56	0.55
7	1.78	1.66	1.56	1.42	0.98
14	2.01	1.76	1.76	1.63	1.14
21	2.24	2.10	2.04	1.72	1.23
28	3.02	2.50	2.24	1.98	1.17
35	3.08	2.86	2.53	2.47	2.08

[a] Ml 0.1N KOH/g fat
[b] Average of duplicates
[c] Ratio of freeze-dried meat added to fresh meat: A = 1:4; B = 1:5; C = 1:6; D = 1:9; and E = 0.

Table 6—Changes in peroxide value during the drying period of sausages containing varying ratios of freeze-dried meat to fresh meat[a,b]

Drying time (day)	Sausage group[c]				
	A	B	C	D	E
0	5.10	4.85	4.85	5.15	5.15
14	6.51	6.85	7.60	6.79	8.70
21	6.02	6.70	8.92	9.50	6.45
28	6.81	7.22	7.54	8.32	8.43
35	6.93	7.52	8.06	9.28	10.30

[a] Ml 0.002N sodium thiosulfate/g fat
[b] Average of duplicates
[c] Ratio of freeze-dried meat added to fresh meat: A = 1:4; B = 1:5; C = 1:6; D = 1:9; and E = 0.

Table 7—Effect of adding varying ratios of freeze-dried meat to fresh meat on changes in consistency of fermented dry sausages

	Drying time (day)	Shear value (lb)[a,b]				
		A	B	C	D	E
Warner-Bratzler[c]	7	0.58 ± 0.2	0.73 ± 0.1	1.82 ± 0.6	3.05 ± 0.6	5.08 ± 0.6
	14	0.95 ± 0.2	0.95 ± 0.1	2.31 ± 0.3	4.30 ± 0.9	6.40 ± 1.2
	21	1.08 ± 0.2	2.45 ± 0.5	3.17 ± 0.6	3.01 ± 0.9	6.56 ± 0.7
	28	1.52 ± 0.3	1.68 ± 0.2	4.50 ± 0.6	3.45 ± 0.1	9.18 ± 0.5
	35	1.02 ± 0.1	1.34 ± 0.2	2.65 ± 0.2	5.08 ± 0.2	7.10 ± 0.4
Allo-Kramer[d]	14	7.93 ± 0.7	10.72 ± 0.6	16.00 ± 0.8	17.43 ± 0.7	28.65 ± 1.1
	21	8.83 ± 1.0	12.20 ± 1.2	17.75 ± 1.6	19.45 ± 1.0	33.60 ± 1.9
	28	12.67 ± 0.7	14.87 ± 1.2	24.61 ± 1.9	27.91 ± 1.5	53.00 ± 2.6
	35	13.75 ± 2.1	18.16 ± 0.8	30.17 ± 1.5	37.42 ± 1.4	54.20 ± 5.8

[a] Each observation is the mean and S.E. of ten shear values from each sausage group.
[b] Ratio of freeze-dried meat added to fresh meat: A = 1:4; B = 1:5; C = 1:6; D = 1:9; and E = 0.
[c] Pounds to shear 1-in. cores
[d] Pounds of pressure to shear 1-cm thick slices of sausage

The development of a desirable flavor and aroma in dry sausage depends on the addition of spices and seasonings, the decrease in pH and the enzymatic reactions that occur during the ripening process. The enzymes responsible for the reactions are indigenous to the meat itself and those produced by microflora growing in the meat. One of the important reactions occurring in the lipids of dry sausage seems to be the progressive breakdown of the various components of the pork fat by lipolytic bacteria (Cantoni et al. 1965, 1966). During the ripening process of dry sausage the quantity of free fatty acids increases. Niinivaara (1968) reported a three-to-four-fold increase in free fatty acids during a 21-day ripening period. Giolitti and Massacra (1963) also reported similar increases in free fatty acids.

Table 5 shows the effect of adding freeze-dried meat to fresh meat on the liberation of free fatty acids during the 35-day drying period. The results show a significant ($P < 0.05$) increase in the quantity of free fatty acids liberated during the drying period, and the increase in free fatty acids was greater in those sausages containing higher proportions of freeze-dried to fresh meat. Cantoni et al. (1965) reported that the release of fatty acids is caused primarily by bacterial lipases, produced mainly by micrococci. Addition of freeze-dried meat in the sausages may have influenced the growth pattern of microorganisms, possibly favoring the growth of micrococci which resulted in the liberation of greater amounts of free fatty acids.

The development of rancidity in dry sausage is also greatly influenced by the type of microflora. Nurmi (1966) reported that micrococci decreased the peroxide number while lactobacilli increased it. The pattern of peroxide values (Table 6) was not as consistent as that of the free fatty acids, but in general, values were higher with the sausages made without or with lesser proportions of freeze-dried meat. In addition to the microbial factor, the decline in pH value and the increase in salt concentration during drying might also have contributed to the differences noted in peroxide values. According to Watts and Peng (1947), rancidity increased when the pH value of pork sausage decreased from 6.5 to 4.8 and the salt content increased.

The development of the proper consistency (firmness) of dry sausage is generally believed to be a result of the evaporation of water from the product. The effect of the addition of freeze-dried meat on the consistency of the sausages is shown in Table 7. The results obtained with the Warner-Bratzler Shear were somewhat irregular due to pieces of connective tissue catching between the blades of the shear, thus giving higher shear values. Nevertheless, a general trend was noted wherein firmness of sausages decreased as the proportion of freeze dried meat to fresh meat increased. The values obtained with the Allo-Kramer Shear were more reproducible. Firmness increased with drying time, and the order of firmness was the same as that of the Warner-Bratzler Shear. Two other important factors, type of microflora and pH, are involved in the development of the proper consistency of dry sausage. Nurmi (1966) reported that sausages inoculated with lactobacilli had distinctly higher tenderometer scores than the corresponding control and micrococci inoculated sausages. He also noted that a rapid decline in pH was closely related with the development of proper consistency in the sausage. In addition to the microbial factor, the softer texture noted in the sausage groups containing the higher proportion of freeze-dried meat might be also attributed to their higher fat and freeze-dried meat content.

One of the important changes occurring during the production of dry sausage is the development of the cured color. For some special applications (dry sausage in particular), only the nitrate salt is employed. In these instances, the products are cured for extended periods of time, thus allowing the reduction of nitrate to nitrite by the appropriate indigenous bacteria. Niinivaara (1955) has successfully used a strain of micrococci (Micrococcus strain M_{53}) for this purpose in dry sausage. The nitrite formed reacts with myoglobin to form nitric oxide myoglobin (undenatured meat pigment), and the optimum pH range for this reaction is 5.5–5.0. In this study, the initial curing reaction apparently was slower in the sausage groups containing the higher proportion of freeze-dried meat. However, after fermentation, all of the sausages developed a good cured color. In-

itially sausages containing the higher proportions of freeze-dried meat were slightly brownish-red in color. The group without freeze-dried meat had a paler cured color which resembled the commercial products. The difference between the sausage group containing no freeze-dried meat and those groups containing lesser proportions of freeze-dried meat was slight.

In addition to color and texture, development of desirable flavor is essential. The use of the lactic acid culture decreased the pH rapidly, and an agreeable aroma and flavor had developed after only 1 wk of drying, although the taste was rated as "somewhat raw." After 14 days of drying, the flavor became much milder and agreeable. None of the product developed an unpalatable flavor during the drying period.

The results of this study show that freeze-dried meat can be added to dry sausage mixes to shorten the drying period; however, with the increase in proportion of freeze-dried meat, there is a loss of quality (consistency and initial cured color development).

A comparison of all the quality attributes indicates that the best ratio of freeze-dried meat to fresh meat is between 1:6 and 1:9. However, practical application of these findings is contingent on several economic factors.

Additional investigations will be conducted to determine the optimum particle size of freeze-dried meat, ratio of freeze-dried meat and optimum environmental conditions needed to speed up the drying process.

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FREEZE DRYING OF BEEF: THEORY AND EXPERIMENT

INTRODUCTION

BEEF has probably received more attention in the freeze-drying field than any other food. Even so, our knowledge of its behavior still contains several gaps. Many investigators have studied only parts of the complete freeze-drying process. Some sets of data are scanty or inaccurate. There are several theoretical treatments of freeze drying. The basic assumptions are usually the same but the formulas obtained differ widely.

Like most of the previous investigations, the present one contains a set of experimental measurements and a theoretical analysis developed to explain what was observed and to help in predicting freeze-drying behavior in other situations. It differs from most previous investigations in that we used specimens of spherical shape, to avoid the problems associated with heat and vapor transfer from the edges of a slice or slab.

The freeze drying of beef is complex in its details but the basic processes involved are relatively simple. It is not difficult to construct a relatively simple theory of freeze drying that can be easily understood and tested. This theory can, if desired, be refined by taking into account processes or effects that were ignored in the first approximation. A theory of freeze drying should give the relations between all of the important parameters of the process. It should allow us to predict drying times from the dimensions of the specimens and the drying conditions. It should allow us to calculate surface temperatures of specimens, so that damage to the foodstuff by overheating can be avoided. It should also allow us to see what has to be done if the process of freeze-drying is to be speeded up. The theory developed in the present paper is believed to combine the best features of previous treatments and to improve on them in several respects.

EXPERIMENTAL

Theory

The theory to be developed in the present paper is similar in many respects to that given by King and his co-workers (Sandall et al., 1967; Clark and King, 1971) but breaks down the heat transfer to the specimen into its conductive and radiative components. Others who have given theoretical analyses include Ginnette et al. (1958); Massey and Sunderland (1967) and Bralsford (1967). There are additional papers containing theory but all of the more important ideas can be found in those referred to. King's (1971) review, which covers all aspects of freeze drying, is a valuable guide to the literature.

The essential processes in freeze drying are the supplying of heat to the surface of the specimen, the flow of heat from this surface to the surface of the ice core, the sublimation of ice at the surface of the core and the outward flow of the water vapor produced. Some authors, such as Bralsford (1967) and Lusk et al. (1964), treat conditions at the specimen surface as known. Others, such as Sandall et al. (1967), assume that the specimen is contained in a drying chamber and that the temperature of the chamber walls is known but not the temperature of the specimen surface. Both groups assume that the pressure inside the drying chamber is known and that this is the pressure at the specimen surface.

A theory in which the temperature of the chamber walls is given seems to us to be much more useful than one in which the temperature of the specimen surface is given, and we have accordingly developed a theory that includes the heat transfer from the surroundings to the specimen as part of the process. This permits the surface temperature of the specimen to be calculated from quantities that can be easily measured and controlled.

One of the simplifications that will be made in the analysis is to treat freeze drying as a quasi-steady process. Most of the temperatures and temperature gradients, pressures and pressure gradients within the system change slowly with time. Some, such as the ice-core temperature, change very little throughout the entire drying period. Others, such as the temperature of the specimen surface, undergo substantial changes. In the quasi-steady approximation, the heat and mass flows at any instant are assumed to be the same as they would be if the slow drifts were not occurring. In general the approximation is a very good one because the heat flows associated with gradual temperature changes are small compared to the main heat flow from specimen surface to ice core.

Consider a spherical specimen of freeze-drying material such as beef, of radius R, suspended at the center of a spherical drying chamber of internal radius R_w, as shown in Figure 1. This model represents a simplification of the actual situation. In the actual apparatus (Fig. 2), the drying chamber was a cylinder with the specimen hanging from a spring at the axis. Errors associated with the acceptance of a spherically-symmetrical model are believed to be small. The point will be discussed later. The partially-dried specimen is assumed to contain a spherical core of ice of radius r_f. That is, we accept the idea of a uniformly retreating ice front (see, for example, King, 1971).

Radiative heat transfer from the wall of the drying chamber (radius R_w, temperature T_w) to the specimen surface (radius R, temperature T_s) obeys the equation

$$\dot{q}_r = -4\pi R^2 \sigma\epsilon(T_w^4 - T_s^4) \quad (1)$$

where $\dot{q}_r$ is the rate of energy flow, σ is the Stefan-Boltzmann constant, and ϵ is the emissivity of the specimen surface. It is not necessary to consider the emissivity of the chamber wall because the area of the wall is considerably larger than the area of the specimen, and because $1-\epsilon$ for the wall is a small quantity (Jakob, 1957).

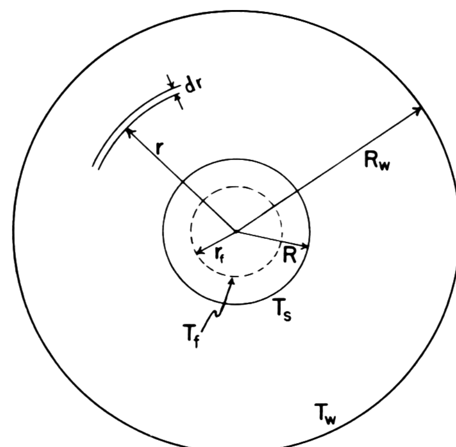

Fig. 1—Accepted model for freeze-drying of spheres.

Conductive heat transfer from wall to specimen is also easily calculated (see for example Jakob, 1949) to be

$$\dot{q}_c = -4\pi k_v \frac{T_w - T_s}{\frac{1}{R} - \frac{1}{R_w}} \tag{2}$$

where $\dot{q}_c$ is the rate of heat flow by conduction and k_v is the thermal conductivity of the water vapor in the chamber. The total heat flow from wall to specimen (the external heat flow) is $\dot{q}_e$, the sum of $\dot{q}_r$ and $\dot{q}_c$

$$\dot{q}_e = -4\pi k_v \frac{T_w - T_s}{\frac{1}{R} - \frac{1}{R_w}} - 4\pi R^2 \sigma\epsilon (T_w^4 - T_s^4). \tag{3}$$

Note that $\dot{q}_r$, $\dot{q}_c$ and $\dot{q}_e$ are all negative quantities, flow being considered positive in the direction of increasing r.

Heat conduction in the dried layer of the specimen is a more complicated process than conduction in the water vapor outside the specimen because of the presence of outflowing water vapor. As the vapor produced by sublimation of the ice core moves outward it absorbs a small portion of the inflowing heat. Consider a spherical shell similar to the one indicated in Figure 1 but located in the dried layer. Across this shell the equation satisfied is

$$d\dot{q} = \dot{m}\, c_{pv}\, dT \tag{4}$$

where $\dot{q}$ without a subscript is the rate of heat flow within the dried layer, $\dot{m}$ is the rate of mass flow across the layer, c_{pv} is the specific heat of the vapor, and dT is the temperature rise across the layer. The mass flow rate $\dot{m} \equiv dm/dt$ is found from measurements of the mass m of the specimen.

Integrating Eq (4) from the surface of the ice core to some larger value of r

$$\dot{q} - \dot{m}\ell = \dot{m}\, c_{pv} (T - T_f) \tag{5}$$

where we have used the fact that at the core surface $\dot{q} = \dot{m}\ell$, with ℓ the latent heat of sublimation of the ice. Note that $\dot{m}$ is always negative, resembling the various heat flows in this respect. If we put $T = T_s$ in Eq (5), $\dot{q}$ becomes $\dot{q}_e$; we then eliminate $\dot{q}_e$ from Eq (3) to obtain

$$\dot{m}\ell = -\dot{m}\, c_{pv} (T_s - T_f) - 4\pi k_v \frac{T_w - T_s}{\frac{1}{R} - \frac{1}{R_w}} - 4\pi R^2 \sigma \epsilon (T_w^4 - T_s^4). \tag{6}$$

An equation giving T as a function of r within the dried layer is needed. Again using standard procedures (Jakob, 1949) but using Eq (5) for $\dot{q}$ we find

$$\dot{m}\ell + \dot{m}\, c_{pv} (T - T_f) = -4\pi r^2 k_b \frac{dT}{dr}. \tag{7}$$

After rearrangement and multiplication by $c_{pv}dr$, one side of this equation is of the form dr/r^2 and the other of the form $d(T - T_f)/(T - T_f)$. Integrating from the core surface to the specimen surface and solving the result for k_b, we find

$$k_b = -\frac{\dot{m}\, c_{pv}}{4\pi} \frac{\frac{1}{r_f} - \frac{1}{R}}{\ln\left[1 + \frac{c_{pv}}{\ell}(T_s - T_f)\right]}. \tag{8}$$

Eq (6) permits the surface temperature of the specimen to be calculated from experimental data, after which Eq (8) permits the thermal conductivity of the freeze-dried layer to be calculated. Our theory takes account of the heat required to sublime the ice and of that required to raise the temperature of the outflowing vapor from T_f to T_s. It does not take account of the heat absorbed by the dried layer as its temperature gradually rises. This is consistent with the quasi-steady approximation we have accepted. If the temperature field were truly steady the outflowing vapor would still absorb heat but the dried layer would not. Later, data and estimates will be given, showing that the heat absorbed by the vapor is much smaller than that used to vaporize the ice, and that the heat absorbed by the dried layer is roughly four times smaller than that absorbed by the vapor. For making these comparisons it is convenient to expand the logarithm of Eq (8) in series. Retaining only the first term,

$$k_b \doteq -\frac{\dot{m}\ell}{4\pi} \frac{\frac{1}{r_f} - \frac{1}{R}}{T_s - T_f}. \tag{9}$$

This equation can alternatively be obtained by putting $c_{pv} = 0$ in Eq (7). By comparing values of k_b calculated from Eq (8) with those calculated from the less accurate Eq (9) the result of ignoring the heat absorbed by the vapor can be found. Note also that a highly accurate value of c_{pv} is not required in Eq (8) because c_{pv} has only a second-order effect in it.

Several of the quantities required in the theory must be taken from the literature or found by auxiliary computations. Among those requiring computation are $\dot{m}$, r_f and R. The drying rate $\dot{m}$ is found from the observed masses by numerical differentiation. The observed masses also give the radius r_f of the ice core. Assuming that the ice to be removed is initially uniformly distributed we find

$$\left(\frac{r_f}{R_o}\right)^3 = \frac{m - m_d}{m_o - m_d} \tag{10}$$

where m_o is the initial mass, m_d the dry mass, m the mass corresponding to r_f and R_o the observed initial radius. The radius R of the specimen does not remain constant; significant shrinkage occurs during drying. Let R_d be the observed radius after drying. Then if we assume that

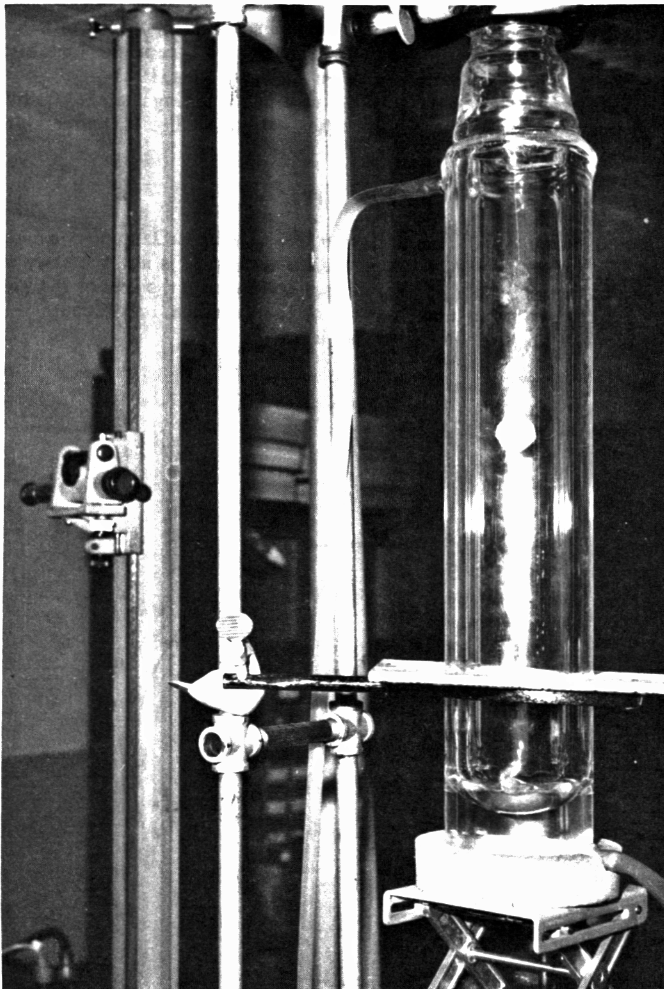

Fig. 2–Photograph showing freeze-drying chamber, specimen and cathetometer.

the dried layer has one constant density and the core has another constant density it is easily shown that

$$\frac{R^3 - R_d^3}{R_o^3 - R_d^3} = \frac{m - m_d}{m_o - m_d} . \quad (11)$$

Note that, for the assumptions made, both r_f^3 and R^3 are linear functions of the mass of the specimen.

The temperature of the ice core surface, T_f, is calculated from P_f, the pressure at that surface, using the vapor pressure-temperature relation of pure ice. However, P_f is not a measured quantity. The pressure P_s in the drying chamber is the known quantity; the pressure drop $P_f - P_s$ in the dried layer must be calculated. Bralsford (1967) has shown that this may be done by accepting a constant permeability G analogous to a thermal conductivity K. Applying his method to a specimen of spherical symmetry we find, analogous to Eq (2)

$$P_f - P_s = -\frac{\dot{m}}{4\pi G}\left(\frac{1}{r_f} - \frac{1}{R}\right) \quad (12)$$

which permits P_f and then T_f to be calculated.

The logarithm in Eq (8) must be accurately calculated, since its value is near zero. It is easily shown that the argument of this logarithm is equal to $\dot{q}_e / \dot{m}\ell$. That is, it is the ratio of the heat flow at the specimen surface to the heat flow at the ice-core surface. This ratio is easily obtained as a by-product when Eq (6) is being solved.

This completes the theoretical analysis. The derivations are based on spherical symmetry. When the theory is applied to beef, which is not isotropic, we obtain a thermal conductivity averaged over the various directions in the specimen in a way that depends on the geometry of the dried layer. The ice core is initially spherical but as drying proceeds it becomes oblate, with the short axis parallel to the beef fibers. In the theory the oblate core is replaced by a spherical core of equal mass and volume. This is done because it greatly simplifies the mathematical analysis. It is believed that the thermal conductivity given by the theory will not differ greatly from an average taken over the three principal axes of the material but a proof of this will not be attempted.

Experiments

The drying chamber and adjacent parts of the apparatus are shown in Figure 2. The drying chamber was a cylindrical, water-jacketed glass enclosure with an inside diameter of 7.0 cm and an inside depth of 44 cm. Vacuum was produced by a 2-stage mechanical pump of 3.3 cfm capacity. Chamber pressures were read, as appropriate, with a mercury manometer and an oil manometer, or a McLeod gage. The measurements made while freeze drying was in process were made with the oil manometer and are accurate to 0.03 torr or better.

Specimens were precooled in dry ice and then installed quickly so that freeze-drying could be started before any melting occurred. Masses were measured with a vitreous silica or a "Ni-Span" spring, the elongations of which were measured with a cathetometer having a least count of 0.005 cm. A new cradle was made up for suspending each specimen from the spring and was weighed before the specimen was put into it. For the first five specimens cradles of AWG 32 Nylon-clad copper wire were used. We then became concerned about the possibility that the copper wire might cause a slight increase in the rate at which heat reached the specimen. The sixth cradle was therefore made from a poor thermal conductor, cotton thread. Results of the change were inconclusive but the use of cotton thread is considered better practice and has been continued in later measurements.

Readings of mass and pressure were normally made at intervals of 5 min or less as freeze-drying was begun. As conditions became more steady the interval between readings was lengthened to 15, 20 and in some cases, to 30 min. The pressure P_s was not held constant at any particular value. This pressure depends on the size of the pump used, the amount of resistance to flow between the drying chamber and the pump, and the size of the specimen being dried. For a normal run with a 5-g specimen the initial value of P_s (excluding transients) was about 0.75 torr. This pressure gradually fell to a very small value as drying took place and $\dot{m}$ became smaller. Dry weights, initial weights, and other data on the specimens are given in Table 1.

The beef specimens all came from a single animal. The semimembranosus muscle was removed from a top round of beef (U.S. Choice, Inspected P.S.D. No. 903) purchased from a local wholesaler. The muscle was trimmed, wrapped in two layers of paper and frozen in an air blast freezer set at about −10°F. Freezing was stated to require about 4 hr. After freezing, the sample was transferred to a refrigerator maintained at about 0°F for storage.

To make the spherical specimens, cubes of appropriate size were cut from the frozen sample with a band saw. Then the corners of the cubes were sawed off, with the specimens held in a specially made plastic jig so that the cuts would be perpendicular to the body diagonals. The resulting 14-sided pieces were then filed to spherical shape by hand. During the sawing and filing the pieces were kept frozen at all times by placing them in dry ice at frequent intervals. The file was also kept cold. The departures of the finished specimens from true spherical shape were small.

The diameter of each specimen was measured with a vernier caliper before freeze-drying; after drying the diameter was again measured, using a traveling microscope. Several measurements along different diameters were made and averaged to obtain the results given in Table 1. Because of shrinkage, the values of R_d are 4.7% lower than the corresponding values of R_o, on the average.

RESULTS

DRYING CURVES (mass vs. time) for the six specimens are shown in Figure 3. Drying times increase with specimen size and decrease with wall temperature, as expected. The initial mass m_o of each specimen was determined by plotting the values of m observed during the early part of the run vs. time and extrapolating back to t = 0. Values found in this way seemed to be more reliable than values found by weighing the specimen just before it was installed.

Drying rates ($\dot{m}$) are shown in Figure 4. The curves fall steadily during most of the drying period but just before $\dot{m} = 0$ is reached they bend sharply and approach the axis of abscissas

Table 1—Dimensions and drying data for the six spherical specimens of raw beef

Specimen	Initial wt	Dry wt	Initial water	Initial radius	Dry radius	Wall temp	Initial ice temp	Initial mass flow	Drying time
	m_o	m_d		R_o	R_d	T_w	T_{fo}	$\dot{m}_o$	t_d
	(g)	(g)	(%)	(cm)	(cm)	(°C)	(°C)	(g/hr)	(hr)
45S	5.190	1.431	72.4	1.060	1.010	65.0	−20.2	1.256	6.81
56S	5.083	1.404	72.4	1.063	1.011	65.0	−20.9	1.209	7.27
57S	4.718	1.234	73.8	1.035	0.982	45.0	−23.3	0.861	9.60
58S	2.504	0.762	69.6	0.835	0.805	65.0	−23.8	0.822	4.71
59S	10.447	2.902	72.2	1.334	1.279	65.0	−18.6	1.856	9.50
60S	10.310	3.455	66.5	1.339	1.290	65.0	−18.8	1.820	8.90

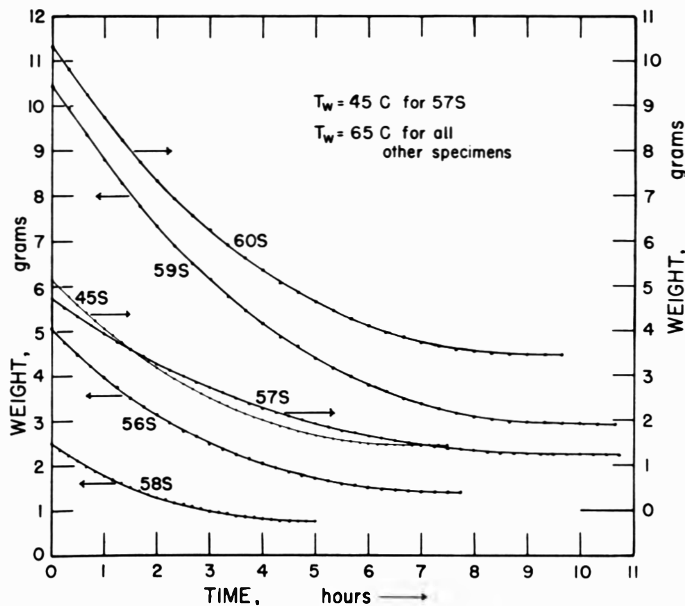

Fig. 3–Weight as a function of time, as freeze-drying progresses. The specimen number of each curve is given beside it.

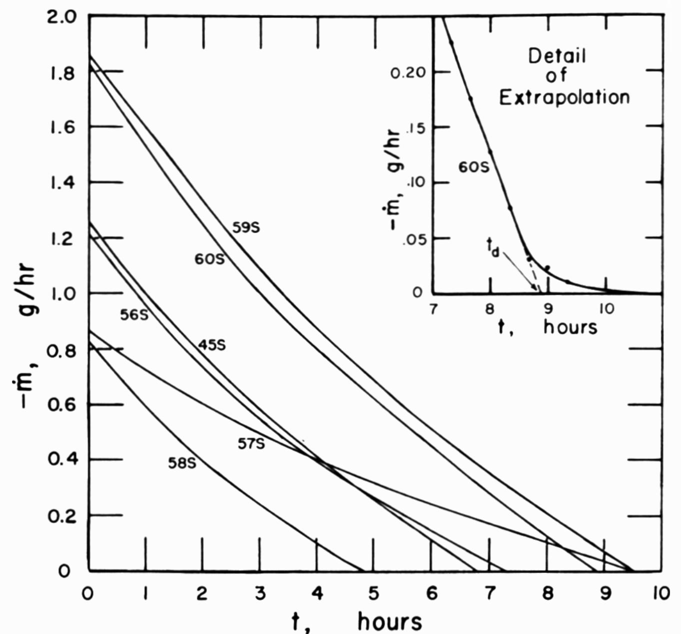

Fig. 4–Rate at which specimens lose weight during freeze drying. The specimen number of each curve is given beside it.

asymptotically, as shown in the insert in Figure 4. The amount of water lost after the sharp bend is passed is negligible in most investigations. Hence we may conveniently establish a drying time t_d for each specimen by extrapolating the main portion of the $\dot{m}$ curve until it cuts the axis of abscissas as shown in the insert in Figure 4. Straight-line extrapolations based on the final 20% of the drying period were satisfactory. Values of t_d found in this way are given in Table 1.

The pressure drop in the dried layer was calculated with G = 18×10^{-6} g/sec cm torr; this value was taken from Bralsford (1967). This permitted P_f and T_f to be found, after which $\dot{m}\ell$ was calculated, using ℓ = 2840j/g. Eq (6) was then solved for T_s, using the following auxiliary data taken from the literature or accepted after preliminary analysis: c_{pv} = 1.86j/gK; k_v = 0.209 mW/cmK; $\sigma = 5.679 \times 10^{-12}$ W/cm^{2}K^4; ϵ = 0.888; and R_w = 4.0 cm. The value of T_w (see Table 1) was 65°C (338.15K) for all specimens except 57S. For this specimen T_w was 45°C; also for this specimen k_v was taken to be 0.198. Some of the reasons for choosing the various values given above will be discussed later. Figure 5 shows the values found for T_s. The average value of T_s at time zero is 251.9K (−21.25°C). All of the curves rise steadily toward the appropriate value of T_w but they are not extended until they reach T_w because our model of the drying process becomes invalid when the ice core disappears or slightly earlier, before T_s reaches T_w.

After T_s has been found, $\dot{q}_e$, $\dot{q}_r$ and $\dot{q}_c$ can be calculated. Figure 6 shows these quantities and several other variables of interest for one typical specimen. It is of interest that conduction in the water vapor outside the specimen accounts for a substantial part of the total heat flow. For example, at t = 3 hr, $\dot{q}_r$ was 71% of $\dot{q}_e$ and $\dot{q}_c$ was 29%. The radius of the ice core, r_f, and its temperature, T_f, are shown in the middle section of Figure 6. Note that T_f remains nearly constant. The final portion of T_f is dashed, to indicate that our model breaks down somewhere in this region. Figure 6 also shows P_f and P_s. Note that P_f can be more than twice P_s, so it is not safe to calculate an ice-core temperature from P_s, ignoring the pressure drop $P_f - P_s$. If this were done at t = 5.25 hr with the data shown in Figure 6, a temperature 9.2K too low would be obtained for the ice core.

The quantity that gives the principal test of our theory of freeze drying is k_b, the thermal conductivity of the freeze-dried layer. It is calculated from Eq (16). In Figure 7, k_b for each of the specimens has been plotted versus the time elapsed since the start of drying. Near t = 0 and again near the end of drying the data tend to scatter or depart from their regular pattern, but there is a long intermediate region in which our model of the drying process is well confirmed. The assumption of a uniformly retreating ice front is likewise confirmed, since our model involves this assumption. In the region of validity of the theory most of the k_b curves are approximately horizontal, with a slight tendency to fall as time increases. The tendency to deviate from the regular pattern would show up in a much more striking manner if we had not, somewhat arbitrarily, decided to reject all the early points for which $R\text{-}r_f$ was less than 0.5 mm and all the late points for which r_f was less than 2 mm. The justification for rejecting the early points is that errors in the thickness determination can cause large errors in k_b here; also there are transient heat flows that take time to die out. The justification for rejecting the late points is that as the ice core grows small it is no longer permissible to ignore the heat absorbed by the dried layer in comparison with that which goes to sublime ice and heat the outflowing vapor.

The curves in Figure 7 are roughly parallel but they show a spread of about 40% from the highest curve to the lowest. Some of this spread is undoubtedly due to natural differences between specimens caused by differing proportions of fat or connective tissue, or by differences in structure. But part of the difference we may expect to be due to differences in the pressure of water vapor within the dried layers of the various specimens. Following the usual procedure we have plotted k_b vs. the average pressure of the water vapor in the dried layer. Figure 8 shows our own data together with those of several previous workers; our own data are indicated by the specimen numbers. The spread of our data, defined as the average depar-

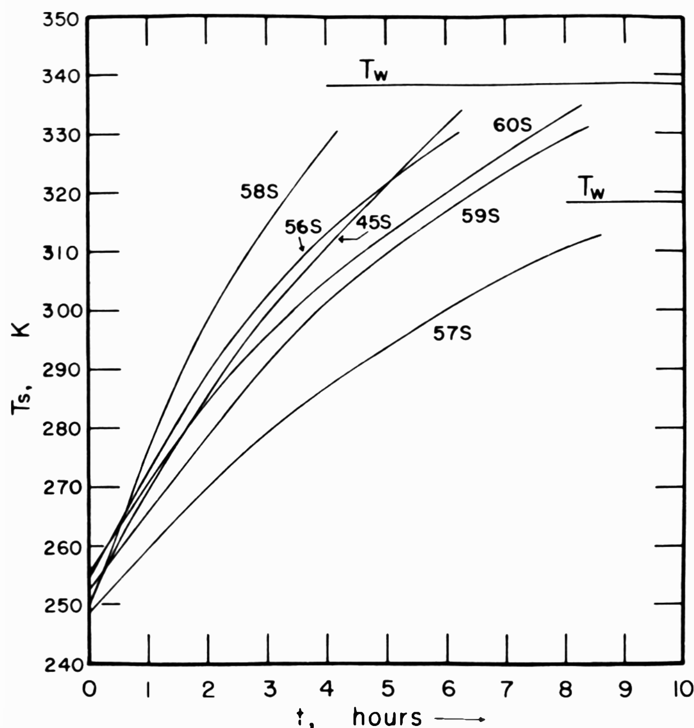

Fig. 5–Calculated surface temperatures for all specimens. As t increases, $T_s \rightarrow T_w$. For specimen 57S, $T_w = 45^\circ C$; for all other specimens $T_w = 65^\circ C$.

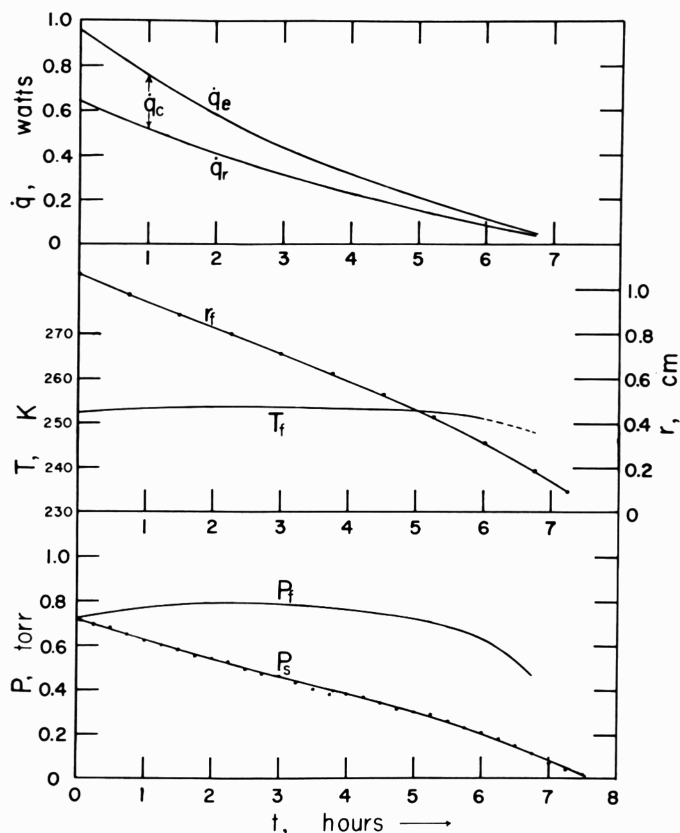

Fig. 6–Various observed and calculated quantities for a typical run (specimen 56S). Top, heat transfer rates between wall and specimen: total ($\dot{q}_e$), radiative ($\dot{q}_r$) and conductive ($\dot{q}_c$); middle, radius and temperature of ice core; and bottom, pressure of water vapor at surface of specimen (P_s) and at surface of ice core (P_f).

ture from a smooth curve drawn to represent them, has been reduced to about 1/3 the spread shown in Figure 7.

The thermal conductivities of porous materials such as freeze-dried beef, when plotted vs. the logarithm of the pressure of the surrounding gas, give characteristic sigmoid curves. Examples of such curves may be found in Harper (1962) and elsewhere. They can be conveniently represented by equations of the form of Eq (13). In the low-pressure region (below about 0.1 torr) each system has a low and constant value of k; again in the high-pressure region (above about 100 torr) the system has a higher and constant value of k. In the intermediate region k rises from one value to the other as described by an equation such as Eq (13). A word of caution is in order, however, when an equation of this form is applied to the beef-water-vapor system. The equation works well when the gas is inert toward the porous material but it may work less well when the gas (water vapor) is strongly attracted to the porous material (freeze-dried beef).

The data plotted in Figure 8 all fall within the intermediate pressure region in which k_b should increase with pressure. In the relatively short range of pressures covered by Figure 8, a sigmoid curve such as Eq (13) shows very little curvature. Our own data show an upward slope as expected, but the slope is a little steeper than we had anticipated. The agreement with previous work is considered satisfactory. This point will be discussed later. We will note here, however, that our data give some indication of a dependence of k_b on the size of the specimen. The smallest specimen (58S) has the lowest value of k_b and the two largest specimens (59S and 60S) have the highest values; this may mean that the theory needs further refinement.

Choice of ϵ, k_v and R_w.

As mentioned earlier, our data were calculated using ϵ = 0.888, k_v = 0.209 mW/cm K (0.198 in one case), and R_w = 4.0 cm. In order to account for all of the heat that flowed from chamber wall to specimen, it was necessary to choose ϵ and k_v 4.5% higher than what we judged to be the most reliable values in the literature, and also to use a value of R_w slightly smaller than we might otherwise have done. The only published values of ϵ for beef are those of Sunderland and his co-workers. Petree and Sunderland (1972) give values of 0.80–0.85 for slowly-frozen, raw, freeze-dried beef but find that samples rapidly frozen by direct contact with dry ice have emissivities about 15% lower. Our own samples would be expected to have emissivities in the higher range. Values of the thermal conductivity of water vapor appropriate for our use are given by Hilsenrath (1955) and in the Landolt-Bornstein Tables (1968). Estimating the effective vapor temperature to be 44°C, a value of k_v was arrived at. This value was then raised 4.5% to give k_v = 0.209, which was used for all specimens except 57S. A similar procedure led to an estimated vapor temperature of 29°C and an accepted value of k_v = 0.198 for 57S. These values of k_v do not change with pressure as freeze drying progresses,

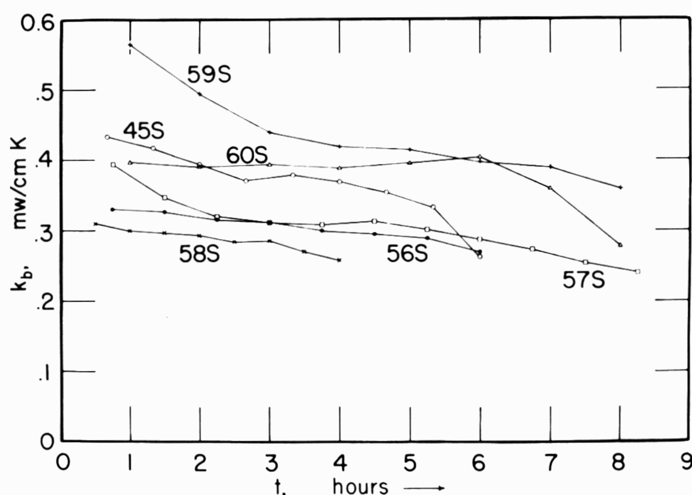

Fig. 7–Thermal conductivity of the freeze-dried layer of raw beef as a function of the time elapsed since the start of drying.

because the distance from specimen surface to chamber wall is always many mean free paths.

Constant values of ϵ and k_v throughout the drying period have been assumed. However, a high value of ϵ, falling to the accepted value during the first half-hour or hour of drying, could be justified almost as well as a constant value. As drying begins there is some ice and frost on the surface of the specimen in addition to the ice in the meat itself. This ice has an emissivity in the range 0.96–0.985 according to the American Institute of Physics Handbook (1957).

The possibility that convection could augment heat transfer to the specimen was seriously considered. At very low gas pressures the density of the convecting medium is very small and even a substantial percentage difference in density will furnish only a small driving force for convection. Jakob (1949) gives a method of calculating the ratio of the apparent conductivity (which includes convection) to the true conductivity (without convection) in a fluid where convection can occur. A rough calculation patterned after Jakob indicated that if the pressure of water vapor in our apparatus were 100 torr we might possibly expect convection to increase the heat transfer by 10% of that due to conduction alone. Our pressures were of the order of 1 torr and since the important parameter (the Grashof number) is proportional to the square of the density there seems to be no possibility that convection would be appreciable in our experiments.

Comparison with previous work

Comparison of the present work with that of previous investigators will be based principally on the values obtained for the thermal conductivity of freeze-dried beef. Figure 8 contains the results from five previous investigations together with our own data. Only Bralsford (1967) gives values for heat flow both parallel and perpendicular to the fiber direction. At a pressure of 0.5 torr his data show $k_\perp$ to be only 65% of $k_\parallel$. The data of Stuart and Closset (1971) are for cooked beef rather than raw beef. All the other plotted data apparently refer to raw beef. This was verified by private communication in one case (Massey and Sunderland, 1967). Bralsford's second paper (1967) contains thermal-conductivity data for freeze-dried cooked beef that are not included in Figure 8. These data indicate that the thermal conductivity is not changed much by cooking; the same conclusion can be drawn from the position of Stuart and Closset's data in Figure 8.

Harper (1962), Harper and Chichester, (1960) and Harper and El Sahrigi (1964) have published thermal-conductivity data for freeze-dried beef, peach, apple and other substances, with air or some other inert gas surrounding the specimen. Unfortunately, no data are given for beef in the presence of water vapor, although Harper and El Sahrigi (1964) speak of a few confirmatory measurements made on this system. By drawing on all three of the references given above it is possible to construct the following equation to represent the thermal conductivity of freeze-dried beef in the presence of water vapor

$$k_b = 0.374 + \frac{0.187}{1 + (1.8/P)} \tag{13}$$

Here k_b is in mW/cm K and P is in torr.

Data obtained by Massey et al. (1967) and by Magnusson (1969) are also shown.

There is some indication that an equation of the form of Eq (13), which works well for inert gases, does not accurately describe the thermal conductivity of freeze-dried beef when the surrounding gas is water vapor. No measurements covering the full range of interest (0.1–100 torr) have been reported, probably because one would have to work at about 50°C to avoid vapor condensation. Bralsford (1967) says that his results for freeze-dried beef surrounded by water vapor "are significantly different from those obtained with noncondensible gases. In particular they show no flattening out over the range of pressures studied and the values lie increasingly far above those for air." Triebes and King (1966) working with freeze-dried cooked turkey meat also found that, when water vapor was employed, the conductivity was frequently greater than would be predicted.

To compare our own data with those of Bralsford (1967) we need to calculate from his values of $k_\perp$ and $k_\parallel$ a suitable average value to be used for a sphere. An average giving equal weight to all directions in a sphere of beef would be $(2k_\perp + k_\parallel)/3$. However, this average can hold for spheres only as drying begins. Because $k_\parallel$ is greater than $k_\perp$ the ice core will develop into an oblate spheroid, of eccentricity departing farther and farther from unity as drying proceeds. This will shift the effective value of k closer to $k_\parallel$ than the value given above. It seems unlikely that such a shift can bring the effective value much above $(k_\perp + k_\parallel)/2$. Hence our data are definitely higher than those of Bralsford. There are small uncertainties in Figure 8 associated with the pressures to be assigned to some of the previously published k_b values. For our own data we have simply used $(P_s + P_f)/2$ but this was not possible in the case of some of the previously published data.

Some of the data in Figure 8 were obtained while freeze drying was in process. The rest were obtained in separate experiments, after freeze drying had been completed. Bralsford indicates that the same results are obtained in the two cases. Our own work does not fully confirm Bralsford in this respect. The value of k_b may actually be somewhat higher while freeze-drying is in progress than it is when drying is complete. It seems reasonable to expect the dried material closest to the ice core to have a higher conductivity than the more remote material that has been dried longer and also has reached a higher temperature. Hence data such as ours, taken while freeze drying is in progress, may actually be better for use in freeze-drying calculations than data taken in separate experiments.

DISCUSSION

THE THEORY of freeze drying presented in this paper includes certain refinements and improvements over previous work. For example, edge and end corrections are avoided by using spherical specimens. Also, heat transfer to the specimens

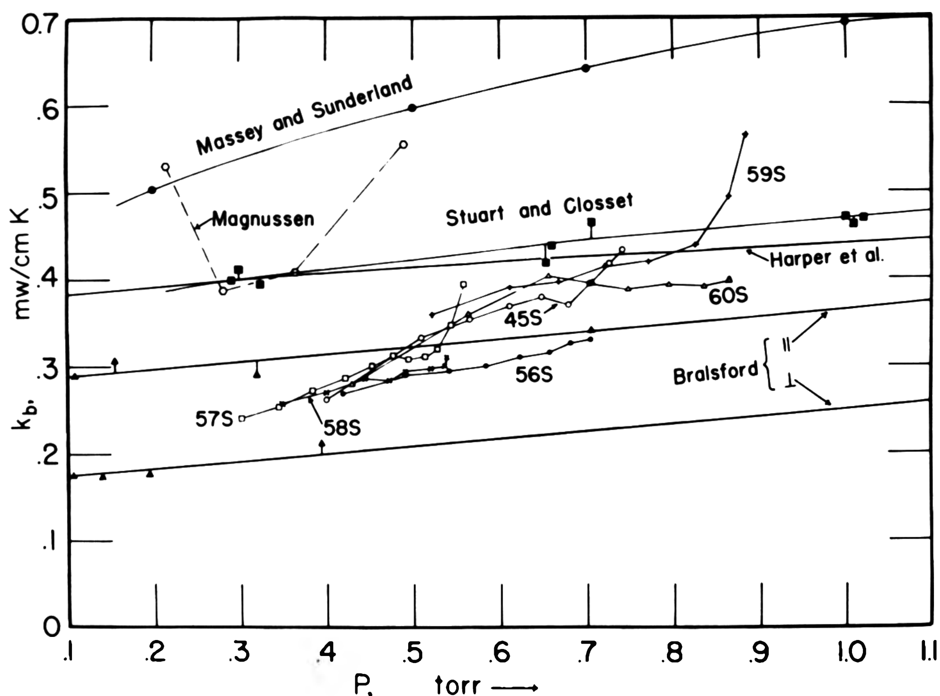

Fig. 8—Thermal conductivity of freeze-dried beef. Results of the present investigation are shown by the curves marked with specimen numbers: 45S, 56S, 57S, 58S, 59S and 60S. The measurements of Stuart and Closset were made on cooked beef; all the rest refer to raw beef. The abscissa is the pressure of water vapor within the sample. If a pressure gradient existed within the sample, the average pressure was used as abscissa whenever the necessary data were given.

is broken down into its radiative and conductive components, which is more precise than using a heat-transfer coefficient. The heat absorbed by the outflowing vapor is accurately accounted for. There are, however, still some improvements that could be made. The theory would be more accurate if the heat absorbed by the dried layer was taken into account. A rough estimate can be made of the error caused by neglecting this heat. The mass of the dried meat is about 1/3 of the mass of the ice that is removed from it, and the specific heat of the dried material is probably somewhat less than that of water vapor. The effect of the heat absorbed by this dried material is therefore probably about 1/4 as large as the heat absorbed by the outflowing vapor. We can find the effect of ignoring the heat absorbed by the vapor, by calculating k_b first from Eq (8) and then from Eq (9) which ignores the vapor. The effect of ignoring the vapor is to lower k_b, on the average, by about 4%. This error, of course, is not present in our data because we use Eq (8). However, it shows us that when we ignore the heat absorbed by the dried layer we are making an error that, on the average, is not much more than 1%.

Another improvement over our treatment would be to use the vapor pressure and the heat of sublimation of the actual beef juices in the calculations, rather than the values for pure ice. Hill and Sunderland (1967) found ℓ for frozen beef juice to be 8% high, and the value for the subliming phase of beef muscle to be 22% high, when compared to ℓ for pure ice. We made a few exploratory measurements, calculating ℓ from vapor-pressure data as Hill and Sunderland (1967) had done, and found ℓ to be only 2.4% above the value for pure ice. Being uncertain of all these values, we decided that the easiest way out was the best, and have treated the subliming material as if it were pure ice, both as regards heat of sublimation and vapor pressure.

It would also improve the reliability of experiments if a spherical drying chamber were used rather than a cylindrical one for which R_w had to be estimated. This has in fact been done in experiments now in progress. The change in the shape of the drying chamber has not had any marked effect on the values of k_b obtained.

CONCLUSIONS

IN SUMMARY, a theory of freeze drying has been developed. It has been well verified by experimental measurements and comparison with previous data. However, there is some tendency for larger specimens to show higher thermal conductivities than smaller specimens, and for the thermal conductivity to fall gradually as the dried layers thicken.

The theory is sufficiently well validated to make it useful in practical work. Surface temperatures of drying material can be calculated and kept below the danger point. Drying times can be calculated. The results are strictly valid only for spheres, but work now in progress indicates that spheres and cubes having equal masses also have nearly equal drying times. The drying times of material other than beef can be computed from the theory, once the thermal conductivity of the material has been determined.

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EFFICACY OF PROTEIN ADDITIVES AS EMULSION STABILIZERS IN FRANKFURTERS

INTRODUCTION

HANSEN (1960) has established that the mechanism of emulsion formation during the comminution of sausage materials involves the creation of fat globules which are subsequently surrounded by membranes formed by muscle proteins between dispersed (fatty) and external (water) phases. Stable emulsions, from which the fat does not separate during subsequent cooking, are of paramount economic importance to the processed meats industry. A number of factors affecting emulsion capacity or stability have been identified. Muscle pH (Trautman, 1966), ionic strength (Hegarty et al., 1963), pH and salt concentration (Swift and Sulzbacher, 1963), level of added water (Swift and Sulzbacher, 1963; Morrison et al., 1971), chopping temperature (Helmer and Saffle, 1963; Townsend et al., 1968, Swift et al., 1961) and processing temperature and humidity (Saffle and Helmer, 1963) are all important considerations in manufacturing frankfurters.

A wide variety of protein additives are presently available for use in frankfurters to reduce formulation costs, to improve processing yields and/or to improve emulsifying capacity. Inklaar and Fortuin (1969) and Pearson et al. (1965) concluded that protein solubility was closely related to emulsifying capacity. Little has been published regarding protein solubility, emulsifying capacity or other functional properties of some of the oilseed protein products. It has been assumed that the oilseed proteins act as emulsifiers, increasing the stability of sausage emulsions, or serve as binding agents, preventing moisture and fat separation from the emulsion during processing. The present study assayed certain protein additives with regard to empirical functional properties and determined their behavior in frankfurter emulsion systems before and after heat treatment.

EXPERIMENTAL

ELEVEN KINDS of protein additives were compared by preparing emulsions and manufacturing frankfurters in a small-scale pilot plant operation. Frankfurters of four different fat contents were manufactured from 10 of the 11 protein additives. Liquid cyclone processed (LCP) cottonseed protein concentrate was used only in frankfurters of high fat content. Fat percentages were calculated prior to manufacture to provide two pairs each of emulsions with low and high fat content. Emulsions containing more than 30% fat were prepared because preliminary investigations indicated that high levels of fat would facilitate evaluation of the influence of protein additives (added at the 3.5% level) on the stability of the frankfurter formulation used in the present study.

The basic formulation for manufacture of frankfurters is presented in Table 1. Beef muscle was obtained from the chuck and round of slaughter cow carcasses, pork muscle was obtained from the shoulder of butcher hog carcasses and fat was derived from the fatback of butcher hog carcasses. Chopping was performed in a small silent cutter (Hely-Joly) in batches of approximately 3.6 kg. Beef muscle, half of the necessary ice and all other ingredients except pork muscle and pork fat were mixed and chopped for 2 min in the silent cutter. Pork muscle, pork fat and the remaining ice were added and the mixture was chopped for an additional 6 min. Total chopping time was 8 min and the final temperature of all batches did not exceed 18°C. Emulsions were sampled for chemical and physical determinations and the remainder was stuffed into 17 mm artificial cel-

Table 1—Basic formulation for the manufacture of the control and experimental frankfurters

Item	Quantity (grams)
Beef muscle	1,000
Pork muscle	1,500
Pork fat[a]	154 (adjustable)
Crushed ice[b]	750 (adjustable)
Salt	75
Dextrose	15
Sodium erythorbate	0.6
Commercial curing agent[c]	9
Commercial seasoning[c]	14
Nonmeat protein additives[d]	105 (adjustable)

[a] Adjusted to obtain specific fat levels in each batch
[b] Adjusted to comply with federal regulations assuming a processing yield of 90%
[c] Obtained from Griffith Laboratories, Chicago. These included wiener seasoning DF-241 (processed from spice extractives of pepper and other spices on a dextrose carrier) and prague powder (a mixture of sodium chloride, sodium nitrite and sodium nitrate).
[d] Adjusted to obtain 3.5% levels of final processed weight

Table 2—Identification of the protein additives used for frankfurter manufacture.

Identity	Code
Soy protein isolate—high nitrogen solubility[a]	SPI-HNS
Soy protein isolate—low nitrogen solubility[a]	SPI-LNS
Soy flour—high nitrogen solubility[a]	SF-HNS
Soy flour—low nitrogen solubility[a]	SF-LNS
Soy protein concentrate—high nitrogen solubility[b]	SPC-HNS
Soy protein concentrate—low nitrogen solubility[a]	SPC-LNS
Glandless cottonseed flour—high nitrogen solubility[c]	CF-HNS
Glandless cottonseed flour—low nitrogen solubility[c]	CF-LNS
Nonfat dry milk—high nitrogen solubility[d]	NFDM-HNS
Fish protein concentrate—low nitrogen solubility[c]	FPC-LNS
Cottonseed protein concentrate—low nitrogen solubility[e]	CSPC-LNS

[a] Chemurgy Div., Central Soy Co., Chicago, Ill.
[b] Griffith Laboratories, Chicago, Ill.
[c] Food Protein Research & Delvelopment Center, Texas A&M University, College Station, Texas
[d] Land O'Lake Creameries, Minneapolis, Minn.
[e] Southern Marketing & Nutrition Research Div., New Orleans, La.

lulose casings and hand-linked on a wire spacer rack to an average length of 12 cm. All of the above operations were performed at a room temperature of 15°C. The smokehouse was preheated at 54°C for 2 hr. Time periods, sources of heat and house temperatures for the processing schedule were as follows: 20 min of dry heat at 54°C; 20 min of dry heat at 65°C; 15 min of 1/3 steam heat and 2/3 dry heat at 82°C; 3 min of steam heat at 82°C; and 15 min of cold water showering. Relative humidity in the smokehouse was between 30 and 50% during the processing cycle. Internal temperatures of the frankfurters after cooking and showering approximated 63°C and 35°C, respectively.

The protein additives listed in Table 2 were added to the control formula prior to chopping so that the finished product would contain them as approximately 3.5% of the total weight of finished frankfurters. Preparation, chopping, stuffing, linking, cooking and smoking procedures were identical to those used for the control batches of frankfurters. The percentages of moisture, fat and protein in nonmeat protein additives, raw meat materials, sausage emulsions and finished frankfurters were determined by AOAC methods (AOAC, 1970). Nitrogen solubility index (NSI) was determined by the method described by Inklaar and Fortuin (1969) and NSI was expressed as the ratio of water-soluble protein to total protein in a sample. The pH value of each protein additive was determined on a slurry of the additive (10g protein additive in 100 ml deionized water) using a Model 12, Corning pH meter.

Water-holding capacity was determined by weighing 1g of each protein additive into a 50 ml plastic centrifuge tube and dispersing it into 30 ml of 3.5% NaCl solution (to approximate the salt concentration of commercial frankfurters). The pH of the dispersion was adjusted to that of the sausage emulsion prepared from that protein additive by the addition of aliquots of either 0.1N HCl or 0.1N NaOH. The centrifuge tube was then agitated for 10 min, heated in a 85°C water bath for 15 min with slow shaking, cooled under running tap water for 10 min and subsequently centrifuged at 5,000 rpm for 15 min at 25°C. The resulting supernatant fluid was decanted and the tube was inverted until it was weighed. An aliquot of 1.5g of each protein additive (0.015g of nonfat dry milk because of its high solubility) was weighed into an aluminum dish and dried for 20 hr at 105°C. Water-holding capacity (WHC) was expressed as the ratio of the wet weight of the additive to the dry weight of the additive.

Emulsifying capacity was determined by a modification of the method of Swift et al. (1961). The method consisted of dispersing 6.7g of the additive into 100 ml of 3.5% salt (NaCl) solution. After shaking for 10 min, the pH was adjusted as previously described, and stirred slowly for 2 min to obtain a uniform dispersion. An emulsion was formed by stirring with maximum speed while corn oil was added at a flow rate of 1 ml per sec. The point at which the emulsion collapsed was determined by use of an electrical device which detected changes in the current flow (in response to changes in viscosity) through the Waring Blendor. Emulsifying capacity was expressed as the number of ml of oil emulsified by 1g of the protein additive. Emulsifying capacities which required less than 100 ml or more than 440 ml of oil for the collapse of the emulsion could not be accurately determined by the device.

Emulsion stability was determined for each of the protein additives by preparing a dispersion of the additive in water with pH adjustments in the manner previously described. 54g of corn oil (the amount of oil necessary to equal 35% in the dispersion) was poured into a Waring Blendor and stirred at maximum speed for 3 min. From the resulting emulsion, 30 ml was poured into a 50 ml centrifuge tube, heated in a 77°C water bath for 40 min and subsequently cooled for 30 min under running tap water. The tube was centrifuged for 15 min at 4,800 rpm and the degree of separation of oil above the supernatant was scored subjectively by use of a 6-point scale (6 = no oil release; 1 = complete oil release).

In order to investigate the behavior of protein additives in a very concentrated emulsion, the following test was conducted with each sample: 20g of protein additive and 30 ml of corn oil were poured into a Waring Blendor with 100 ml of 3.5% salt solution and stirred at maximum speed for 90 sec. Samples of the resulting slurry were examined under a microscope using a magnification of 160 (10× ocular, 16× objective). This test was performed because the ratio of moisture to protein additive in commercially prepared sausage emulsions is considerably lower than that usually used in model systems for testing emulsifying capacity and emulsion stability.

Emulsion stability for low fat-content frankfurter batches was measured by use of the Morrison et al. (1971) modification of the Townsend et al. (1968) procedure. This method involved weighing 30g of emulsion into a glass bottle, heating in a water bath at 75°C for 15 min and decanting the resulting fluid. Emulsion stability is expressed as the ratio of weights before and after cooking. This modification was chosen because of its ability to magnify subtle differences in emulsion stability. The procedure of Saffle and Helmer (1963) was used for high fat-content frankfurter batches. This method includes weighing 9g of emulsion into a modified Paley bottle and heating in a 70°C water bath for 30 min. Emulsion stability is determined by percent of fat separation as read directly from the scale on the neck of the bottle. The latter method was selected because preliminary tests revealed that lower mean differences were obtained between the values obtained from sample replicates.

Degree of fat separation was evaluated for the finished products in each batch. Individual frankfurters were scored by visual evaluation conducted by a 2-member panel by use of an expanded 6-point scale (60 = no fatting-out; 10 = extreme fatting-out). Three frankfurters were randomly selected for vacuum packaging (polyvinylidene chloride–polyvinyl chloride bags) and subsequent cooking for 15 min at 88°C. The average percentage of weight loss (measured to the nearest gram) was used as a measure

Table 3–Characteristics and functional properties of the protein additives

Protein additive	Total protein[a] (%)	Soluble protein[b] (%)	Nitrogen solubility index[c]	pH[d]	Water-holding capacity[e]	Emulsifying capacity[f]	Emulsion stability[g]
Soy protein isolate (HNS)	89.7	55.1	61	6.7	6.5	32	6.0
Soy protein isolate (LNS)	90.8	2.9	3	5.2	5.4	46	6.0
Soy flour (HNS)	50.7	35.8	71	6.7	6.1	>66	6.0
Soy flour (LNS)	50.2	2.4	5	6.7	4.9	<15	2.0
Soy protein concentrate (HNS)	67.2	27.3	41	6.9	5.9	>66	5.5
Soy protein concentrate (LNS)	66.2	3.4	5	6.6	4.7	<15	4.5
Glandless cottonseed flour (HNS)	52.6	15.6	30	6.6	6.8	>66	6.0
Glandless cottonseed flour (LNS)	54.3	5.8	11	6.6	5.5	49	4.0
Nonfat dry milk (HNS)	35.4	34.2	99	6.7	4.7	61	5.5
Fish protein concentrate (LNS)	84.6	1.2	1	6.4	4.8	<15	1.0
Cottonseed protein concentrate (LNS)	62.5	10.5	17	6.5	5.7	>66	5.0

[a] Determined by use of the macro-Kjeldahl method of AOAC (1970).
[b] Determined by the method of Inklaar and Fortuin (1969).
[c] $NSI = \frac{\%\ soluble\ protein}{\%\ total\ protein} \times 100$
[d] By use of a 10% dispersion of protein additive in deionized water
[e] Ratio of wet weight to dry weight of the protein additive
[f] The number of ml of corn oil emulsified by 1g of the protein additive
[g] Scored by use of a 6-point scale (6 = no oil release; 1 = complete oil release).

of cooking stability. Weight losses were assumed to result from both moisture and fat release. Each batch of low fat-content frankfurters was evaluated for color by use of a 9-point rating scale (9 = like extremely; 1 = dislike extremely) and for peelability using a 6-point scale (6 = extremely easy to peel; 1 = extremely difficult to peel).

Consumer evaluations of cooked frankfurters of low fat content were conducted by use of a 30-member untrained panel. The panelists evaluated the color, texture, juiciness, flavor, and overall satisfaction of the frankfurters according to a 9-point rating scale (9 = like extremely; 1 = dislike extremely). Comparisons of emulsion stability for frankfurter batches were made by comparative rank because different methods (inversely related with regard to stability and numerical magnitude) were used for determining stability in the two treatment groups. Furthermore, the differences in stability between treatment groups within a serial set of batches depended largely on the fat content in the emulsion. Relationships between the rankings from frankfurters of different fat contents were determined by the Spearman's rank correlation method (Ostle, 1963).

RESULTS & DISCUSSION

CHARACTERISTICS and functional properties of the 11 protein additives are presented in Table 3. Nitrogen solubility index (NSI) was highest for NFDM-HNS and SF-HNS and lowest for SPI-LNS, SF-LNS, SPC-LNS and FPC-LNS. High nitrogen solubility (HNS) samples of soy flour, soy protein concentrate and glandless cottonseed flour had higher values for nitrogen solubility index (NSI), water holding capacity, emulsifying capacity and emulsion stability than did their counterpart samples (SF-HNS vs SF-LNS, SPC-HNS vs SPC-LNS and CF-HNS vs CF-LNS). Such was not the case for sample of soy protein isolate (SPI-HNS vs SPI-LNS) when comparing emulsifying capacity and emulsion stability. If SPI-HNS and SPI-LNS were not considered, higher NSI values among the 11 protein additives were generally associated with higher values for emulsifying capacity and emulsion stability. FPC-LNS had the lowest NSI and among the lowest values for water-holding capacity, emulsifying capacity and emulsion stability.

Simple correlation coefficients between certain characteristics and functional properties of protein additives are presented in Table 4. With the exception of its relationship ($P < 0.05$) to water-holding capacity, NSI evidenced little relationship to the other characteristics or functional properties of protein additives. The latter result was not unexpected since NSI is indicative of water soluble protein content, while Swift et al. (1961) and Swift and Sulzbacher (1963) have demonstrated the superior emulsifying characteristics of salt soluble proteins.

Characteristics of the 11 batches of frankfurters containing less than 30% fat are presented in Table 5. Moisture content of frankfurters differed little among batches and, with the exception of CF-LNS, closely paralleled corresponding percentages of processing shrinkage. Frankfurters containing nonfat dry milk had the lowest shrinkage and those containing cottonseed flour (LNS) had the highest percent shrink. The batch containing SPI-LNS had the lowest emulsion pH (reflecting the acidity of the additive alone, Table 3) and the lowest cooking yield. The pH values of all of the other emulsions were higher than that of the control batch (Table 5) and emulsion pH generally paralleled the pH of the additives alone (Table 3). Cooking yield is determined as the percent of solid remaining after cooking and low cooking yields are believed to result from low stability of the emulsion (see results in Table 7). The most desirably colored frankfurters were those containing SPI-LNS. This finding may have resulted from the low pH of this protein additive (Table 3), since low pH enhances the reduction of nitrate and nitrite to nitric oxide and thus accelerates the formation of nitrosomyoglobin. The peelability of frankfurters is more closely related to processing technique than to emulsion characteristics (Saffle et al., 1967), but in the present study those frankfurters containing SPI-HNS, SPI-LNS, SF-LNS and SPC-LNS were the most difficult to peel.

Organoleptic ratings for cooked frankfurters from each test batch are presented in Table 6. Composite scores indicate that frankfurters prepared from SPI-LNS, SPC-LNS and FPC-LNS were considerably less desirable than control frankfurters. The emulsion containing SPI-LNS was very low in stability (Table 7). Consequently, the finished products had sustained greater losses of moisture and fat during cooking and consumer acceptability was lowered. None of the batches containing protein additives had higher composite scores than control samples. Mean separation analyses for overall satisfaction ratings revealed that frankfurters containing SPI-HNS, SPI-LNS, SPC-HNS, SPC-LNS and FPC-LNS were less satisfactory than were control frankfurters (Table 6). The undesirable flavor of frank-

Table 4—Simple correlation coefficients between certain characteristics and functional properties of protein additives

Trait	Water-holding capacity	Emulsifying capacity	Emulsion stability
Nitrogen solubility index	0.65*	0.51	0.52
Water holding capacity		0.52	0.53
Emulsifying capacity			0.83**

** $P < 0.01$
* $P < 0.05$

Table 5-Characteristics of 11 batches of low fat-content frankfurters

Protein additive	Emulsion pH[a]	Processing shrinkage[b] (%)	Moisture[c] (%)	Cooking yield[d] (%)	Color score[e]	Peelability score[f]
SPI-HNS	6.4	8	55	99	5.5	3.0
SPI-LNS	6.0	9	54	86	7.5	3.2
SF-HNS	6.4	10	54	95	4.5	5.2
SF-LNS	6.3	10	53	94	5.0	3.5
SPC-HNS	6.4	10	54	92	6.5	5.3
SPC-LNS	6.4	6	57	96	5.0	3.3
CF-HNS	6.4	10	54	94	5.5	5.1
CF-LNS	6.3	11	57	94	4.5	5.5
NFDM-HNS	6.5	6	56	94	5.0	5.0
FPC-LNS	6.3	8	55	94	2.0	5.5
None	6.2	8	55	96	5.7	5.4

[a] Measured by use of a 10% dispersion of each sausage emulsion in deionized water
[b] Percent weight loss during processing in the smokehouse and storing in a 4.5°C cooler for 16 hr
[c] Percent moisture in the frankfurters as determined by AOAC (1970) method
[d] Percent weight remaining after cooking vacuum packaged frankfurters in water for 15 min at 88°C.
[e] Subjectively scored prior to peeling according to a 9-point scale (9 = like extremely; 1 = dislike extremely)
[f] Scored according to a 6-point scale (6 = extremely easy to peel; 1 = extremely difficult to peel)

furters containing SPI-LNS, SPC-LNS and FPC-LNS was primarily responsible for the low overall satisfaction ratings. Frankfurters containing soy flour were rated higher in organoleptic quality than those containing either soy protein isolate or soy protein concentrate. None of the protein additives increased organoleptic quality compared to control frankfurters based on consideration of scores for juiciness, flavor or overall satisfaction.

Effects of protein additives on the stability of frankfurters differing in fat content are presented in Table 7 and simple correlation coefficients between emulsion stability, visual score and cooking stability are presented in Table 8. These data indicate that higher values for emulsion stability are not necessarily indicative of more desirable appearance in finished frankfurters. Emulsion stability was significantly related ($P < 0.01$) to cooking stability and thus to yields of the finished frankfurters (Tables 7 and 8).

Comparative rank and rank correlation coefficients for emulsion stability are presented in Table 9. While no significant correlation ($r = 0.27$) was observed between the ranking of emulsion stability between the two series of low fat-content frankfurters, there was very good agreement ($r = 0.80$) between the results among high fat-content frankfurters. Among frankfurters containing less than 30% fat, and when used at the level of 3.5% in the finished product, the protein additives appeared to have little influence upon emulsion stability. Among frankfurters with more than 30% fat, SPC-LNS and FPC-LNS increased the stability of emulsions. The effect of soy protein flour of either high or low nitrogen solubility upon the stability of the emulsions was not consistent. Emulsions containing SPI-LNS ranked lowest in stability in every comparison, which may have resulted from the exceptionally low pH (5.2) of this protein additive. The extractability of the salt-soluble protein of muscle contributes significantly to the stability of sausage emulsions (Hegarty et al., 1963) and may be lowered considerably (Trautman, 1966) during comminution. In the present study, the pH at the end of the first 2 min of chopping in the silent cutter was 5.3. The assumed relationship between pH and stability was verified in this laboratory in association with studies of "simulated frankfurters" prepared by replacing meat proteins with textured soy protein isolate of pH 5.3. When the pH of the soy protein isolate was increased to 7.0 by the addition of a mixture of phosphates (i.e., 30% of sodium pyrophosphate and 70% of sodium metaphosphate) at the level of 0.5% of the total batch weight, the stability increased and consumer acceptability was also enhanced

Table 6—Mean organoleptic ratings and composite scores for each batch of low fat-content frankfurters

Protein additive	Organoleptic rating[a]					Composite score[b]
	Color	Texture	Juiciness	Flavor	Overall satisfaction	
None	5.9	6.4	7.1	7.1	6.9[c]	33.4
SPI-HNS	5.6	6.7	7.1	6.3	6.3[d]	32.0
SPI-LNS	5.4	3.6	3.4	4.3	3.9[d]	20.6
SF-HNS	5.9	6.1	6.5	6.8	6.8[c]	32.1
SF-LNS	5.7	6.7	6.7	7.1	6.8[c]	33.0
SPC-HNS	5.8	6.8	5.8	6.3	6.0[d]	30.7
SPC-LNS	4.9	5.4	4.9	4.1	4.3[d]	23.6
CF-HNS	6.0	6.1	6.7	6.6	6.7[c]	32.1
CF-LNS	5.9	6.5	6.6	6.8	6.8[c]	32.6
NFDM-HNS	6.0	6.1	7.0	6.9	6.8[c]	32.8
FPC-LNS	5.1	4.3	4.8	3.5	3.6[d]	21.3

[a] Scored according to a 9-point scale (9 = like extremely; 1 = dislike extremely) by a 30-member untrained panel
[b] Composite score was determined by combining the numerical ratings for color, texture, juiciness, flavor and overall satisfaction
[c,d] Means bearing different superscripts differ significantly ($P < 0.01$)

Table 7—Effect of protein additives on the stability of frankfurters differing in fat content

Sample	Low fat-content emulsions				High fat-content emulsions					
	23.9 ± 0.5% fat		26.4 ± 0.5% fat		34.3 ± 0.5% fat			35.1 ± 0.5% fat		
	Emulsion stability[a]	Visual score[b]	Emulsion stability[a]	Visual score[b]	Emulsion stability[c]	Cooking stability[d]	Visual score[b]	Emulsion stability[c]	Cooking stability[d]	Visual score[b]
SPI-HNS	92	56	86	25	26	88	40	65	68	12
SPI-LNS	67	45	77	36	61	72	40	82	53	11
SF-HNS	89	55	92	48	4	94	55	60	72	29
SF-LNS	87	50	87	43	36	77	45	56	67	36
SPC-HNS	77	55	91	55	60	74	40	71	65	27
SPC-LNS	91	50	92	30	7	85	55	58	77	32
CF-HNS	88	50	86	25	44	87	50	73	61	26
CF-LNS	87	48	90	55	51	82	40	72	65	28
NFDM-HNS	85	50	90	50	25	84	57	71	61	39
FPC-LNS	88	55	93	50	3	93	59	37	86	30
CSPC-LNS	–	–	–	–	13	90	50	62	66	27
Control	92	55	87	53	9	81	57	59	78	55

[a] Calculated as $\frac{\text{Weight of emulsion after cooking}}{\text{Weight of emulsion before cooking}} \times 100$ by the modified method of Townsend et al. (1968)
[b] Scored by the use of a 6-point scale ranging from 10 = extreme fatting-out to 60 = no fatting-out
[c] By use of the method developed by Saffle et al. (1967) and expressed as percent of the total fat in the emulsion which was released upon heating
[d] Calculated as $\frac{\text{Weight of frankfurter after cooking}}{\text{Weight of frankfurter before cooking}} \times 100$

because much more fat and moisture were retained upon cooking than was the case for control frankfurters.

In order to increase the stability of an emulsion, protein additives must be able to contribute to stability without competing for available moisture with the muscle proteins. In the emulsion systems used in manufacturing frankfurters the ratio of the protein additives to the available moisture necessary for them to form "classical emulsions" is by far lower than optimum. Ratios of 15:1 or 20:1 (moisture:protein) have been utilized to determine the functional properties of protein additives in laboratory model systems by previous workers (Swift et al., 1961). In the present study, the amount of moisture was not a limiting factor for determining "empirical" functional properties. However, when actual sausage emulsions were prepared the ratio of the weight of total added moisture to that of the protein additives approximated 5:1. Within this concentrated system the empirical functional properties previously determined (Table 3) were of little predictive value for estimating the actual contribution of protein additives to the stability of the frankfurter emulsion.

Based on the present study, the conclusions previously advanced by Pearson et al. (1965) and Inklaar and Fortuin (1969) could not be confirmed. On the contrary, the fish protein concentrate which was expected originally to have the poorest emulsification properties appeared to contribute most to increasing stability. Protein additives other than FPC and SPC of low nitrogen solubility, had generally detrimental or no distinct effects upon the characteristics of the resulting emulsions.

The stabilizing mechanism of fish protein concentrate and soy protein concentrate of low nitrogen solubility is assumed to occur in the manner described below. It is possible that very effective stabilization against coalescence of an emulsion can be attained by using certain very finely divided powders. The chemical nature of the particles may not be as critical as their surface properties. According to Kitchener and Mussellwhite (1968), the requirements to perform such a function are: (1) that the particle size must be very small compared with the droplet size; (2) that the particle must exhibit a substantial angle of contact at the 3-phase oil-water/solid) boundary; and (3) that the hydrophilic-lipophilic balance of the solid must be adequate to allow it to form a solid phase between water and oil.

Table 8—Simple correlation coefficients between emulsion stability, visual score and cooking stability for certain frankfurter preparations

Fat content	Fat level (%)	Relationship	Correlation coefficient
Low	23.9 ± 0.5	ES[a] vs VS[b]	0.69*
Low	26.4 ± 0.5	ES vs VS	0.39
High	34.3 ± 0.5	ES vs CS[c]	–0.77**
		ES vs VS	–0.49
High	35.1 ± 0.5	ES vs CS	–0.92**
		ES vs VS	–0.37

[a] ES = emulsion stability
[b] VS = visual score
[c] CS = cooking stability
** P<0.01
* P<0.05

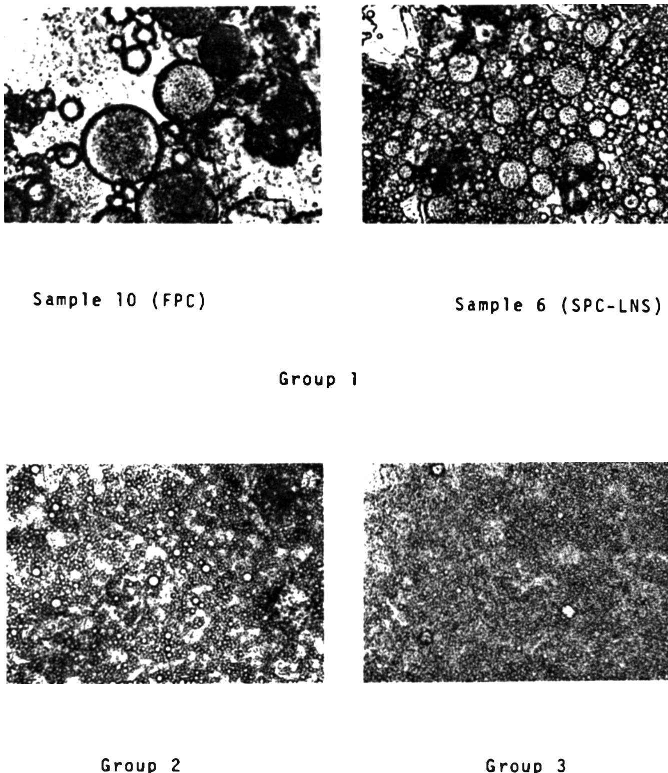

Fig. 1—Classification of the protein additives according to their appearance under the microscope (160× magnification).

Table 9—Comparative rank and rank correlation coefficients for emulsion stability

	Comparative rank according to emulsion stability			
Protein additive	24% fat	26% fat	34% fat	35% fat
SPI-HNS	1	9	7	7
SPI-LNS	11	11	12	12
SF-HNS	4	3	2	5
SF-LNS	8	8	8	2
SPC-HNS	10	4	11	9
SPC-LNS	3	2	3	3
CF-HNS	5	10	9	11
CF-LNS	7	5	10	10
NFDM-HNS	9	6	6	8
FPC-LNS	6	1	1	1
CSPC-LNS	–	–	5	6
None	2	7	4	4
$(r_s)^a$	0.27		0.80**	

[a] Spearmans rank correlation coefficient was computed between relative rankings assigned for emulsion stability between frankfurter emulsions with 24 and 26% fat and between frankfurter emulsions with 34 and 35% fat.
** P<0.01

In other words, the interfacial tension between the oil and water phases must be greater than the sum of those between the solid and water phases and solid and oil phases. Such solids provide an interfacial "film" of considerable strength and stability which serves to stabilize such emulsions.

Using subjective appraisals based on appearance under the microscope, samples of the protein additives could be classified into three different groups according to their behavior in the concentrated system. The first group was comprised of FPC-LNS and SPC-LNS and formed emulsions which were stabilized by the solid particles. As can be seen in Figure 1, FPC-LNS formed an emulsion with larger droplets of much higher density of attached protein particles than did SPC-LNS. However, the latter protein additive also evidenced formation of an emulsion with a high concentration of the solid particles. These samples are generally thought to meet the requirements as described above which are necessary for the formation of a "3-phase emulsion."

SPI-LNS, SF-LNS, SPC-HNS, CF-LNS and CSPC-LNS were classified into the second group. These proteins were simply dispersed in the aqueous phase and oil was collected as small droplets. The oil globules had no noticeable amount of solid particles attached to them.

The last group comprises SPI-HNS, SF-HNS, CF-HNS and NFDM-HNS. The protein additives of this group displayed neither any sign of emulsion formation nor immiscible oil droplets within the aqueous phase. Referring again to Table 7, it may be noted that only the samples of the first group (Group I) behaved positively in promoting stability of sausage emulsions. All of the other samples exerted generally detrimental or, at most, no distinct effect upon stability.

It may be hypothesized that the detrimental effects of samples in groups 2 and 3 upon stability resulted from the fact that the protein additives take up some of the water from the matrix of soluble muscle proteins and water by their hydrophilic properties and that the emulsion becomes unstable. On the other hand, the mode of action of the protein additives in Group I (those which stabilized sausage emulsions) assumes that the protein particles (acting as finely divided solids) have the ability to form a "3-phase emulsion" by attaching to the fat globules which are, in turn, encapsulated by the protein-water matrix. The particles are concentrated on the boundary between the internal or oil phase and the external or water phase. Since the solid is wetted by the external phase, interfacial area between internal and external phase would be minimized. When all other factors are identical, it is evident that an emulsion system which has a lower interfacial area for phasic contact will be more stable than one in which this parameter is larger (Fig. 2).

The FPC-LNS used in the present study was sifted through openings of 177 micron, 80 mesh. Ackerman et al. (1971) have suggested that the presence of fat globules 200 microns or more in diameter is indicative of emulsion instability. Since 177 microns is actually large in relation to the size of fat globules found in frankfurters (Borchert et al., 1967). this may suggest an alternative hypothesis for the mechanism of FPC-LNS in stabilizing emulsions which would be based on prevention of coalescence of fat droplets. The fact that the proposed solid-phase mechanism failed in a more dilute situation (measures of emulsifying capacity) may lend credence to the alternative hypothesis.

Unfortunately, both SPC-LNS and FPC-LNS significantly decreased the organoleptic quality when they were used as additives at the level of 3.5% in the finished products (Table 6) and their use in frankfurter emulsions would not be recommended.

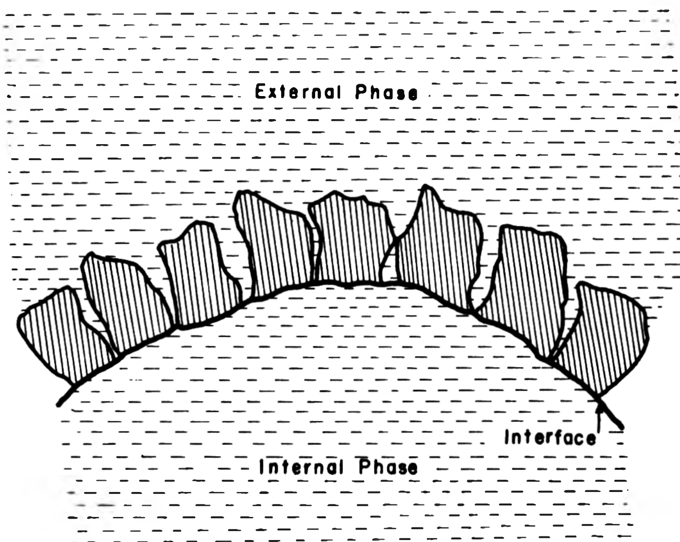

Fig. 2–Stabilization of an emulsion by finely-divided solids in which solid particles in the external phase reduce the interfacial contact area (Becher, 1956 citing original research by Thomas, 1927).

CONCLUSIONS

ON THE BASIS of these studies the following conclusions were reached:

(1) Comparisons of functional properties of protein additives (i.e., water-holding capacity, emulsifying capacity and emulsion stability) with NSI values under pH and salt concentration conditions similar to those of actual frankfurter emulsions revealed that higher NSI was not clearly indicative of the ability to form a stable emulsion. Moreover, higher values for emulsion stability were not necessarily indicative of more desirable appearance in finished frankfurters.

(2) Frankfurters prepared from SPI-HNS, SPI-LNS, FPC-LNS, SPC-HNS and SPC-LNS were less desirable in palatability than control frankfurters. Frankfurters prepared from FPC-LNS had unsatisfactory color and those prepared from SPI-HNS, SPI-LNS, SF-LNS and SPC-LNS were difficult to peel in relation to control frankfurters. Correspondingly, evaluations of organoleptic and processing characteristics, as well as emulsification properties, must be considered in selecting protein additives for frankfurter manufacture.

(3) Among frankfurters with less than 30% fat, the protein additives seemed to exert little influence upon stability. Among high fat-content frankfurters, the data indicated a significant influence of protein additives upon emulsion stability. It appears that emulsion stability is very sensitive to low pH, since conditions of low pH bring the salt soluble proteins nearer their isoelectric point and thereby decrease their solubility.

(4) Microscopic observations led to the hypothesis that very small particles (as finely-divided solids) can play a role in emulsion stabilization based more on their physical state than on their solubility. An alternative mechanism (solids preventing coalescence of fat droplets) may also be involved in explaining the effect of certain protein additives.

(5) It is possible that the detrimental

effect of certain protein additives on emulsion stability results from their competition for available moisture with the matrix of soluble meat proteins and water surrounding fat globules.

(6) These data support the belief that muscle proteins are excellent emulsion stabilizers and that they are better than most of the vegetable proteins offered to the trade for that purpose.

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BATCH DRY RENDERING: AN INVESTIGATION OF HEAT TRANSFER TO BOILING WATER/TALLOW EMULSIONS

INTRODUCTION

IN AN INVESTIGATION of batch dry rendering at a Melbourne abattoir, Herbert and Norgate (1971) found that the measured heat transfer coefficients were at a maximum at the start of a cooking cycle, decreased rapidly at a given stage of evaporation and then assumed a low value which was maintained until the end of the cook.

Herbert and Norgate (1971) postulated that the initially high coefficients resulted from the evaporation of water from a water/tallow emulsion in which water was the continuous phase. Progressive evaporation of water compacted the emulsion until it inverted to tallow-continuous. The rapid decrease in the overall coefficient was attributed to the inversion process, and the final low coefficients taken as characteristic of heat transfer to a tallow-continuous system.

The difficulties of identifying the composition and nature of a liquid phase in the dry rendering cooker made impracticable an "in situ" evaluation of their hypothesis. Furthermore, a literature search produced no direct evidence against which the hypothesis could be tested. Consequently, it was decided to examine the general problem of heat transfer to boiling emulsions in a simplified laboratory apparatus and to correlate the results with those from the batch dry rendering investigation.

EXPERIMENTAL

THE CONSTRUCTION of the laboratory heat transfer vessel is shown in Figure 1. The vertical, coil-heated design avoided the analytical difficulties of a variable heat transfer area as encountered in the horizontal, jacket-heated cookers used for commercial dry rendering operations (Herbert et al., 1971).

The auxiliary equipment layout is shown in Figure 2. Steam pressure to the silver heating coil was maintained at the set pressure by means of a pneumatic controller. Heat losses from the apparatus were minimized by the use of electrically heated tapes and fiberglass insulation on all exposed hot surfaces. Temperatures were measured by copper-constantan thermocouples, located as shown. The electrical conductivity probe was operated at 1000 Hz to avoid electrode polarization.

Materials

The oil phase used was an inedible beef/mutton tallow, produced at the same Melbourne by-products plant at which the full scale dry rendering cooker investigated by Herbert et al. (1971) was installed. The physical properties are given in Table 1.

The water phase was deionized water made electrically conducting by the addition of 1g/liter of sodium nitrate.

Procedure

The emulsions were formed in the heat transfer vessel by first adding a known weight of water, setting the stirrer to the required speed, warming the water to approx 140°F and then pouring in a known weight of molten tallow. After closing the charging port, steam was re-admitted to the heating coil and the run assumed to start from the collection of the first drop of distillate; thereafter distillate volume was read at regular intervals. Simultaneous recordings were also made of the vessel temperatures, steam coil inlet temperatures and the electrical conductivity of the emulsion. The runs lasted from 30–70 min and were terminated when either all, or a predetermined volume, of the water had distilled over. Between

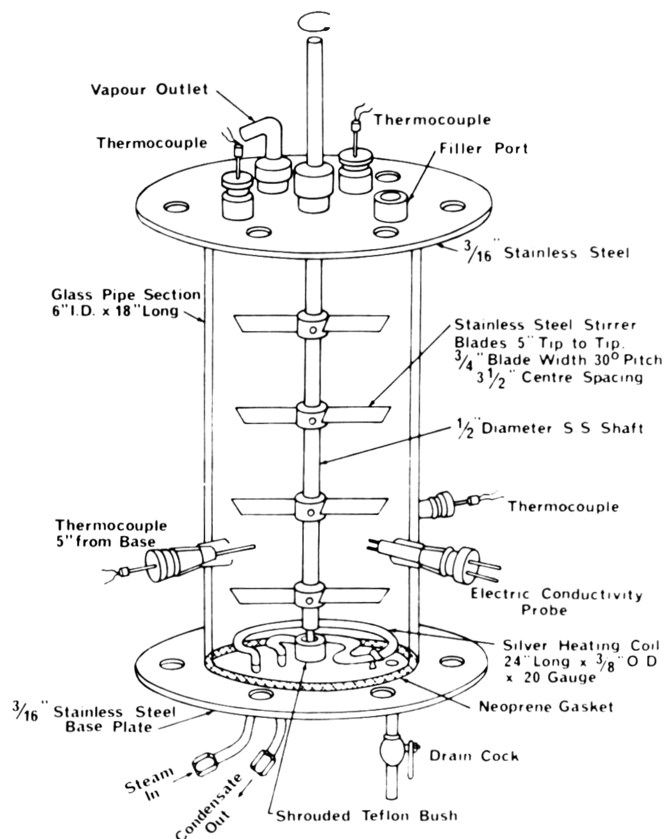

Fig. 1—Heat transfer vessel.

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runs, the vessel was cleaned by boiling out with deionized water for 2 or 3 min at a high stirring rate.

The present results relate to runs carried out at a constant stirring speed (440 rpm) and constant steam coil pressure (20 psig). The emulsion composition range from 0–100% water was covered by a series of overlapping runs, terminated by a run in which deionized water only was evaporated.

Accuracy of primary measurements

Emulsion temperature: ± 0.4°F

Steam coil temperature: ± 0.4°F

Steam coil pressure: ± 0.25 psig

Electrical conductivity: 1% of full scale deflection

Stirring speed: ± 5 rpm

Condensate volumes: ± 5 ml

Weight of water and tallow charged to the heat transfer vessel: ± 1g

Evaluation of results

Relevant data from the runs, (such as steam coil temperature, emulsion temperature, emulsion electrical conductivity, volumes of tallow and water charged and volume of distillate collected), were punched onto cards and processed by PROGRAM TTRAN on a CDC 3200 computer. Print out included time averaged values of the overall heat transfer coefficient, the electrical conductivity ratio (conductivity at time Θ/conductivity at time zero) and the composition of the remaining emulsion. In the heat transfer calculations account was taken of the measured heat loss from the apparatus, averaging 770 Btu/hr over a wide range of experimental conditions.

Overall heat transfer coefficient, U_o, is defined as:

$$U_o = \frac{Q}{A(t_s - t_e)}$$

where A = external area of silver heating coil (ft²); Q = total heat load across the coil (Btu/hr); t_s = temperature of steam at inlet to heating coil (°F); t_e = temperature of emulsion (°F); and U_o = overall heat transfer coefficient (Btu/ft² hr°F).

Table 1—Physical properties of inedible beef/mutton tallow

Property	Value
Softening point	39°C
Melting point	45–46°C
Density, g/ml	0.860 (100°C) 0.872 (80°C) 0.880 (60°C)
Kinemetic viscosity (centistokes)	14.6 (63°C)
Acid value (mg KOH/g sample)	0.86

RESULTS & DISCUSSION

EXPERIMENTALLY determined values of U_o, the electrical conductivity ratio and the emulsion boiling temperatures are plotted as functions of emulsion composition in Figure 3 (a)–(c).

For ease of discussion, the results have been divided into four regions, each region representing different ranges of emulsion composition.

Region I (92–100 wt% water)

When 8 wt% tallow was added to boiling water, U_o decreased from 2280 to approx 1200 Btu/ft² hr °F. The magnitude of the decrease suggested some effect associated with the surface of the silver heating coil and support for this viewpoint was found in the work of Jakob (1958), who studied the mechanism of steam bubble formation on wetted and nonwetted surfaces. He found that bubbles formed on wetted surfaces (such as clean chromium plate) were small and became readily detached from the surface, leading to a high rate of heat transfer. However, if the surface was made nonwetting by smearing it with a thin layer of oil, the bubbles became much larger. They spread over the surface to form a vapor blanket between the surface and the bulk of the liquid and hence the rate of heat transfer decreased. Applying this to the present system, the addition of tallow to water presumably converted the silver coil surface from wetting to nonwetting. The nature of the bubble formation mechanism was thus changed and the value of U_o correspondingly reduced.

The small amount of tallow had no significant effect upon either the boiling point or electrical conductivity of the system, both remaining the same as for boiling water alone.

Region II (42–92 wt% water)

Evaporation of the emulsion from 92 wt% water to 42 wt% water proceeded at substantially constant values of heat transfer rates, electrical conductivity and boiling temperature. Physically, Region II corresponded to a system of free flowing, spherical tallow droplets suspended in a continuous medium of water. Evaporation was by direct vaporization of water at the coil surface. The apparent constancy of the electrical conductivity ratio in Region II (and also part of Region III, see below) may be explained in terms of two contra-acting processes:

(1) The removal of water by evaporation which increased the concentration of the salt in the remaining water phase and hence increased the absolute conductivity of the water phase.

(2) The removal of water, however, also increased the relative percentage of tallow remaining in the emulsion and consequently increased the length of the electrical pathway between the electrodes of the conductivity probe. Thus, at a fixed voltage, the apparent conductivity of the emulsion would have decreased.

Taken together, (1) and (2) apparently maintained a state of equilibrium such that the measured conductivity ratio for this region remained substantially constant.

Region III (13–42 wt% water)

Progressive evaporation of water at water contents below 42 wt% was accompanied by a decrease in heat transfer coefficient; the electrical conductivity ratio

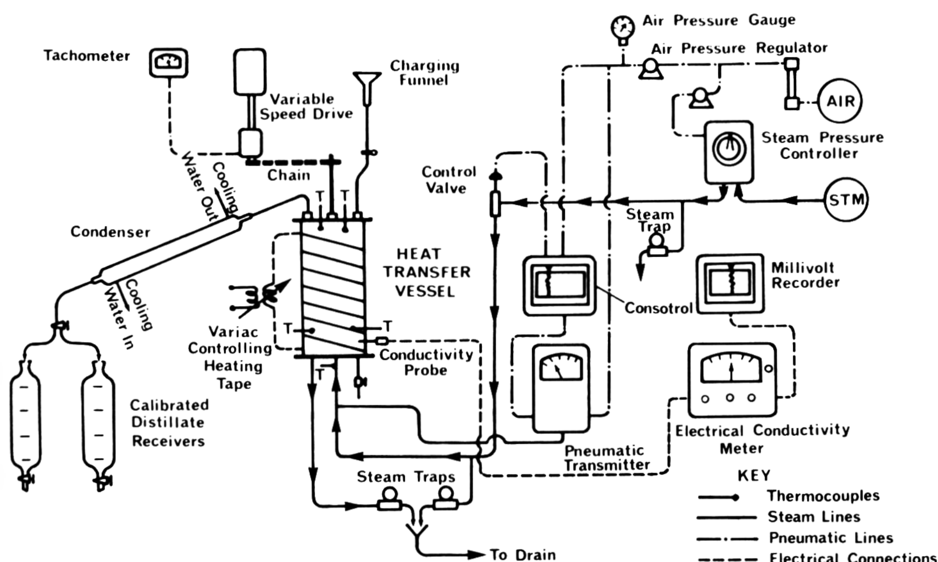

Fig. 2—Auxiliary equipment layout.

remained constant at water contents greater than 20 wt% but at lower water contents, decreased rapidly to a value approaching zero at 13 wt%. Emulsion temperatures remained at 212°F until the water content reached 16 wt%, and increased thereafter.

These observations are consistent with the assumption that below 42 wt% water content the emulsion progressively changed from the free flowing system of Region II into a packed emulsion, in which the tallow droplets packed into an arrangement of polyhedra separated by a film of water (Sherman, 1968). The emulsion was stabilized by the proteinaceous material present in the original tallow and remained water continuous until the water content had dimished to approx 16 wt%, when a rapid decline in conductivity ratio indicated that the emulsion had inverted to tallow-continuous.

Packing of the emulsion led to an increase in viscosity and consequently a decrease in heat transfer coefficient (Rohsenow, 1952).

For values of water content down to 16 wt%, the emulsion existed in a state of dynamic equilibrium in which the surface tension forces promoting coalescence of the dispersed tallow phase were balanced by the effects of agitation continuously creating new tallow droplets (Herbert et al., 1970). However, as the water content decreased below 16 wt%, the coalescence forces gradually predominated and the emulsion inverted from water-continuous to tallow-continuous. The inversion process was not instantaneous, and over the range of 16–13 wt% water content, both tallow-continuous and water-continuous emulsions were observed to co-exist in the heat transfer vessel.

Region IV (less than 13 wt% water)

At water contents below 13 wt% the emulsion was tallow-continuous, i.e., it existed entirely as water droplets suspended in tallow. Further evaporation increased the temperature of the emulsion until, at zero water content, the tallow temperature approached steam coil temperature (260°F).

The elevation of emulsion temperature in this region was attributable to the change in heat transfer mechanism accompanying the inversion. In tallow-continuous systems, the tallow served as a heat transfer medium between the heating coil and the suspended water droplets. Since the boiling point of the water remained the same after inversion as before, the temperature of the tallow (and hence of the average emulsion) must inevitably have risen for boiling to continue.

The value of the overall heat transfer coefficient in these circumstances would be largely governed by the rate of convective heat transfer from the heating surface to the tallow. It was found to remain virtually constant for the whole of the tallow-continuous region and be lower than the values obtained for the water-continuous systems.

The other major change on inversion was in the electrical conductivity. Tallow is a poor electrical conductor and, after inversion, the conductivity of the emulsion became near zero. This served as a useful index in characterizing the completion of the inversion process.

Comparison with plant data

Results from one of the full scale batch rendering plant runs are shown in Figures 4 (a)–(c). The methods of operating the cooker and evaluating the data were basically those described by Herbert and Norgate (1971). In the run described here, the cooker was not pressurized, i.e., the batch was cooked out under 'open vent' and the electrical conductivity of the cooker contents could be measured by means of a probe inserted flush with the inside wall of the cooker. The cooking charge totalled 5872 lb, comprized equally of hard and soft offal and contained 50.4 wt% water.

Comparing Figures 3 and 4, it can be seen that the curves of plant scale heat transfer coefficients and electrical conductivities versus wt% water are similar to those observed in Regions III and IV of the laboratory results. Although the absolute values of the overall heat transfer coefficients in the two pieces of equipment are different (because of the high thermal conductivity of the silver heating coil in the laboratory apparatus when compared with the mild steel shell and shaft heaters in the cooker), it nevertheless appears that the heat transfer mechanisms in both the cooker and laboratory apparatus follow part of the same pattern. Thus, a water-continuous 'packed' emulsion is initially formed as the liquid phase in the early stages of cooking, when tallow and water are liberated from the offal. Evaporation of the water leads to further packing and a decline in heat transfer rates. After inversion, the heat transfer coefficient becomes nearly constant and is governed by the rate of convective heat transfer from the heated sections of the cooker to the tallow.

As a corollary, it can be further deduced that liquid phase properties are the dominant factor in characterizing the rate of heat transfer in batch dry rendering. Because of the marked dependence of the overall heat transfer coefficient on liquid phase properties, it seems unlikely that

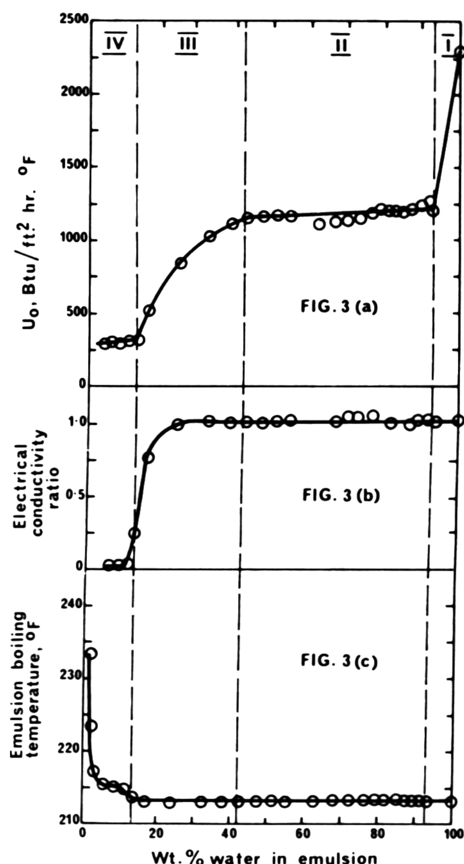

Fig. 3–Heat transfer to boiling water/tallow emulsions–Laboratory apparatus.

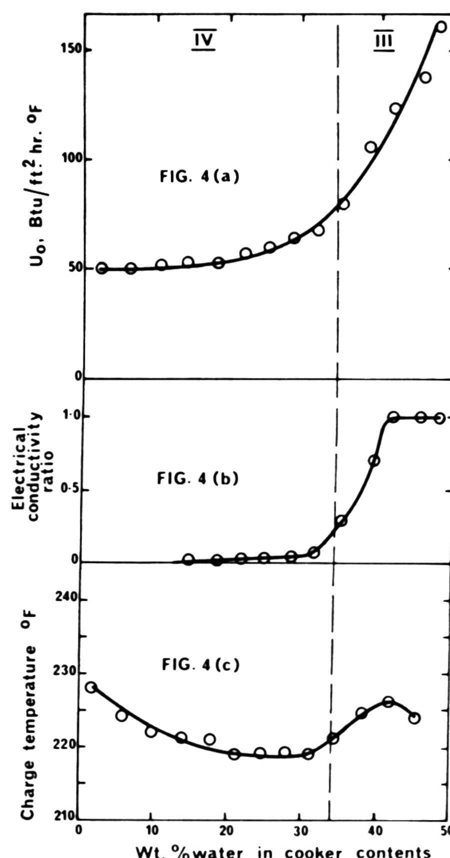

Fig. 4–Heat transfer to boiling water/tallow emulsions–Commercial dry rendering cooker.

direct heat transfer from the heated wall of the cooker to the offal solids plays a significant part in the heat transfer mechanism occurring in batch dry rendering. The offal solids appear to affect the heat transfer mechanism mainly in that they have a capacity to hold water and tallow and so influence the liquid phase composition. Hence the water content at the inversion point in the plant run (Fig. 4) was 34% compared to 16% for the laboratory run (Fig. 3). However, if the water content of the solid phase is taken into account, the liquid phase composition at inversion in the plant run was 18%, which is in good agreement with the laboratory result. The bulk of the water contained in the solid particles did not evaporate until all the water in the liquid phase had disappeared.

One respect in which the laboratory and plant results were markedly different was in the temperature response at inversion. In the laboratory apparatus, the average emulsion temperature *increased* on inversion, whereas in the cooker it *decreased*. The explanation for this may be attributed to the method of venting steam from the cooker. The diameter of the vent pipe was such that back pressure was generated, sufficient to elevate the initial boiling temperature of the emulsion to 232°F. When the rate of heat transfer decreased, the volume of steam evolved was reduced causing the back pressure in the line and the boiling temperature of the emulsion to decrease. At inversion, the overall rate of decrease in boiling temperature due to the diminishing back pressure was greater than the rate of increase in temperature due to the inversion. Consequently the emulsion temperature did not increase until the later stages of cooking, when most of the water had been evaporated.

CONCLUSIONS

(a) The rate of heat transfer to boiling water/tallow emulsions depends upon the emulsion composition. It is highest in free flowing, water-continuous emulsions and lower in tallow-continuous emulsions. In stabilized emulsions, the two regions are linked by a packed, water-continuous stage in which progressive reduction of water content rapidly decreases the heat transfer coefficient.

(b) A comparison of laboratory and plant data has shown that a packed, water-continuous emulsion is formed in the initial stages of the dry rendering cycle. Progressive evaporation of this emulsion leads to a decrease in heat transfer coefficient, which continues until the emulsion inverts to tallow-continuous. After inversion, the heat transfer coefficients remain substantially constant at a value determined by the rate of conductive heat transfer between the heated wall of the cooker and the tallow-continuous phase.

(c) As a corollary to conclusion (b), it appears that direct heat transfer from the heated portions of the cooker to the offal solids plays only a minor role in determining overall heat transfer rates in normal batch dry rendering operations.

(d) The inversion from water- to tallow-continuous emulsions can be additionally identified by a rapid decrease in the electrical conductivity of the liquid phase and a small rise in the average emulsion temperature. However, on a plant scale of operation, this latter effect can be obscured by other factors such as the variable back pressure generated in the cooker vent pipe.

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Additional data and results for runs will be supplied on request to the authors.

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HOT WATER AND MICROWAVE ENERGY FOR PRECOOKING CHICKEN PARTS: EFFECTS ON YIELD AND ORGANOLEPTIC QUALITY

INTRODUCTION

IN RECENT YEARS, the increasing demand by consumers for more convenience food products has stimulated the search by marketing specialists for quality food products which require a minimum of preparation time. Thus, the further processing of poultry has increased, and as evidence of this new trend, almost one-fourth of the poultry processors in 1970 had further processing activities (National Broiler Council, 1971).

The further processing of poultry into precooked frozen chicken has stimulated a great interest in the poultry industry. Large quantities of this product are consumed in institutional outlets. The changing market patterns in the poultry industry have given rise to faster and more efficient methods of processing food items. Microwave energy with the rapidity of the method has attracted the attention of many companies involved in the precooking of chicken.

Weight losses during precooking are of great economical concern to poultry processors. The effect of cooking methods on shrinkage, moisture and ether extractable content of broiler legs and thighs was investigated by Mostert and Stadelman (1964). Higher cooking losses were obtained in thighs than legs regardless of the method of cookery. Smith et al. (1966) found that chicken parts precooked by microwave were exceptionally good products. Cooked yield for the microwave cooking of breaded parts was 93% as compared to 86% for the steam cooking. Cooking in microwave oven was reported by Monk et al. (1964) to have considerably less drip losses for both broilers and hens than cooking by boiling or steam pressure.

In determining the best methods for cooking poultry, the organoleptic quality of the product must not be overlooked. Baker and Darfler (1968) showed that flavor appeared to be associated with tenderness and juiciness both in light and dark meats of poultry. There is some disagreement among workers as to the effect of different cooking methods on tenderness of poultry. Mickelberry and Stadelman (1960) reported that microwave cooked chicken was less tender than chicken cooked in a conventional oven. May et al. (1962) also observed that broilers and roasters cooked in a microwave oven had slightly higher shear values than the same class of poultry cooked by a moist heat method. In contrast, Goodwin et al. (1962) found no differences between the microwave and conventional method of cooking on the tenderness of precooked turkey meat. Smith et al. (1966) found that chicken parts precooked by microwave were significantly more tender than chicken precooked with steam. Cipra et al. (1971) reported no significant taste panel differences for tenderness and juiciness of turkey roasts between microwave and gas oven methods. However, meat cooked by microwave possessed a more intense turkey flavor.

Hot water has long been used as a means of cooking food. Continuous and batch hot water cookers have been used by processors for precooking chicken. Some companies have recently changed from water to microwave precooking operations because of the increased savings in time and labor. However, there is little information available comparing microwave and water precooked chicken on the basis of yield and organoleptic quality.

The objectives of this study were: (1) to select an optimum cooking time and temperature combination for cut-up chicken parts precooked by water; (2) to select an optimum cooking time and oven load combination for cut-up chicken parts precooked by microwaves; and (3) to compare the effects of two precooking methods—microwave and water—on the yield and organoleptic quality of poultry meat.

EXPERIMENTAL

Source of chicken

Fresh commercially dressed broilers with a weight range of 908–1021g (2–2 1/4 lb) were used for this study. Carcasses were cut up into eight parts by cutting each half into front and hind quarters and further separating into breast, wing, thigh and drumstick portions.

Cooking time study

Chicken parts of the same type were removed from the refrigerator and placed in water at room temperature for 10 min to establish a constant temperature of approximately 15.6°C. The parts were then removed from the water, blotted dry, weighed and properly labeled for designating the time of removal from cooking.

Using a nylon casted line, the parts were suspended in a water-filled steam-jacketed kettle. Parts were cooked in 85.0, 87.8 and 90.6°C water while the water was mechanically agitated. Two pieces of chicken were removed at various times and immersed in an ice water slush for 5 min to stop the cooking process. Doneness was determined subjectively, but thermocouples were also used to measure endpoint temperature in order that the reliability of this measurement for doneness could be assessed.

This procedure was replicated five times in order to establish approximate cooking times. Three cooking times were chosen for each part in which they were judged done in all five replications, and a total of ten pieces were then cooked to determine the final cooking time.

Cooking times in a home style microwave oven (Kenmore Model 103.9927101, Sears, Roebuck and Co.) were determined for oven loads of one, two, three and four parts. The part or parts were removed from the oven at 1 min intervals, immersed in an ice water slush and judged for doneness. This procedure was replicated sufficiently in order to establish the correct cooking time for the four types of cut-up parts of each of the oven loads.

Yield study

40 parts of each type were weighed and placed in identified stockinettes. The stockinettes were placed in water, and the parts cooked at their appropriate times for each of the three temperatures. The parts were weighed and the percent cooking loss determined.

A total of 40 parts of each type were cooked two at a time in the microwave oven to determine the percent drip and volatile fraction losses. The samples were cooked on glass saucers in order to measure amount of drip loss. Volatile fraction losses were calculated by subtracting drip loss from the total cooking loss.

Organoleptic evaluation

Taste panel evaluations of both white and dark meat and cooking methods were made in midmorning and midafternoon for four consecutive days, resulting in four replications for each type of meat and cooking method.

The cooked product was immediately frozen by immersing in food grade Freon (DuPont Co., Delaware) at a temperature of −30 ± 5°C for 5 min. After 1 week's storage at −24°C, frozen thigh and breast samples were battered and breaded with a commercial product (Batter

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Table 1–Weight ranges, mean weights and standard deviations of broiler parts from 908–1021g carcasses[a]

Part	Weight range (g)	Mean (g)	S.D.
Breast	122–225	163	22
Thigh	114–175	140	15
Drumstick	61–86	76	9
Wing	54–78	66	11

[a] Total of 500 of each type part used in these experiments.

Table 2–Precooking temperatures and times of chicken parts in water[a]

Precooking temp (°C)	Cooking times for parts (min)			
	Breast	Thigh	Drumstick	Wing
85.0	24	34	24	17
87.8	20	28	20	15
90.6	18	22	18	13

[a] All parts cooked at each of these times were judged fully cooked.

G70-14, Golden Dipt. Co., Illinois) and deep-fat fried at 190.6°C for 6 min in a General Electric Mark 313 Deep-Fat Fryer. Coded sampes were scored for flavor, juiciness and tenderness on a 9-point scale by six experienced panelists.

Statistical analyses

Statistical analyses of data were made according to those procedures described by Steel and Torrie (1960). Duncan's new multiple range test (1955) was used to determine whether significant differences occurred between the means.

RESULTS & DISCUSSIONS

Optimum precooking temperature in water

Weight ranges, mean weights and standard deviations of broiler parts used in this study are given in Table 1. Based on the large number of parts studied, a processor could predict, with a fair degree of accuracy, the average weight and variation of these same parts for a carcass of this size.

The times and temperatures for precooking chicken parts in water are summarized in Table 2. These times were chosen because preliminary experiments indicated that they resulted in a highly acceptable product with a completely "done" appearance. Total precooking time decreased for all parts with increase in temperature. However, the thigh sustained a larger decrease in cooking time in comparison to the other parts. Both the breast and drumstick were fully cooked at the same time for all three temperatures.

Measuring internal temperature with thermocouples was not an effective means of evaluating doneness. Although chicken is usually considered fully cooked when an internal temperature of 82.2°C is attained, chicken parts with internal temperatures as high as 87.8°C as measured with a recording potentiometer were observed to be not fully cooked. In contrast, parts judged "done" by subjective determination were frequently recorded in the 76.7–82.2°C range on the potentiometer. This observation appears to indicate that exact anatomical thermocouple placement is necessary for objectively determining doneness in cut-up poultry parts. Goertz and Watson (1964) found similar results with turkey which was considered underdone when cooked to an endpoint temperature of 85°C. Stone and May (1969) also observed this phenomenon when the chicken was cooked to an 88°C endpoint at 121°C.

Total cooking losses for the various cut-up parts precooked in water at 85.0, 87.8 and 90.6°C are presented in Table 3. Cooking losses for the breast were significantly affected ($P < 0.05$) by the three precooking temperatures used; whereas the thigh, drumstick and wing were not affected.

Results of the taste panel evaluations of flavor, juiciness and tenderness of the breast and thigh samples cooked at these temperatures are shown in Table 4.

Taste panel evaluations of white and dark meat indicated no significant difference in palatability due to the three precooking temperatures. Although differences were not significant, the 87.8°C cooking treatment received the best scores for flavor, juiciness and tenderness for both types of meat. Juiciness-tenderness and juiciness-flavor scores were apparently correlated. The overwhelming preference by the panelists for dark meat in this study may be attributed to smaller overall cooking losses and the greater fat content for the thighs in comparison tc other parts.

Due to more favorable palatability scores and yields, 87.8°C was chosen as the optimum cooking temperature to be used in the comparative precooking studies with water and microwave energy.

Effect of microwave oven load on total cooking loss, doneness and cooking time

The effects of microwave oven load on total cooking loss, doneness and cooking time of cut-up breasts plotted in Figure 1 showed a linear relationship between total cooking loss and cooking time for various oven loads at the point of complete doneness. Similar results were observed with the thigh pieces. As oven load and cooking time increased, only a very slight increase in cooking loss was observed for both parts at the point of doneness. However, this relationship did not exist with undercooked parts, and up to the point of doneness, an increase in oven load at the same cooking time gave a corresponding decrease in cooking loss. However, at the stage of total doneness, cooking time appears to become more important in its effect on losses.

The slight differences in cooking loss did not prove to be a restriction in determining the optimum microwave oven load for cut-up chicken parts. In the comparative study, all parts were cooked two

Table 3–Mean[a] percent total cooking loss of cut-up chicken parts precooked in water at 85.0, 87.8 and 90.6°C

Precooking Temp (°C)	Cooking loss (%)							
	Breast		Thigh		Drumstick		Wing	
	Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
85.0	15.7[b]	2.8	12.3[a]	2.7	5.1[a]	1.4	3.5[a]	1.8
87.8	15.3[b]	2.3	12.5[a]	2.8	5.2[a]	1.2	3.6[a]	2.0
90.6	17.2[a]	3.3	12.8[a]	2.4	5.4[a]	1.7	3.7[a]	2.1

[a] Means within a column and without a common superscript are significantly different ($P < 0.05$). Each mean represents 40 observations.

Table 4–Mean[a] taste panel scores[b] for white and dark meat precooked in water in different treatment temperature[c]

	Palatability factors		
	Flavor	Juiciness	Tenderness
Precooking temp (°C)			
85.0	3.02[a]	2.73[a]	3.12[a]
87.8	2.81[a]	2.35[a]	2.88[a]
90.6	2.88[a]	2.62[a]	3.15[a]
Meat type			
White	3.18[a]	3.08[a]	3.40[a]
Dark	2.62[b]	2.06[b]	2.69[b]

[a] Means within a column grouping and without a common superscript are significantly different ($P < 0.01$).
[b] Possible scores ranged from 1 to 9 with lower scores indicating greater acceptability.
[c] Scores in top part of table were obtained from a composite sample of meat cooked at each temperature.

Table 5–Mean[a] percent cooking loss of cut-up chicken parts precooked in a microwave oven and by water

Cut-up parts	Cooking loss (%)					
	Microwave				Water	
	Volatile fraction	Drip	Total		Total	
	Mean	Mean	Mean	S.D.	Mean	S.D.
Breast	17.5	5.0	22.5[a]	3.9	16.0[b]	3.7
Thigh	20.5	5.8	26.3[a]	4.7	13.0[b]	3.3
Drumstick	14.7	5.6	20.3[a]	3.3	5.9[b]	2.1
Wing	15.6	4.1	19.7[a]	3.8	3.9[b]	1.5

[a] Mean totals within a row and without a common superscript are significantly different ($P < 0.01$). Each mean represents 40 observations.

Table 6–Mean[a] taste panel scores[b] for dark and white meat precooked in a microwave oven and by water

Palatability factors	Precooking treatment × type of meat			
	Microwave dark	Water dark	Microwave white	Water white
Flavor	2.12[ab]	1.96[a]	2.79[c]	2.38[b]
Juiciness	2.12[b]	1.62[a]	2.96[c]	2.33[b]
Tenderness	1.96[a]	1.88[a]	3.71[b]	2.00[a]

[a] Means within a row and without a common superscript are significantly different ($P < 0.05$).
[b] Possible scores ranged from 1 to 9 with lower scores indicating greater acceptability.

at a time because of the greater conveniences in handling and shorter cooking times. Cooking time for both the drumstick and wing portions at this oven load was three minutes; whereas, the breast and thigh were cooked for 6 min. All of these times were considerably less than those found for the water precooked parts.

Comparison between microwave energy and hot water for precooking

Results shown in Table 5 reveal the influence of precooking method on the yield of cut-up chicken parts. Total cooking losses were significantly affected ($P < 0.01$) by the methods of precooking. Mean total cooking losses for all four parts precooked in the microwave oven were much greater than those precooked in water. These findings are in agreement with those for various meat products as reported by Kylen et al. (1964). The difference in losses between methods of precooking was much less for breasts than for the other parts. Higher cooking losses were obtained in thighs as compared to drumstick with both methods of precooking.

Volatile fraction accounted for the greatest amount of total cooking loss for parts precooked in the microwave oven. Similar results were reported by Cipra et al. (1971) for microwave cooked turkey roasts.

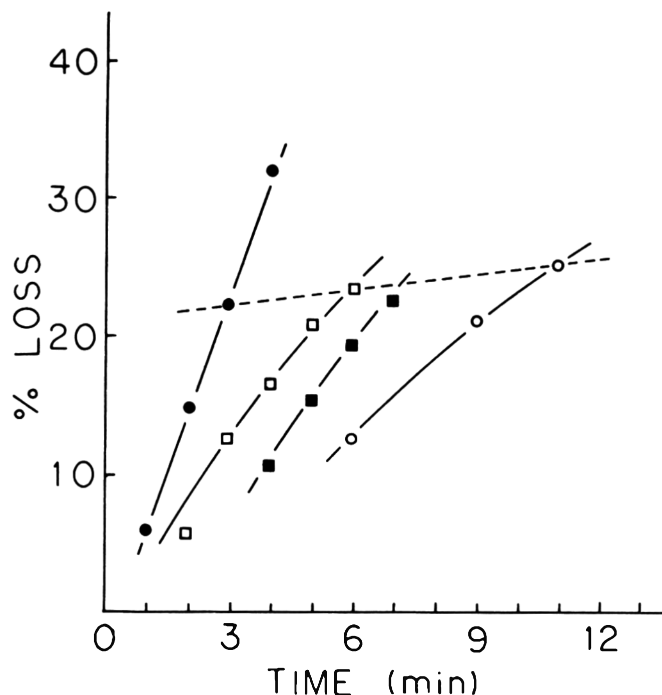

Fig. 1–Effect of microwave oven load on total cooking loss, cooking time and doneness of cut-up chicken breasts. (–●–●– 1 Part; –□–□– 2 Parts; –■–■– 3 Parts; –○–○– 4 Parts; and ------- Observed doneness)

No detectable difference in adhering ability of batter and breading to parts precooked in water as compared to those precooked by microwave was observed.

Results of the taste panel evaluations for flavor, juiciness and tenderness of dark and white meat precooked in water and a microwave oven are given in Table 6. Palatability for both dark and white meat were significantly ($P < 0.05$) affected by method of precooking. Dark meat always received the best scores for flavor, juiciness and tenderness in comparison to white meat. Cash and Carlin (1968) found dark turkey meat to consistently receive higher taste panel scores for both flavor and juiciness than white meat.

Microwave cooked white meat received the lowest acceptability scores than any of the other precooking methods or type of meat combinations. Although not always significant, water cooked dark meat was found to be more acceptable for all of the palataiblity factors evaluated. White meat precooked in water was significantly ($P < 0.05$) more acceptable in flavor, juiciness and tenderness than that cooked in the microwave oven. There was no significant difference between microwave and water cooked dark meat for either flavor or tenderness scores. Juiciness of dark meat was significantly ($P < 0.05$) affected by the precooking treatments. Dark meat precooked in water received the best score.

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AUTOLYSIS AS A FACTOR IN THE PRODUCTION OF PROTEIN ISOLATES FROM WHOLE FISH

INTRODUCTION

THE PRESENCE of proteolytic enzymes in the viscera of fish has been demonstrated in a number of ways. Stern and Lockhart (1953) presented evidence on the occurrence of proteolytic activity at 50°C and approximately pH 8. These workers, as well as Cunningham and Shuttleworth (1950), suggested the use of the fish enzyme as leather bates. The preparation, properties and crystallization of pepsin from the stomachs of tuna was reported by Norris and Mathles (1953).

The enzymes of fish muscle have not been investigated to the extent as the viscera enzymes. Siebert (1962) associated fish spoilage with fish muscle catheptic enzymes. Groninger (1964) also obtained a cathepsin from tuna, and recently Ting et al. (1968) prepared partially purified salmon muscle cathepsins. The preparation of Ting et al. showed optimum enzyme activity at pH 3.7.

Autolytic enzyme activity has been suggested as the reason for lower FPC yields from iced than from fresh fish when the isopropanol extraction procedure was used. (Dubrow et al., 1971). Dubrow and Hammerle (1969) cited evidence of lower FPC yields with increased storage time of comminuted fish in 91% IPA. They attributed the lower yields of FPC to autolytic enzyme activity during storage of fish in the 91% IPA. Koury et al. (1971) studied the catheptic enzyme activity of herring and both inside (Puget Sound) and outside (Pacific Ocean) hake. The whole fish all evidenced proteolytic activity under the experimental conditions used by the workers. Eviscerated herring showed no activity, but eviscerated outside hake had essentially the same pattern of enzyme activity as whole outside hake. Koury et al. (1971) also demonstrated a decreased yield of inside hake FPC, prepared by an IPA extraction process, with increased digestion time of comminuted fish prior to the IPA extractions. Decreases in FPC yield were the greatest during the first 20 min of digestion.

Essentially all of the features ascribed to the autolytic proteolytic enzymes of fish have been undesirable features, other than the potential use of fish viscera or offal for leather bates. Also, on the desirable side are those reports, such as by Lum (1969), in which the autolytic enzymes perform a useful function in producing liquid fish products. Combinations of the autolytic enzymes along with added pre-formed enzymes have been used (Meinke, 1959) to enzymatically eviscerate and clean fish, and to provide the basic technique for the processing of fish for poultry feeds (Keyes and Meinke, 1966).

EXPERIMENTAL

A GENERAL experimental approach was employed in obtaining the experimental data. The general procedure was the same as employed in a previous publication (Meinke et al., 1971) and briefly was as follows:

1. Raw fish or fish fractions were reduced to a hamburger grind by passing through a food chopper fitted with an end-plate drilled with 1/8 in. diameter holes.
2. Weighed quantities of comminuted raw material were slurried in a known volume of extracting medium. The volume of extraction medium was composed of the water (volatile matter) of the raw material, a volume of hydrochloric acid or sodium hydroxide solution used to provide a given pH, and water added to give the desired ratio of raw material to extracting medium. This ratio, in general, was 10g raw material per 100 ml extracting medium.
3. The slurries were agitated at 20°C for 30 min and then centrifuged for 20 min at 2000 rpm (approximately 900-X gravity).
4. The centrifuged supernatant extract was decanted through a 230 mesh stainless steel screen to remove surface scum solids.
5. Volumes of the screened extracts were determined and stored at 5°C for subsequent Kjeldahl nitrogen determinations. Raw materials were subjected to Kjeldahl nitrogen determinations. Throughout this paper the term protein means Kjeldahl nitrogen × 6.25.

Any modifications of this general procedure and definition of raw materials will be provided in subsequent sections of this publication.

RESULTS & DISCUSSION

THE DATA of Figure 1 represent the solubility of carp (*Cyrinus carpio*) protein (Nitrogen × 6.25) as a function of pH. Curve A is for whole ground carp, and Curve B is a calculated composite representation derived from solubility data obtained on eviscerated carp carcass and carp viscera. The carp viscera was composed of the internal organs and gills, and the carp carcass was the remaining fish structure.

As in the previous publication with other species of fish (Meinke et al., 1971), the whole carp protein solubility data gave a characteristic "V" shaped curve with minimum mean protein solubility (mean isoelectric point) between pH 5 to 6. The intercepts of the dashed line extrapolations of the curves of Figure 1 suggest a mean protein solubility at or near pH 5.5. It may be noted that essentially 90% of the carp protein was in solution at pH 3 and 11. Assuming such extracts are adjusted to pH 5.5, it may be estimated that essentially 65% of the carp protein would be recoverable as a crude protein isolate curd and 25% of the fish protein would be present in the whey. The remaining 15% of the fish protein is relegated to the sludge fraction produced when extracts are recovered.

Curve B of Figure 1 is also an expres-

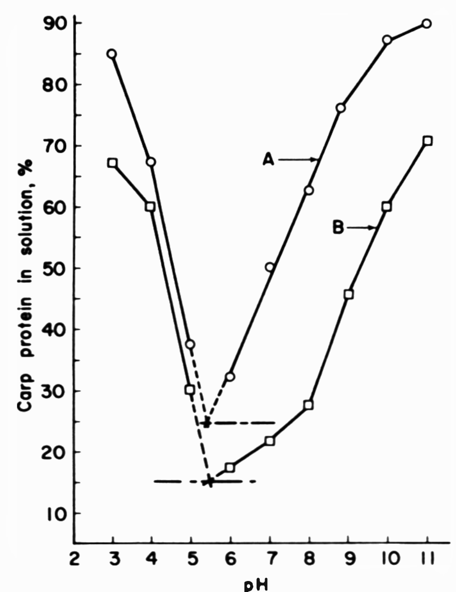

Fig. 1—Solubility of whole carp protein: (a) Whole fish, experimental; (b) Whole fish, calculated from data of Figure 2.

sion of the protein solubility of whole carp protein as a function of pH. By fractionation of the fish, it was possible to establish solubility data for the eviscerated carp in the absence of autolytic viscera enzymes. However, the viscera extractions were in the presence of enzymes. Data for Curve B of Figure 1, as mentioned above, were calculated from the carcass and viscera solubility data (Fig. 2) and the weight percent contribution of each fraction to the whole carp.

It is important to mention that the solubility studies involved two distinct process phases. The sample preparation and sampling stages initiated at 5°C and ended at around 20°C over a period of 1 to 1-1/2 hr. During at least 1 hr, the viscera was intimately mixed with the carcass when whole fish were ground. Thus, autolysis of the comminuted fish, pH 6.6–6.8, was initiated and carried out for approximately 1 hr prior to the 30 min extraction and 30 min centrifuging stages used in the experimental approach. In studies on the eviscerated carcass (Fig. 2), the sample preparation was free of autolytic activity due to viscera enzymes and was essentially free of muscle catheptic enzyme activity. The catheptic enzyme activity of muscle and viscera is optimum in the acid pH range and, therefore, was more effective during the extractions and centrifugations.

Figure 3 presents data relative to the solubility (protein in solution) and distribution of the soluble protein of crappie fillets to crude isolate (curd) and centrifugate (whey) from the recovery of the curds as a function of digestion time prior to pH adjustment (3 and 10) for protein extraction. The viscera/fillet weight ratio used was equal to the viscera/whole fish ratio obtained on evisceration of the fish.

The first zero digestion time data points were calculated from independent solubility runs on the fillet and viscera fractions as per Curve B of Figure 1. The second viscera zero digestion time points were obtained by mixing ground viscera with the ground crappie fillets and slurry media at pH 3 and 10. The slurry pH adjustment was done as rapidly as possible. Slurries of fillet and viscera were also set up for 1/2-, 1- and 4-hr digestions at 22°C and native pH of around 6.8, prior to adjustment to both pH 3 and 10. Protein was extracted by 30 min agitations followed by 30 min centrifugations. Protein assays on the digests were used to develop the data of Figure 3. An increase in digestion time increased protein in solution and curd protein yields. However, an expected increase in whey protein due to autolytic enzyme action was not obtained. The reason for this is not apparent.

The last data presentation, Figure 4, is for Pacific anchovies, a marine fish which could have potential for a fish protein concentrate program. Curve A is the conventional solubility curve obtained by the technique obtained earlier. This curve again discloses an approach to 90% anchovy protein (N × 6.25) solubilization at both pH extremes. The curve also indicates that 30% of the fish protein is in solution at the mean isoelectric point, around pH 5.6.

However, the food grade, flash frozen anchovies used to obtain the data of Figure 4 would have marginal value for an isolate approach based on process conditions, 22°C extraction and mean isoelectric precipitation, used in this paper. This is evidenced by Curves B and C. For this study, 80g of comminuted frozen anchovy were slurried in 800 ml of extracting medium and stirred (no vortex) for 30 min. The digest was clarified by centrifugation and then decanted. 50-ml aliquots of the two extracts were then adjusted to pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5 and 7.0. The pH adjustments caused curd formation. The curds were removed by centrifuging, and aliquots of the clear supernatant extracts were assayed for nitrogen by the Kjeldahl method.

Curve B shows that 63% of the protein of the pH 10 extract is relegated to the whey and only 37% recoverable as a mucoid, cream-like curd at the isoelectric point. Similar values for the pH 3 extract are 46 and 54%, respectively, for the whey and curd protein distribution at the isoelectric point. With a 90% extraction of fish protein at pH 3 and 10, Curve A suggests a potential 60% extraction of fish protein at pH 3 and 10, Curve A suggests a potential 60% recovery of the anchovy protein in the curd for processing into a purified protein isolate. The 37 and 54% curd values for the pH 10 and pH 3 extracts, respectively, translate to approximately 33 and 49% on fish or raw material basis. These recoveries, as evidenced by Figure 4 data, would be realized at variable minimum mean protein solubility points, 4.5 for the alkaline extract and 5.0 for the acid extract. The solubility curve indicates a minimum mean protein solubility at pH 5.5.

The 60% recovery of protein as an iso-

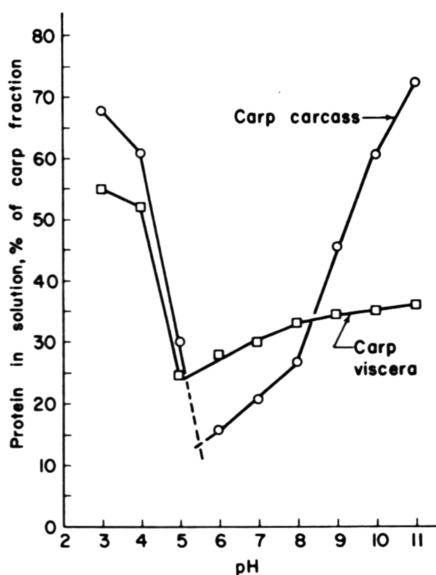

Fig. 2—Protein solubility of carp fractions.

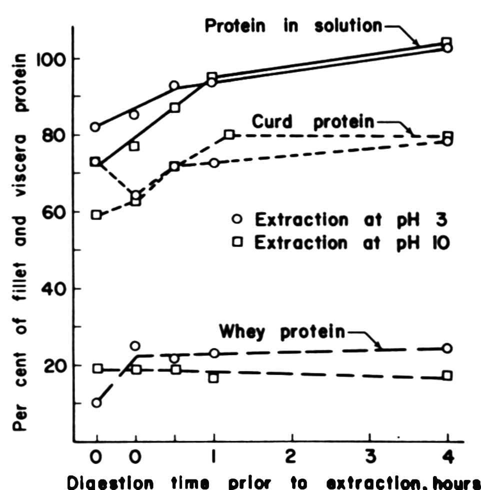

Fig. 3—Crappie fillet-viscera protein solubility and distribution.

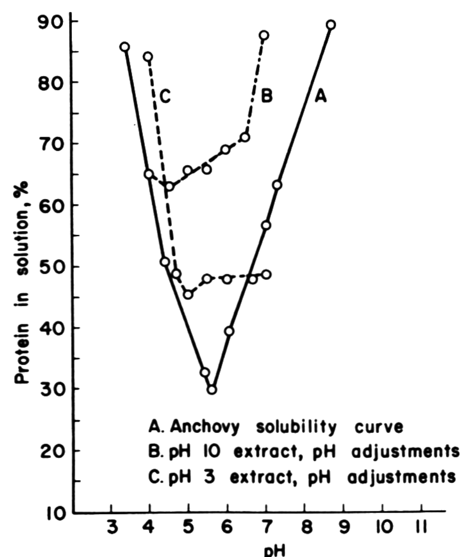

Fig. 4—Protein solubilities in anchovy and anchovy extracts.

late suggested by Curve A (Fig. 4) is a reasonable yield and could be tolerated. However, the 33 and 49% protein yields projected by Curves B and C, respectively, would not be desirable if only the isolate, prepared by the isoelectric precipitation method, is considered as the primary product. That is, the autolytic proteolytic enzymes of the anchovy degrade or modify the fish protein in both acid and alkaline extraction media. The protein modification decreases curd (isolate) yields by the isoelectric precipitation process under consideration. These data obtained with the anchovy cast some doubt on the merits of using whole anchovy or perhaps certain other whole fishes for the production of unmodified protein isolates as the sole protein preparation. This does not preclude the possible use of whole fish for approaches producing both modified isolates and soluble (whey) protein products. The dual protein product concept has been demonstrated by the work of Cheftel et al. (1971) with their enzymatic digestion investigations on isopropanol extracted fish protein concentrate.

CONCLUSIONS

THE USE OF protein (N × 6.25) solubility curves to project potential yield of protein isolate from the whole-carcass plus viscera-fish is subject to considerable error. This is true because the protein solubilized (protein in solution) has been modified or degraded by the autolytic proteases of the fish. Thus, the solubility profiles, protein in solution versus pH, reflect the solubility of the degraded protein rather than the native protein of the fish. The modified protein does not respond to mean isoelectric pH precipitation and, therefore, more protein is recovered as a soluble protein in the liquid (whey) remaining after curd (protein isolate) recovery at the pH of minimum mean protein solubility.

The modification of the protein of whole fish, as well as eviscerated fish, by native or autolytic enzymes enhances the overall extraction of the fish protein at pH values of around 3 and 10. This enhanced protein extraction, obtained by pre-digesting the fish products prior to extractions at pH 3 or 10, result in higher yields of curd protein than obtained when no pre-digestion period is used.

The term "no pre-digestion" is in reality a theoretical nonattainable condition as related to fish. As soon as fish are caught, the autolytic enzymes can start digestion of the fish protein. Refrigerated brine or icing are several approaches to preserving the fish on the fishing vessel and land based holding facilities. The preservation methods decrease but do not stop autolytic enzyme activity. Thus, a pre-digestion time is experienced in raw material prior to entering an isolate processing plant. In the plant, other pre-digestion times arise during processing from fish to final isolate. Grinding of the fish mixes viscera with flesh, and autolysis is intensified because of intimate contact of enzymes and flesh. Mechanism of slurry formation and pH adjustment may also add to pre-digestion time during which the fish are essentially at their native pH of 6.6 to 7.0. Extraction procedures, at least as suggested by the experimental data, would involve extractions at either pH 3 or 10. Alkaline extraction would favor further protein modification by autolytic enzymes of the viscera which exhibit greater activity at alkaline pH values. Acid extractions would favor enzyme activity of the catheptic enzymes found in varying amounts in fish muscle from different species of fish. Finally, heating cannot be employed to destroy enzyme activity because it also denatures and reduces the solubility of the proteins of the fish. Thus in the absence of enzyme deactivation procedures, continued variable autoytic enzyme activity may be anticipated during purification procedures applied to the crude curd isolate for the final production of a dry de-fatted oderless water-soluble protein isolate.

Limitations on the use of whole fish or eviscerated fish could be obviated to a degree by using lower extraction conditions, 5°C rather than the 22°C conditions employed in this study. This situation adds to engineering and processing costs. Also, evisceration of fish by mechanical means would aid in resolving the problem but would add to raw material costs and would not resolve the problem associated with the tissue autolytic enzymes. In fact, both low temperature and evisceration approaches were conducted in the laboratory. However, these conditions did not resolve another problem which has been mentioned, namely, the cream to mucoid character of the curds obtained by the isoelectric point precipitation technique. Granular-fibrous curds to creams were obtained from whole fish at 5°C. The mucoid creams retain larger quantities of whey and thus purification is more difficult than with firm granular curds. The condensed phosphate-protein curd reportedly produces a firm particle which lends itself to further purification—as by solvent extraction.

Autolytic protease (proteolytic) activity varies from fish to fish, and therefore the problems associated with the production of protein isolates by the isoelectric precipitation method can vary. Future research, at least based on the laboratory data reported, should not be directed only to the production of isolates. Concentrates produced from the modified proteins extracted from the whole fish are one possiblity. Another apparent research study would be an evaluation of the economics and feasibility of production of a water soluble protein concentrate from the whey and an isolate from the protein extracted from fish at either pH 3 or 10. This approach would utilize autolysis as an aid in recovering protein from raw fish and also would provide two protein products of varying functional properties. In fact, Cheftel et al. (1971) demonstrated the merits of such a dual product concept in their enzymatic investigations on isopropanol extracted fish protein concentrate.

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SKIM MILK PROTEIN RECOVERY AND PURIFICATION BY ULTRAFILTRATION
Influence of Temperature on Permeation Rate and Retention

INTRODUCTION

PROTEIN CONCENTRATES obtained by ultrafiltration of skim milk may be used for the production of highly pure protein powders (Peri et al., 1973b) or for cheese manufacture (Maubois and Mocquot, 1971).

The following points are of critical importance in determining the feasibility and economy of the process: (a) maximum protein retention; (b) minimum retention of lactose and other low molecular weight contaminants; (c) maximum permeation rate; (d) minimum protein denaturation; (e) minimum microbial contamination and growth during the process.

Points (a) and (b) are mainly related to membrane characteristics. Points (c), (d) and (e) depend strongly on operating conditions. Among them the choice of the proper temperature is of primary importance.

Low temperatures (2–5°C) allow a satisfactory control of microbial growth without denaturation of proteins, but result in very low permeation rates, especially at high protein concentrations (Pastore, 1972; Peri et al., 1973). This effect is due to the increase in viscosity that enhances the concentration polarization phenomena at the membrane-solution interface.

Temperatures high enough to stop microbial growth (> 60°C) cause protein denaturation and may negatively affect membrane life and performance, especially with high flux cellulose acetate membranes.

Temperatures in the range of 50–55°C are relatively safe for both proteins and membranes and have been extensively used in ultrafiltration of milk (Maubois and Mocquot, 1971) and whey (Horton et al., 1972). However, at these temperatures, thermophylic bacteria may grow and therefore the time of operation must be appropriately limited (Pastore, 1972).

In this paper we summarize the results of experiments carried out at two temperatures: 5 and 50°C. The permeation rates and retentions are reported with particular reference to the behavior of nitrogen compounds of both the protein and non-protein fractions.

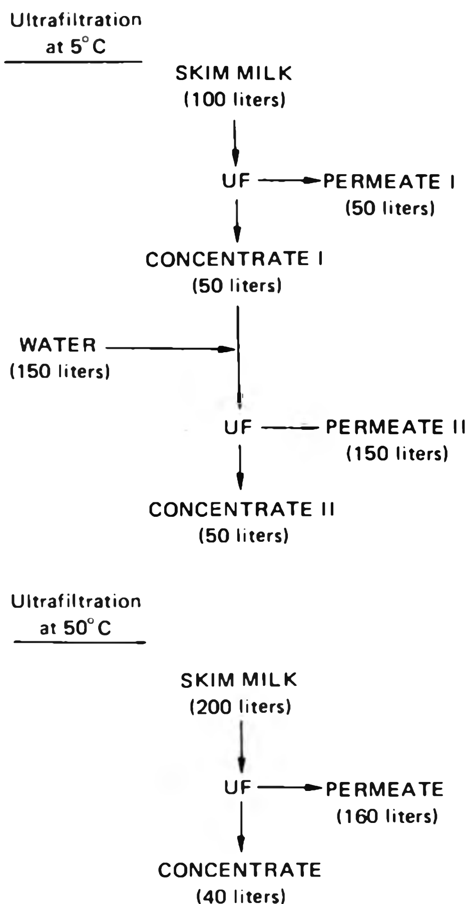

Fig. 1—Procedure followed in ultrafiltration experiments on skim milk, at 5°C and 50°C.

EXPERIMENTAL

THE ULTRAFILTRATION experiments were carried out on skim milk (less than 0.1% fat) pasteurized according to the traditional HTST procedure (78°C/15"), using a pilot plant model UF-22S (Abcor Inc., Mass.) in a batch mode as previously described (Peri et al., 1973b).

The temperature was maintained constant during the runs at 5 and 50°C (± 1°C), by circulating chilled or hot water in the feed tank jacket.

Permeation rates were measured during the runs and samples for analyses were taken at selected intervals, cooled and kept at 4°C before the analyses, with Na merthiolate (100 ppm) added.

The results reported in this paper were obtained in two typical ultrafiltration experiments carried out according to the procedure shown in schematic form in Figure 1. In both experiments the final concentrate contained about 70% protein on a dry basis.

This level of purification was obtained by direct ultrafiltration at 50°C and with a washing step at 5°C.

Samples of skim milk, concentrates and permeates were analyzed according to the following methods:

Total solids

Analyzed by the gravimetric method, drying in an oven at 100°C.

Total potassium, sodium, calcium and magnesium

Analyzed by atomic absorption (SP-90 Pye Unicam Atomic Absorption Spectrophotometer) after dry ashing (500°C maximum temperature).

Total phosphorus

Analyzed by the colorimetric method established by the International Dairy Federation (1967).

Total nitrogen and nitrogen fractions

Analyzed by a micro-Kjeldahl procedure according to Rowland (1938).

Lactose

Analyzed by the AOAC polarimetric method (1970).

Free amino acids

Free amino acids were extracted with picric acid (Resmini et al., 1969) and analyzed in a Beckman-Unichrom analyzer using a single column and replacing NaOH by a third buffer. (The following buffers were employed: Buffer A: 0.2N sodium citrate buffer pH 3.22 ± 0.01; Buffer B: 0.3N sodium citrate buffer pH 3.80 ± 0.02; Buffer C: 0.2N sodium citrate buffer pH 4.00 ± 0.02, containing 0.6M NaCl.) After eluting the acidic and neutral amino acids, this buffer carries through the basic amino acids and, at the same time, regenerates the resin. By applying this procedure the run time is reduced from 11.5 hr to about 7 hr. An Infotromics CRS 110 AB integrator was used to integrate the area of each amino acid peak.

Protein retention and denaturation

Samples were also freeze dried and analyzed by disc-electrophoresis (Davis, 1964; Ornstein,

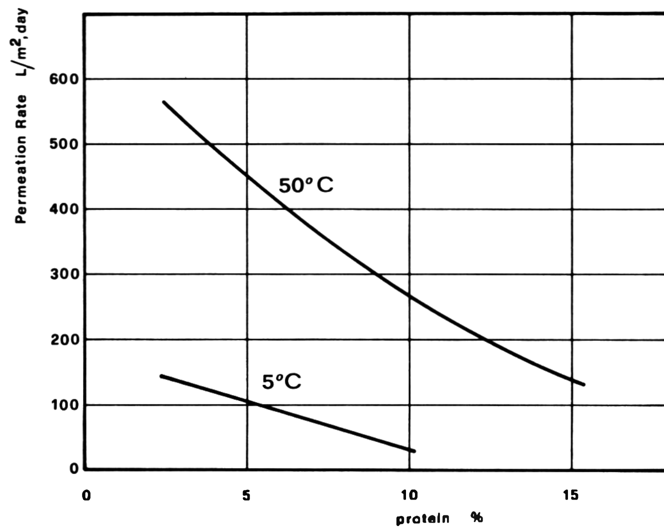

Fig. 2–Permeation rate (liters per square meter per day) vs. protein concentration in the ultrafiltration of skim milk at 5°C and 50°C. Data obtained in batch operation at 2.04 atm outlet pressure and 1.5 m/s flow velocity.

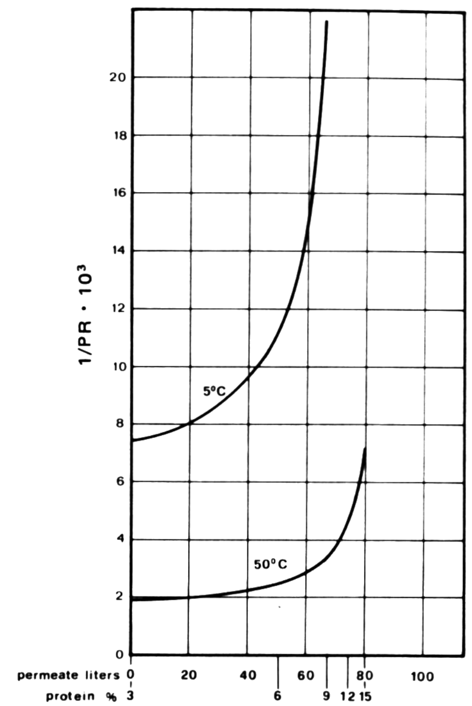

Fig. 3–Plot of reciprocal of permeation rate vs. permeate volumes and protein concentration. Data calculated from curves in Figure 2.

1964) for qualitative evaluation of protein retention and denaturation.

Permeates were dialyzed before freeze drying to eliminate soluble constituents and increase the relative concentration of proteins.

RESULTS & DISCUSSION

THE PERMEATION RATES at 5 and 50°C are reported as a function of protein concentration in Figure 2.

The permeation rate at 50°C is four- to fivefold higher than at 5°C. In both cases it decreases with increasing protein concentration.

The increasing difficulty of the operation is better visualized by plotting the reciprocal of permeation rate vs. permeate volumes, as represented in Figure 3. The area under the curves is proportional to the time necessary to achieve a given protein concentration or reduction of volume (Peri et al. 1973). The steepening of the curves clearly indicates that the separation of the same volume of permeate takes progressively longer times.

The results of analyses of skim milk, concentrates and permeates obtained in the two experiments described in Figure 1 are reported in Tables 1 and 2.

Figures 4 and 5 are pictures of disc-electrophoresis patterns of proteins in various samples.

The analyses of free amino acids are reported in Table 3.

A slight permeation of whey proteins takes place at 50°C. Figure 5 clearly shows that both α-lactalbumin and β-lactoglobulins are lost in the process. However, this loss accounts for only about 1% of the total nitrogen as indicated by the analytical data reported in Table 2.

At 5°C the retention of proteins is complete except for a minor loss of α-lactalbumin during the second step of the process, after dilution with water.

The different retention of whey proteins at the two temperatures may probably be attributed to variation in porosity of the membranes or to the different incidence of concentration polarization and membrane plugging at the two temperatures. The differences in retention may be shown quantitatively by comparing the percent distribution of the nitrogen fractions in the skim milk and in the concentrates.

At 5°C both casein and whey proteins

Table 1–Analyses of skim milk, concentrates and permeates obtained in the ultrafiltration experiment at 5°C, following the procedure schematized in Figure 1

	Total solids g/100g	Lactose g/100g	Total nitrogen g/100g	Casein nitrogen g/100g	%	Whey-protein nitrogen g/100g	%	Nonprotein nitrogen g/100g	%	Ash g/100g	Sodium mg/100g	Potassium mg/100g	Calcium mg/100g
Skim milk	8.60	4.69	0.437	0.344	76	0.066	15	0.028	9	0.62	49.5	168	131
Concentrate I	12.44	5.05	0.939	0.734	77	0.172	18	0.033	5	0.83	59.3	160	215
Permeate I	4.00	3.12	0.025	0.00	–	0.00	–	0.025	–	0.37	32.7	103	29.4
Concentrate II	9.13	2.02	0.967	0.782	81	0.169	18	0.015	1	0.61	27.0	59.8	181
Permeate II	1.10	0.97	0.006	0.00	–	0.00	–	0.007	–	0.09	9.1	28.5	–

increase, while the nonprotein fraction decreases (Table 1). At 50°C the percent of casein increases, while that of nonprotein nitrogen and whey proteins decreases (Table 2).

The electrophoretic pattern of the initial skim milk and of the final concentrate are very similar at both temperatures, showing no significant qualitative changes or denaturation of proteins.

Complete analysis of free amino acids and peptides in skim milk, concentrates and permeates shows that the retention of these compounds is very low at 5°C and nearly zero at 50°C.

A significant increase of NH_3 during the runs and a decrease of the total amount of several amino acids indicate that a limited enzymatic or microbial degradation of these compounds takes place during ultrafiltration. As for other constituents, retention of minerals is higher at 5°C than at 50°C.

The amount of calcium, phosphorus and magnesium in the permeates obtained at 50°C accounts respectively for 46, 34 and 70% of the amount found in the initial skim milk. These values are in good agreement with the distribution of percent soluble calcium, phosphorus and magnesium reported in the literature (Verma and Sommer, 1957), meaning that, at 50°C, the soluble fraction of minerals freely permeates through the membrane, while the colloidal fraction is completely retained. At the same temperature potassium shows almost zero retention.

At 5°C lactose and soluble minerals are partially retained; because the data reported in the literature show that the amount of dialyzable and ultrafilterable salts increases with decreasing temperature (Davies and White, 1960), we tend to explain our results on the basis of the different hydrodynamic conditions at the membrane-solution interphase at the two temperatures. In particular the polarized layer that is formed at low temperature may contribute to substantially increase the nominal retention of the membrane.

CONCLUSIONS

ACCORDING to the results reported in this paper, ultrafiltration of skim milk at 50°C has two main advantages over ultrafiltration at 5°C: (a) higher permeation rates and (b) lower retention of low molecular weight contaminants such as lactose and salts. These advantages clearly

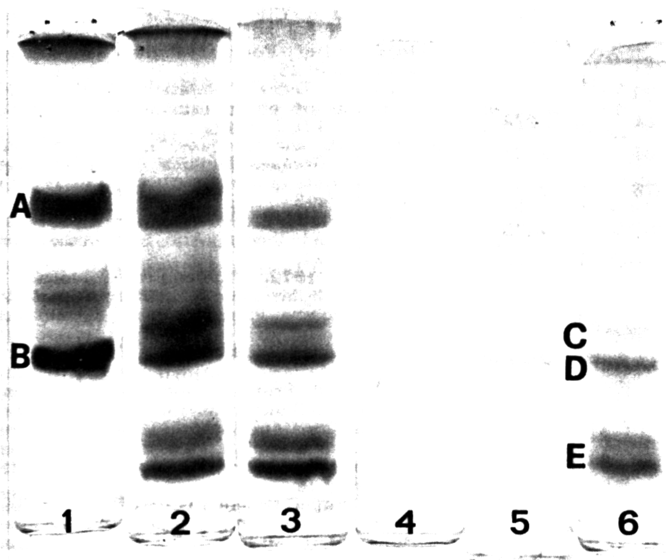

Fig. 4—Electrophoretic pattern of proteins in skim milk, concentrate and permeates obtained by ultrafiltration at 5°C.

1. *Sodium caseinate*
2. *Initial skim milk*
3. *Concentrate II (final)*
4. *Permeate I*
5. *Permeate II*
6. *Whey proteins standard*

A. *β-casein*
B. *α-casein*
C. *Blood serum albumin*
D. *α-lactalbumin*
E. *β-lactoglobulins*

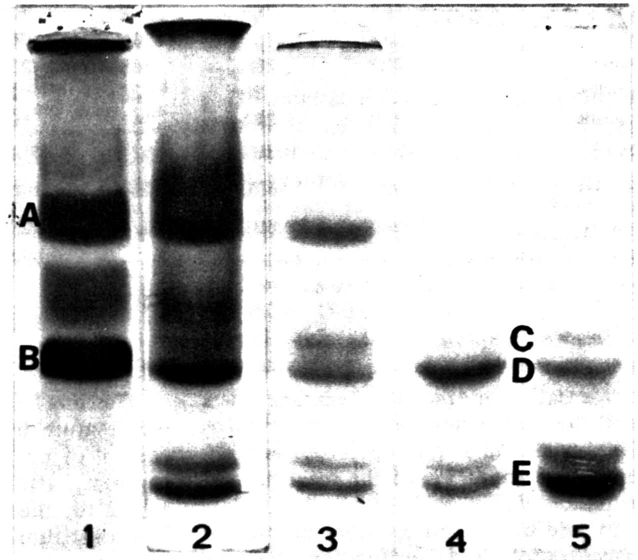

Fig. 5—Electrophoretic pattern of proteins in skim milk, concentrate and permeate obtained by ultrafiltration at 50°C.

1. *Sodium caseinate*
2. *Initial skim milk*
3. *Concentrate*
4. *Permeate*
5. *Whey proteins standard*

A. *β-Casein*
B. *α-casein*
C. *Blood serum albumin*
D. *α-lactalbumin*
E. *β-lactoglobulins*

Table 2—Analyses of skim milk, concentrate and permeate obtained in the ultrafiltration experiment at 50°C, following the procedure schematized in Figure 1

	Total solids g/100g	Lactose g/100g	Total nitrogen g/100g	Casein nitrogen g/100g	%	Whey-protein nitrogen g/100g	%	Non-protein nitrogen g/100g		Ash g/100g	Potassium mg/100g	Calcium mg/100g	Magnesium mg/100g	Phosphorus mg/100g
Skim milk	8.99	5.05	0.502	0.390	78	0.091	18	0.021	4	0.81	148	114	10.0	101
Concentrate	24.00	5.62	2.230	1.903	85	0.283	13	0.044	2	1.67	155	490	22.2	281
Permeate	5.57	5.26	0.029	0.00	–	0.006	–	0.023	–	0.47	140	52.3	7.0	34.1

Table 3–Free amino acids in skim milk, concentrates and permeates obtained by ultrafiltration at 5°C and 50°C (mcg/100ml)

	5°C			50°C		
	Skim milk	Conc I	Permeate I	Skim milk	Conc	Permeate
Hydroxyproline	77	–	–	33	–	46
Aspartic acid	431	564	210	250	254	71
Threonine	268	442	177	64	70	70
Serine	72	67	45	113	122	107
Aspar. + Glutam.	346	604	200	133	69	109
Sarcosine	12	8	5	–	–	–
Proline	268	228	214	229	180	148
Glutamic acid	2534	2590	2006	3746	3900	3746
Glycine	631	643	507	693	795	633
Alanine	339	345	268	268	282	191
α-aminobut.acid	23	16	20	7	8	–
Valine	88	107	65	125	184	108
Methionine	–	–	–	41	–	13
Isoleucine	67	78	56	26	49	18
Leucine	150	275	131	54	65	53
Tyrosine	42	–	32	21	13	23
Phenylalanine	45	57	21	18	4	12
γ-aminobut.acid	1068	776	828	607	1215	477
Ornithine	158	265	139	102	116	129
NH_3	433	2750	368	555	848	1037
Lysine	239	286	166	230	291	322
Histidine	32	36	25	27	33	44
Arginine	200	224	141	193	184	274
Total I	7524	10361	5624	7553	8682	7631
Peptides	2925	3630	1454	2681	2075	2739
Total II	10449	13991	7078	10234	10757	11370

compensate for the disadvantage of a small loss of whey proteins that takes place at the higher temperature and accounts for about 1% of the total protein content.

In addition, a higher level of protein concentration and purification may be achieved at 50°C by simple ultrafiltration, without the water washing step. In experiments carried out with the same apparatus and in the same operating conditions, at 50°C a maximum protein concentration of about 24% was obtained, with 30% total solids, corresponding to about 80% of protein on a dry basis (Grassi and Peri, unpublished data). At this concentration of protein permeation rate was still as high as about 85 liters/m^2, day.

The most critical point in the use of temperature in the range of 50°C is the control of microbial growth. At this temperature, after an initial lag phase of 1.5–2 hr, thermophylic bacteria develop at a logarithmic rate (Pastore, 1972; Peri, 1972). However, studies carried out in this laboratory have shown that microbial growth may be easily kept under control in batch ultrafiltration by choosing an appropriate length of time of operation (Pastore, 1972).

When operating on a continuous mode the concentrate substraction compensates for the increase in microbial population and the net result depends on the relative importance of average residence time, residence time distribution and specific microbial growth rate (Peri, 1972). In general, in the continuous ultrafiltration with commercially available plants the control of microbial growth doesn't constitute a limiting factor in the 20-hr daily operation.

As a general conclusion of our experiments we consider that ultrafiltration of skim milk at 50°C is more economical and more effective in protein purification than ultrafiltration at low temperature (5°C).

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REDUCTION OF CHILLING INJURY OF CITRUS FRUITS IN COLD STORAGE BY INTERMITTENT WARMING

INTRODUCTION

CITRUS FRUITS stored for long periods are held at low temperatures to avoid decay, but caution must be taken to avoid chilling injury. Hruschka et al. (1969) and Lieberman et al. (1958, 1959) determined that injury due to chilling of commodities such as potato tubers and sweet potatoes, respectively, could be reduced by interrupting cold storage by short periods of warm storage. In a comprehensive work on storage diseases of grapefruit, Brooks and McColloch (1936) found that pitting of grapefruit stored at 32° or 36°F could be lessened by intermittent warming. Although these authors suggested further checking of the results, commercial interests have not taken advantage of these findings. To study further the effects of short warming periods on the development of peel injury which frequently leads to subsequent decay, both grapefruit and oranges were subjected to continuous cold storage and to intermittent warming.

MATERIALS & METHODS

THE ORANGES and grapefruit used in these tests were harvested from commercial groves in central Florida. All fruits were washed and waxed before storage. The fungicide, when applied, was thiabendazole (TBZ) (2-(4'-thiazolyl) benzimidazole). Pitting (sunken darkened areas) was considered slight if the total affected area was 1/2" to 3/4" diam, and moderate to severe if larger. Brown staining (tan to brown discoloration of the peel) was considered slight if less than one-fourth of the total area was affected and moderate to severe if greater. Fruits were removed periodically from storage to 70°F for 1 day (8 hr).

Marsh grapefruit were stored in 50-fruit lots, Temple oranges in 80-fruit lots and Valencia oranges in 100-fruit lots.

Fruits were inspected for chilling injury and decay on removal from storage and after holding at 70°F for 1–3 wk. Juice of composite 8- to 10-fruit samples was analyzed for ethanol and acetaldehyde by a gas chromatographic method (Davis and Chace, 1969), for solids by refractive index, for acid by titration and for pH with an expanded scale pH meter.

RESULTS & DISCUSSION

MARSH GRAPEFRUIT, harvested in January, 1970, were stored without fungicidal treatment for 8 wk at 40°F, a temperature conducive to pitting. On removal from storage, fruit held continuously at 40° had a total of 32% pitting with 13% moderate to severe (Table 1). The lot which was removed and warmed weekly had a total of 16% pitting with only 4% considered moderate to severe. Warming at 2-wk intervals had little beneficial effect. After a 1-wk holding period at 70°F following storage, the fruit which had been warmed each week had slightly less decay than the fruit which had been held continuously at 40°F prior to removal to 70°F. Decay was caused by either *Penicillium digitatum* Sacc. or stem-end rot organisms, primarily *Phomopsis citri* Fawc.

To minimize the decay factor, Marsh grapefruit harvested in May, 1970, were treated with TBZ before storage. Only a small amount of pitting was observed at the end of the 8-wk storage period, but symptoms of brown staining (scald), considered to be another injury due to chilling (Brooks and McColloch, 1936; Friend and Bach, 1932; Smoot et al., 1971) and possibly related to acetaldehyde toxicity (Pantastico et al., 1968) were evident (Table 2). Fruits held at 40°F continuously had a total of 17% brown staining, while those warmed each week had none. After a 1-wk holding period, pitting developed in fruits held continuously at

Table 1—Effect of intermittent warming of Marsh grapefruit stored 8 wk at 40°F, January, 1970. Washed and waxed only[a]

Storage condition	% Pitting		% Decay	
	Slight	Moderate to severe	Penicillium	Stem-end rot
On removal from 40°F storage				
Fruit warmed at 70° for 8 hr each wk	12	4	2	0
Fruit warmed at 70° for 8 hr each 2 wk	16	12	0	0
Fruit stored at 40° continuously	19	13	2	0
After 1 wk at 70°				
Fruit warmed at 70° for 8 hr each wk	12	0	4	10
Fruit warmed at 70° for 8 hr each 2 wk	12	2	0	18
Fruit stored at 40° continuously	12	10	6	14

[a] Figures represent one lot of 50 fruit for each storage condition.

Table 2—Effect of intermittent warming of Marsh grapefruit stored 8 wk at 40°F, May, 1970. Washed, waxed and treated with TBZ[a]

Storage condition	% Pitting		% Brown staining	
	Slight	Moderate to severe	Slight	Moderate to severe
On removal from 40°F storage				
Fruit warmed at 70° for 8 hr each wk	0	0	0	0
Fruit warmed at 70° for 8 hr each 2 wk	2	0	4	0
Fruit stored at 40° continuously	2	0	12	5
After 1 wk at 70°				
Fruit warmed at 70° for 8 hr each wk	0	0	4	0
Fruit warmed at 70° for 8 hr each 2 wk	4	0	10	8
Fruit stored at 40° continuously	12	26	0	18

[a] Figures represent one lot of 50 fruit for each storage condition.

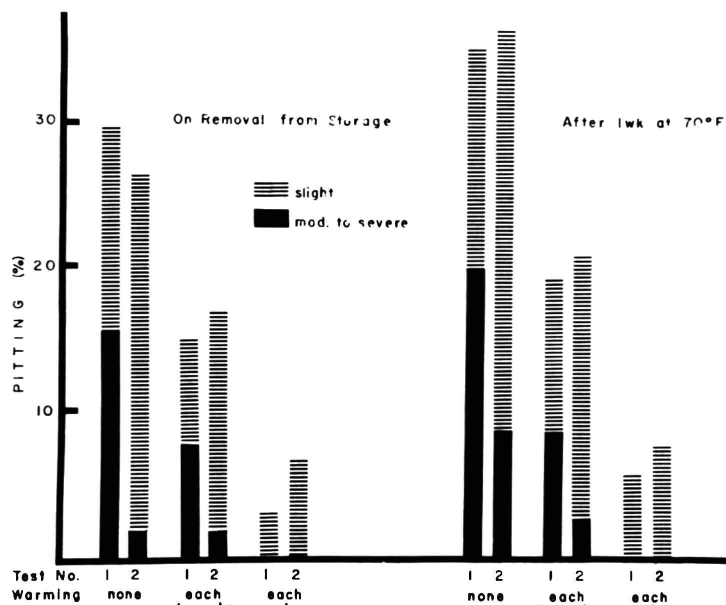

Fig. 1–Effect of intermittent warming of Marsh grapefruit stored 8 wk at 40°F, November, 1970 (Test 1) and January, 1971 (Test 2). Washed, waxed and treated with TBZ. (For both tests, bars represent an average of four lots of fruit, 50 fruit per lot, for each storage condition.)

40°F, and brown staining increased in severity.

Two additional and more extensive tests with grapefruit were performed during the 1970-1971 season, with confirmatory results (Fig. 1). Less pitting occurred in fruits warmed at 70°F for 1 day each wk than in those warmed each 2 wk. Fruits stored at 40°F continuously had the greatest amount of pitting. No brown staining occurred in either test. There were no discernible differences in internal quality when evaluated by analyzing juice samples for ethanol, acetaldehyde, solids, acid, or pH. This indicates that the primary effect of intermittent warming is on the rind rather than on internal quality. Early grapefruit (Test 1) developed more moderate to severe pitting than did those harvested 2 months later (Test 2), but in each case there was only slight pitting in fruits warmed each wk. After a 1-wk holding period at 70°F following cold storage, pitting increased in fruits which had been held at 40°F continuously or warmed at 2-wk intervals. Fruits warmed weekly did not develop any severe pitting nor did slight pitting increase appreciably.

Temple oranges stored continuously at 34°F for 10 wk (Table 3) developed a total of 10% pitting in one test. In a second test, pitting was a minor factor, but brown staining averaged 14% after a 2-wk holding period at 70°F. Both symptoms of chilling injury were lessened by warming each 2 wk and were virtually eliminated by weekly warming periods.

Valencia oranges treated with TBZ and stored at 34°F for 12 wk had no pitting and only a minor amount of aging (depressed areas near stem end) on removal from storage (Table 4). No decay was evident on removal of the fruit from storage. After 1 wk at 70°F, decay developed up to 7% in fruit held without warming. After 2 wk at 70°F, fruits which had been warmed each wk still had only 4% decay, while those held continuously at 34°F had 14% decay. This may indicate that chilling injury had occurred but without visible symptoms such as pitting, and that intermittent warming had prevented the injury.

Analysis of juice showed no significant change in ethanol, acetaldehyde, solids, acid, or pH attributable to intermittent warming. Fruits stored continuously at 34°F had slightly higher but not significant ethanol and acetaldehyde contents than fruits which had been warmed intermittently.

The basic causes of chilling injury in citrus fruit are still obscure. Humidity (Brooks and McColloch, 1936), accumu-

Table 3–Effect of intermittent warming of Temple oranges stored 10 wk at 34°F, January, 1971 (Test 1) and February, 1971 (Test 2). Washed, waxed and treated with TBZ[a]

	% Pitting after removal + 1 wk at 70°		% Brown staining after removal + 2 wk at 70°
Storage condition	Slight	Moderate to severe	Moderate to severe
Fruit warmed at 70° for 8 hr each wk			
Test no. 1	1	0	0
Test no. 2	0	0	0
Fruit warmed at 70° for 8 hr each 2 wk			
Test no. 1	3	0	0
Test no. 2	0	0	7
Fruit stored at 34° continuously			
Test no. 1	8	2	0
Test no. 2	2	2	14

[a] For both tests, figures represent an average of three lots of fruit, 80 fruits per lot, for each storage condition.

Table 4–Effect of intermittent warming of Valencia oranges stored 12 wk at 34°F, April, 1970. Washed, waxed, and treated with TBZ[a]

	% Aging on	% Decay (Phomopsis citri)		
Storage condition	removal from 34° storage	On removal from 34° storage	After 1 wk at 70°	After 2 wk at 70°
Fruit warmed at 70° for 8 hr each wk	0a	0	2d	4bcd
Fruit warmed at 70° for 8 hr each 2 wk	2a	0	3cd	8bc
Fruit warmed at 70° for 8 hr each 3 wk	1a	0	4bcd	7bcd
Fruit warmed at 70° for 8 hr each 4 wk	0a	0	4bcd	10ab
Fruit stored at 34° continuously	1a	0	7bcd	14a

[a] Figures represent an average of three lots of 100 fruit per lot for each storage condition. Letters following averages represent Duncan Multiple Range Test significance values at 5% level. Figures followed by no letters in common are significantly different.

lation of acids (Demoise and Miller, 1959), and failure of the ATP-ADP energy transfer system (Pantastico et al., 1968; Vakis et al., 1971) have been cited as possible causal factors, and further investigation in this area is needed. The problem is complicated by the fact that chilling injury is seldom exactly reproducible due to seasonal variations, maturity and preharvest environmental conditions. In addition, it manifests itself in various forms such as pitting, aging and rind staining. Intermittent warming offers one approach to the solution of the problem commercially, and further investigations are now in progress to optimize the duration and degree of warming required. There is apparently a time-temperature relationship in the chilling injury process, and this should furnish insight into the fundamental causal factors.

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CHANGES IN TITRATABLE ACIDITY, °BRIX, pH, POTASSIUM CONTENT, MALATE AND TARTRATE DURING BERRY DEVELOPMENT OF THOMPSON SEEDLESS GRAPES

INTRODUCTION

MALIC AND TARTARIC acids are the predominant acids in the grape. The acids contribute organoleptically to the acceptability of the fresh grape and to the quality of wine or juice produced from grapes and also retard microbial spoilage and stabilize color. A must of low pH is preferred for wine making and selection of wine grapes is based on total acidity, malic and tartaric acid contents, and pH. The ultimate pH of the grape berry depends on total acidity, relative concentrations of malic and tartaric acids, and the extent of salt formation.

Kliewer et al. (1967) analyzed 78 varieties of vinifera grapes at two stages of berry development and found that malate and tartrate concentrations varied widely among varieties, but in all cases acids decreased on ripening. Kliewer (1966) analyzed Thompson Seedless grapes at four stages of berry development and found that the rate of decrease of malates during ripening was greater than that of tartrates. Du Plessis (1968) reached the same conclusion with four South African grape cultivars. Kliewer (1971) recently observed that the rate of decrease of acids was dependent on temperature and cultivar, but relatively independent of light intensity.

Several maturity studies have been conducted on European grapes prior to 1940 (Amerine and Winkler, 1942). No systematic study has been done on the changes in malic and tartaric acids, and potassium content and their relationship to pH and titratable acidity during berry development of grapes. The present study is undertaken to determine the pH, °Brix, titratable acidity and potassium content and simultaneous determination of malic and tartaric acids by gas-liquid chromatography during berry development of Thompson Seedless grapes.

EXPERIMENTAL

ONE ROW consisting of 28 vines of Thompson Seedless grapes planted in 1961 at the University of Arizona Experimental Farm at Tucson was selected for this investigation. Cultural management of this farm (fertility, irrigation, pruning, etc.) was similar to that used on commercial table grape vineyards in Arizona. Sample taking was initiated as soon as the berries were sufficiently mature to yield enough juice for analytical purposes. Twice a week, one cluster of approximately the same stage of maturity was picked from each vine from June through July 1972 (a total of 392 clusters). The berries were removed from the clusters and a random sample of 30 berries from each cluster was freeze dried and stored frozen. Another random sample of 30–50 berries from each cluster was used for the determination of average weight, average volume, pH, °Brix and titratable acidity.

Volume, pH, °Brix and titratable acidity

The volume of a weighed sample of 30–50 berries was determined by displacement of water in a measuring cylinder. The berries were blotted dry after draining the water, homog-

Table 1—Average values of berry weight, berry density, pH, °Brix, and titratable acidity of Thompson Seedless grapes in Tucson for the 1972 season

Date	Berry wt	Density	pH	°Brix	Titratable acidity 0.1N NaOH/100 ml juice
June 5	1.169	1.008	2.68	9.2	476.7
June 8	1.470	1.036	2.89	10.9	345.3
June 12	1.532	1.037	2.93	12.3	273.2
June 15	1.763	1.039	3.04	13.2	218.3
June 19	1.750	1.046	3.17	15.6	182.7
June 22	1.874	1.045	3.23	15.5	149.2
June 26	1.882	1.055	3.37	16.9	135.9
June 29	1.752	1.060	3.45	18.4	122.8
July 3	1.951	1.065	3.54	19.2	106.1
July 7	2.048	1.049	3.57	19.2	93.7
July 11	2.007	1.064	3.69	19.9	82.0
July 17	2.053	1.073	3.69	19.7	83.1
July 24	2.052	1.080	3.79	21.9	72.2

Table 2—The relationships among titratable acidity, potassium content and organic acids of Thompson Seedless grape juice during berry development

Titratable acidity 0.1 NaOH/100 ml juice	Potassium mg/100 ml juice	Malic and tartaric acid content g/100 ml juice: Total malate	H_2Ma	KHMa	K_2Ma	Total tartrate	H_2Ta	KHTa	K_2Ta
513.2	162.1	2.45	2.10	0.37	0.001	1.20	0.81	0.38	0.008
357.4	179.1	1.60	1.28	0.31	0.001	1.07	0.64	0.42	0.012
297.6	220.1	1.30	1.02	0.28	0.001	1.04	0.59	0.44	0.014
261.8	222.5	0.99	0.71	0.28	0.002	1.00	0.48	0.50	0.023
198.8	266.7	0.60	0.37	0.23	0.003	0.98	0.35	0.59	0.043
151.6	250.3	0.42	0.25	0.17	0.002	0.93	0.31	0.57	0.047
111.6	292.1	0.31	0.14	0.15	0.004	0.80	0.17	0.55	0.068
120.8	299.6	0.32	0.16	0.16	0.003	0.85	0.22	0.57	0.065
101.8	264.0	0.25	0.10	0.14	0.004	0.83	0.16	0.58	0.094
90.4	300.2	0.20	0.066	0.13	0.005	0.82	0.11	0.58	0.130
77.8	324.7	0.19	0.058	0.13	0.005	0.90	0.11	0.63	0.160
66.4	352.2	0.18	0.051	0.13	0.006	0.95	0.10	0.66	0.190

enized in a Waring Blendor and filtered through filter paper. After measuring the pH and °Brix of the filtrate, an aliquot was titrated with 0.1N NaOH to pH 8.0. Correlation coefficients (r) between °Brix and pH, °Brix and log titratable acidity, and pH and log titratable acidity were calculated from the data obtained from all the samples (392 clusters).

Potassium, malates and tartrates

The freeze-dried samples of grapes were grouped into classes of the same titratable acidity regardless of the time of their picking. The grapes (25–30g) were homogenized in a Waring Blendor with distilled water and filtered through filter paper. The filtrate (juice) was then diluted to the °Brix prior to freeze drying and an aliquot titrated with 0.1N NaOH to check abnormalities, if any, in the sampling. The potassium content of the juice was determined with a Perkin-Elmer 290 B Atomic Absorption Spectrophotometer after appropriate dilution. The total malates and tartrates were determined by the method of Philip and Nelson (1973). The free malic and tartaric acids, potassium acid malate and tartrate, and potassium malate and tartrate were calculated as described by Amerine and Winkler (1942).

RESULTS

THE SUMMARY of data on maturity studies with Thompson Seedless grapes for the season 1972 is shown in Table 1. The pH and °Brix increased steadily while the titratable acidity decreased during berry development. The average berry weight and volume doubled during the period of investigation.

Potassium, malate and tartrate contents

Table 2 shows the potassium content, free malic and tartaric acids, potassium acid malate and tartrate, potassium malate and tartrate, and total malates and tartrates during berry development of Thompson Seedless grapes. In the early stages of berry development malates predominate over tartrates whereas this condition is reversed toward the later stages. At pH 3.80 and 21.6 °Brix, 70% of the total tartrates and malates are present as their acid salts which contribute 65% to the total acidity. In the early stages of berry development, the tartrates and malates are present mostly as free acids. The juice content of a single berry increases as the berry develops and if this fact is taken into consideration, there is no significant change in the tartrate concentration during berry development. The lower amounts of tartrate obtained for some samples may be an analytical error due to incomplete extraction of less soluble tartrate salts. The malate concentration during the same period of maturity decreases rapidly. The potassium content increases linearly with °Brix and it appears that potassium which is translocated from the plant, has a possible relationship in controlling pH and acidity in grape berries.

The physiological role of large amounts of tartrate in the grape is unknown. Organic acids are early products of photosynthesis and during the early period of fruit ripening, acids accumulate in the grape berry (Stafford and Loewus, 1958). During the later stages of berry development, where sugar synthesis is active, very little synthesis of organic acids occurs (Saito and Kasai, 1968). Malate, being a member of Krebs cycle, is readily utilized by the berry. The results of the present study indicate that tartrates are not metabolized during berry develop-

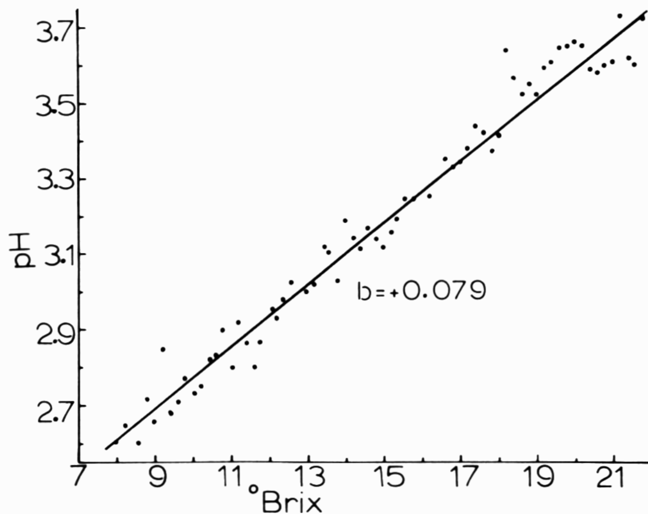

Fig. 1–Regression of mean pH on °Brix. Mean pH values of juice samples of same °Brix were plotted against °Brix. The sample size varies between 10 and 25.

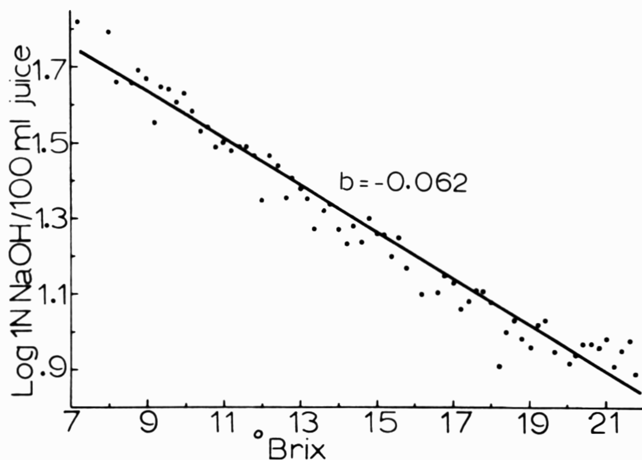

Fig. 2–Regression of mean log titratable acidity on °Brix. Mean log titratable acidity of juice samples of same °Brix were plotted against °Brix. The sample size varies between 10 and 25.

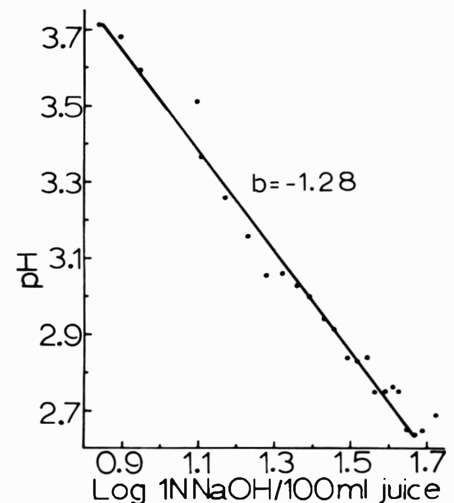

Fig. 3–Regression of mean pH on log titratable acidity. Mean pH values of juice samples of same titratable acidity were plotted against log titratable acidity. The sample size varies between 10 and 25.

ment, either due to the lack of enzymes or due to the formation of insoluble salts less susceptible to enzymic degradation.

Titratable acidity, pH and °Brix

The linear relationships between pH and °Brix (r = 0.94), °Brix and log titratable acidity (r = 0.94), and pH and log titratable acidity (r = 0.98) of Thompson Seedless grape juice during berry development are shown in Figures 1, 2 and 3, respectively. Although a survey by Amerine and Winkler (1942) indicated the lack of well defined correlation between pH and titratable acidity, a high correlation (r = 0.98) between pH and log titratable acidity is obtained in this study. The pH increases steadily and titratable acidity decreases logarithmetically (Fig. 1 and 2) during berry development. Since the total tartrate concentration remains unchanged, the log decrease in titratable acidity is due to the rapid decrease in malate as well as due to the formation of potassium salts. The pH and acidity of the ripe grape berry depend to a large degree on the extent of salt formation which, in turn, is related to the potassium content. The increase in potassium content during berry development is directly related to °Brix and pH (Table 2). Mattick et al. (1972) found a similar relationship between pH and potassium content of Concord grape juice.

Arizona grapes are early season grapes and are considered to be commercially ripe at 16 °Brix for consumption as table grapes. Acidity is an important factor in the quality of table grapes. The ratio of soluble solids to acid is currently used for classification of wine grapes. The relationship between potassium content and pH indicates the possibility of a simpler method of classification of wine grapes based on the potassium content.

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COMPACTION BEHAVIOR OF GROUND CORN

INTRODUCTION

COMPRESSIBILITY of powder foods under axial loads (loads applied only in one direction) is important in the development of pelleted foods and other applications requiring basic information on compaction of these materials. In this work, a theory of compaction is developed on the basis of bulk compressibility of the materials.

On a particle-to-particle scale, two processes of compaction are differentiated by whether or not it is large or small voids (relative to particle size) that are being filled as discussed by Cooper and Eaton (1962). According to Heckel (1961a, b), the process of compaction involves three stages namely: filling the die, rearranging of particles in the die and deformation of the particles. Seelig and Wulff (1946) discussed the process of compaction by three basic mechanisms: sliding, elastic and plastic deformation, and fragmentation.

Bulk compressibility may be defined as

$$B_c = -\left[(dV/V)/dp\right]_m \qquad (1)$$

where dV is change in volume, V is initial volume, m is moisture content and dp is change in hydrostatic stress. Equation (1) is true only when hydrostatic stress is applied. In the case of axial loading, B_c will not be the bulk compressibility of the food material. For our purpose we call it the compressibility factor, denoted by B_{cf}. Therefore, Equation (1) becomes

$$B_{cf} = -\left[(dV/V)/dP\right]_m \qquad (2)$$

where P is axial pressure.

The relationship between mass (M), volume (V) and density (D) of a food material is given by

$$D = M/V \qquad (3)$$

Integrating Equation (2), assuming B_{cf} constant where pressure increases to P with volume V_o to V at constant moisture content, and substituting Equation (3) we obtain

$$\ln D = B_{cf}P + \ln D_o \qquad (4)$$

where D_o is the relative apparent density of the food material. Replacing the term $\ln D_o$ with a constant A, Equation (4) can then be written as

$$\ln D = B_{cf}P + A \qquad (5)$$

The constant A (packing factor) and B_{cf} (compressibility factor) can be determined from the intercept and slope, respectively from a plot of ln D versus P.

MATERIALS & METHODS

THE CORN USED in this study was a midwest, open variety supplied by Robinson Hybrid Corn Co., Delware, Ohio. The pedigree of the variety was (WF9MSTXH7IXOI.43RFXB37RF). The endosperm was separated from whole corn by hand dissecting after soaking overnight. The corn samples were dried and ground to a size less than 60-mesh.

Samples of ground corn were dried in a vacuum oven for 72 hr at 75°C. Samples of whole corn and endosperm were conditioned for moisture with a series of relative humidities from 11–97%. The desired relative humidities were maintained in desiccators by means of saturated salt solutions according to methods by Wexler and Hasegawa (1954), Carr and Harris (1949) and Wink and Sears (1950).

The desiccators were evacuated to accelerate moisture transfer and kept in a controlled-temperature chamber at 78°F. The samples attained the equilibrium moisture content in 2–3 weeks, depending on the humidity shift. Mold growth was prevented in the high humidity desiccators by placing an open dish of toluene in it.

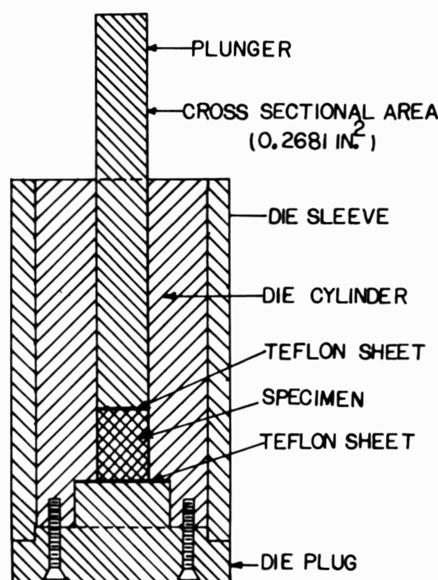

Fig. 1–Die for compressibility factor determination.

Samples in the desiccators were transferred from corn baskets to the sealed boxes, so that they would not lose any moisture in the new air. The moisture content was determined by an air oven method (103°C and 72 hr), given by researchers at USDA (1959). All moisture percentages are expressed on a dry matter basis.

The load cell of the Ametek Testing Machine was calibrated by a prooving ring. The loading rate was fixed to 0.056 in. per minute.

The ground corn was compressed in the die shown in Figure 1. The die of cross sectional area 0.2681 in.^{2}, consisted of a mild steel plug, stainless steel plunger, aluminum cylinder and aluminum sleeve. The top surface of the plug and bottom end of the plunger were lined with teflon sheets. The plug was screwed to the cylinder by two countersunk head screws.

To find the initial and final length of the specimen a dial indicator–adjusted to zero with the die empty–was used. The die was then filled with a 4g sample of corn using a funnel, and the plunger was driven by slight hand pressure. This specimen was then compressed by a 60-lb initial load and the initial length of the specimen was recorded. The specimen was loaded to the required pressure with a given loading rate. The specimen was pressed out after removing the plug screws and the die was cleaned for the next test.

The lengths of the specimen at several pressures in three to five replications of each test level were added and averaged in order to obtain a "mean curve." The density of the specimen was calculated for each pressure. The values of compressibility factor and packing factor were determined by the least square method of Steel and Torrie (1960).

RESULTS & DISCUSSION

THE DENSITY-PRESSURE relationship under axial compression was obtained for ground whole corn and endosperm. From density-pressure data it was found that change of density with pressure at any given pressure was proportional to the density of the given powder food. The data adapted well to the predicted (Equation 5) since a straight line was obtained when density and pressure were plotted on semilogarithmic paper as shown in Figure 2. The coefficients of correlation and coefficients of variability for the above fit ranged from 0.94–0.99 and 2.3–8.1%, respectively. Furthermore, A in equation (5) was a measure of low pressure densification which included the following: (1) densification due to individual particle motion; (2) densification brought about

Table 1—Compressibility factor and packing factor for ground whole corn under axial compression (Initial load of packing 60 lb)

Moisture content %	Packing factor g/cc	Compressibility factor ($\times 10^{-5}$ psi)	Maximum pressure psi
45.38	1.172	42.85	1063
31.34	1.064	22.38	2070
26.00	1.059	15.67	2070
21.28	1.069	9.84	3096
16.02	1.035	5.40	5147
14.67	1.072	4.54	5147
7.12	1.076	3.45	5147
6.57	1.069	3.42	5147
1.75	1.076	3.29	5147

Table 2—Compressibility factor and packing factor for ground corn endosperm under axial compression (Initial load of packing 60 lb)

Moisture content %	Packing factor g/cc	Compressibility factor ($\times 10^{-5}$ psi)	Maximum pressure psi
30.44	1.028	16.97	2070
26.54	1.021	12.15	3095
20.32	1.035	5.30	5147
18.82	1.082	5.22	5147
16.71	1.082	4.39	5304
15.07	1.040	4.01	5304
13.23	1.038	3.48	5147
9.08	1.059	2.42	10705
8.25	1.059	2.40	10705
4.75	1.073	1.82	10705
3.75	1.086	1.81	10705

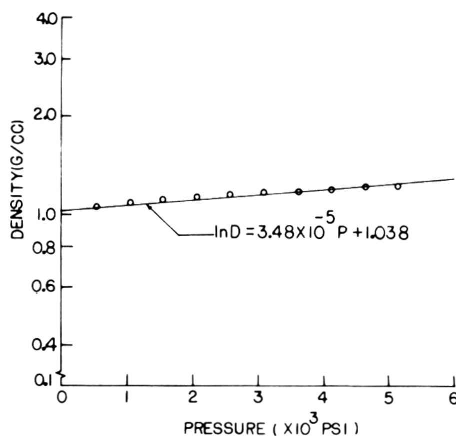

Fig. 2—Pressure density relationship for ground endosperm at moisture content 13.23%.

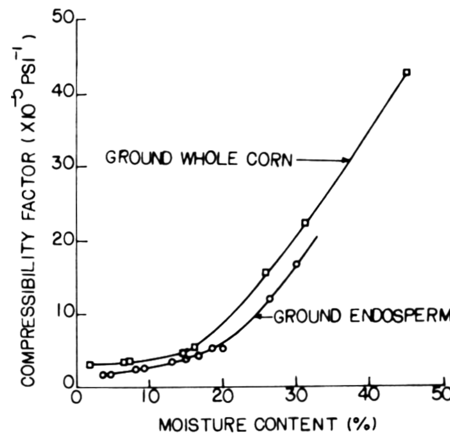

Fig. 3—Compressibility factor at various moisture contents for ground whole corn and endosperm.

by filling the die; and (3) initial load, if any. In the same equation, B_{cf} was a measure of the ability of the powder food to densify by plastic deformation. For this reason, A was called the packing factor and B_{cf} the compressibility factor.

The values of packing factor and compressibility factor for ground whole corn and endosperm on various moisture contents are given in Tables 1 and 2, respectively. The compressibility factor was plotted against the moisture content for ground whole corn and endosperm and is shown in Figure 3. The packing factor ranged from 1.021–1.086 for corn endosperm and 1.035–1.172 for whole corn. There was no definite trend for packing factor.

The compressibility factor of ground whole corn as well as corn endosperm increased with an increase in moisture content. This phenomenon could be explained on the basis of molecular adsorption of water. In molecular adsorption, hydroxyl anions (-OH) in the molecular structure offer active polar sites of bonding energy for the water molecule. At a moisture content close to saturation, water molecules have satisfied the active polar sites of the hydroxyl anions. Additional moisture can be held by the formation of chains of water molecules due to their dipole nature. When water chains are formed, the bonding forces between the hydroxyl anions at the ends of the chains become smaller. In the shear the water molecule in the chain can jump from one anion group to another. This jump is not completely elastic because some energy is lost in the process. The slipping of the anion bond between neighboring chains was the most plausible explanation which could be given for increasing plasticity with increasing moisture content. An increase in the compressiblity factor would be expected with increasing moisture content (increasing plasticity).

In comparing the compressibility factor values in Tables 1 and 2, it was observed that the compressibility factors increased more rapidly for the whole corn than for the endosperm. This suggested that deformation at higher moisture contents was predominantly plastic due to the effect of the moisture content of the germ which was lower than that of endosperm around 80% relative humidity as given by Kumar (1972).

The ability to express the compaction behavior of powder foods in terms of the constant A and B_{cf} had several advantages. The mathematical expression of density-pressure relationship of a given powder food permitted the analytical determination of the density values in the range of pressures investigated. It also permitted extrapolation to pressure in excess of those available experimentally. However, the most important fact was that the constants quantitatively described the compaction behavior of a powder food under axial loads.

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HOT AIR TOASTING AND ROLLING WHOLE WHEAT
Effect on Organoleptic, Physical and Nutritional Quality

INTRODUCTION

CEREALS are efficient and inexpensive sources of calories, protein and certain vitamins and minerals. However, the raw flavor and consistency of cereals must be modified by cooking either at a central processing plant or in the home before they are palatable (Osman, 1967). Those cereals which receive most of their processing in a central plant save the consumer both time, an important factor in highly industrialized nations, and fuel which is often expensive and scarce for individuals in developing nations.

In this paper, we have chosen the word instant or instantized to describe a product sufficiently precooked to be palatable as is or by simply adding hot or boiling water to it without additional cooking. These instantized products can be finished products or precooked ingredients for instant formulations. The processing of cereals must be inexpensive and impart desirable flavors and convenient forms with minimal nutritional loss.

High temperature-short time heating of whole or debranned grain in air followed by flaking, milling, grinding or other conversions appeared an economically favorable approach to preparing products such as instant rolled wheat, grits or pregelatinized flour. A continuous, high capacity, hot air toasting machine designed to heat and expand grains for improved feed efficiency by means of recirculating hot air (Rockwell et al., 1968), produced a tasty flaked wheat product when used with conventional flaking rolls. Cost for toasting and rolling grain for feed, using this equipment, were estimated at $1.25–2.25 per ton depending on production rate. In addition, commercial units of 4 tons per hour capacity for feedlot use have been built (Walker, 1970). We saw no reason why this type of machine could not be used to produce precooked food products. Many uses for such products are possible, for example, in dry gravy mixes, dried soups, blended food products such as WSB which is used in the U.S. Food for Peace Program (Horan, 1973), meat extenders, engineered foods and porridges.

In a fluidized bed toaster, Walker et al, (1970), found that temperatures above 446°F gave satisfactory popping or expansion of various grains for feed use. At 473°F, they found grain was expanded in 30 sec and longer time only increased charring. Wheat, barley and dent corn displayed a puff index of 1.5–2.0, with all kernels expanding to about the same extent. They also showed that starch digestibilities (in vitro, diastase) increased as the moisture content of the wheat at time of toasting was increased up to 25% moisture.

Osner and Johnson (1968) in a review article point out, however, that food processing causes some degradation of proteins though not necessarily harmful. Mild degradation may improve digestibility. Nutritional losses occur when individual amino acids are destroyed or made unavailable. Interaction (especially lysine) with carbohydrate or lipids is known to cause such losses. Moisture and pH have been observed to affect loss rate but no generalized trends are apparent from limited data available. Blamberg (1970) observed marked differences in protein efficiencies among certain dry breakfast cereals which receive toasting. Formulation and heat damage were suggested as the two factors causing differences.

The purposes of our present investigation were to determine if the hot air toasting approach could indeed produce acceptable instantized food products with minimal nutritional loss and to explore a range of processing conditions and resulting product characteristics.

EXPERIMENTAL

Processing

A continuous, gas fired, hot air toaster of about 1 ton per hour capacity (shown diagramatically in Fig. 1) was used in this work. Air, heated in the center, circulates through the toasting compartments which revolve about the center. Retention time of the charge of wheat in the toasting compartments is determined by the speed with which the compartments revolve from the feed opening to the exit opening. An air temperature of 620°F at the slot was used throughout our work. The toaster operation and its characteristics have been discussed in more detail by Rockwell et al. (1968).

The wheat was moistened and allowed to temper overnight before processing. The processing sequence was: Feeder, hot air toaster, a cooling water spray at the toaster outlet, conveyer to the rolls, rolls, collecting bin, and shallow metal cooling trays. Feed rate was 800 lb of wheat per hour for all runs. Approximately 10% cooling water was sprayed on the wheat as it fell from the toaster exit. The sprayed wheat was conveyed in 1-1/2 min to the flaking rolls, which were smooth iron rolls set at 0.006 in. clearance at room temperature.

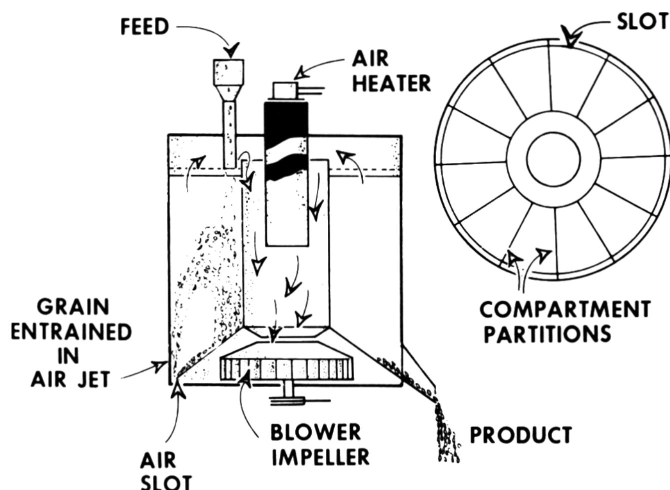

Fig. 1—Cutaway diagrams of the hot air toaster. Side view of vertical cross section on left; top view of horizontal cross section on right.

Batches of Gaines variety wheat, 30–40 lb each, with initial moistures at 10% and 20% (equilibrated) were toasted 10–40 sec. In addition, a 70-lb batch was processed at 30% moisture for 30 sec.

The temperature of the wheat leaving the toaster (before the spray) was measured by quickly thrusting a bare 26 gauge thermocouple directly into a quart sized sample from the middle of a run. This (unflaked) sample was cooled in an air stream and color lightness was determined using a Hunter D-25 color meter. Puff index was calculated by dividing the volume per gram of toasted whole wheat by the volume per gram of the original wheat.

Organoleptic

Three samples, arbitrarily selected, were hydrated with boiling water and tasted as a breakfast cereal by a small group to determine in a general way whether an acceptable product had been produced. Samples were rated good or bad, with comments. Next a series of small panels (three to seven persons) was held to determine what good or bad flavors were present and to relate changes in flavor or mouth feel to changes in processing conditions. Panelists were asked to place a series of samples in order based on flavor development, and then to describe the flavors in each sample. We were interested particularly in the point of raw flavor loss, but also in other flavors and other characteristics that the panelists might feel affect product acceptability. Some variations were made in the method of sample preparation for panels assessing specific flavors since preparation does affect flavor. Product forms on which at least some organoleptic tests were run included the flakes, toasted whole grain (eaten as is), flours from milling the toasted whole grain in a Brabender Junior mill and grits made from grinding the flakes in a Wiley mill.

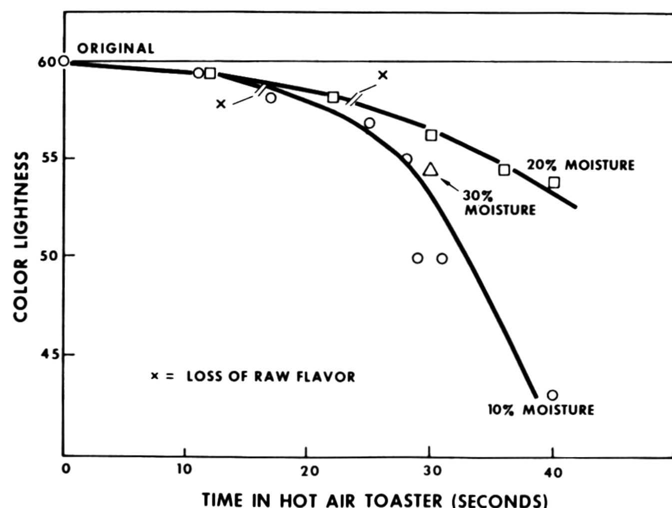

Fig. 2—Effect of toasting moisture and time on color (Hunter L value or lightness) of whole kernel wheat before rolling.

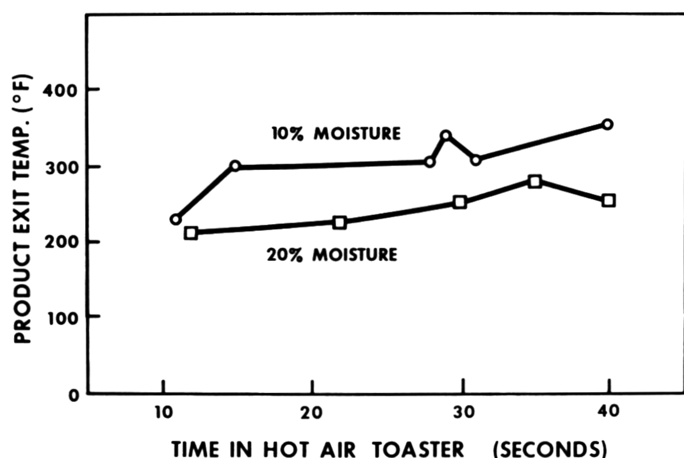

Fig. 3—Effect of toasting moisture and time on exit temperature of wheat from toaster.

Physical evaluation

Flaked samples were subjected to sieve analysis using 100g samples, in duplicate, on 8-in. screens, shaking for 5 min on a Rotap. Flake thickness values were obtained by taking the mean of thickness measurements using a platform micrometer on 10 flakes chosen at random from each sample.

When consistency tests were run using flakes it became evident that variations in the character (thickness, etc) of the flakes tended to interfere with the assessment of the effects of the other steps in the processing. Since the manufacture of flakes was incidental to the evaluation of the toasting, the remaining tests were run on flakes ground on a Wiley mill to pass 30 or 40 mesh.

A Bostwick consistometer (Bookwalter et al., 1968) was used to measure the consistency of sample slurries according to the following procedure: The appropriate weight of the ground (40 mesh) sample was added to 100g of water, mixed for 1 min in a 250 ml beaker, allowed to stand 4 min, stirred again briefly, poured into the Bostwick, and the top struck level with a spatula. The Bostwick trip lever was released within 15 sec, and the distance in cm traveled by the slurry in exactly 1 min recorded. Starch gelatinization determined by presence or absence of birefringence was measured using a Zeiss Standard WL polarizing microscope. Starch damage (Williams and Fegol, 1969), water absorption index (modification of Anderson et al., 1969) and soluble solids (Kite et al., 1957) were also measured.

Since the use of hot water, such as is used to make breakfast gruels, imposes a cooking effect on the product in addition to the cooking received in product manufacture, the tests were first run using cold water to measure the effects due to manufacture only. Then Bostwick, water absorption and soluble solids tests were repeated using hot (boiling) water, which better simulated a typical preparation procedure for eating or a situation in which the product would become part of a formulation subject to additional processing. During the 5 min hydration time for the Bostwick test the slurry temperature dropped to just below 70°C which approximates serving temperature. Water absorption and soluble solids were measured after cooling to room temperature. Selected samples were run on the Brabender viscoamylograph.

Nutritional tests

Thiamine was determined by the thiochrome method (AACC, 1962). Phytic acid was determined by the method of Wheeler and Ferrel (1971). Protein Efficiency Ratios (PER's) were determined using S-D strain rats by the AOAC method (1965). All diets were adjusted to 10% protein (N × 6.25) and PER's adjusted to 2.50 for casein diet. Original wheat was 15% protein, dry basis. Digestiblities of the diets were determined during the second trial week. The PER and Digestibility values for the processed samples were compared statistically with those for the unprocessed wheat at the 0.05 and 0.01 probability levels, using Student's t-tables.

Enzyme activities

Lipoxygenase activity of the ground, toasted flakes was determined by measuring the oxygen uptake using sodium linoleate as the substrate (Wallace and Wheeler, 1972). Esterase activity was determined using 4-methylumbelliferone caprylate as the substrate by measure-

ing the fluorescent cleavage product, 4-methylumbelliferone, in Tris buffer at pH 7.4 (Jacks and Kircher, 1967). A spot test was used to determine peroxidase activity using pyrogallol as hydrogen donor (Evans, 1970).

RESULTS & DISCUSSION

Organoleptic

The general reaction to the three flaked samples initially hydrated with boiling water was favorable. The flakes were said to possess a flavor of mild toast as well as an intriguing and very pleasant toasted aroma, although one of the three was slightly raw and required about 1-1/2 min further cooking to develop a pleasing flavor.

When the panels were expanded in an attempt to discover flavor patterns, relate them to processing and pick exact points of flavor change, some difficulties were experienced. All panelists arranged the samples in the same order, which corresponded to the actual processing time as shown in Figure 2. They described the flavor development with increasing processing time as: raw, bland, mild toast, heavy toast and a few described one sample (10% moisture, 40 sec) as having some burnt flavor. Aromas were light and full toast. It became obvious, however, through the comments of the judges that the flavor changes were gradual. Although opinions varied on the exact points of flavor change, all judges agreed on the changes taking place. Variations in sample preparations only made matters more complex since addition of heat, salt, etc. modified the flavors being assessed. Thus the points for raw flavor loss shown in Figure 2 actually represent the areas of flavor change from raw to bland. The 20% moisture samples required several seconds more processing time to destroy raw flavor than did the 10% samples.

Physical tests

Figure 3 shows the temperature of 10% and 20% moisture wheat batches, sampled at the toaster exit before the cooling water spray. The 30% moisture sample, processed 30 sec, left the toaster at 230°F. The color lightness of these unflaked samples after cooling is shown in Figure 2. It can be seen that toasting time does affect sample color with the 20 and 30% moisture samples remaining lighter than the 10% ones. In these experiments all hot air toasting treatments resulted in a puff index of about 1.5, that is, a 50% increase in the volume of the wheat.

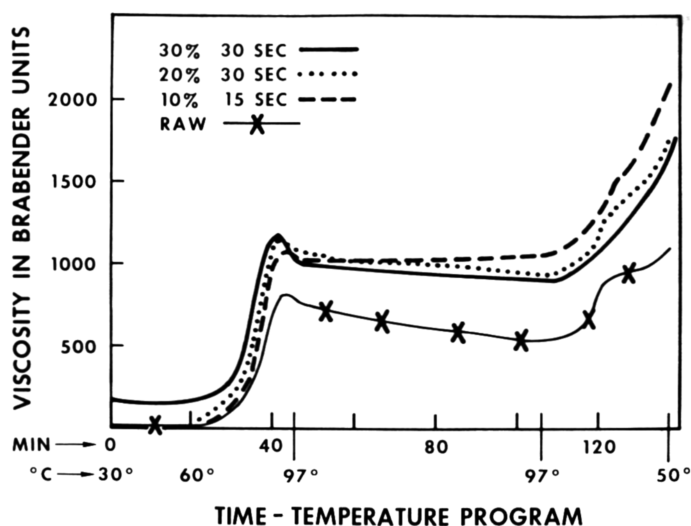

Fig. 4—Effects of hot air toasting and rolling on viscoamylograph characteristics using 65g of ground material (14% moisture) in 450 ml water. Program: heat at 1-1/2°C per min to 97°C; hold at 97°C for 1 hr; cool at 1-1/2°C per min to 50°C. Initial moisture shown in %, toasting time shown in sec.

Sieve analysis of the flakes showed a wide variation in the amount of fines produced (percent through 45 mesh) (Table 1). Correlation coefficients were developed for fines against each of the other factors in the table, first using total data, and then initial moisture groups separately. Amount of fines correlated (negatively) with initial and final moisture and flake thickness, but only when total data were used. This indicates that higher initial moisture (higher gelatinazation level) promotes greater flake integrity. Fines production was excessive for all the samples without raw flavor and would have to be reduced by some means if flakes were the desired product form.

Flake thickness (Table 1) was less than 0.025 in. for all the dry toasted series. The thickest flakes (more than 0.030 in.) resulted from high initial moistures with shorter toasting times and were considered raw to taste by most panelists.

The consistency values (Table 2, Col. I) of the ground product when mixed with cold water fell into four distinct groups corresponding to the initial moistures of the grain at toasting, beginning with the unprocessed sample, which made the thinnest slurry, and ending with the 30% processed sample, which formed a solid gel. Variations within each group were minor when compared to the large differences between groups. One cause of these differences is indicated by the birefringence values for the starch observed in dilute slurries of the samples (Col. II). Loss of birefringence is the first step in the gelatinization sequence. Thus gelatinization, which enables starch to absorb water, was more developed in the wet processed groups than in the dry or unprocessed, and the average for each group followed the group pattern of the cold Bostwick. Since wheat grain is about 60%

Table 1—Physical measurements made on the toasted wheat flakes

	Initial moisture[a]												
	10%							20%					30%
Toasting time (sec)	11	15	25	28	29	31	40	12	22	30	35	40	30
Final flake moisture[a] (%)	5.9	4.4	5.3	5.3	3.1	5.0	2.8	10.4	8.1	3.1	5.4	5.1	9.2
Fines (% thru 45 mesh)	18.5	23	23	20	16.5	19	16.5	5	13	13	13	21	3.5
Avg flake thickness (in/1000)	24.4	23	22	22	18.5	22.5	22.8	53	40.8	–	26.4	28.9	34.8

[a] Moisture determinations (AACC, 1962) were made on the grain before toasting and on the product immediately after cooling and before storage at 0°F. The final moisture values reflect changes in moisture due to water spray and uncontrolled cooling as well as due to toasting.

Table 2–Physical characteristics resulting from various toasting conditions after hydration with cold water

Wheat sample	I Bostwick[a] (33g per 100g water) cm	II Starch granules displaying birefringence %	III Damaged starch Absorbance with iodine[b]	III Damaged starch %[c]	IV Water absorption index[d] (2.5g per 40g water)	V Soluble solids (10g per 200g water) %
Original	(5 sec)	88	0.05	< 1	2.1	9
Processed						
Moist. Sec.						
10% – 11	(40 sec)	83	–	–	2.8	9
15	24	64	0.11	4	2.9	10
25	23	57	–	–	2.8	8
28	23	75	–	–	3.0	7
29	23	44	–	–	2.9	8
31	22	80	0.08	3	2.9	8
40	(15 sec)	67	0.08	3	3.0	8
20% – 12	3	10	–	–	3.7	8
22	3	26	0.46	22	3.9	8
30	3	10	0.36	18	3.9	9
35	3	15	–	–	4.0	9
40	4	10	0.40	20	3.7	9
30% – 30	0	3	0.73	38	4.5	10

[a] Ground to pass a 40 mesh sieve. When values are given in seconds, this equals time for slurry to flow the full 24 cm flow-path, otherwise value is distance traveled in exactly 1 min.
[b] Williams and Fegol (1969).
[c] % (Farrand) as calculated from the regression equation of Williams and Fegol (1969) between absorbance and percent Farrand.
[d] $\frac{\text{Total solids + water in ppt}}{\text{Total solids}} = \frac{\text{Wt. of wet ppt + wt of dry supernatant}}{\text{Total solids}}$

Table 3–Physical characteristics after hydration with boiling water

Wheat sample	Bostwick[a] I (20g per 100g water) cm	Bostwick[a] II (15g per 100g water) cm	III Water absorption index,[b,c] (2.5g per 40g water)	IV Soluble solids[c] (10g per 200g water) %
Original	10	21-1/2	6.3	16
Processed				
Moist. Sec.				
10% – 11	3	12	6.5	19
15	3	7	7.2	18
25	2	9	6.8	19
28	1	8	6.9	19
29	1	5	6.6	19
31	1	8	7.4	20
40	1	8	6.6	18
20% – 12	7	21	7.0	15
22	4	14	7.3	16
30	4	13	6.2	19
35	2	11	6.2	22
40	3	11	6.1	19
30% – 30	3	13	6.2	18

[a] Ground to pass a 40 mesh sieve. When values are given in seconds, this equals time for slurry to flow the full 24 cm flow-path, otherwise value is distance traveled in exactly 1 min.
[b] $\frac{\text{Total solids + water in ppt}}{\text{Total solids}} = \frac{\text{wt of wet ppt + wt of dry supernatant}}{\text{Total solids}}$
[c] Rapidly boiling water added, cooled 30 min with frequent stirring, centrifuged at 20°C

starch, it is to be expected that starch modification would have a major effect on the consistency variations in the toasted products, though changes in other components due to toasting might also contribute. Two common quantitative tests for starch modification, Water Absorption and Damaged Starch, shown in Col. III and IV (Table 2), followed the same pattern. Col. V shows that processing did not increase the solubility in water of any of the samples, which in turn indicates that neither dextrinization (suggested by Bostwick value for 10% moisture, 40 sec sample), nor the gelatinization sequence was far enough along in these samples to release molecular fragments soluble in water.

The gelatinization sequence initiated in the toasting treatment was carried on further when boiling water was added (Table 3). This is indicated by the high Bostwick values at greater dilutions compared with Table 2 where cold consistencies were measured (Col. I and II). The large consistency differences between toasted groups noted in Table 2 have been eliminated. All toasted samples had a greater consistency than the unprocessed wheat. Water absorption index (Col. III) shows the same effects. Preparation in boiling water compared to cold water doubled the soluble solids (Col. IV). However, there is no significant effect of toasting treatments on percent soluble solids as measured here.

When samples from each moisture group were run on a Brabender viscoamylograph so that these viscosity changes are seen as functions of preparation temperature and time (Fig. 4), the absolute viscosity values and the order among samples are seen to depend on the particular point in the program at which the measurements are compared. Similar variations must be expected if sample preparation for eating or reprocessing methods differ. The major effect of additional heating, however, is to nearly eliminate the large viscosity differences between toasted groups and to increase the viscosity of all samples to a much higher level. The difference in the viscosities of the toasted and untoasted samples (Fig. 4) is probably due to the action of amylase in the untoasted sample. The large cold water differences do not register on the viscoamylograph (30°C) due to the high dilution used here.

Nutritional

Figure 5 shows that thiamine was more labile when the wheat was processed dry. A study of the temperature of wheat leaving the toaster (Fig. 3) shows the samples processed at 10% initial moisture left the toaster some 10–60°F hotter than the 20% samples. Quite possibly, these temperature differences are related to rate of thiamine destruction. The first 10% moisture sample without raw flavor

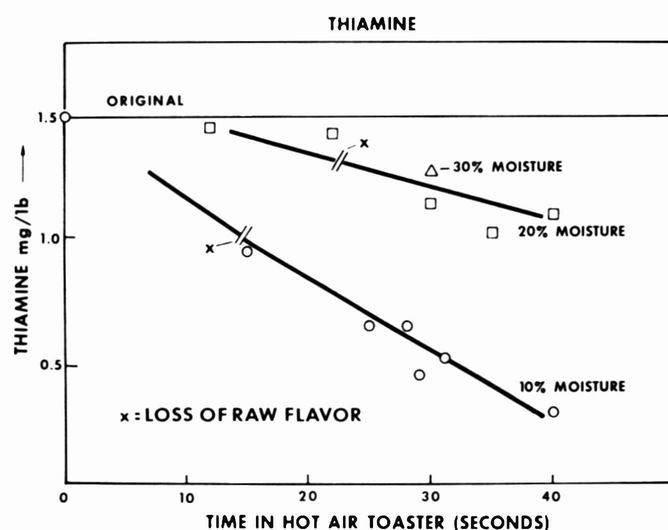

Fig. 5—Effect of toasting moisture and time on thiamine content of wheat.

Table 4—Effects of processing on PER and Digestibility

	Casein control	Original wheat	Toasted at 10% moisture		Toasted at 20% moisture		
			15 sec	40 sec	22 sec	30 sec	40 sec
PER	2.50	1.25	1.27	0.88[b]	1.37	1.31	1.27
Digestibility[a] %	95.7	89.7	90.6	90.8	90.6	90.6	91.0

[a] $\frac{\text{Feed intake} - \text{Fecal wt}}{\text{Feed intake}} \times 100\%$ = Digestibility of total PER diet. Wheat comprises about 67% of the diet (dry basis).

[b] Significantly lower than original wheat at $P < 0.01$

(15 sec) displayed a 35% thiamine loss while the first 20% sample without raw flavor (30 sec) lost 23% of its thiamine. Phytic acid values showed no significant changes due to processing. Both the original and processed wheat contained 0.2% phytate phosphorous.

None of the diets containing toasted wheat where significantly different in either PER or digestibility than the diet containing the unprocessed wheat, at either 0.05 or 0.01 probability level, except for the PER of the highly toasted 10% moisture, 40 sec one, which was significantly lower at $p < 0.01$ (Table 4). Since the 15 sec toasting at 10% initial moisture, and 30 sec toasting at 20% moisture produced cooked flavors, it is evident that the instantized product we had set out to make can indeed be produced by this method without protein damage. The fact that toasting at 20% initial moisture for times long beyond the point of raw flavor loss caused no loss in PER indicates a possible protective role for the water and suggests that the choice of gelatinization level which is determined by the initial moisture level can be extended upwards without nutritional detriment. The thiamine results also support this thought.

None of the processed samples showed enzyme activity when tested for lipoxygenase, esterase or peroxidase.

Other product forms

Although flakes received the greatest emphasis, they were not the only product form evaluated. Flaked samples ground to a grit retained the same flavors that were present in the flakes, though physical aspects such as mouth feel were of course, altered. A flour milled from the toasted whole wheat samples had the general appearance of first clears flour and flavor modifications similar to those of the flakes. These alternate forms show the range of uses to which toasted wheat could be put; for example, (1) a hot breakfast cereal can be made from the flakes alone; (2) the grits could easily be incorporated in formulation with soy, dried milk, or other nutritious cereal complements; while (3) the toasted flour might be used as the basis for beverage products, or instant sauces. Further work would be necessary to develop such products and to determine their storage stability or shelflife.

CONCLUSIONS

TOASTED, rolled wheat products possessing attractive toasted flavors have been prepared, with no protein damage (PER) and small reduction in thiamine. Flavor development depended on toasting time as well as initial grain moisture, while level of starch modification (gelatinization) varied only with initial moisture level. Thus flavor and consistency can be developed independently in toasting whereas they develop simultaneously in conventional cooking. Higher gelatinization levels appear linked with greater nutrient (thiamine) retention. The higher moisture needed for gelatinization probably reduces the high product temperature which would otherwise cause nutrient loss. The effect of gelatinization level on organoleptic appeal was not clear, though the gelatinization levels themselves were measured objectively. The effects of additional cooking (boiling) were to raise the viscosity of all samples to much higher levels, eliminating the large differences between processed groups, and to remove residual raw flavors from undertoasted samples. Hot air toasting allowed higher cooked viscosities, compared with no toasting, possibly due to destruction of enzymes which otherwise act during cooking.

Toasted whole grain, flakes, grits and a flour milled from the grain displayed the same flavor development pattern described for the flakes, that is, raw, bland, light toast and heavier toast. Physical aspects differed with product form, as did response to further cooking.

Acceptable products with varying properties were produced over a range of initial moistures and toasting times, indicating the possibility of optimizing or tailoring conditions for a particular product use.

Very low processing costs for hot air toasting, availability of various commercial equipment, retention of protein quality, the ability to produce products of various desired consistencies and with a bland or a highly pleasing toasted flavor, and numerous conceivable markets for toasted wheat products provides substantial incentive to improve and eventually utilize such a process.

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Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Dept. of Agriculture to the exclusion of others that may be suitable.

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INHIBITION OF STAPHYLOCOCCAL ENTEROTOXIN PRODUCTION IN CONVENIENCE FOODS

INTRODUCTION

TECHNICAL INFORMATION is needed by the food industry to establish food processing, packaging and storage conditions which will prevent the production of staphylococcal enterotoxin in foods. Previous research on staphylococcal food poisoning has been primarily concerned with the presence and growth of *Staphylococcus aureus* in foods. Markus and Silverman (1968, 1969) found that enterotoxin can be produced by nongrowing and nonreplicating cells. Therefore, research on cell growth or members of staphylocci alone is of little value in determining the presence of enterotoxin. In our laboratory we investigated the effect of combinations of environmental conditions on enterotoxin production in food slurries. The fluorescent antibody technique (FAT) of Stark and Middaugh (1970) was developed to detect enterotoxin producing *S. aureus* cells. A previous investigation in our laboratory demonstrated that individual environmental conditions were more inhibitory to enterotoxin production than to cell growth in a defined growth medium (Stark, 1970). Atmospheres of CO_2 and N_2 have unusual effects on cell fluorescence and enterotoxin production in culture medium (Stark and Middaugh, 1970). Thatcher et al. (1962) investigated the effect of gases on enterotoxin production in vacuum-packed bacon. Enterotoxin was found to be produced under 5% CO_2, in O_2 and in N_2.

The present paper presents data showing the effects of combinations of environmental conditions on staphyloccocal enterotoxin B production in food slurries. The foods were used as slurries to optimize opportunities for bacterial growth. The environmental conditions tested in cheese, ham and shrimp slurries were temperature of incubation, initial pH, NaCl concentration and an atmosphere of CO_2, N_2, or air.

EXPERIMENTAL

Materials

Staphylococcus aureus strain 243 was used throughout this study. Strain 243, the prototype strain for enterotoxin B, was obtained through the courtesy of M.S. Bergdoll (Food Research Institute, Madison, Wisc.). Highly purified enterotoxin B was supplied through the courtesy of E.J. Shantz (U.S. Army Laboratories, Fort Detrick, Md.).

Rabbit antiserum for enterotoxin B was prepared by injecting enterotoxin with incomplete Freund adjuvant. The freshly prepared antiserum was used for gel-diffusion tubes. Antiserum prepared and conjugated with fluorescein isothiocyanate by Stark (1970) was used for the FAT.

Methods

Detection of enterotoxin by gel-diffusion. The Oakley-Fulthrope double gel-diffusion method was used to verify enterotoxin detection by the FAT. The tubes were prepared according to Hall et al. (1963) as modified by Stark and Middaugh (1970) using Noble agar (Difco) and sodium barbitol (0.8g) in 100 ml of water (pH 7.4 buffer). Cells were centrifuged from the aqueous food slurries, and portions (0.2 ml) of the supernatant fluid were added to tubes and incubated at 30°C for 48 hr.

Staining of fixed smears. Smears were prepared and stained by the method of Stark and Middaugh (1970). Cells were observed after staining and the degree of cell fluorescence was determined with a graded density filter system, on a +1 to +4 scale (Heidelbauer and Middaugh, 1972). The fluorescence values presented in tables of results are averages of six replications.

All preparations were observed by using an American Optical Microscope and Fluorlume illuminator with an Osram HBO 200 W maximal-pressure mercury-vapor arc with a UGI (Corning No. 5840) as the exciter filter, and a Schott GG-99 as the barrier filter.

Production of enterotoxin under combinations of environmental conditions. Homogenized cheddar cheese (10% w/v) was used to insure growth and enterotoxin production. Additional foods included homogenized ham (10% w/v) and homogenized frozen shrimp (10% w/v). Fisher Brain Heart Infusion broth (BHI) (Fisher Scientific Co., Fairlawn, N.J.) was used throughout as a control medium for enterotoxin production and for inoculum preparation. The food slurries and BHI were sterilized at 121°C for 15 min before use.

The inherent NaCl concentrations in the food slurries were determined by modification of the Mohr titration method (Snell and Biffen, 1944).

Erlenmeyer shake flasks (250 ml) containing 70 ml of food slurries or BHI broth were used in all experiments except those involving special atmosphere control, in which case similar flasks with a side arm cleaning port fitted with a serum vial-syringe plug and a silicone rubber stopper were used. The syringe plug allowed the evacuation of the flask and the addition of measured amounts of the gases and sampling. All culture flasks were inoculated with 10^6 organisms of an 18-hr BHI broth culture. The test cultures were shaken throughout incubation in all cases. Throughout the investigation samples were taken at 16, 24 and 40 hr of incubation and checked for cell fluorescence and enterotoxin production. Growth in all food slurries was determined with staphylococcus 110 medium (Difco) using the Standard Plate Count Method.

The investigation was planned as a multiple-stage factoral design which in one experiment would yield the optimum and minimum conditions affecting enterotoxin production by particular combinations of variable conditions. A 3 × 3 × 3 design was chosen which allowed the testing of pairs of variables and three levels of each variable. The resultant nine sets of variables were duplicated and the experiments were repeated three times. Three levels of each variable were used to represent the expected optimum, maximum and minimum levels affecting toxin production by that variable. Three sets of variables determined to be optimum were carried over to the next stage to be combined with three levels of another variable.

First, combinations of three temperatures of incubation, and three pH's were tested. Secondly, three optima from this temperature-pH test were evaluated with three NaCl concentrations. Then three optima from step two were combined with three levels of adjusted CO_2 and air atmosphere (Stark and Middaugh, 1970). In test four, the same three optima from step two were combined with three adjusted N_2 and air atmospheres. Finally, representative combinations of conditions were tested with both ham and shrimp slurries.

In each case the NaCl was added to the medium prior to sterilization. The pH was adjusted aseptically after sterilization. The atmospheres of CO_2 and N_2 were also aseptically adjusted after sterilization (Stark and Middaugh, 1970).

In the experiments with controlled atmospheres, samples were aseptically withdrawn with sterile syringes via the side-arm stopper.

RESULTS

Effect of temperature of incubation vs. initial pH on cell fluorescence and enterotoxin production

Incubation temperatures of 30°C, 37°C and 44°C were tested with pH's of 5, 7 and 9. At a temperature of 44°C enterotoxin production was limited. The temperature of 37°C was the optimum. An apparent buffering action in the

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cheese slurries caused a pH drop to 5 within 6–9 hr. Detectable amounts of enterotoxin were produced in the flasks with an initial pH of 9. This could be explained by the fact the pH 9 flasks took longer to drop to pH 5, allowing some enterotoxin production. Stark and Middaugh (1970) found a pH of 5.5 to inhibit toxin production. Because of the pH drop, all three pH's were carried over for the second set of experiments along with an incubation temperature of 37°C. Table 1 shows the effects of these combinations on enterotoxin production. As can be seen by the last two sets of variables, the fluorescent antibody technique indicated cell-associated enterotoxin which was not indicated to be in the supernatant fluid by the immuno-gel diffusion tests. This was demonstrated throughout the experiments. All fluorescence has been found to precede enterotoxin detection in the supernatant fluid by up to 4 hr (Stark and Middaugh, 1970).

Effect of temperature–pH vs. NaCl concentration

The conditions tested at 37°C were pH 9, 7 and 5 vs. total NaCl concentrations of 2, 6 and 10%. From Table 2 it can be seen that growth conditions of pH 7–2% NaCl and pH 9–2% NaCl (both at 37°C) gave the best cell fluorescence. Fair cell fluorescence was also observed with growth conditions of pH 7 and pH 9–6% NaCl. The only positive gel-diffusion tubes were flasks with pH 9–2% NaCl. This was probably due again to the pH drop. The enterotoxin levels detected by the cell fluorescence were too low to detect by gel-diffusion. The three combinations of conditions selected to be used in the next experiment were pH 7–2% NaCl, pH 9–2% NaCl and pH 7–6% NaCl (all at 37°C).

Effect of temperature–pH–NaCl vs. atmospheres of CO_2 and air

25%, 50% and 100% CO_2 (percent of the atmosphere) in air were tested against the previously mentioned conditions. Results indicate (Table 3) that cell fluorescence is limited to flasks containing 2% NaCl and 50% and below added CO_2. This agrees with Stark and Middaugh (1970) and also shows that 6% total NaCl inhibits cell fluorescence and enterotoxin productions in this combination as shown by the gel-diffusion tubes. All of the gel-diffusion tubes were positive except those from flasks with 6% NaCl concentration.

Effect of temperature–pH–NaCl vs. N_2 and air

The same combination of conditions was tested here as in the CO_2 experiment with the same levels of N_2 substituted for the CO_2. The results corroborate previous work (Stark and Middaugh, 1970) with good cell fluorescence in the 16-hr samples and negative enterotoxin production as detected by gel-diffusion tubes from 100% N_2 atmosphere flasks. Inhibition of cell fluorescence and enterotoxin production was observed only with 6% NaCl (Table 4).

Effect of combinations of environmental conditions on enterotoxin production in shrimp slurry

The shrimp and ham slurries were used to see if the effects of the combinations of conditions were similar in other high

Table 1–Effect of temperature of incubation vs. initial pH on cell fluorescence and enterotoxin production by S. aureus Strain 243 in cheese slurry

Conditions		Cell fluorescence			Enterotoxin B Gel-diff.		
Temp	pH	16 hr	24 hr	40 hr	16 hr	24 hr	40 hr
44	9	+0.3	+0.5	+0.5	+	+	+
44	7	+0.25	0	+0.25	–	–	–
44	5	+1.75	+1.0	+0.75	–	–	–
37	9	+1.3	+1.75	+2.0	+	+	+
37	7	+2.5	+2.5	+1.5	–	–	–
37	5	+2.0	+2.0	+1.5	–	–	–
37	BHI Control	+3.0	+2.5	+3.0	+	+	+

Table 2–Effect of temperature-pH vs. NaCl on cell fluorescence and enterotoxin production by S. aureus Strain 243 in cheese slurry

Conditions			Cell fluorescence			Enterotoxin B Gel-diff.		
Temp	pH	NaCl (%)	16 hr	24 hr	40 hr	16 hr	24 hr	40 hr
37	7	2	+1.75	+2.2	+2.0	–	–	–
37	9	2	+2.25	+2.5	+2.3	–	+	+
37	7	6	+1.5	+1.75	+1.0	–	–	–
37	9	6	+1.6	+1.5	+1.75	–	+	+
37	BHI	Control	+3.2	+3.0	+3.2	+	+	+

Table 3–Effect of temperature–pH–NaCl vs. CO_2 and air atmosphere on cell fluorescense and enterotoxin production by S. aureus Strain 243 in cheese slurry

Conditions				Cell fluorescence			Enterotoxin B Gel-diff.		
Temp	pH	NaCl (%)	CO_2 (%)	16 hr	24 hr	40 hr	16 hr	24 hr	40 hr
37	7	2	50	1.75	1.3	0.2	+	+	+
37	9	2	25	1.6	0.5	0.2	+	+	+
37	9	2	50	1.6	0.5	0.2	+	+	+
37	7	6	50	0.3	0.5	0.4	–	–	–
37	BHI Control			3.5	3.0	3.4	+	+	+

Table 4–Effect of temperature–pH–NaCl vs. N_2 and air atmosphere on cell fluorescence and enterotoxin production by S. aureus Strain 243 in cheese slurry

Conditions				Cell fluorescence			Enterotoxin B Gel-diff.		
Temp	pH	NaCl (%)	N_2 (%)	16 hr	24 hr	40 hr	16 hr	24 hr	40 hr
37	7	2	50	2.8	1.5	0.2	+	+	–
37	7	2	100	2.5	2.3	1.3	–	–	–
37	7	6	50	0.4	0.2	0	–	–	–
37	7	6	100	0.6	0.5	0.3	–	–	–
37	BHI Control			3.3	3.0	7.8	+	+	+

Table 5—Effect of temperature, pH, NaCl, CO_2 and N_2 on cell fluorescence and enterotoxin production by S. aureus Strain 243 in incubation temperature of 37°C in all cases

Conditions				Cell fluorescence			Enterotoxin B Gel-diff.		
pH	NaCl (%)	CO_2 (%)	N_2 (%)	16 hr	24 hr	40 hr	16 hr	24 hr	40 hr
7	0			2.5	2.9	1.2	–	–	–
9	0			4.0	3.0	1.5	–	–	–
9	4			4.0	3.5	2.0	–	–	–
9	0			2.0	0	0	+	+	+
9	0	50	50	4.0	4.0	0	–	–	–
	BHI Control			3.5	4.0	4.0	+	+	+
	Shrimp Control			3.0	3.0	3.0	–	–	–

Table 6—Effect of temperature, pH, NaCl, CO_2 and N_2 on cell fluorescence and enterotoxin production by S. aureus Strain 243 in ham slurry

Conditions				Cell fluorescence			Enterotoxin B Gel-diff.		
pH	NaCl (%)	CO_2 (%)	N_2 (%)	16 hr	24 hr	40 hr	16 hr	24 hr	40 hr
9	3.6			3.0	4.0	3.5	–	–	–
9	6			0.5	2.0	2.0	–	–	–
9	3.6		50	0	1.2	3.2	–	–	–
9	3.6		100	0	0	4.0	–	–	–
7	3.6	25		0	0	0	+	+	+
9	3.6	50		0	0.6	0.6	+	+	+
		Ham Control		0.5	0.5	0	–	–	–
		BHI Control		3.0	2.3	2.3	+	+	+

protein foods. Good cell fluorescence was recorded from both pH 7 and 9 with 0% NaCl and from pH 9 with 4% NaCl flasks. The effect of CO_2 was similar to the cheese tests. Fair cell fluorescence was recorded with pH 9–0% NaCl and 50% and less added N_2 growth conditions (Table 5). Only spotty results were obtained with the gel-diffusion tubes, apparently the enterotoxin is absorbed into the food particles and not left in the filtrate. The most consistent positive gel-diffusion tubes were from growth conditions of pH 9–0% NaCl and 50% added CO_2.

The pH change in the shrimp slurries was insignificant, unlike that of the cheese.

Ham slurry

The NaCl concentration present in the ham slurry used was 3.6% (w/v). Amounts of NaCl were added to obtain concentrations of 6% and 8% in addition to the inherent level. These NaCl concentrations were tested with pH's of 5, 7 and 9 at 37°C.

Table 6 shows that peak cell fluorescence was recorded with the combinations of pH 9–3.6% NaCl with fair cell fluorescence with pH 9–6% NaCl growth conditions. Good cell fluorescence was also recorded with pH 9–3.6% NaCl with 50% and 100% added N_2. Very little to no fluorescence was recorded under the effect of CO_2 atmospheres.

One interesting point was the lack of all fluorescence in the inoculated ham slurry control flask which was not adjusted in any way. The original initial pH was 6.1 and the NaCl concentration was 3.6%. Apparently, this pH was too low; as seen from the adjusted flasks where all cell fluorescence was poor with pH 7 growth conditions.

The overall cell fluorescence was progressively better up to the 40-hr sample whereas the cell fluorescence was progressively poorer in the previous experiments. Apparently enterotoxin production in ham is slower.

The gel-diffusion results (Table 6) demonstrate that the only consistent positive tubes are those from flasks with adjusted CO_2 atmospheres.

DISCUSSION

THE EFFECTS of combinations of temperature of incubation, initial pH, NaCl concentration and atmospheres of CO_2, N_2 and air on cell fluorescence and enterotoxin production in cheese, shrimp and ham slurries has been shown. The fluorescent antibody technique was found to be reliable for rapidly detecting enterotoxin B produced in these food slurries except when influenced by high concentrations of CO_2 or N_2 atmospheres.

Enterotoxin production in cheese slurry (10% w/v) was inhibited by incubation temperatures of 44°C in combination with initial pH below 7. Enterotoxin was inhibited by temperatures of 37°C pH 7 and NaCl concentrations of 6% and above. The production of enterotoxin was inhibited in shrimp slurry (10% w/v) by pH's below 7 and by 8% NaCl at an incubation temperature of 37°C. In ham slurry (10% w/v) enterotoxin was inhibited by pH's below 9 and NaCl concentration above 6%, incubated at 37°C.

An atmosphere of CO_2 was found to inhibit cell fluorescence above 50% added CO_2 without affecting enterotoxin production or detection by the double gel-diffusion method. Carbon dioxide did not make the other environmental conditions more limiting. This is in exact agreement with the finding of Stark and Middaugh (1970). A N_2 atmosphere was found to inhibit cell fluorescence when combined with NaCl concentrations of 6% and above. Enterotoxin detection by double gel-diffusion was inhibited by added N_2 concentration above 50%. In combination with 6% NaCl enterotoxin production was inhibited at all N_2 levels (25%, 50% and 100%). The same effect of N_2 on cell fluorescence and enterotoxin production was shown by Stark and Middaugh (1970). However, the inhibition of all fluorescence by 6% NaCl indicates that flourescence of enterotoxin B in the presence of N_2 is not independent of all other environmental conditions affecting enterotoxin production.

The results of this investigation can help food processors eliminate toxin production during preparation, packaging and transportation of their products. Also, investigators of food poisoning cases can know under what combinations of conditions to expect toxin production in particular foods.

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EFFECT OF ROASTING ON AFLATOXIN CONTENT OF ARTIFICIALLY CONTAMINATED PECANS

INTRODUCTION

IN THE PAST 3 years, aflatoxin-producing strains of *Aspergillus flavus* Link ex Fries and *Aspergillus parasiticus* Speare were isolated from moldy pecans. (*Carya illinoensis* Koch) (Lillard et al., 1970; Doupnik and Bell, 1971). When incubated under improper conditions, both in-shell and shelled pecans supported extensive mold growth and toxin production (Lillard et al., 1970). This observation has prompted concern regarding potential toxin contamination in processed pecans and therefore studies were initiated to control mold growth on pecans more effectively during production (Taylor and Worley, 1972) and storage (Heaton, 1972). In addition, measures that could eliminate moldy or toxin contaminated nuts during shelling and sorting operations were investigated (Tyson, 1972).

Shelled pecans represent up to 85% of the pecan crop. Such nutmeats are traded in the form of halves, pieces, granules and meal and, in turn, are introduced into a wide variety of different products either commercially or in the home. It has been estimated that about 60% of all pecan meats serve as ingredients in bakery goods, confections and, to a small extent, as raw material for pecan oil recovery (Woodroof and Heaton, 1961). The remaining 40% are roasted or toasted and utilized for ice cream formulations, for salted nuts or for the preparation of pecan butter. Woodroof (1967) lists three major procedures for roasting pecan meats: dry roasting at 191°C for 12 to 15 min, roasting in vegetable oil at 191°C for 6 min and roasting in margarine at 107°C for 60 min.

Several attempts have been made to apply dry or moist heat as a means of detoxifying aflatoxin contaminated peanuts and cottonseeds (Dollear, 1969). Toxin degradation can be achieved to a certain extent although these compounds are rather heat stable (Dollear, 1969). Lee et al. (1968) showed that dry roasting of naturally contaminated single peanut halves at 150°C for 30 min lowered the content of aflatoxin B_1 and B_2 by 80% and 60%, respectively. Later Lee et al. (1969) reported that 65% of B_1 and G_1 in laboratory inoculated batches of peanuts was reduced during oil roasting at 163°C for 7 min or at 174°C for 3 min. Waltking (1971) roasted batches of naturally contaminated peanuts containing 200–400 ppb aflatoxin B_1 and G_1 and 20–40 ppb B_2 and G_2 at 204°C (no time mentioned) and found an average loss of 40–50% of B_1 and G_1 and of 20–40% of B_2 and G_2.

Pecans differ from peanuts in size, shape and chemical composition and are roasted commercially under different conditions than peanuts. For this reason, the laboratory scale study in this report was initiated to determine the effect of dry roasting and roasting in vegetable oil and margarine on the aflatoxin content of artificially contaminated pecan halves and meal. At the same time, the sterilizing effect of roasting on the mold used for inoculation was evaluated.

The observation of Cucullu et al. (1966) and Whitaker et al. (1970) with peanuts and that of Whitten and Smith (1972) with cottonseeds that natural contaminations occur in a very small proportion of those seeds with high toxin concentrations, led the present investigators to conjecture that a similar situation might prevail with pecan meats. Thus pecan halves with comparatively high initial toxin content were prepared. In pecan meal, natural contamination will probably be more uniform but at a lower level. Therefore only small toxin quantities were introduced to fresh pecan meal.

EXPERIMENTAL

Preparation of samples

Halves of an improved pecan variety (Fancy Mammoth Stuart, 240 halves per lb) and of seedling pecans (Fancy Medium Eastern Seedling, 500 halves per lb) of the 1971 pecan crop were used. For each roasting experiment 500g of halves were dipped into a 1% solution of sodium hypochlorite for 45 sec, washed in sterile water, blotted dry with sterile cheese cloths and transferred into a 2800 ml Fernbach flask. The halves were inoculated by adding 50 ml of an aqueous spore suspension containing a total of 5 million spores of *Aspergillus parasiticus* NRRL 2999 and repeatedly tumbled in the flask for an even dispersion of the spores. The halves were incubated at 25°C for 2, 3 or 4 days to obtain meats with different toxin concentrations. The halves were then dried at 50°C to 3–4% moisture content, a level recommended by Heaton and Woodroof (1970), and stored at 4°C until used.

Pecan meal (Fancy Grade) was artificially contaminated with a crude aflatoxin extract prepared as follows: yeast extract-sucrose broth (2g and 20g per liter) was inoculated with *A. parasiticus* NRRL 2999 and incubated for 7 days at 27°C. The aflatoxins were extracted with chloroform, and 45 ml of the concentrated extract were sprayed on 500g of meal. The meal was stirred for 30 min and dried for 2 hr at 50°C. Lower toxin concentrations were obtained by subsequent blending with non-contaminated meal.

Roasting procedures

A small baking and broiling oven with a 16 × 11 × 6-in. chamber (Model 303.69190, Sears, Roebuck & Co.) was preheated to 199°C. 170–200g of seedling halves (190–200 halves) or 200–300g of Stuart halves (110–160 halves) from one 500g inoculation batch were spread in the oven in a single layer on a screen. 150g of meat was spread on aluminum foil in 0.5-in. layer. A stainless steel plate between the heating elements and the screen or foil prevented direct radiation and insured more even temperature distribution. Halves and meal were roasted for 15 min at 191±10°C air temperature. Since the air temperature dropped below 191°C during the loading of the oven, the 2–4 min necessary for the oven to return to this temperature was not included as roasting time.

800–900g margarine (J.M. Filbert, Inc., Baltimore, Md.) were preheated in a pan to 117°C, the same amount of coconut fat (Durkee Konut, SMC Corp., Cleveland, Ohio) to 199°C. 150–350g Stuart or seedling halves from one 500-g inoculation batch were weighed into a "French fry" basket and roasted for 60 min at 107±10°C in margarine and for 6 min at 191±5°C in oil. Again, the 1–2 min required for the oil or margarine to return to the desired temperature after immersing the halves was not counted as roasting time.

Actual temperatures of halves and of the meal layer during roasting were measured with an iron-constantan thermocouple (0.027 OD, insulation included) which was placed into the center of a half or the meal layer.

Sampling and analytical procedures

After each roasting experiment, four 40-g samples of roasted halves (i.e., about 40 seedling or 20 Stuart halves) and four 40-g samples

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of fresh halves of the same inoculation batch were placed in separate quart size Mason jars and immediately covered with 200 ml acetonitrile-water (8:2, v/v). Then the jars were placed in a freezer at −18°C. In the same way, two 50-g samples each of fresh and roasted pecan meal of every roasting test were transferred into quart size Mason jars with 150 ml acetonitrile-water (8:2, v/v) and stored at −18°C. All samples were brought to room temperature for extraction and blended with a high speed blender (I. Sorvall Inc., Newtown, Conn.) for 3 min. The extracts were filtered through a Whatman #1 filter paper and the aflatoxins partitioned into benzene according to Cucullu et al. (1972).

In one dry-roasting experiment, aflatoxins from 40 fresh and from 40 roasted seedling halves were assayed. For this study the nutmeats were assayed individually by extracting with acetone-water (7:3, v/v) utilizing the method proposed by Cucullu et al. (1966) for single peanuts.

The extraction procedure of Parker and Melnick (1966) was suitable for assaying of aflatoxins from oil and margarine. Two 50-g samples were weighed into separatory funnels immediately after each roasting test and extracted when still warm.

The aflatoxins in all extracts were separated by thin-layer chromatography on Adsorbosil-1 (Applied Science Labs, State College, Pa.) using chloroform-acetone (88:12, v/v) as the developing solvent in a nonequilibrated tank. The developing time was 30–40 min at 25°C. Aflatoxin spots were quantitated with a fluorodensitometer (Photovolt Inc., New York, N.Y.) by comparing fluorescence of samples with that of standards obtained from the USDA Southern Marketing & Nutrition Research Div., New Orleans, La.

Test for mold growth after roasting

Stuart and seedling halves with high content of *A. parasiticus* NRRL 2999 were prepared as previously described except that the drying temperature was held at 30–40°C rather than at 50°C. 20 roasted halves were selected from (1) a dry roasting; (2) a roasting in oil treatment; and (3) a roasting in margarine trial and transferred to the surface of plates containing Czapek-Dox agar (Difco). The plates were incubated at 27°C for 21 days and examined for occurrence of mold growth.

RESULTS & DISCUSSION

ANALYSES of 40 single fresh and 40 single dry-roasted seedling halves revealed (Table 1) that the initial toxin distribution within one inoculation batch was not uniform. This was partly due to the fact that mold growth occurred mainly in the embryo area and other places where the testae of the halves were ruptured, and that the size of these ruptures varied greatly. Uneven dispersion of the spores throughout the batch probably contributed to the variations. Nonetheless, the average toxin degradation in this particular experiment was statistically highly significant (Table 2). Degradation, calculated on the basis of initial and final toxin concentrations, was 89% for B_1 and 85% for G_1. Due to a weight loss of 7% during roasting actual degradation based on total toxin quantities initially introduced is even higher, 90% for B_1 and 86% for G_1. It should also be noted that the values in Table 1 for toxin concentrations of fresh and roasted halves cannot be paired, since one half could be analyzed only before or after treatment.

Despite the large variation within one inoculation batch, assaying four 40-g samples of fresh and roasted halves of each roasting trial proved to be a suitable procedure for achieving conclusive results. Degradation recorded with this method for dry roasting of halves using different toxin levels are presented in Table 3. The concentration of aflatoxin B_1 and G_1 in seedling halves was reduced by 70–90%; in Stuart halves the reduction varied from 30–60%. Table 3 also shows a reduction of 50–70% in pecan meal roasted under similar conditions.

Within the three products, the extent of degradation did not depend on the initial toxin concentration. The overall reduction in Stuart halves and in meal was significantly lower than that in seedling halves. Differences of temperatures in the three products, none of which reached the oven temperature of 191°C during the 15 min roasting time, explain the different extent of reduction (Fig. 1). Stuart halves have almost twice the volume of medium halves and therefore a lower rate of heat penetration. Temperature curves are also somewhat lower in the center of the particular meal layer chosen for this study. That reduction rates depend on product temperatures was shown by

Table 1—Aflatoxin concentration of 40 fresh and 40 dry-roasted seedling halves from the same inoculation

Toxin concentration (µg/g)							
Fresh[a]		Roasted[b]		Fresh[a]		Roasted[b]	
B_1	G_1	B_1	G_1	B_1	G_1	B_1	G_1
6	7	.05	.1	508	653	24	46
30	29	.1	.5	522	657	35	57
67	33	.2	.9	573	701	39	77
68	87	.4	1.5	584	800	41	77
84	142	.4	2.0	665	867	49	87
101	225	.7	5.0	740	890	56	89
138	241	1.4	6.0	758	972	64	92
159	254	1.5	7.0	763	996	70	94
180	282	3.0	7.4	787	1007	71	104
198	315	3.1	8.8	816	1024	75	141
201	320	4.3	13	823	1059	77	147
243	343	6.0	16	863	1128	93	198
244	356	6.0	22	900	1169	100	214
292	399	7.0	23	935	1231	102	270
336	402	11	24	989	1424	125	275
355	480	13	30	1143	1605	136	348
424	492	16	31	1241	1734	139	364
463	540	18	31	1256	1741	147	543
500	575	19	32	1586	2377	435	560
507	611	21	43	1600	2430	436	637

[a] 3-day incubation
[b] Dry roasting at 191°C for 15 min

Table 2—Reduction of aflatoxin concentration and weight loss during dry roasting[a]

			Fresh	Roasted	
B_1	Mean concentration	(µg/g)	567[b]	61[b]	
	Standard deviation	(µg/g)	412	98	
	Reduction in % of initial conc.				89
G_1	Mean concentration	(µg/g)	765[b]	118[b]	
	Standard deviation	(µg/g)	603	164	
	Reduction in % of initial conc.				85
Average weight per half		(g)	0.99	0.92	
Weight loss in % of fresh weight					7

[a] Calculations from data shown in Table 1
[b] Difference between fresh and roasted significant at the 1% level (Student's t test; Beyer, 1968)

roasting seedling halves at 144, 171 and 191°C for 15 min. The concentration of aflatoxin B_1 and G_1 was reduced by 15, 40, and 80% respectively. These observations also indicate that a large amount of the toxin is located within the halves and not only on the surface because surface temperatures and thus degradation of toxin on the surface would not vary with different sizes of halves containing 4% or less moisture. The distribution pattern was confirmed when surface areas were assayed for toxin separately from the rest of the halves: Fresh and dry-roasted seedling halves (191°C for 15 min) were gently shaken with three portions of acetonitrile-water (8:2, v/v) for 1 min each time and only then blended for further analysis. Of the total toxin content, 0.5% was found in the washing of fresh halves and 0.2% in that of roasted halves. Therefore, only about 0.5% of the total toxin in fresh meats or 0.2% in roasted meats was located on or very near the surface of the halves. The relation between roasting time and toxin degradation was not investigated; it is obvious that total reduction increases with longer treatment periods according to the reduction rate at a particular temperature.

Almost no difference between temperature curves of seedling and Stuart halves was observed when those nutmeats were roasted in oil and margarine (Fig. 1). In contrast to the dry roasting, all the pecan meats reached the temperature of the oil and margarine bath within a short time. In these treatments, the difference of pecan size does not play an important role. Table 4 shows that the toxin reduction in two sizes of halves were almost identical, i.e., 65% (seedling) and 60% (Stuart) for roasting in oil, 60% (seedling) and 59% (Stuart) in margarine. The reduction of toxin concentration during oil and margarine roasting was about equal. The much lower margarine temperature 107°C compared to that of the oil bath (191°C) was compensated by the much longer treatment time (60 min vs. 6 min).

The percentages are based on toxin concentration in fresh and roasted pecans

Table 3—Reduction of aflatoxin concentration in halves and meal during dry roasting for 15 min at 191°C

	Initial toxin conc (µg/kg)		Reduction of initial conc (%)		
	B_1	G_1	B_1	G_1	Total
Seedling halves	306,000[a]	467,000[a]	71 ± 4[b]	82 ± 5[b]	
(average weight	216,000	370,000	81 ± 4	78 ± 5	
0.9g per half)	88,000	42,000	83 ± 3	84 ± 4	
	77,000	67,000	91 ± 8	76 ± 11	
	6,700	6,400	71 ± 7	78 ± 5	
			80	80	80
Stuart halves	230,000	175,000	36 ± 18	36 ± 19	
(average weight	104,000	155,000	68 ± 12	54 ± 13	
1.9g per half)	4,680	5,680	52 ± 8	30 ± 10	
			52	40	46
Meal	5,750	4,650	65	66	
	1,650	1,620	70	67	
	555	585	52	45	
	165	220	48	57	
			60	60	60

[a] Mean of 4 samples for halves and of 2 samples for meal; the extract of each sample and the aflatoxin standards were spotted twice on the same TLC plate.
[b] Calculation of confidence limits from standard deviations and according to the rules of error propagation (Bevington, 1969).

Table 4—Reduction of aflatoxin concentration in halves during roasting in oil and margarine

	Initial toxin conc (µg/kg)		Reduction of initial conc (%)		
	B_1	G_1	B_1	G_1	Total
Seedling halves	328,000[a]	502,000[a]	49 ± 6[b]	58 ± 6[b]	
in oil (6 min	185,000	327,000	77 ± 7	76 ± 7	
at 191°C)	89,000	247,000	52 ± 19	74 ± 15	
	1,440	2,420	68 ± 8	66 ± 5	
			62	68	65
Stuart halves	110,000	150,000	62 ± 6	35 ± 7	
in oil (6 min	2,000	2,710	60 ± 8	52 ± 7	
at 191°C)					
			61	58	60
Seedling halves	105,000	99,000	64 ± 8	67 ± 13	
in margarine	2,370	3,510	58 ± 10	51 ± 9	
(60 min at 107°C)					
			61	59	60
Stuart halves	104,000	155,000	55 ± 5	54 ± 5	
in margarine	4,160	5,680	65 ± 7	63 ± 6	
(60 min at 107°C)					
			60	58	59

[a] Mean of 4 samples; the extracts of each sample and aflatoxin standards were spotted twice on the same TLC plate.
[b] Calculation of confidence limits from standard deviations and according to the rules of error propagation (Bevington, 1969).

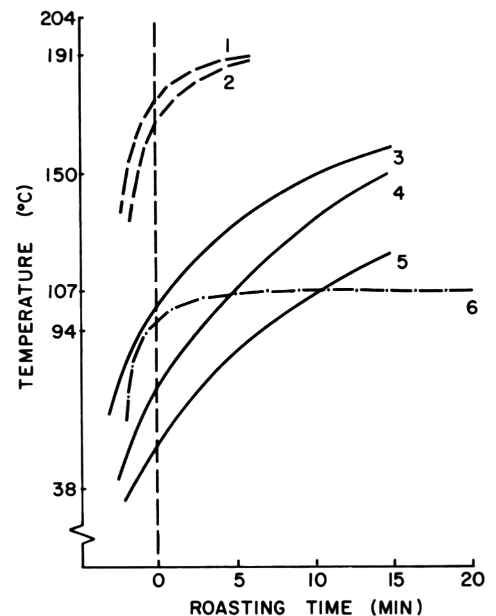

Fig. 1—Internal temperature during roasting of seedling and stuart halves and meal: (1) Seedling halves in oil, 191°C; (2) Stuart halves in oil, 191°C; (3) Seedling halves, dry, 191°C; (4) Meal, dry, 191°C; (5) Stuart halves, dry, 191°C; and (6) Seedling and Stuart halves in margarine, 107°C. At zero roasting time, the air, oil or margarine reached the desired temperature indicated above.

Table 5–Average mass balances for roasting of seedling halves in oil and margarine

	% of amount introduced	
	B_1	G_1
300g fresh halves	100	100
800g fresh oil	0	0
1100g	100	100
315g roasted halves	40	39
776g oil after roasting	3	4
1091g	43	43
Actual toxin degradation	57	57
150g fresh halves	100	100
900g fresh margarine	0	0
1050g	100	100
196g roasted halves	45	45
721g margarine after roasting[a]	3	5
917g	48	50
Actual toxin degradation	50	50

[a] Part of loss due to water evaporation

regardless of weight changes due to uptake of oil by the nuts and are therefore somewhat misleading. Aflatoxin could be recovered from the oil and margarine bath after roasting. Average mass balances for halves, oil, margarine and aflatoxins B_1 and G_1 were calculated (Table 5). Whereas the reduction during roasting in oil, based on the concentrations of toxin in seedling halves, was 65%, only 57% of the initially introduced toxin quantity was actually degraded. Roasting in margarine reduced the toxin concentration in seedling halves by 60%, but degraded only 50% of the total introduced toxin amount. Immediately after roasting, 2–4% of the toxin was found in the oil and margarine respectively. Experiments with Stuart halves gave similar results. Parpia and Sreenivasamurthy (1971) indicated that the degradation rate of aflatoxin in hot oil is comparable to that in pecans treated under the same conditions. Yet these authors state that aflatoxin-free products pick up significant amounts of toxin when fried in contaminated oil.

A nonaflatoxin greenish fluorescent artifact, which Lee et al. (1969) found during their roasting trials on chromatograms at R_f values of aflatoxin G_2, was often observed in the present study. With this exception, naturally occurring substances in fresh and roasted pecans other than aflatoxins did not interfere in the methods used.

No growth of *A. parasiticus* or other molds occurred on any of the Stuart or seedling halves that had been incubated after roasting. Apparently the heat treatment utilized in all three roasting methods was sufficient to effectively inactivate *A. parasiticus* or *A. flavus*. Mycelium and conidia of aspergilli are substantially less resistant to dry heat than certain bacterial spores. Pitt and Christian (1970) found a similar difference in heat resistance between aspergilli and bacterial spores in aqueous systems.

Findings in the present study suggest that roasting significantly lowers the concentration of aflatoxin B_1 and G_1 in contaminated pecan halves and meal. The extent of reduction depends upon the type of product and the kind of roasting. Vegetative cells or spores of *A. flavus* and of *A. parasiticus* on pecan halves are destroyed during roasting. Yet, potential toxicity or carcinogenicity of degradation products of aflatoxins remain as questions which require further investigation before applying roasting as a detoxification procedure.

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COMPARATIVE EFFECTS OF ETHYLENE OXIDE, GAMMA IRRADIATION AND MICROWAVE TREATMENTS ON SELECTED SPICES

INTRODUCTION

MOST SPICES, imported from tropical countries, are very high in bacterial count and thus may contribute to the spoilage of food products in which they are used (Jensen, 1954). The spoilage activities of spices in meat products such as spiced meat steaks, canned foods and different sausages have been reported (Yesair et al., 1942).

Today, ethylene oxide treatment is used to achieve 90% reduction in the bacterial population of many spices. However, depending on the microbial load of spices and the process, this treatment could be costly and time consuming. Furthermore, the undesirable effects of ethylene oxide treatment on the flavor and color qualities of spices has been known in some cases and as a result, spices such as onion and garlic have generally not been treated with ethylene oxide to prevent losses in flavor (Neale, 1963, personal communication).

Microwave energy and ionizing radiation are two methods whose potential use in the food industry has been tested and appreciated in many food institutions. The purpose of this study was to ascertain the efficacy of both microwave and gamma irradiation as means of sterilizing six ground spices as compared to the conventional ethylene oxide method.

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MATERIALS & METHODS

THE SIX GROUND spices were selected for their extensive use in the food industry and relatively high bacterial contamination. The ethylene oxide treatment was carried out on spices in small polyethylene bags according to commercial practices. Black pepper, paprika, oregano and celery seeds were treated with ethylene oxide for 16 hr while allspice and garlic were treated for 12 and 5 hr, respectively. Gamma irradiation was performed on spices in test tubes submerged in water at a constant temperature of 24°C. With respect to microwave treatment, samples were placed in glass tubes and subjected to different microwave units at various holding times.

The ethylene oxide sterilizer resembled an autoclave with a volume of 160 cu ft, maximum capacity of 3000 lb, an operating temperature of 135°F and humidity equal to that of 150 ml of water. The gas, consisting of a mixture of 10% ethylene oxide and 90% carbon dioxide by weight was introduced to the chamber under 25 in. of vacuum until a positive pressure of 8 lb was established. The gamma irradiation unit consisted of a gamma cell 220 (^{60}Co) with a maximum dose rate of 1.6 Mrads per hour. The two microwave units consisted of an S-band 2.45 Ghz continuous wave oscillator with a power output of 1kw, and an X-band 8.5 Ghz pulsed oscillator with average power of 25 watts.

Microbiological examinations consisting of total count, thermophilic count and aerobic spore count were performed on spices according to the standard APHA (1966) method.

The water-soluble and water-insoluble color of paprika were determined by the Official Analytical Methods of the American Spice Trade Association. The color change in ground paprika was determined using a Hunter Color Difference Meter (model D-25).

The preparation and smoking of garlic sausage were done according to commercial practices. The finished product was divided into three groups for incubation at 8°C for 2 wk, 24°C for 1 wk and 35°C for 2 days. Microbial examinations consisting of total count, yeast and mold count, and greening bacteria were performed on the product before and after smoking and during incubation at various conditions.

The flavor evaluation of the product was investigated by a panel of 10 judges. The methods used in this evaluation were the ranking difference and scoring difference tests obtained from the *Methods in Sensory Evaluation of Food* (Larmond, 1967).

RESULTS

THE TOTAL bacterial examination of raw spices indicated varying degrees of contamination. Similar results were obtained for the thermophilic count and the aerobic spore count which were relatively lower among the six spices (Table 1).

Paprika and oregano were the only spices free from bacteria after the ethylene oxide treatment. Thermophillic bacteria were found only in black pepper and garlic. The aerobic spore formers were not found in any of the six spices after fumigation (Table 1).

The gamma irradiation treatment of spices indicated that different doses were required for the total destruction of microorganisms (Fig. 1). However, thermophilic bacteria and aerobic spore formers required lower doses of radiation for total destruction (Fig. 2 and 3).

With respect to microwave treatment,

Table 1—The comparative effect of ethylene oxide and gamma irradiation on the bacterial flora of selected raw spices

	Treatments (Number of organisms per gram)								
	Raw			Ethylene oxide			Gamma irradiation		
Spices	Total count	Thermophilic	Aerobic spores	Total count	Thermophilic	Aerobic spores	Total count	Thermophilic	Aerobic spores
B. pepper	4.0×10^6	1.58×10^6	6.34×10^4	1.48×10^3	4.3×10^2	0.0	0.0	0.0	0.0
Paprika	9.86×10^6	3.24×10^5	3.0×10^3	0.0	0.0	0.0	0.0	0.0	0.0
Oregano	3.26×10^4	1.8×10^3	1.0×10^2	0.0	0.0	0.0	0.0	0.0	0.0
Allspice	1.74×10^6	1.5×10^6	1.05×10^2	4.25×10	0.0	0.0	0.0	0.0	0.0
Celery seeds	3.7×10^5	1.3×10^5	3.94×10^3	0.8×10	0.0	0.0	0.0	0.0	0.0
Garlic	4.65×10^4	9.0×10^2	0.0	1.45×10^4	3.5×10^2	0.0	0.0	0.0	0.0

it was found that generated heat was localized due to conduction and was insufficient for the sterilization of spices. The results indicated no significant change in the bacterial population of spices following microwave treatment.

Among the six spices, the volatile oil content of black pepper and allspice were reduced by more than half after gas fumigation, while gamma irradiated spices indicated no change in volatile oils after radiation. The nonvolatile oil content of spices were similarly affected by the ethylene oxide. Gamma irradiated spices on the other hand, indicated a slight change which was found to be insignificant (Table 2).

Table 2–The comparative effect of ethylene oxide and gamma irradiation on the volatile and nonvolatile oil content of six raw spices

	Treatments (Volatile and nonvolatile oils, %)					
	Raw		Ethylene oxide		Gamma irradiation	
Spices	Volatile oil	Nonvol. oil	Volatile oil	Nonvol. oil	Volatile oil	Nonvol. oil
B. pepper	3.6	10.436	1.6	9.210	3.6	9.363
Paprika	–	14.345	–	11.380	–	15.010
Oregano	3.3	10.401	3.2	8.060	3.33	10.248
Allspice	6.16	10.910	1.6	6.130	6.16	9.650
Celery seeds	1.73	23.420	1.73	21.360	1.73	23.100
Garlic	–	1.340	–	0.607	–	1.040

The results of the Hunter color determination of ground paprika indicated lower values for brightness, redness and yellowness of ethylene oxide treated samples as compared to the untreated and gamma irradiated samples (Table 3). Visually, ethylene oxide treated samples were dull and darker in color while no noticeable difference in color was observed between the untreated and gamma irradiated samples. The water-soluble and water-insoluble color of the ethylene oxide treated paprika indicated a different O.D. value than that of the raw paprika. Gamma irradiated samples, on the other hand, indicated no change in the O.D. values.

The total bacterial count of different sausages prior to smoking was highest when the samples were made from raw spices. Sausages made with gamma irradiated spices had the lowest level of contamination. During the incubation of sausages at various temperatures and periods of storage, the bacterial population of sausages had increased relative to each condition (Table 4).

The studies performed on the type of colonies indicated that after smoking, the developed colonies had mainly originated from raw spices. The morphological and biochemical examinations of these colonies identified them as *Bacillus mycoides* and *Bacillus stearothermophilus* which were found in considerable amounts in raw black pepper. The examination of spices and sausages for the greening bacteria indicated that mainly subsurface colonies were responsible for such a discoloration. The morphological examinations of these colonies revealed gram positive motile rods of a microaerophilic nature which were found in the greatest proportion in garlic.

The flavor evaluation of sausages followed by the analysis of variance indicated that the panel had no specific preference for any one type of sausage over

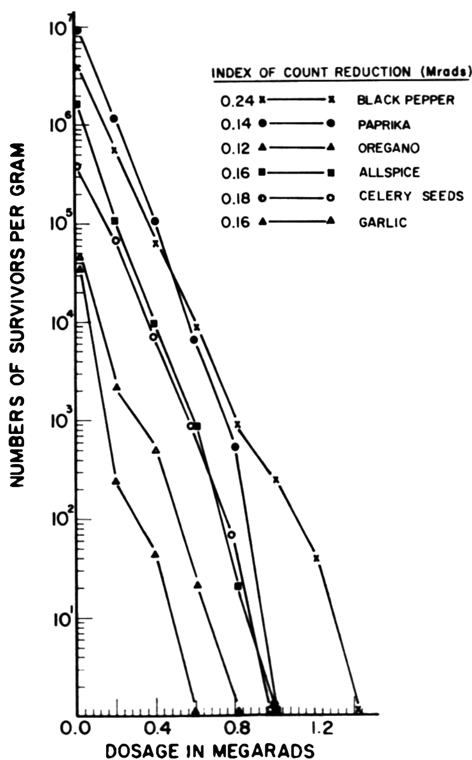

Fig. 1–The effect of gamma irradiation on the total bacterial flora of selected raw spices.

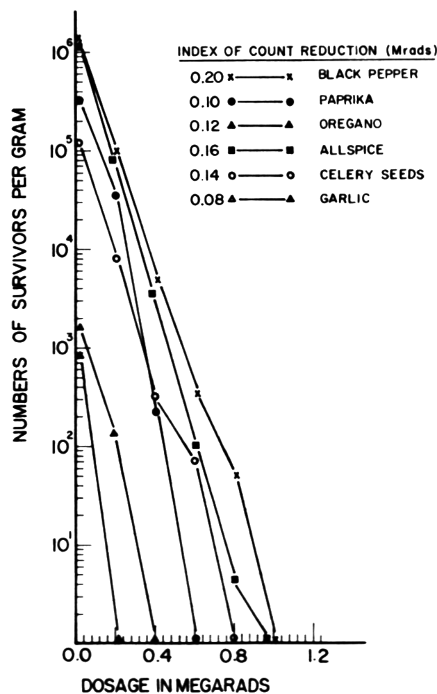

Fig. 2–The effect of gamma irradiation on the thermophilic bacteria of selected raw spices.

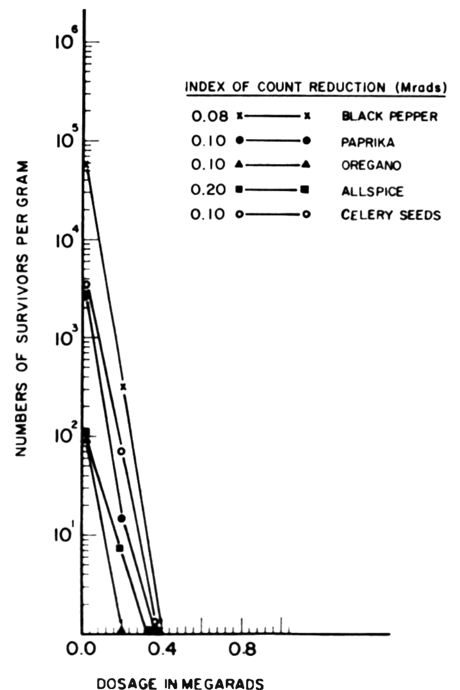

Fig. 3–The effect of gamma irradiation on the aerobic spore count of selected raw spices.

the other. The ANOVA on the ranking evaluation, however, indicated that the panel preferred sausages made with gamma irradiated spices over those made with ethylene oxide treated spices.

DISCUSSION

TODAY, safety in the bacteriological quality of spices is achieved by subjecting spices to ethylene oxide treatment and obtaining 90% reduction in the bacterial population. However, depending on the process used, a heavily contaminated spice usually requires many hours of treatment to bring about complete destruction of microorganisms. Since this is not feasible, many spices contain microorganisms in varying quantities, depending on the duration of treatment. The least reduction in the bacterial population was indicated by garlic after the ethylene oxide treatment. This means that a 5-hr treatment was not sufficient to adequately reduce the bacterial population of garlic. More drastic treatment of this spice with ethylene oxide is deemed undesirable because of the possible loss of flavor quality (Neale, 1963, personal communication).

In our investigation, it was found that those samples which contained high thermophilic and spore forming bacteria required higher radiation doses for complete destruction of the microorganisms. In these samples, there appears to be a correlation between high thermophilic and spore count and total bacterial count. On the basis of our findings and the index of count reduction, it was possible to establish an irradiation procedure by which sterility could be achieved in any spice, once the contamination level was known.

The most important property of the microwave energy is the ability to rapidly generate heat in moist products. As a result of a low moisture content, the spices were incapable of being heated sufficiently. Therefore, variation in the frequency, distance from the source, shape and type of container, as well as the potential power of the microwave sources did not affect the samples microbiologically. Consequently, it was concluded that microwave treatment was not effective in reducing the microbial population of spices.

During the storage of sausages at 8°C and room temperature, the bacterial population of sausages increased slowly. However, it should be noted that on a commercial basis, a greater increase in the bacterial population could be expected due to the excessive water content of sausages and the higher relative humidity of the low temperature storage room.

Table 3—The comparative effect of ethylene oxide and gamma irradiation on the color of paprika

Treatment	Color of ground paprika (brightness) L	(redness) +a	(yellowness) +b	Water soluble color O.D. at 450 nm	Water insoluble color (A.S.T.A. units)
Raw paprika	24.8	22.7	12.2	0.23	85.7
Ethylene oxide treated paprika	22.26	16.3	9.7	0.33	81.0
Gamma irradiated paprika	24.9	23.6	12.3	0.23	85.7

Table 4—The effect of smoking and storage on the total bacterial counts of different garlic sausages

Sample condition	Total bacterial count (No/g) Unsmoked	Smoked	35°C/2 days	24°C/1 wk	8°C/2 wk
Meat and gamma-irradiated spices	9.37×10^4	8.3×10^3	6.6×10^6	3.9×10^4	9.1×10^3
Meat and ethylene oxide treated spices	1.05×10^5	4.55×10^4	1.24×10^8	1.5×10^5	5.6×10^4
Meat and raw spices	3.35×10^5	1.2×10^5	1.31×10^9	4.93×10^6	2.87×10^5

In regard to ranking difference, it should be noted that the panel's preference for sausages made with gamma irradiated spices may not be due mainly to the effect of the treatment, as other factors which may be present could have easily influenced the panel's judgement on the ranking difference test. Nevertheless, there still remains the possibility of enhancing the flavor characteristics of spices by the irradiation treatment. This however, remains to be seen until extensive research is done with the aid of more accurate analytical means and toxicological studies, to establish the relative importance of these changes.

CONCLUSION

IN OUR STUDY, it was shown that gamma irradiation was more effective than ethylene oxide in reducing the bacterial population of spices. In addition, it was found that the ethylene oxide treatment reduced the oil content of spices and affected the color of paprika; whereas with gamma irradiation these changes were found to be insignificant. It is needless to say that other fumigation practices may prove to be more advantageous in reducing some of these changes in spices.

Processing by ionizing radiation is technologically feasible. However, of the factors that could affect commercialization of food irradiation, the most important are investment and operating costs. Therefore, if the radiation step can fit into an operating process line, the additional capital cost would be relatively inexpensive (USDC, 1968).

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A Research Note
REACTION PRODUCTS OF HISTIDINE WITH AUTOXIDIZED METHYL LINOLEATE

INTRODUCTION

PEROXIDATION of lipids in biological systems and in foods results in reactions with proteins and amino acids. We found previously (Clark, 1965; Karel et al., 1966; Tjhio and Karel, 1969) that histidine, lysine and methionine affected oxidation of lipids, and underwent changes as a result of their reactions with peroxidizing lipids. Histidine has been found to be sensitive to other oxidizing conditions. Tomita et al. (1969) isolated and identified several products of its photooxidation, and Schubert et al. (1968) reported formation of cytotoxic compounds from histidine exposed either to hydrogen peroxide or to ionizing radiations.

As part of our study of lipid-protein interaction, we characterized products formed by reactions between histidine and peroxidizing lipids.

EXPERIMENTAL

HISTIDINE was reacted with autoxidized methyl linoleate in one of the following systems: (a) dry filter paper containing the reactants; (b) anhydrous mixture of methyl linoleate and histidine in air, or a mixture of linoleate hydroperoxides and histidine in air; and (c) concentrated aqueous dispersion.

Materials

Methyl linoleate (Hormel Institute, Minneapolis, Minn.) was further purified by distillation. L-Histidine (Calbiochem, Los Angeles, Calif.) was used without further purification.

Methyl linoleate hydroperoxides were prepared by the method of Banks et al. (1959).

Experiments on filter paper

L-Histidine (39–156 mg) dissolved in a minimum volume of water was dispersed dropwise on a filter paper (11 cm in diameter, weighing about 825 mg). The filter paper was dried in air (4 hr), then in a vacuum desiccator to a constant weight. Methyl linoleate (308 mg) dissolved in *n*-hexane (1 ml) was then dispersed on the paper, and the solvent was removed by vacuum evaporation.

After the incubation in air at 37°C, a small piece of the filter paper was extracted with aqueous methanol (1:1 v/v). The extract was spotted on thin-layer plates (silica gel G) (Tjhio and Karel, 1969). The solvent system consisted of 2-propanol:concentrated ammonium hydroxide:water (8:2:1), and spots were visualized with ninhydrin or with Pauly reagent. [Diazotized sulfanilic acid in alkaline medium gives red color with imidazole-containing compounds, with at least one unsubstituted ring carbon atom (Bailey, 1962).]

The rest of the filter paper was extracted with 1N HCl, and chromatographed on a 2.0-cm × 30-cm column packed with Dowex-50 (H+ form, 1010g), with 4N HCl and water used respectively in a gradient chromatographic technique. A total of 14 fractions, each containing 0.5 ml, were collected.

Reaction in a stirred anhydrous mixture

Histidine (155 mg) and methyl linoleate (308 mg) were magnetically stirred in a 25-ml round-bottomed flask for 14 days at 37°C. The reaction products were extracted with a mixture of light petroleum ether (b.p. 30–60°C) and benzene (1:3, v/v) and then with aqueous methanol (25%, v/v). The petroleum ether: benzene fraction was concentrated under reduced pressure and the residue (250 mg) was examined on TLC (alumina) with iodine vapor as reagent.

Reaction in the presence of water

A mixture of 200 mg histidine, 400 mg methyl linoleate and 1 ml water was stirred for 6 days at room temperature with occasional addition of a few drops of water. The filtrate on TLC (silica) showed two ninhydrin-positive spots.

A similar experiment was carried out in the presence of ferrous sulfate (10^{-7} M). The reaction mixture turned brown, and TLC (silica) showed four ninhydrin-positive spots. When cobalt nitrate was used in place of ferrous sulfate, two ninhydrin-positive spots were obtained.

RESULTS & DISCUSSION

REACTIONS between histidine and autoxidizing methyl linoleate resulted in the formation of several compounds, whose nature depended on experimental conditions. Table 1 shows the R_f value on TLC (silica gel) of these compounds. Four of the isolated products were identified: I is aspartic acid; III is histidine; V is hista-

Table 1—R_f values of histidine-derived compounds isolated from reactions mixtures

Reaction	R_f of various reaction products[a]					
	I	II	III	IV	V	VI
On filter paper		0.56(n,s)	0.59(n,s)		0.68(n,s)	0.83(n)
In anhydrous mixture		0.56(n,s)	0.58(n,s)			
In aqueous system			0.58(n,s)		0.68(n,s)	
In presence of Fe	0.40(n)		0.59(n,s)	0.65(n)	0.67(n,s)	
In presence of Co			0.58(n,s)		0.68(n,s)	

[a] n = ninhydrin positive; s = Pauly reaction positive. R_fs of authentic amino acids: histidine = 0.58; histamine = 0.68; aspartic acid = 0.41; valine = 0.65; ethylamine = 0.81

mine and VI is ethylamine.

In the reactions taking place on filter paper, histamine and ethylamine were identified as reaction products. As mentioned above, 14 fractions were collected from the column chromatography of the material extracted from paper. Fractions 3 and 4 showed a single ninhydrin-positive spot, but failed to produce a red spot with Pauly reagent. These fractions (3 and 4) were pooled, concentrated under reduced pressure, and treated with picric acid. On cooling, picrate separated as yellow crystals, m.p. 163–164°C (10.0 mg). Mixture with the authentic picrate of ethylamine did not depress the m.p.

Fractions 6, 7 and 8 gave positive Pauly reactions. When pooled and evaporated to dryness under reduced pressure, these fractions produced white crystals (45 mg), m.p. 263–238°C. A picrate derivative of this product had an m.p. of 144–145°C. Mixture with the authentic picrate derivative of histamine did not depress the m.p.

The identification of histamine and ethylamine was also confirmed by comparison of R_fs, of infrared spectra, and of NMR spectra with authentic compounds. They represent reaction products V and VI shown in Table 1. Product III is unreacted histidine.

An uncharacterized histidine-like compound was also isolated (product II in Table 1). This compound moved more slowly than histidine on TLC and took longer than histidine to generate color when reacted with N-methylmaleimide. The product was contained in fractions 10, 11, 12, 13 and 14 from the small column, which were were combined and evaporated to dryness under reduced pressure. A residue (125 mg) was obtained which showed two closely running spots on TLC. The residue was dissolved in water (1.0 ml), made basic with pyridine, and the separated solid was filtered off and washed with water. On TLC, this product moved more slowly than histidine and gave a positive Pauly reaction. Its molecular weight determined in water was 186, which is lower than a possible dimeric compound of histidine.

Reactions in anhydrous mixtures showed the formation of the slow-moving compound (II), but not of histamine or ethylamine. Reactions in aqueous dispersion, on the other hand, resulted in formation of histamine (V), but not the slow-moving derivative (II).

Decarboxylation of histidine by chemical means and by the use of metal ions as catalyst in aqueous solution have been reported by Andrews and Grundemeier (1966), by Goudot (1955) and by Hearon (1947). We observed that the formation of histamine was greatly increased by carrying out the reaction in an aqueous medium in the presence of metal ions.

Reactions in aqueous systems in presence of iron gave, in addition to histidine (III) and histamine (V), aspartic acid (I) and another ninhydrin-positive compound (IV). Production of aspartic acid in photooxidation of histidine has been reported by Tomita et al. (1969). The R_f of compound IV corresponded to that of valine.

Ethylamine (I) was obtained only by extraction with dilute hydrochloric acid from filter paper. Thinking that it might be formed from aspartic acid, we allowed aspartic acid and methyl linoleate to react on filter paper at 37°C for 2 wk and we extracted with water. No ethylamine was obtained. Extraction from filter paper with dilute hydrochloric acid, however, gave ethylamine.

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A Research Note
STABILITY OF BUTYLHYDROXYANISOLE (BHA) IN WATER UNDER STRESSES OF STERILIZATION

INTRODUCTION

THE IDENTIFICATION of degradation and transformation products of antioxidants as caused by typical processing conditions is of great importance to the food industry, particularly in view of the present consumer interest in food additives in general.

Exact pathways of the decomposition of BHA are unknown at this time (Hathway, 1966) yet it is one of the most frequently used and most powerful antioxidants.

To date, our knowledge about other antioxidants is also very limited. The thermal, in vitro decomposition of tocopherols resulted in the formation of a dimer and quinone production (Telegdy et al., 1971). Fifteen products were reported by Tatum et al. (1969) when a 35% solution of ascorbic acid in water was heated for 5 hr at 100°C. The effect of irradiation on vitamin E. has been reported (Diehl, 1969). Piulskaya (1961) reported on the effect of thermal processing on the amount of antioxidant remaining after treatment but did not detail any antioxidant formation products. A study on the photodegradation of BHA showed the production of two dimers (Kurechi, 1967).

Most antioxidants are incorporated into food systems at a concentration between 0.01–0.02% (based on lipid content of food moiety). Therefore, it is important that any study of the reactivity of these compounds be conducted at concentrations compatible with these systems. This factor was taken into consideration as we studied the behavior of BHA under thermal processing conditions.

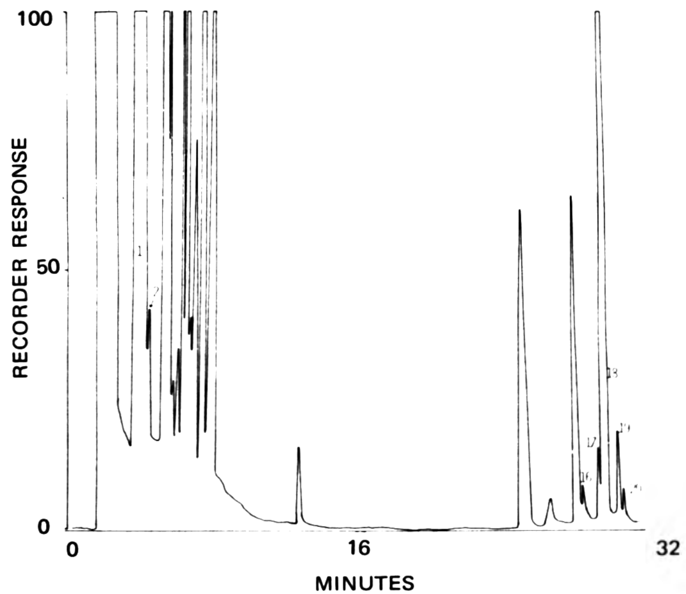

Fig. 1—Typical GLC separation of BHA and its reaction products.

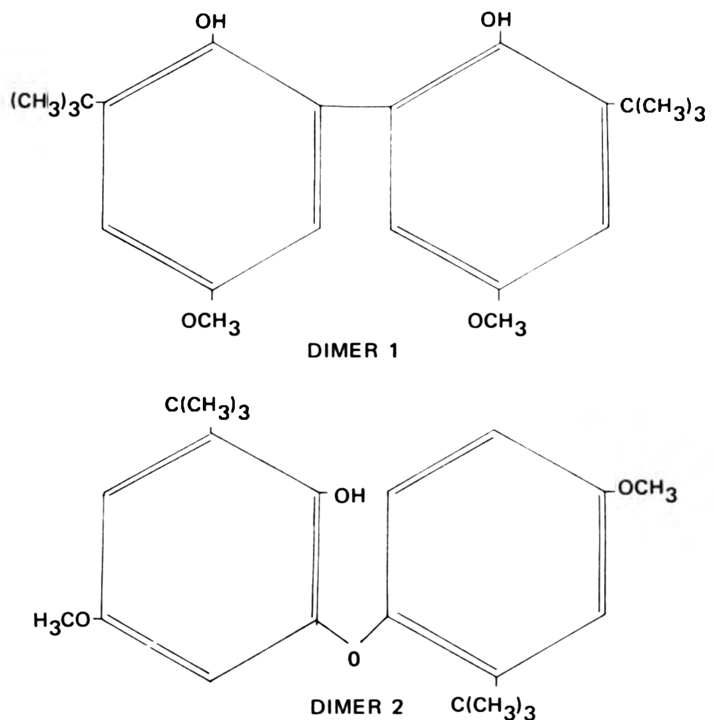

Fig. 2—Structures of primary dimers.

EXPERIMENTAL

Sample treatment and extraction

20 ml glass ampules were used as reaction vessles. 20 ml of distilled-demineralized water and 0.6g of BHA were combined in each ampule. At reaction temperatures only approximately 0.75% of this will go into solution, producing a 0.02% aqueous solution of BHA. This concentration represents the maximum concentration of BHA in foods permitted by law. The additional BHA was introduced to raise BHA-2 levels high enough for quantitation. The tops were hermetically sealed with a minimum amount of air remaining due to the

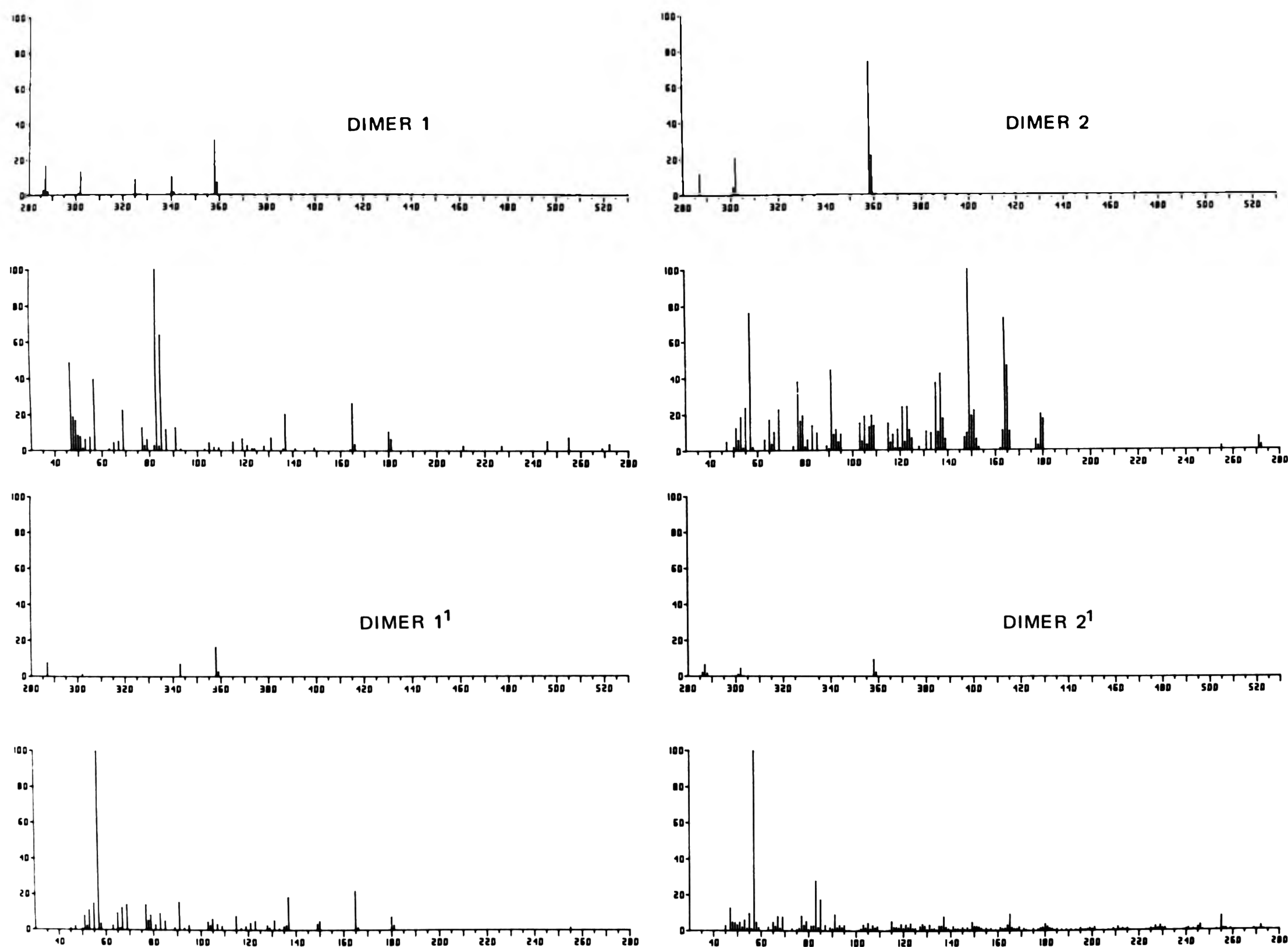

Fig. 3–Mass spectrometer scans of the four dimers associated with BHA reactivity. Dimer 1: 2-2'-dihydroxy-5,5'-dimethoxy-3-3'-di-tert-butyl biphenyl. Dimer 1[1]: 2-2'-dihydroxy-5,5'-dimethoxy-3'-4-di-tert-butyl biphenyl. Dimer 2: 2',3-di-tert-butyl-2-hydroxy-4',5-dimethoxy biphenyl ether. Dimer 2[1]: 2',4-di-tert-butyl-2-hydroxy-4',5-dimethoxy biphenyl ether.

sample volume and the ampules were autoclaved at 15 psi steam for 15 min.

Extraction of the reacted mixtures was done with two 20-ml portions of redistilled chloroform. The chloroform layers were combined and evaporated under reduced pressure at room temperature to a volume of 1 ml.

Gas-liquid chromatography (GLC)– Mass spectrometer (MS) analysis

A Beckman GC-45 equipped with a 4 ft by 1/16 in OD stainless steel column packed with 1% OV-7 on 60-80 mesh HMDS treated Chromosorb-W was used. The carrier gas was helium at a flow rate of 42 ml/min. 5 μl of sample was used for injection. The first 10 min of each run was held at 100°C. Then the temperature was programmed to reach 300°C in the next 22 min. The injection port was 275°C and the detector 280°C.

The unit was connected to an A.E.I. MS 12 medium-resolution mass spectrometer through an all glass system, including magnetically operated glass valves and a fritted interface. The MS was interfaced to a PDP-8 digital computer (4K Core) which had two 32K disks for data and program storage.

As each peak passed out of the column, the flow was split and the majority of the sample was passed into the MS. Every 10 sec a printout was produced of the relative mass particles produced.

RESULTS & DISCUSSION

20 SEPARATE PEAKS were visually identifiable by GLC. A typical GLC scan of BHA and its reaction products is shown in Figure 1. Two of these peaks represent the isomeric forms of BHA (Peak 1: 3-tert-butyl-4-hydroxyanisole and Peak 2: 2-tert-butyl-4-hydroxyanisole), as verified by use of commercial standards. A third peak (Peak 16) with a mass of 196 amu is postulated to represent the oxidized form of both of the above.

The most significant finding was the separation of two of the compounds (Peaks 18 and 19) as representing dimer structures identical to those found by Kurechi (1967) resulting from the photodegradation of BHA. The structures of these compounds are shown on Figure 2. G.C. peaks (17, 20) have the same mass as 18, 19 (359 amu) but their M.S. scans differ (Figure 3). The proposed structures of these dimers correspond to isomeric forms of 2-2'-dihyroxy-5,5'-dimethoxy-3-3'-di-tert-butyl-biphenyl and 2',3-di-tert-butyl-2-hydroxy-4',5-dimethoxy-biphenyl ether.

Further work is being carried out to completely identify all reaction products found and to investigate BHA and other antioxidant reactions as influenced by other factors: pH and reactive food ingredients such as amino acids and reducing sugar. These findings will be reported in a later publication.

CONCLUSION

UNDER NORMAL circumstances of concentration and time and temperature of thermal processing the food antioxidant BHA undergoes extensive rearrangement and condensation. The possible health significance of these newly formed products is not known at this time. However, rat feeding studies involving the synthesized dimers is currently under way.

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A Research Note
A COMPARISON OF CHILLED-HOLDING VERSUS FROZEN STORAGE ON QUALITY AND WHOLESOMENESS OF SOME PREPARED FOODS

INTRODUCTION

A DESIRE to store precooked foods for 2–3 wk under refrigeration without spoilage, and to reheat them without the adverse effects sometimes experienced with frozen foods, were among the objectives of the Swedish "Nacka System" (Bjorkman and Delphin, 1966). In this method, foods are cooked as usual except that all parts of the food are made to reach a temperature of at least 80°C. The hot food is transferred into plastic bags which are then evacuated, sealed and placed in boiling water for 3 min, after which they are cooled with running water to a temperature of 10°C. After drying, packages are stored in a refrigerator at a temperature of 3°C or less. To heat the contents, bags are placed in boiling water for 30 min. Thus the Nacka method combines the effects of a pasteurizing heat treatment and cold storage to preserve foods and is similar to the "Frigicanning" method reported by Kohman (1960). It is also the basis for the "A.G.S. System" described by McGuckian (1970).

The purpose of these experiments was to spot check, on a very limited basis, whether the pasteurization treatment is adequate to destroy *Salmonella* of *Clostridium perfringens* if these are present in the food after its preparation; how the retention of thiamine, riboflavin and ascorbic acid in chilled samples compares with that of frozen samples; and how the palatability of stored chilled foods compares with that of frozen controls.

EXPERIMENTAL

COMMERCIAL RECIPES were used to prepare the three products studied: Chicken a la king (made with white chicken meat, white sauce, carrots, onions, potatoes, peas and spices); Codfish in cream sauce (made with frozen codfish fillets and a sauce consisting of chicken stock, water, flour, shortening, cream, starch, butter, flour and seasonings); and Broccoli and cream sauce (made with frozen broccoli and a cream sauce of the same composition as that used for codfish).

Individual portions (12 oz for chicken and cod and 7 oz for broccoli) were packed in 6 in. × 11 in. Mylar-polyethylene film pouches, 0.0044 in. thick. The method described by Bjorkman and Delphin (1966) was closely adhered to with respect to filling, vacuumizing, pasteurizing and cooling.

Chilled samples were stored at 2°C and frozen samples (which were not vacuumized or pasteurized) at −23°C.

Samples of Chicken a la king and Codfish in cream sauce for bacteriological study were inoculated with 1 ml each of saline suspensions of *C. perfringens* and *Salmonella* Type Paratyphi B. Samples of chicken a la king and broccoli in cream sauce were prepared by weighing each ingredient to the nearest gram to insure identical composition in replicate samples. Samples were stored for up to 45 days, assays being carried out immediately after preparation and after 15, 30 and 45 days.

Samples for vitamin assay were not heated, frozen samples being thawed at room temperature. Chilled samples for bacteriological and organoleptic evaluation were heated for 30 min in boiling water. Frozen samples were thawed overnight in a refrigerator and the contents of the pouches heated in individual pans, with occasional stirring, just enough to bring the temperature to 68°C (Tressler and Evers, 1957). Duplicate samples were used for each examination.

Methods used for the isolation and identification of both were standard qualitative tests (Anon, 1969). Thiamine was determined by the thiochrome method, riboflavin by the fluorometric method and ascorbic acid by the method of Loeffler and Ponting (Tressler and Evers, 1957). Organoleptic evaluation was carried out by a bench panel which ranked frozen and chilled samples along with fresh controls and determined the acceptability of stored samples.

RESULTS & DISCUSSION

Results of the bacteriological and vitamin assays are shown in Tables 1 and 2, respectively.

After 15 and 30 days of storage, all the reheated refrigerated samples gave negative results for the two organisms, while frozen samples gave negative results for *C. perfringens* but positive results for *Salmonella*. When samples stored for 45 days were examined, two sets of samples were examined unheated (i.e. thawed) while two sets were heated to a temperature higher than that used after 15 and 30 days (93.3°C). The absence of *Salmonella* in the reheated, 45-day old frozen samples may have resulted from the more severe heat treatment. Refrigerated samples gave negative results in all cases, indicating that the preparation, handling

Table 1—Results of examination for C. perfringens and Salmonella in chilled and frozen samples of precooked entrees

	Storage time (days)									
			15		30		45			
	0[a]		After reheating		After reheating		Prior to reheating		After reheating	
	C.P.	Sal.	C.P.	Sal.	C.P.	Sal.	C.P.	Sal.	C.P.	Sal.
Refrigerated										
Chicken a la king	+	+	–	–	–	–	–	–	–	–
	+	+	–	–	–	–	–	–	–	–
Codfish in cream	+	+	–	–	–	–	–	–	–	–
sauce	+	+	–	–	–	–	–	–	–	–
Frozen										
Chicken a la king	+	+	–	+	–	+	–	+	–	–
	+	+	–	+	–	+	–	+	–	–
Codfish in cream	+	+	–	+	–	+	–	+	–	–
sauce	+	+	–	+	–	+	–	+	–	–

[a] After filling pouches and before evacuation or pasteurization

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[2] Present address: Food Science Associates, PO Box 265, 145 Palisade St., Dobbs Ferry, NY 10522

Table 2—Ascorbic acid, thiamine and riboflavin content and retention in chicken a la king and broccoli in cream sauce

Storage time (days)	Frozen samples		Refrigerated samples	
	Chicken a la king	Broccoli in cream sauce	Chicken a la king	Broccoli in cream sauce
	Ascorbic Acid Content–(mg %) and (Retention in %)			
0	35.6 (100.0)	54.2 (100.0)	24.7 (69.4)	37.7 (69.5)
	s=2.6	s=2.4	s=1.9	s=3.3
15	22.4 (62.9)	31.8 (58.6)	15.2 (42.7)	21.2 (39.1)
	s=2.4	s=4.3	s=2.8	s=2.9
30	15.3 (42.9)	27.1 (50.0)	10.9 (30.6)	16.4 (30.3)
	s=1.0	s=4.3	s=1.8	s=2.5
45	13.8 (38.7)	22.4 (41.3)	10.1 (28.3)	15.3 (28.2)
	s=1.8	s=4.4	s=1.6	s=2.5
	Thiamine Content–(μg %) and (Retention in %)			
0	84.9 (100.0)	60 (100.0)	82.2 (96.7)	56 (93.3)
	s=0.05	s=0.02	s=0.5	s=0.03
15	71.1 (83.6)	45 (75.0)	76.1 (89.6)	49 (81.7)
	s=2.6	s=0.03	s=2.6	s=0.03
30	64.5 (75.9)	44 (73.3)	73.6 (86.6)	47 (78.3)
	s=2.7	s=0.03	s=2.6	s=0.02
45	65.4 (76.9)	41 (68.3)	71.6 (84.3)	46 (76.7)
	s=2.0	s=0.03	s=2.7	s=0.04
	Riboflavin Content–(μg %) and (Retention in %)			
0	89 (100.0)	12 (100.0)	89 (100.0)	12 (100.0)
	s=0	s=0.4	s=1	s=0.1
15	79 (88.7)	9 (75.0)	81 (91.0)	10 (83.3)
	s=1.0	s=2.1	s=0.7	s=1.0
30	79 (88.7)	9 (75.0)	81 (91.0)	10 (83.3)
	s=0.17	s=2.1	s=0.7	s=1.0
45	79 (88.7)	9 (75.0)	82 (92.1)	11 (91.7)
	s=0.5	s=2.1	s=1.3	s=1.2

and pasteurization procedures prescribed by the Nacka system effectively destroyed both the *Salmonella* and the vegetative *C. perfringens* with which they had been inoculated. The absence of *C. perfringens* and the presence of *Salmonella* in all stored frozen samples confirm the known lethality of freezing upon *C. perfringens* and the known ability of *Salmonella* to survive frozen storage.

With respect to vitamins it will be noted that about one-third of the ascorbic acid present was lost in the initial processing of chilled products, and retention in the frozen samples was significantly greater than in the chilled samples throughout the storage period. (Ascorbic acid content of chicken a la king resulted from ingredients in the sauce, not chicken). Comparable thiamine and riboflavin values were found in frozen and chilled samples before storage, but beyond the 15th day refrigerated samples showed slightly higher levels of thiamine retention than frozen controls while fairly constant values for riboflavin were obtained beyond 15 days. After 15 days of storage the panel could not detect any significant difference in appearance, flavor and consistency between the frozen and refrigerated samples, while fresh controls were graded superior to either of the stored samples. After 30 days, refrigerated samples were no longer deemed acceptable by the panel, while frozen samples although inferior to fresh, were acceptable.

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A Research Note

MICROBIOLOGICAL COMPARISON OF STEAM- (AT SUB-ATMOSPHERIC PRESSURE) AND IMMERSION-SCALDED BROILERS

INTRODUCTION

BACTERIOLOGICAL problems inherent in commercial scalding of poultry by submersion in water as commonly practiced are generally recognized. Lillard (1973) showed that water scalding of broilers by submersion results in contamination of the respiratory and circulatory systems and, consequently, the edible parts with microorganisms, including *C. perfringens*, present in the scald water. Since contaminants disseminated to the edible parts of the carcass via the respiratory and circulatory systems during submersion scalding probably are not removed or destroyed during processing, a feasible alternate method is needed.

Steam scalding at atmospheric pressure has been used successfully in turkey- and fowl-processing plants but has not proven entirely satisfactory for broilers which require lower, well-controlled temperatures to maintain skin quality and appearance.

Klose et al. (1971) and Kaufman et al. (1972) suggested the use of steam at sub-atmospheric pressures for scalding broilers. Their results show that contamination of air sacs with aerobic microorganisms is lower for birds scalded with steam at sub-atmospheric pressures than for birds scalded by submersion in water. The present study was undertaken to compare the efficacy of water scalding and sub-atmospheric steam scalding of broilers with regard to contamination of edible parts of broiler carcasses with both aerobic and anaerobic microorganisms.

MATERIALS & METHODS

BROILERS were taken from a U.S. Dept. of Agriculture-inspected commercial processing plant. From four to six broilers were taken at each of three sampling points on each sampling day: (1) live broilers at the dock for sub-atmospheric steam scalding in our pilot plant; (2) broilers from the 75-sec bleeding line prior to water scalding; and (3) carcasses immediately after water scalding at 58°C for 120 sec. All birds were stunned by electric shock and killed by an automatic killer using the standard cut.

Sub-atmospheric steam scalding was accomplished by 2-min exposure of 60-sec-bled birds in a chamber having a flowing steam atmosphere maintained at 53°C and an absolute steam pressure of 2.1 lb/sq in. (0.14 kg/sq cm) (Klose et al., 1971). The 60 sec bleeding time was used as a more severe treatment than the 75 sec bleeding time used in the processing plant. Adequacy of this scalding procedure for complete feather removal without serious skin abrasion was confirmed on each sampling day by including at least three birds that were scalded and picked in a free-floating, cyclic, rubber-fingered picking machine. Birds used for bacteriological sampling were scalded but not picked. Samples of hearts, liver and flesh were taken as described by Lillard (1973). Lungs were removed dorsally after flame-sterilizing the surface of the skinned carcass with a bunsen burner. *C. perfringens* was isolated by enrichment techniques described by Lillard (1971). 1-g samples were used in making decimal dilutions for total counts. Peptone (0.5%) was used as a diluent and pour plates (Plate Count Agar, Difco) were incubated at 35°C for 48 hr.

Table 1—Incidence of *C. perfringens* in broiler parts prior to scalding and after water or sub-atmospheric steam scalding

	Sampling point					
	Bleeding line (prior to scalding)		After water scalding		After sub-atmospheric steam scalding	
Sample	No. positive/total	%	No. positive/total	%	No. positive/total	%
Heart	0/26	0	1/26	3.8	0/37	0
Liver	0/26	0	0/26	0	0/37	0
Lungs	2/26	7.7	15/26	57.7	2/37	5.4
Flesh	0/26	0	0/26	0	0/37	0

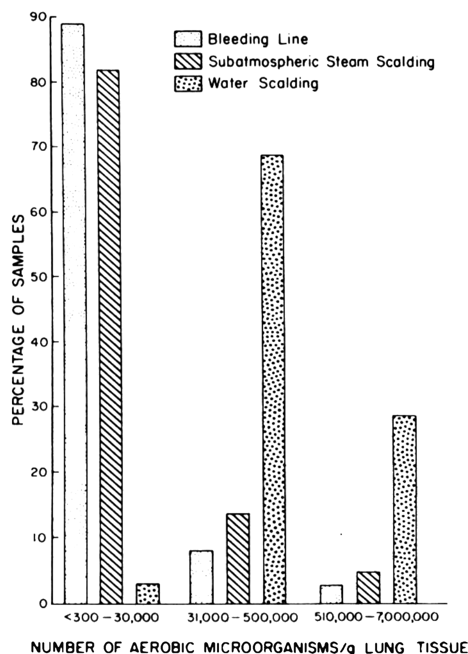

Fig. 1—Distribution of counts of aerobic microorganisms from lungs of broilers prior to scalding, after water scalding and after sub-atmospheric scalding.

RESULTS & DISCUSSION

BECAUSE it was shown that contamination of the respiratory system (lungs) of broilers with microorganisms present in scald water is the most likely cause of contamination of the circulatory system and edible parts of poultry (Lillard, 1973), and that *C. perfringens* is present in water from commercial scald tanks (Lillard 1971, 1973), lungs of broilers were selected as samples indicative of the degree of contamination due to scalding. Although lungs are removed during processing, portions of the connected air sac system remain with the edible carcass and contain contaminants introduced through the lungs.

Table 1 shows that *C. perfringens* is isolated more frequently from lungs of water-scalded broilers than from lungs of broilers prior to water scalding or of broilers scalded by steam at sub-atmospheric pressures. About 58% of lungs

(15/26) from water-scalded broilers were positive for *C. perfringens,* whereas only 7.7% of lungs (2/26) sampled prior to scalding, and 5.4% of lungs (2/37) sampled after sub-atmospheric steam scalding were positive for *C. perfringens.* The difference between 15/26 and 2/26 is statistically significant at the 0.1% level. *C. perfringens* was isolated frequently from scald water in this processing plant. On sampling days when *C. perfringens* was not isolated from scald water, lungs of all water-scalded birds were negative.

C. perfringens was not isolated from samples of liver or flesh of broilers taken prior to scalding nor after sub-atmospheric steam or water scalding. Only one of the 26 samples of hearts was positive after water scalding, whereas none of the 26 hearts sampled from the bleeding line and none of the 37 hearts sampled after sub-atmospheric steam scalding was positive for *C. perfringens.*

The highest counts of aerobic microorganisms from lung samples were obtained from samples that also showed a high incidence of *C. perfringens.* Figure 1 shows the distribution of counts from lung samples of broilers prior to scalding, after water scalding and after scalding with steam at sub-atmospheric pressure, corresponding to mean logs and standard errors respectively of 3.51 ± 0.24, 5.53 ± 0.24 and 3.25 ± 0.24. Statistical analysis of the data using both parametric and nonparametric tests (analysis of variance of the logarithms and Friedman's test extended to the case of several observations per experimental unit) showed a significant difference at the 0.1% level in counts from lungs of water-scalded broilers compared to counts from lungs of broilers from the bleeding line or after scalding with steam at sub-atmospheric pressure. These results confirm and extend results reported by Kaufman et al. (1972). No significant difference was found in counts between lungs of broilers from the bleeding line and from lungs after sub-atmospheric steam scalding. About 89% of lungs from the bleeding line (32/36) and 82% of lungs after sub-atmospheric steam scalding (36/44) had counts below 30,000/g whereas 97% (34/35) of lung samples from water-scalded broilers had counts over 30,000/g.

Total counts from samples of flesh, heart and liver were almost all <300/g. These samples were taken from each of 30 broilers prior to scalding, 30 after water scalding and 36 after sub-atmospheric steam scalding. These results confirm the previous report by Lillard (1973) which showed that, though contaminants present in scald water may be disseminated in the carcass during scalding, the level found in edible parts was low and enrichment techniques had to be used for detection. The incidence of *C. perfringens* isolated from edible parts of water-scalded carcasses in this commercial processing plant also was lower than that reported in the previous study (Lillard, 1973). This suggests great variability in the contaminating effect of different immersion-scalding systems. Although the level of contamination in edible parts is low, the high level of contamination in lungs of immersion-scalded broilers should be of some concern. Even though lungs are normally removed, air sacs are left in the carcass.

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A Research Note
THE RATE OF COAGULATION OF ZEIN

INTRODUCTION

COAGULATION PROPERTIES of zein were studied because of the unique water insoluble film and fiber forming properties of zein which could be utilized effectively in novel, high technology edible items. Utilization of corn protein will become a more significant issue when corn is considered as a source of protein, such as in high lysine corn and when a better milling process to preserve nature protein is developed.

Hermans (1947) has analyzed coupled diffusion and chemical reaction which results in precipitation or coagulation. This analysis was applied by Booth (1967) to the extruded single filaments of polyacrylonitrile in aqueous zinc chloride solutions. Paul (1968) performed a model study on acrylic polymer in dimethylacetamide to elucidate the mechanisms involved in the coagulation process. However, no such study was performed on protein systems.

EXPERIMENTAL

Preparation of samples

Dopes of zein (Freeman Industries F-200) in 95% denatured ethyl alcohol were prepared at 30% and 39% dry weight concentrations. These two concentrations are equivalent to 29.4% and 38.4% protein based on the suppliers information (Zein F-200 Data Sheet, Freeman Industries).

Coagulation bath

The coagulating medium was an aqueous HCl solution at pH 3. The bath temperature was maintained at 32°, 75°, 96° and 116°F.

Measurement of coagulation rate

The dope was placed on a microscope slide and another slide was placed on top to obtain a uniform thin layer of dope between the two slides. The coagulating solution is poured into the dish containing the slides with the dope. A sharp boundary line formed between the creamy coagulated region and the yellowish uncoagulated region. The advancement of this boundary line was measured at regular time intervals until a constant value was reached. Measurements were made with a vernier caliper accurate to 0.005 cm. The experiment was performed in duplicate and the average deviation between the duplicates was ± 5%.

RESULTS & DISCUSSION

THE ADVANCEMENT of the coagulation boundary (ℓ) is rapid at the beginning then gradually tapers off. The rate of coagulation appears to be faster at 29.4% than at 38.4% protein concentration at the temperatures studied (Fig. 1).

The thickness of the coagulated layer (ℓ) was also plotted against the square root of time (Fig. 1). Considering the fact that these ℓ vs. $t^{1/2}$ curves indicate linear relationship at the initial stage, diffusion velocity, $D_v = \ell^2/4t$ was obtained from this curve (Booth, 1967; Paul, 1968)

The values of D_v obtained in this study (Fig. 2) show that at 32°F, the rate of diffusion for both 29.4% and 38.4% protein concentration are about the same. The diffusion rate increases with the temperature up to around 96°F, then decreases at the temperature around 116°F. Except at 32°F, the diffusion velocities for 29.4% concentration were greater than that for the 38.4% protein sample.

In the coagulation process, low molecular weight solutes from coagulation bath diffuse through an increasing thickness of coagulating protein layer. At the initial stage of the coagulation process, the diffusion is faster because the solute has to diffuse through a smaller length of the more resistant coagulated portion while later more protein coagulates and the barrier to diffusion becomes greater. Apparently, this resistance to diffusion of the coagulants by 38.4% sample is greater than at 29.4% sample as could be ex-

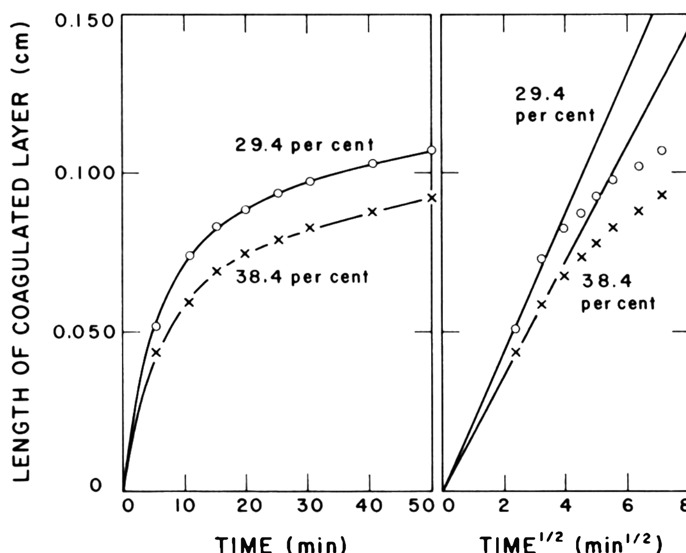

Fig. 1—Length of coagulated layer vs. time and square root of time.

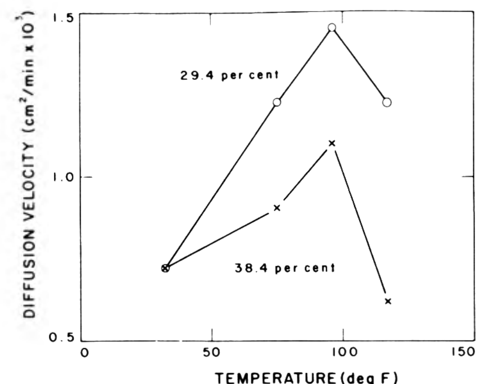

Fig. 2—Diffusion velocity vs. temperature.

pected (Li and Gainer, 1968). As the temperature goes up from 32°F to 75°F to 96°F, diffusion of the coagulant solutes increases, hence the increase in the rate of coagulation. However, there is a sudden drop in the diffusion velocities from 96°F to 116°F which would indicate a conflicting mechanism like thermal coagulation.

CONCLUSIONS

THE COAGULATION data were analyzed assuming a diffusion-limited chemical reaction and values of a parameter D_v which describes the boundary advancement were obtained. D_v ranged from $6.40 \times 10^{-4} cm^2/min$ to $14.40 \times 10^{-4} cm^2/min$ (with maximum around 96°F) for 29.4% and 38.4% protein dope at 32°F, 70°F, 96°F and 116°F.

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A Research Note

CHILLING INJURY OF GREEN BANANA FRUIT: KINETIC ANOMOLIES OF IAA OXIDASE AT CHILLING TEMPERATURES

INTRODUCTION

THERE IS considerable evidence that chilling injury in vegetative tissues relates to phase changes of membrane lipids with a resulting disruption of membrane integrity and functions. (Lyons and Asmundson, 1965; Lyons and Raison, 1970; Raison et al., 1971. Chilled banana fruit accumulate acetaldehyde and ethanol in the peel and pulp and α-keto acids in the peel (Murata, 1969). The accumulation of such toxic metabolites is presumably a reflection of the disjuction of mitochondrial integrity.

Certain plant tissues are capable of adapting to low temperature stress. This process, referred to as "cold hardening," appears to involve a stabilization of membrane structure and cellular functions. Relatively little is known of the role of plant hormones in stress response. This is contrary to the extensive work relating hormones to homeostasis in mammalian systems. Tumanov and Trunova (1958) found that auxin decreases on hardening of coleoptiles and that the ability of juvenile tissues to harden was reduced if indole-3-acetic acid was added to tissue cultures. This suggests that auxin metabolism is an essential element of cold acclimation. We had previously reported that the peroxidase recovered from green banana fruit declined during the initial stages of chilling and subsequently increased after approximately 7 days at 5°C when severe chilling was manifest (Haard and Timbie, 1973). The present communication deals with the direct influence of chilling temperatures on the affinity of banana peroxidase for auxin in vitro.

EXPERIMENTAL

Source of Fruit

Preclimacteric banana fruit, No. 2 according to the scheme of Loesecke (1950), were generously provided by the United Fruit Company.

Isolation of IAA oxidase

Cell-free extracts were prepared from liquid nitrogen powders of green pulp by the method described for "soluble peroxidase" (Haard, 1973). A 50-ml sample of the cell-free extract was passed through a column containing Sephadex G-50 coarse (500 ml bed volume). The protein fraction eluted with distilled water was dialyzed against 100 volumes of Tris-HCl (0.01M, pH 8.0) at 4°C for 18 hr. The resulting dialyzate was concentrated to a final volume of about 10 ml by lyophilization and the concentrated sample was dialyzed against 100 volumes of Tris-HCl buffer.

IAA oxidase assay

The oxidatic metabolism of IAA was monitored with the assay medium and dimethylaminocinnimaldehyde stop procedure described by Meudt and Gaines (1967). The concentration of IAA in the reaction vessel and the assay temperature were varied as indicated in the results section. The pH optimum of banana IAA oxidase was previously shown to be 6.0 (Haard, 1973).

RESULTS & DISCUSSION

SUBSTRATE-VELOCITY PLOTS of IAA oxidase showed characteristic hyperbolic change in the 0.05–1 mM range of IAA when the assay temperature was 15–30°C (Fig. 1). The Q_{10} of this reaction was quite small in this temperature range. The relatively low temperature coefficient for this reaction may relate to a depression of oxygen solubility at increased temperatures, or may simply be characteristic of the enzyme. At assay temperatures below 15°C there was progressive inhibition of activity at low substrate concentrations such that at 5°C there was negligible oxidation of substrate at concentrations of 0.2 mM and less. There was also a substantial reduction in the rate of IAA oxidation at higher substrate concentrates when the assay temperature was 5°C (Q_{10} = 2.5 from 5–15°C).

Raa (1971) investigated IAA oxidase from normal root and club-root cabbage and reported that both tissues contained an allosteric IAA oxidase and IAA oxidase with normal kinetic properties. In normal cabbage, the allosteric enzyme

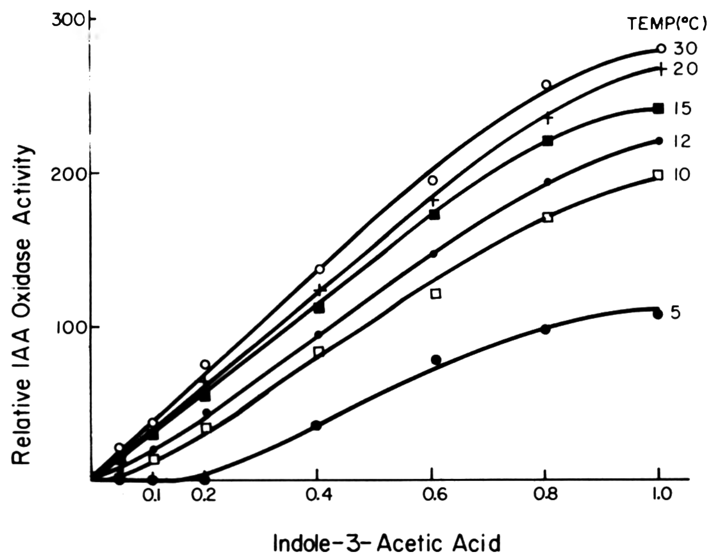

Fig. 1—Indole-3-acetic and oxidase activity of cell free extract from green banana fruit at auxin concentrations ranging from 0.05–1.0 mM. Cofactor and buffer concentrations were constant (see Meudt and Gaines, 1967) and not limiting. Assay time was 15 min; parallel results were obtained at assay times of 5, 10, 30 and 60 min. A 10 μl sample of extract was added to each reaction tube to initiate the reaction (total vol = 3.0 ml). 100 units of activity represent a $\Delta A562_{nm}$ = 0.01 in 1 min by 10 μl of extract in a 3.0 ml reaction mixture. The data are for duplicate assays of one experiment and are representative of four similar trials.

was associated with cell fractions sedimenting at 20,000 and 105,000 × G and in club-root it was soluble. Although Raa assayed at 22°C the sigmoidal nature of substrate-velocity plots observed by us at low temperatures was similar to that exhibited by the "allosteric enzyme" in cabbage. There appears to be no other information available on the response of IAA oxidase to low temperature.

The response of the IAA oxidase reaction to low temperatures may have a causal relation with chilling injury because (1) auxin catabolixm has been related to cold acclimation in hardy tissues (Tumanov and Trunova, 1958); (2) the break in linearity of substrate velocity plots occurred at temperatures coincidental with those known to promote chilling of banana; and (3) auxins appear to exert these physiological effects at low concentrations (Price, 1970).

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A Research Note
ANTHOCYANINS OF BLACK GRAPES OF 10 CLONES OF Vitis rotundifolia, MICHX.

INTRODUCTION

PRODUCTION of muscadine grapes (*Vitis rotundifolia*, Michx.) for wine is increasing rapidly in the coastal plain and piedmont of North Carolina. In order to expand this industry, cultivars with fruits which make fine-quality red wines as well as white or rosé wines are needed. Unpublished studies of black-fruited clones in the grape breeding program at North Carolina have indicated that as few as 30% produce wine with a good-quality color. Liao and Luh (1970) and Ribéreau-Gayon (1959) reported that color of wines is associated with anthocyanin (Acy) composition of the grapes from which they are made. Reports of the Acy contained in black muscadine grapes are few, however. Brown (1940), using analytical techniques of that period, identified the Acy in black grapes of the cultivar 'Hunt' as the 3,5-diglucoside of petunidin. Ribéreau-Gayon (1959, 1964) analyzed muscadine grapes sent to him from the United States and reported contents of nonacylated 3,5-diglucosides of malvidin (Mv), peonidin (Pn), petunidin (Pt), cyanidin (Cy) and delphinidin (Dp). However, he made no mention of the number or type of clones that were analyzed. Therefore, there remains a degree of doubt that muscadine grapes of a wide range of genotypes all contain these same Acy.

The objective of the present study therefore was to identify the Acy in fruit of a large number of clones of muscadine grapes representing a wide range of genotypes of the species *V. rotundifolia*, Michx.

MATERIALS & METHODS

SAMPLES of ripe berries (50g fresh weight) of seven cultivars (Albemarle, Burgaw, Duplin, Hunt, Memory, Noble and Tarheel) and three selections (NC 148-1, NC 153-1 and NC 154-2) from the North Carolina grape breeding program were harvested from experimental vineyards at Clayton, N.C. and frozen. Later, the hulls were removed and homogenized with 1% HCl in methanol. The homogenate was repeatedly filtered and washed with additional 1% HCl in methanol until the residue was colorless.

The combined extracts from each sample were streaked on sheets of Whatman No. 3 paper (46 × 57 cm). Papers were developed for 29 hr with 1% HCl solvent (Ballinger et al., 1970; 1972). Bands were eluted with MAW (methanol, acetic acid, water; 90:5:5, v/v), streaked on other paper sheets (same size), developed for 36 hr in BAW (1-butanol, acetic acid, water; 4:1:5, v/v, upper layer) eluted, restreaked, and developed for 11 hr with 15% HAc (acetic acid, water) (Ballinger et al., 1970; 1972). The Acy were eluted with MAW, dried and stored in a darkened vacuum desiccator until identified.

The purified Acy were identified using previously described, conventional techniques (Ballinger et al., 1970; 1972) such as spectral analysis (including $AlCl_3$ additions); partial hydrolysis; acid hydrolysis with subsequent identification of sugars and aglycones; and co-chromatography of each unknown Acy with Mv-3,5-diglucoside as a marker using four solvent systems (BAW, BuHCl, 1% HCl and HAc: HCl). The Acy were analyzed for acylation by spectral analysis and by extracting the cooled hydrolysate after partial acid hydrolysis of each Acy was completed, three times with ether (Liao and Luh, 1970). The combined ether extracts plus solutions of commercially-obtained caffeic, p-coumaric, ferulic and chlorogenic acids were spotted on three 46 × 57 cm sheets of Whatman No. 1 paper. The papers were developed in BAW, 2% HAc (acetic acid, water) and the upper layer of Ben:HAc:W (benzene, acetic acid, water; 2:2:1, v/v).

RESULTS & DISCUSSION

BERRIES from each of the 10 samples of black muscadine grapes contained the same Acy. Each extract separated into five distinct bands. Very faint sixth and seventh bands were sometimes evident but these were considered products of hydrolysis during extraction and development by the acidic solvent systems. Partial-hydrolysis tests indicated that the Acy in the five bands were 3,5-diglycosides (two intermediate spots on the chromatograms) (Abe and Hayashi, 1956). Complete hydrolysis and co-chromatography of the resulting sugars and aglycones with known markers indicated that the five Acy contained Dp, Pt, Cy, Mv, Pn and only one sugar, glucose. Co-chromatography of the ether extract of the hydrolysate mixtures indicated no acylation.

A comparison of the R_f's of the Acy, co-chromatographed with Mv-3,5-diglucoside as a marker with those of the literature (Harborne, 1967), as well as the λ max; the ratio of O.D. 440/O.D. max, and $AlCl_3$ shifts of the λ max of the spectral curves, indicated that the 5 Acy in each sample of these black muscadine grapes were 3,5-diglucosides of Dp, Pt, Cy, Mv and Pn (Table 1). The absence of peaks at 300–330 nm on the spectral curves confirmed that the separated and purified Acy were not acylated.

These findings, based upon a wide range of genotypes of muscadine grapes confirm and extend those of Ribéreau-Gayon (1959, 1964) which were based on an unspecified number or type of clones.

Table 1–Properties of anthocyanins isolated from hulls of berries of Vitis rotundifolia[a]

Pigment	λ max (nm) MeOH 0.01% HCl UV	Vis	Δ max (nm) $AlCl_3$	E440/Emax × 100	$R_f \times 100$[b] BAW	BuHCl	1% HCl	HOAC-HCl
Delphinidin-3,5-diglucoside	278	536	43	11	14.0	03.0	08.0	30.0
Petunidin-3,5-diglucoside	278	536	37	11	23.0	03.7	08.0	32.0
Cyanidin-3,5-diglucoside	275	526	42	13	27.0	05.7	15.4	39.5
Malvidin-3,5-diglucoside	278	536	0	11	30.0	03.0	13.4	43.0
Peonidin-3,5-diglucoside	275	526	0	14	30.6	09.0	16.6	45.0

[a] Averages of readings from Acy of berry hulls of 10 clones
[b] Determined using Whatman No. 1 paper

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A Research Note
SPECTROPHOTOMETRIC DETERMINATION OF CAFFEINE IN NIGERIAN KOLA NUTS

INTRODUCTION

ALTHOUGH kola nuts contain xanthine stimulants like caffeine, theophylline and theobromine (Fleischer, 1956; Kamp, 1960; Rafal and Tadeusz, 1966; Van Pinxteren and Schallenberg-Heertjes, 1953), there are no reports on the presence of these stimulants in Nigerian kola nuts. Most Nigerians chew kola nuts because it produces effects such as sleeplessness, prolonged capacity for work and increased mental efficiency when chewed in moderate quantities. Similar pharmacological effects have been reported for caffeine (Bowman et al., 1971). In Nigeria, it is commonly believed that the stimulating effects of the commonly edible kola nuts decrease in the order stated: *Cola acuminata*, *Cola nitida rubra* and *Cola nitida alba*. A study on the caffeine contents of the two commonly edible species of Nigerian kola nuts—*C. acuminata and C. nitida*—as well as a wild species, *C. verticillata*, which because of its slimy taste is not eaten by most Nigerians, is reported in this paper. This investigation not only provides some information about the caffeine contents of Nigerian kola nuts, it also provides a scientific basis for the acceptance of the local belief on the comparative stimulating effects of Nigerian kola nuts.

EXPERIMENTAL

Grinding of kola nuts

The samples of kola nuts (purchased from Ebute-Ero market, Lagos) were separately grated into coarse particles, dried to a constant weight in an oven at 105°C and ground to a fine powder.

Extraction

The procedure of extraction was a modification of the method reported by Van Pixteren and Schallenberg-Heertjes (1953). A 100g sample of kola nut powder was shaken vigorously in a 2-liter flask with 1 liter of chloroform and 100 ml of 25% aqueous ammonia, for 1 hr. The mixture was magnetically stirred for 24 hr and filtered on a 32.0 cm Whatman Grade I filter paper. The residue was washed thoroughly with about 250 ml of chloroform and the washing added to the filtrate. The filtrate was gently evaporated to dryness, so as not to sublime the ingredients present.

Preparation of calibration curve for caffeine in chloroform

The absorption peak for pure caffeine (BDH Chemical Ltd., England) in spectroscopic chloroform (BDH Chemical Ltd., England) was determined by observing the ultraviolet spectrum recorded with a Unicam SP 800 Ultraviolet spectrophotometer. It was found to be 276 mμ.

Very pure caffeine was obtained by placing several grams of the crystalline commercial caffeine in an evaporating dish covered by a watch glass and heating over a hot plate. The sublimed caffeine was collected from the watch glass and dried at 110°C for 1 hr. Chloroform solutions containing from 5–25 mg per liter of the pure sublimed caffeine were examined with a Unicam SP 500 Series 2 Ultraviolet and Visible spectrophotometer. Readings were made at 276 mμ. A plot of absorbancy vs. concentration gave a straight line showing excellent conformity with Beer's Law.

Purification and spectrophotometric determination of caffeine in the kola nut extracts

The procedure used for purification and spectrophotometric determination of caffeine was essentially that reported by Shingler and Carlton (1959) with slight modifications.

An air bubble free 70 × 1 cm column of silica gel was prepared and washed with spectroscopic chloroform until no absorbance was registered by the eluate at 276 mμ using a Unicam SP 500 Series 2 Ultraviolet and Visible spectrophotometer. 250 mg of the kola nut extract was chromatographed on the column at an elution rate of 100 ml/hr using spectroscopic chloroform as the eluent. Several 50 ml fractions were collected until all the caffeine had passed from the column. The caffeine in each fraction was spectrophotometrically determined by measuring the difference in absorbance at 310 mμ and 276 mμ using either spectroscopic chloroform or the first 50 ml fraction, since both show no absorbance, as the reference.

Recovery studies

Fractions with optical density values greater than 0.025 (corresponding to 0.5 mg/liter) were combined and gently evaporated to dryness, to avoid sublimation of the ingredients. The dry crystalline material obtained was washed with small amounts of petroleum ether (60–80°C) to remove colored impurities which contaminated it, then recrystallized from benzene/petroleum ether 100–120°C, (20:80 v/v) mixture, weighed and its melting point determined.

Thin layer chromatography

Silica gel G Merck (Brinkman Co., New York) was used to prepare the thin layer chromatographic plates according to the procedure outlined by Randerath (1966). The thin layer chromatography plates were spotted with chloroform solution of the recovered material and eluted separately with petroleum ether (60–80°C); 96% ethanol; benzene; chloroform; and 96% ethanol/chloroform (1:9 v/v) mixture. The plates were dried and developed in a tank of iodine vapor.

Spectral studies

The ultraviolet spectrum of the recovered material was recorded in water using a Unicam SP 800 Ultraviolet spectrophotometer. The infra-red spectrum was examined as a mull, using Nujol as the mulling oil. The spectrum was recorded with a Unicam SP 1200 Infra-red spectrophotometer. The nuclear magnetic resonance spectrum of caffeine dissolved in deutero-chloroform was recorded with a Varian T60 NMR spectrophotometer.

Table 1—Caffeine contents of Nigerian kola nuts

Sample of kola nuts	Weight of caffeine spectrophotometrically determined per 100g of powdered kola nuts	Weight of recovered recrystallized caffeine after chromatography per 100g of powdered kola nuts
Cola acuminata	2.192 ± 0.001g	2.052 ± 0.005g
Cola nitida rubra	1.910 ± 0.001g	1.795 ± 0.005g
Cola nitida alba	1.401 ± 0.001g	1.300 ± 0.005g
Cola verticillata	1.043 ± 0.001g	0.964 ± 0.005g

RESULTS & DISCUSSION

THE RESULTS summarized in Table 1 prove satisfactory when recovery studies were carried out. The homogeneity of the recovered and recrystallized material was

indicated by the presence of only a single spot in the thin layer chromatogram after eluting the plate with various solvents. The infra-red, ultraviolet, nuclear magnetic resonance spectral data and the melting point of the recovered material were found to be in excellent agreement with the spectral data and melting point reported for caffeine (Bhancca et al., 1962; Ikan, 1966; Kamp, 1960; Leal et al., 1961), hence the recovered and recrystallized material is pure caffeine.

The results in Table 1 show that *C. acuminata* contains more caffeine than *C. nitida rubra* which is richer in caffeine content than *C. nitida alba* justifying the local belief that the stimulating power of commonly edible Nigerian kola nuts decreases in the order of *C. acuminata, C. Nitida rubra* and *C. nitida alba*.

CONCLUSION

THIS INVESTIGATION shows that Nigerian kola nuts vary in caffeine content. The variation not only occurs from one species to another but also between varieties of the same species as exemplified by the difference in caffeine contents of *C. nitida rubra* and *C. alba.*

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A Research Note

SPECTROPHOTOMETRIC ANALYSES OF ORANGE JUICES AND CORRESPONDING ORANGE PULP WASHES

INTRODUCTION

ORANGE PULP WASH (water extraction of soluble fruit solids from orange pulp) is a by-product obtained during the processing and production of orange concentrate. Over the years pulp wash production and use has increased; however, methods of evaluating the product are lacking and much in need.

A comprehensive comparison of aqueous extracts of orange pulp and reconstituted orange juice concentrates was conducted at the Agricultural Research & Education Center, Lake Alfred, Fla. (Hill and Patrick, 1959; Huggart et al., 1959; Rouse et al., 1959; Wenzel, 1959). Eight chemical and physical characteristics were investigated. Their results showed that the characteristics of water extracts of orange pulp varied greatly with pulp washing procedures and with the variety of fruit used. However, water-soluble pectin and flavonoids were always found in larger quantities in the pulp wash samples than in their corresponding orange juices. Sawyer (1963) reported on an extensive investigation of the chemical composition of pulp extract, and fresh and concentrated orange juices. He reported that pulp extracts were generally lower in citric and ascorbic acid and higher in nicotinic acid, nitrogen content and pentose equivalent figures than fresh juices. Differences in ash composition were also noted. From results of this type he indicated that a general composition of a juice may be constructed.

Hendrickson et al. (1958) reported that addition of rag and pulp to grapefruit juice greatly increased the ultraviolet absorption. Petrus and Dougherty (1973) investigated the combined visible and ultraviolet absorption of alcoholic solutions of Florida orange juices. They reported that the alcoholic solutions of the rag and albedo components showed very strong ultraviolet absorption but little, if any, visible absorption. However, alcoholic solutions of the juice component indicated it to be the major contributor to absorption in the visible region. The juice component also revealed strong ultraviolet absorption.

The purpose of the work presented herein is to describe a method for the estimation of the orange juice content of orange pulp washes from their combined visible and ultraviolet absorption.

EXPERIMENTAL

Sample collection and preparation

Samples of mid- and late season frozen orange concentrates and their respective frozen orange pulp wash concentrates were obtained from four commercial processors. The samples were collected so that an orange concentrate and an orange pulp wash concentrate were obtained from the same fruit lot.

All orange concentrates and pulp wash concentrates were reconstituted to 12.8° Brix. The orange juice sample or pulp wash sample was diluted with an equal volume of distilled water. 5 ml of the diluted sample were made up to 50 ml with absolute alcohol. The alcoholic solution was then placed in the dark until the floc-

Table 1—Absorption characteristics of midseason orange juices and corresponding pulp washes

Plant	Juice[a]	443 nm	325 nm	280 nm	% Orange Juice[b]
1	OJ	0.092	0.812	1.578	
	PW	0.051	1.455	2.400	55
2	OJ	0.095	0.860	1.560	
	PW	0.047	1.400	2.252	49
2	OJ	0.107	0.918	1.575	
	PW	0.051	1.390	2.176	48
3	OJ	0.084	0.849	1.527	
	PW	0.062	1.175	2.055	74
4	OJ	0.100	0.813	1.530	
	PW	0.045	1.178	2.001	45

[a] OJ = Parent orange juice; PW = Pulp wash
[b] Absorbance ratio at 443 nm of PW/OJ X 100

Table 2—Absorption characteristics of late season orange juices and corresponding pulp washes

Plant	Juice[a]	443 nm	325 nm	280 nm	% Orange Juice[b]
1	OJ	0.118	0.835	1.282	
	PW	0.050	1.735	2.404	42
1	OJ	0.122	0.792	1.285	
	PW	0.059	1.611	2.404	48
2	OJ	0.161	0.892	1.318	
	PW	0.075	1.422	2.010	47
2	OJ	0.158	0.951	1.475	
	PW	0.062	1.388	2.055	39
3	OJ	0.158	0.842	1.270	
	PW	0.079	1.288	1.835	50
3	OJ	0.139	0.880	1.278	
	PW	0.072	1.427	1.950	52
4	OJ	0.156	0.898	1.360	
	PW	0.065	1.235	1.718	42

[a] OJ = Parent orange; PW = Pulp wash
[b] Absorbance ratio at 443 nm of PW/OJ X 100

culent precipitate formed. The precipitate was removed by centrifugation to obtain a crystal-clear solution.

A Coleman Model 124 Recording Spectrophotometer was used to scan the clear alcoholic solutions from 600–200 nm using 90% ethanol as a reference.

RESULTS & DISCUSSION

ABSORPTION MAXIMA, obtained on alcoholic solutions of the reconstituted orange juices and pulp washes, were observed at 465, 443 and 425 nm of the visible spectrum and at 325, 280 and 245 nm of the ultraviolet spectrum and were in accordance with previous investigations (Petrus and Dougherty, 1973). The visible absorption was due mainly to the carotenoids present and the ultraviolet absorption due to the polyphenols (325 nm), flavonoids (280 nm) and ascorbic acid (245 nm). To simplify the discussion only the most intense and resolved absorption maxima are presented.

The spectral shape for the orange juice and pulp wash solutions were quite similar. However, there were major differences in the absorption intensities. Tables 1 and 2 present the absorption data, at the various wavelengths of interest, for the parent orange juices (OJ) and pulp washes (PW). It is evident from the tables that the visible absorption (443 nm) of the pulp wash sample decreased and the ultraviolet absorption (325, 280 nm) increased dramatically from that of the parent orange juice.

Water extraction of orange pulp, during the pulp washing process, would tend to incorporate more rag and pulp and their water extractable soluble solids into the product. This should increase the ultraviolet absorption and decrease the visible absorption since experimental results have shown that alcoholic solutions of the rag and albedo components have little, if any, visible absorption but very strong ultraviolet absorption (Petrus and Dougherty, 1973). In contrast, however, the orange juice component was observed to be the major contributor to the visible obsorption and also showed strong ultraviolet absorption (Petrus and Dougherty, 1973). Since the orange juice component was observed to be the major contributor to the visible absorption, and all pulp wash samples examined exhibited a decrease in the visible absorption (443 nm) from that of the parent orange juice, pulp wash may be considered a product containing some orange juice. Absorbances for known concentrations of alcoholic solutions of any given orange juice obeyed Beer's law for all of the observed absorption maxima. If it may be assumed that the parent orange juices are pure juices, then the orange juice concentration in a pulp wash product may be estimated from the visible absorption at 443 nm. This is simply the absorbance ratio (at 443 nm) of PW/OJ × 100.

The orange juice contents (% orange juice) of the mid- and late season pulp wash products are presented in Tables 1 and 2. The midseason pulp wash indicated an orange juice content range from 45–74% while the late season had a range from 39–52%. The plant-to-plant variation in the orange juice content of pulp wash may be dependent upon a number of factors such as: extractor pressure, pulp washing procedures and variety and maturity of the fruit used.

CONCLUSIONS

THE COMBINED visible and ultraviolet absorption characteristics of alcoholic solutions of orange pulp washes differ considerably from their parent orange juices. The pulp washes are characterized by weak visible and very strong ultraviolet absorption. The weak visible absorption of the pulp wash solutions is due to the concentration of parent orange juice present, while the very strong ultraviolet absorption is the result of the parent orange juice present and incorporation of rag and pulp and their water extracted soluble solids into the product during the pulp washing process. It has been shown that the visible absorption of the pulp wash and parent orange juice may be used to estimate the orange juice content of the pulp wash. It has also been shown that this orange juice content may vary between processors.

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A Research Note
AMYLOSE CONTENT AND PUFFED VOLUME OF PARBOILED RICE

INTRODUCTION

SHORT-GRAIN low amylose rice is used in gun-puffed and oven-puffed breakfast cereals in the U.S.A. (Brockington and Kelly, 1972). "Pinipig" or parboiled waxy rice is used for making puffed rice in the Philippines (Brockington and Kelly, 1972). The process consists of parboiling steeped rough rice in earthen pots over a fire, and either milling or flattening the parboiled rice. The milled rice is then puffed by heating with a small amount of vegetable oil. The flattened brown rice is processed by gun puffing.

We recently found that the equilibrium water content of steeped brown rice at ambient temperature was negatively correlated with amylose content but not correlated with gelatinization temperature in low- and high-amylose lines from the same cross (Kongseree and Juliano, 1972). Bhattacharya et al. (1972), however, found that grains of semidwarf rices have a higher water content than grains of the tall Indian varieties regardless of gelatinization temperature. Waxy rice has been reported to give higher popped volume than nonwaxy rice (Mottern et al., 1967). In contrast, volume expansion of milled rice during boiling is positively correlated with amylose content (Juliano et al., 1965). Because waxy and low-amylose rice is preferred for making puffed rice and since puffed volume is affected by the degree of parboiling (Roberts et al., 1954), we investigated these properties in rice samples differing in amylose content.

EXPERIMENTAL

ROUGH RICE SAMPLES were obtained from the 1972 dry season crop of the IRRI farm. All samples were from semidwarf varieties and lines, except Malagkit Sungsong. The samples were steeped in water at 60–63°C for 6–7 hr, and parboiled for 14 min in an autoclave at 100°C and 0 psig steam pressure (Raghavendra Rao and Juliano, 1970). The samples were air dried at 25–27°C, dehulled with a Satake dehuller and milled to 10% bran-polish removal with a McGill mill. The milled parboiled samples were stored at 25–27°C for 3–4 wk to equilibrate them to a constant moisture content of about 12% and then puffed by placing them in the air outlet of an air oven for 16–20 sec at 250°C (Roberts et al., 1954).

The equilibrium water content of brown rice was determined on duplicate 15-g samples prepared by vacuum infiltration in water for 10 min and soaking for 18 hr in distilled water at 25–27°C (Kongseree and Juliano, 1972). The samples were blotted with a moist chamois, weighed, and dried for 16 hr at 100°C. Water content was determined from the weight lost after drying and expressed on a wet weight basis. Water content was also determined on rough rice samples steeped at 60–63°C. Amylose content was determined on milled rice by the method of Juliano (1971). Volume of parboiled and puffed parboiled milled rice was measured by the xylene displacement method (Reyes et al., 1965) and expressed as volume expansion ratio.

To determine the effect of water content during parboiling on puffed volume, rough rice samples of IR20 (nonwaxy) and IR833-6-2 (waxy) were steeped at 60–63°C for 0.33, 3.75 and 6.75 hr, tempered in bottles for 12 hr and parboiled, milled and puffed as described. Water content was also determined on the tempered samples.

RESULTS & DISCUSSION

THE EQUILIBRIUM water content of steeped rice differed among varieties and lines and tended to be highest in waxy rice, followed in order by nonwaxy rices with low (< 20%) and intermediate (20–25%) amylose, and then by those with moderately high (25–27%) and high (> 27%) amylose (Table 1). Rough rice steeped at 60–63°C generally had higher water content than brown rice steeped at 25–27°C, particularly samples with low final gelatinization temperature (< 70°C). Only IR20 had intermediate gelatinization temperature (70–74°C). This difference is probably due to the swelling of the grains at the higher temperatures. Protein content (6.2–10.3% at 14% moisture) was not related to equilibrium water content. This negative relationship between amylose content and water content of steeped rice had previously been reported by Kongseree and Juliano (1972). Bhattacharya et al. (1972) also reported that steeped waxy rice had higher water content than for nonwaxy rice.

The higher water content of waxy rice may be due to the less compact packing of its starch granules, as shown by their lower absolute density (Reyes et al., 1965), and to the presence of micropores on their surface below 9% moisture (Watabe and Okamoto, 1960). It is possible that porosity of the starch granules decreases with an increase in amylose content.

Among the nonwaxy rices of similar amylose content, IR8, the only sample with an opaque or white-belly portion, had a higher water content than rices with translucent grains. Water evidently enters the air spaces in the opaque portions of the IR8 grain during steeping,

Table 1—Effect of amylose content on water content of steeped brown rice and volume expansion of milled parboiled rice on oven puffing

Variety or line	Amylose Content (%)[a]	Equilibrium water content (%)[b] Brown rice 25–27°C	Equilibrium water content (%)[b] Rough rice 60–63°C	Volume expansion ratio
Malagkit Sungsong	waxy	35.2	38.7	1.61
IR833-6-2	waxy	35.1	38.2	1.52
IR24	18	30.7	31.6	1.29
IR480-5-9	24	30.5	32.4	1.25
IR20	26	28.8	29.0	1.10
IR22	28	28.8	31.3	1.05
IR8[c]	28	29.7	32.3	1.10
LSD (5%)		1.0	1.0	0.059

[a] Dry basis
[b] Wet basis
[c] With opaque portions

thus, increasing its water content. Since all samples except Malagkit Sungsong are semidwarf varieties, higher water content was not due to the dwarfing gene, as suggested by Bhattacharya et al. (1972), but to the presence of opaque portions with air spaces in some rice varieties, regardless of plant height.

Volume expansion during puffing differed among the rice samples; it was highest for waxy rice (Table 1). Puffed volume was greater in steeped rices with water contents above 30% at 28–30°C (samples with less than 25% amylose) than in samples with less than 30% water (samples with more than 25% amylose). Brockington and Kelly (1972) reported that waxy rice overpuffs and disintegrates during puffing under conditions that are suitable for low-amylose nonwaxy rice. The volume expansion ratios reported here are lower than those of Roberts et al. (1954) since our values are based on absolute rather than bulk volume.

Samples of IR20 and IR833-6-2 rices parboiled at different moisture contents but puffed under the same condition showed that the water content of the rice on parboiling determined the puffed volume (Fig. 1). The two samples showed little differences in puffed volume up to about 30% water content. Since volume expansion is low for rice with 30% water parboiled at 100°C and the degree of parboiling directly affects puffed volume (Roberts et al., 1954), the water content of rice during steaming must be the major influence on the degree of parboiling. A milled parboiled rice sample of Malagkit Sungsong processed in a local "pinipig" factory gave a volume expansion ratio of 2.76 in our laboratory, showing that the temperature used for parboiling also affects puffed volume. Our data indicate that in addition to parboiling conditions, amylose content of rice influences the puffed volume of the resulting milled parboiled rice by affecting the degree of parboiling of grains processed under identical conditions.

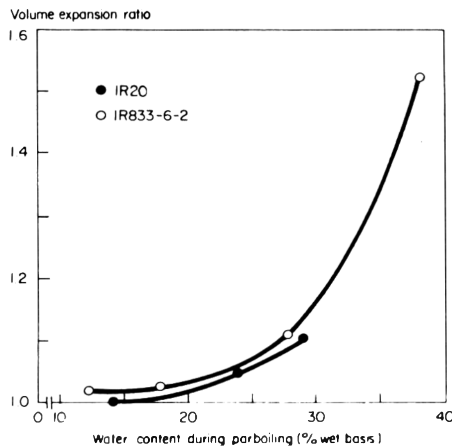

Fig. 1—Water content of rough rice during parboiling in relation to volume expansion ratio on puffing of milled parboiled rice of a nonwaxy (IR20) and a waxy (IR833-6-2) rice.

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