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ABSTRACTS:
IN THIS ISSUE

COMPARISON OF FROZEN, FOAM-SPRAY-DRIED, FREEZE-DRIED, AND SPRAY-DRIED EGGS. 8. Coagulation Patterns of Slurries Prepared with Milk and Whole Eggs, Yolks or Albumen. K. FUNK & M.E. ZABIK. *J. Food Sci.* **36,** 715–717 (1971)–Coagulation patterns of slurries prepared with milk and frozen, foam-spray-, freeze- and spray-dried whole eggs, yolks and albumen were studied. All slurries were heated and stirred at constant rates using a Brabender Visco/Amylo/Graph. Ranked in order of initial gelation temperatures were slurries prepared with frozen, freeze-, spray- and foam-spray-dried whole eggs. Slurries prepared with frozen yolks or albumen showed initial gelation at lower temperatures than those prepared with dried yolks or albumen. Viscosity data indicate detrimental effects of drying to gelation properties of whole eggs, yolks and albumen.

ACTION OF EMULSIFIERS IN ICE CREAM UTILIZING THE HLB CONCEPT. R. GOVIN & J.G. LEEDER. *J. Food Sci.* **36,** 718–722 (1971)–Experiments showed that the level of milk solids-not-fat normally used in ice creams, alone, had sufficient emulsifying capacity to emulsify the fat present in a standard ice cream mix. The added chemical emulsifier, regardless of its HLB, contributed little to the stability of the mix. This was true even where butteroil was used as a source of fat. Fat deemulsification during freezing increased with the HLB of the emulsifier while the dryness showed no meaningful trend. The meaning of the terms dryness, stiffness, melt resistance and stand-up quality has been clarified.

STABILITY OF MILK FAT-WATER EMULSIONS CONTAINING SINGLE AND BINARY EMULSIFIERS. T.C. TITUS & J.B. MICKLE. *J. Food Sci.* 36, 723–724 (1971)–The stability of 25% milk fat-water emulsions containing sorbitan-fatty acid emulsifiers were compared to determine the influence of single vs. binary emulsifier systems, and saturated vs. unsaturated emulsifier side chains. These variables were compared at emulsifier HLB values of 9.5–10.0 and at usage levels of 0.5–2.0% of the fat. There were no statistical differences ($P > 0.05$) between single and binary emulsifier mixtures or between emulsifiers with saturated or unsaturated side chains. There also was no significant difference ($P > 0.05$) between emulsifier HLB numbers of 9.5 and 10.0. However, as the amount of emulsifier in the system was increased the stability of the emulsion increased ($P < 0.01$).

EXTRACTABILITY OF COCONUT PROTEINS. A.S. SAMSON S.J., R.N. KHAUND, C.M. CATER & K.F. MATTIL. *J. Food Sci.* **36,** 725–728 (1971)–Studies were made on nitrogen (protein) solubility of coconut meal in aqueous media over a range of pH's. The point of minimum solubility was found at pH 3.9 and nitrogen solubility increased toward the acidic and alkaline sides. Solubility characteristics in different salt solutions were also determined. Comparisons were made with coconut meal prepared from commercial desiccated coconut and sun-dried copra. Variables affecting extractability of coconut protein in coconut meal were also examined: temperature of extraction, solvent:meal ratios, length of time of extraction and effect of different amounts of added salts. Osborne classification studies of coconut meal indicate that 90% of the proteins would be classified as albumins and globulins.

AN ENZYMATIC PROCESS FOR A PROTEIN-CONTAINING BEVERAGE BASED ON SOYBEAN PROTEIN AND LEMON JUICE. H. SUGIMOTO, J.P. VAN BUREN & W.B. ROBINSON. *J. Food Sci.* **36,** 729–731 (1971)–An acidic enzymatic process for a bland soybean protein hydrolysate is described. Cooked suspension (5.0%) of isolated soybean protein is mixed with small amounts of an acid-protease preparation from *Trametes sanguinea*, adjusted to pH 3.3–3.5 with concentrated lemon juice and incubated at 50°C for 8–10 hr. After stopping the proteolysis by heat treatment, the clear supernatant is separated from any insoluble residue by centrifugation. A solubilized nitrogen recovery of 88–90% is obtained. After being diluted about twice and sweetened with sugar, the hydrolysate constitutes a completely clear, slightly yellowish lemonade-like flavored beverage. The data on chemical analysis, gel-filtration and rheological behavior of the hydrolysate are described and discussed.

STORAGE STABILITY OF CSM: ALTERNATE FORMULATIONS FOR CORN-SOY-MILK. G.N. BOOKWALTER, H.A. MOSER, W.F. KWOLEK, V.F. PFEIFER & E.L. GRIFFIN JR. *J. Food Sci.* **36,** 732–736 (1971)–Corn-soy-milk (CSM), a high-protein food supplement for children, contains pregelatinized corn meal, soy flour, nonfat dry milk, vitamins and minerals. To increase the choice of possible ingredients, several formulations were studied. Flavor and chemical tests after storage at 77, 100, and 120°F were made on experimental samples containing corn meals and soy flours of different compositions, dry whey, increased levels of nonfat dry milk, sucrose and dextrose hydrate. Stability was adequate for all formulations tested except those containing dextrose hydrate or unprocessed whole corn meal. At storage temperatures of 100°F or above, substantial losses in available lysine occurred in the samples containing dextrose hydrate.

STORAGE STABILITY OF CSM: INCREASING FAT TO 6% IN CORN-SOY-MILK BLENDS. G.N. BOOKWALTER, H.A. MOSER, L.T. BLACK & E.L. GRIFFIN JR. *J. Food Sci.* 36, 737–741 (1971)–CSM is a high-protein food supplement for infants and preschool children; it consists of partially gelatinized corn meal, toasted soy flour and nonfat dry milk and is fortified with vitamins and minerals. The original CSM formulation contained only 2% fat. Storage stability characteristics were determined for CSM blends containing as much as 6% fat. The fat sources tested were processed corn germ, full-fat soy flour, refined soybean oil, expeller crude corn oil and a combination of high-fat corn meal with full-fat soy flour. Flavor and chemical tests were made on blends stored at 77, 100 and 120°F and compared with those on CSM containing 2% fat. Stability was similar or improved in the formulations containing higher fat levels. One sample of crude corn oil was satisfactory while another caused off-flavors in the experimental blends.

BLANCHING OF WHITE POTATOES BY MICROWAVE ENERGY FOLLOWED BY BOILING WATER. S.C. CHEN, J.L. COLLINS, I.E. McCARTY & M.R. JOHNSTON. *J. Food Sci.* **36,** 742–743 (1971)–Potatoes, when subjected to microwave energy, became hot first at the core with a heat gradient developing toward the periphery. Boiling water produced a heat gradient from the periphery toward the core. Peroxidase was inactivated initially in two zones, one at the core and one at the periphery. The minimum time required for complete inactivation of peroxidase and the subsequent shear values were as follows: 1.5 min microwaves and 3 min boiling water, 119 lb force; and 2 min microwaves and 2 min boiling water, 124 lb force. Peroxidase was not completely inactivated by microwaves at 1 min or less when followed by heating in boiling water for 5 min.

SPECIFIC GRAVITY DETERMINATION WITH A UNIVERSAL TESTING MACHINE. R.G. HULSEY, P.E. NELSON & C.G. HAUGH. *J. Food Sci.* **36,** 744–746 (1971)–A rapid, precise technique for determining specific gravity of fruits and vegetables was developed. The method utilized a universal testing machine. Variables were studied and optimized. The experimental work was performed primarily with tomatoes. Calcula-

tion of specific gravity involved determining fruit volume and weight. Other volume measurement techniques investigated proved inaccurate or too time-consuming. An Instron machine measured the buoyancy force of a fruit submerged in a standard density fluid. The buoyancy force is directly related to the fruit volume. Error analysis showed relatively low sensitivity to errors in weight measurement, but was influenced mainly by errors in determining fluid density.

COMPUTER EVALUATION OF IRRADIATION PROCESSES IN CYLINDRICAL CONTAINERS WITH GAMMA SOURCES. K.S. PUROHIT, J.E. MANSON & J.W. ZAHRADNIK. *J. Food Sci.* **36,** 747–749 (1971)–A numerical technique is presented for evaluating food irradiation processes using cylindrical gamma sources. Dose rate distributions obtained by dividing a container into finite concentric rings permit the use of a nonuniform initial spore load distribution and the determination of both number and distribution of survivors. The double integral giving dose rates was computed using several methods. The computations gave survivors significantly different from those obtained by using approximations given in literature. Accurate evaluations require utilization of dose rate distributions in radial and axial directions.

THEORETICAL EVALUATION OF COMBINED IRRADIATION AND THERMAL PROCESSES IN CYLINDRICAL CONTAINERS WITH GAMMA SOURCES. K.S. PUROHIT, J.E. MANSON & J.W. ZAHRADNIK. *J. Food Sci.* **36,** 750–751 (1971)–A digital computer technique is presented for studying combined effects of heat and radiation in food sterilization, using steam retorts and cylindrical gamma sources. Temperature and dose rate distributions in cylindrical containers are computed using numerical methods. Distributions due to preliminary treatments–survivor and degree of spore sensitization–are inputs to subsequent treatment. The heat-radiation sequence was studied using data available, which indicates no synergism. This additive combination served as control to demonstrate synergism for radiation-heat sequence. Synergy, as influenced by irradiation exposure time, source strength, thermal process time and retort temperature was studied.

EFFECT OF RADURIZATION ON SHEAR RESISTANCE AND FRAGMENTATION OF CHICKEN MUSCLE. R.C. WHITING & J.F. RICHARDS. *J. Food Sci.* **36,** 752–755 (1971)–Excised pectoralis muscles of chicken received radurizing doses of γ-radiation at varying times postmortem to determine the effect on shear resistance, myofibrillar fragmentation (F-ratio) and pH. Irradiation doses between 0.1 and 0.3 Mrad produced decreases in F-ratio and increases in shear resistance. The magnitude of the changes was directly proportional to the duration of postmortem aging prior to irradiation. Muscle pH subsequent to irradiation between 2 and 12 hr post-mortem was not significantly affected by any of the dose-time treatment combinations. Shear resistance and F-ratio changes were strongly correlated on an average basis ($r = +.857$; $n = 6$) but not on an individual sample basis.

PALATABILITY OF BONELESS FRESH PORK HAM AND LEG OF LAMB PREPARED BY INTERRUPTED COOKING PRIOR TO FROZEN STORAGE. B.M. KORSCHGEN & R.E. BALDWIN. *J. Food Sci.* **36,** 756–759 (1971)–Boneless fresh pork hams and legs of lamb were scored acceptable or above in palatability when prepared by the "roasteak" procedure, involving preroasting to 44°C, slicing and chilling or freezing before grilling. Mean scores for flavor and juiciness were higher for frozen-antioxidant-dipped pork slices than for those not treated with antioxidant. However, control slices (zero storage) were not significantly different in flavor from treated samples. Control slices of lamb were significantly better than frozen lamb roasteaks for all attributes, and storage or antioxidant treatment caused no significant difference.

AN EMULSION METHOD FOR RAPID DETERMINATION OF FAT IN RAW MEATS. J.R. MOREAU & M.T. LAVOIE. *J. Food Sci.* **36,** 760–763 (1971)–A new assay for fat determination in raw meats was developed. It involves preparing an emulsion from the meat to be analyzed. A portion is then purposely broken down to separate the fat portion which is subsequently measured in graduated Babcock bottles. The method takes about 10 min and results correlate with those obtained with Salwin's modified Babcock method taking about 20–25 min and with the official ether extraction method taking about 24 hr. The new method permits analyzing large samples of almost any desirable and practical size and applies directly to meat emulsions prepared in manufacturing sausage products, for quality control purposes.

VACUUM PACKAGING OF LAMB. 1. Microbial Considerations. J.O. REAGAN, L.E. JEREMIAH, G.C. SMITH & Z.L. CARPENTER. *J. Food Sci.* **36,** 764–766 (1971) Paired loins from 148 lamb carcasses were utilized to determine the subsequent case-life of chops from loins that had been stored in vacuum packages. Longer periods of vacuum packaged storage were associated with higher initial psychrotrophic counts from subsequent loin chops. Comparisons between vacuum packaged loins that were stored at 0°C vs. 7°C indicated that storage temperatures of 7°C resulted in chops which exhibited decreased retail case-life. 8 days appears to be a reasonable maximum period for the storage of loins in vacuum packages. The average case-life of fresh chops (fabricated 8 days postmortem and displayed immediately) exceeded the average case-life of chops from vacuum packaged loins (packaged after 8 days postmortem storage, placed in vacuum packages for 8 days, fabricated and subsequently displayed) by 1¼ days. These data reveal that the vacuum packaging of primal cuts does not compensate for improper conditions of refrigeration.

EFFECT OF MARBLING AND OTHER VARIABLES ON CASE LIFE OF NEW YORK STEAKS. W.H. KENNICK, D.K. BUCK, L.S. McGILL, N.A. HARTMANN JR. & R.S. TURNER. *J. Food Sci.* **36,** 767–769 (1971)–The effects of type of aging (air vs. vacuum) and degree of marbling on case life of New York steaks were investigated. Color description and color desirability of all steaks were evaluated daily by a three-member panel. Case life was terminated when a visible spot resulting from formation of metmyoglobin appeared on the surface of the meat. There was no difference in case life attributable to type of aging. Degree of marbling had a significant positive linear effect on color description scores at 24 and 48 hr post-cutting, but did not affect color desirability score. Marbling had a significant curvilinear effect on case life with slight (the least) and slightly abundant (the greatest) amounts having the shortest case life.

QUALITY TRAITS ASSOCIATED WITH CONSUMER PREFERENCE FOR BEEF. M.E. JUILLERAT & R.F. KELLY. *J. Food Sci.* **36,** 770–773 (1971)–Procedures used and results of research to relate beef carcass or muscle characteristics to consumer preferences in top round of beef with a 950-family consumer panel are described. Paired comparisons were used and maximum-likelihood estimates obtained of the probability

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a given class would receive top rating. Probability sets for all characteristics were similar and in the order usually expected by food scientists. Descriptive grades are suggested, however, for the probability is .60 to .70 that consumers in this study will rank on top some steak class other than what present grades and meat scientists consider "best." The major application of results is in developing hypotheses for future research.

EFFECTS OF HETEROTROPHIC AND AUTOTROPHIC GROWTH CONDITIONS ON THE COMPOSITION OF Chlorella sorokiniana. R.L. MILLER, H.E. WICKLINE & B. RICHARDSON. *J. Food Sci.* **36,** 774–777 (1971)–The effects of heterotrophic and autotrophic growth conditions on the composition of *Chlorella* are discussed in relation to the potential food value of algae. Cells from the two culture modes differ appreciably in proximate analysis and elemental composition; however, fiber content and vitamin and amino acid composition are essentially unchanged. On this basis it appears that heterotrophic algae, which are less expensive to produce, can be used to examine the acute toxicity problem with bacteria-free cells. In addition, heterotrophic algae may also be used to predict results that would be obtained with autotrophic material in studies of processing techniques designed to improve protein quality and digestibility.

TEMPERATURE CYCLING EFFECTS ON BACTERIAL GROWTH. 1. Pseudomonas fluorescens. A.J. HOWELL, R.L. SAFFLE & J.J. POWERS. *J. Food Sci.* **36,** 778–780 (1971)–Virtually all previous research involving temperature effects on organisms has been carried out at constant temperatures even though the temperatures occurring in nature are constantly fluctuating. An investigation to determine the effect mild cycling of temperature has on *Pseudomonas fluorescens* showed that in general, cycled-down growth responses appear to be greater than those for the cycled-up responses. At temperatures both above and below the optimum, the cycled-down organisms produced greater growth responses than the cycled-up organisms. The constant values were only a small amount greater than the cycled-down values.

EFFECT OF EDTA ON THE GERMINATION OF AND OUTGROWTH FROM SPORES OF Clostridium botulinum 62-A. F.G. WINARNO, C.R. STUMBO & K.M. HAYES. *J. Food Sci.* **36,** 781–785 (1971)–EDTA, in concentrations above 2.5 mM, was found inhibitory to germination of and outgrowth from spores of *C. botulinum* Type A and to toxin production in a fish homogenate. Inhibitory action was influenced by pH of the medium in the range pH 6.5–8.1, the action increasing with pH. It was influenced by Mg and Ca concentrations in the medium, equimolar concentrations of added $CaCl_2$ and $MgCl_2$ completely erasing the growth inhibitory action. Initial spore concentration also influenced inhibitory efficacy–the higher the spore concentration, the higher the EDTA concentration required for inhibition. There was no evidence that EDTA, in any concentration used, promoted spore germination. Release of Ca, Mg and DPA from incubating spores was suppressed to varying extents by 5.0 and 10 mM EDTA.

INFLUENCE OF SEX AND POSTMORTEM AGING ON INTRAMUSCULAR AND SUBCUTANEOUS BOVINE LIPIDS. R.L. HOOD & E. ALLEN. *J. Food Sci.* **36,** 786–790 (1971)–Wholesale beef ribs from bulls, steers and heifers were used to study the changes in subcutaneous (SQ) and longissimus dorsi intramuscular (IM) lipids at 2, 7, 14 and 21 days post-mortem (PM). Cholesterol levels, phospholipid levels and IM phospholipid fatty acid composition did not change with PM aging. The progressively increasing free fatty acid (FFA) levels observed with time PM were paralled by fatty acid composition changes. Differences were observed in the quantity and composition of the fatty acids from the lipid classes for the three sex groups. Rib steaks from heifers had significantly ($P < .05$) higher sensory panel scores for aroma and lower IM and SQ levels of FFA when compared to bulls. A significant ($P < .01$) correlation of −0.49 was obtained between aroma score and IM levels of FFA.

THE RELATION OF ATPase ACTIVITY AND CALCIUM UPTAKE TO POSTMORTEM GLYCOLYSIS. K. KRZYWICKI. *J. Food Sci.* **36,** 791–794 (1971)–Calcium uptake and Ca^{2+}-activated ATPase activity have been investigated in relation to pH_1 in pork muscle Longissimus dorsi, sampled 35 min postmortem. Both the rate of calcium uptake and enzyme activity were negatively, and significantly, correlated with pH_1 except when the latter value dropped below 5.9. At $pH_1 < 5.9$, impairment of reticular function and of enzyme activity became apparent. As pH_1 is a guide to the rate of postmortem glycolysis, the significance of the findings is discussed with respect to accelerated glycolysis and the development of the PSE condition in pork.

LIPIDS OF CURED CENTENNIAL SWEET POTATOES. W.M. WALTER JR., A.P. HANSEN & A.E. PURCELL. *J. Food Sci.* **36,** 795–797 (1971)–The lipids of cured Centennial sweet potatoes were identified and quantitated using a combination of column and thin layer chromatography. It was found that 2.7% of the dry weight was made up of lipids which were shown to consist of 42.1% neutral lipids, 30.8% glycolipids and 27.1% phospholipids. Triglycerides and steryl esters were the major lipids of the neutral fraction. Among the phospholipids, phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol were the most abundant. Galactolipids and steryl glucosides were also present. Fatty acid analysis of the three major lipid groups showed that stearic, palmitic, oleic, linoleic and linolenic acids were the most abundant.

ANTIOXIDANT POTENTIAL OF TEMPEH AS COMPARED TO TOCOPHEROL. L.V. PACKETT, L.H. CHEN & J.Y. LIU. *J. Food Sci.* **36,** 798–799 (1971)–The antioxidant potential of tempeh to preserve tocopherol-stripped corn oil was studied. 25 and 50% levels by weight of tempeh were mixed into corn oil and incubated at 37°C for a maximum of 6 wk. Peroxide values were determined periodically. Results showed that tempeh can effectively prevent lipid oxidation. 50% tempeh in corn oil showed higher antioxidant potential than 25% tempeh, 0.01% alpha-tocopherol, or 0.03% alpha-tocopherol. This study substantiates the antioxidant potential of tempeh and suggests its use with other foodstuffs to help preserve the lipids contained therein.

HYDRATION OF SODIUM CHLORIDE BOUND BY CASEIN AT MEDIUM WATER ACTIVITIES. S. GÁL & D. BÁNKAY. *J. Food Sci.* **36,** 800–803 (1971)–The binding of ions by proteins and their hydration were studied by water vapor sorption measurements with model mixtures of sodium chloride and casein at 25°C between the water activities of 0.50 and 0.75. The hydration, as calculated from the numerical equations of the isopsychric curves, increases progressively with increasing water activity, and also with the amount of salt added up to the point of complete saturation of the protein with ion pairs.

CAROTENOID TRANSFORMATIONS IN RIPENING APRICOTS AND PEACHES. T. KATAYAMA, T.O.M. NAKAYAMA, T.H. LEE & C.O. CHICHESTER. *J. Food Sci.* **36,** 804–806 (1971)–A study of the major carotenoids of the ripening apricot and peach showed that β-carotene was the predominant pigment of apricot tissue and accumulated rapidly during ripening, while in peach tissue, β-carotene along with β-cryptoxanthin and violaxanthin were synthesized in almost equal amounts during ripening. In the latter tissue small amounts of zeaxanthin were detected midpoint in the ripening sequence. [2-^{14}C]MVA injected into both peach and apricot tissue labeled β-carotene, β-cryptoxanthin, lutein and also violaxanthin in peach tissue. In peach tissue, the amount of incorporation of [2-^{14}C]MVA into the above carotenoids was at a maximum during the first part of ripening and then declined with senescence especially in the xanthophylls lutein and violaxanthin.

GAMMA GLUTAMYL TRANSPEPTIDASE OF SPROUTED ONION. S. SCHWIMMER & S.J. AUSTIN. *J. Food Sci.* **36,** 807–811 (1971)–An enzyme capable of liberating p-nitroaniline from γ-L-glutamyl p-nitroanilide has been purified 800-fold from sprouted onions. The enzyme acts optimally at pH 9.0, is activated by amino acids and inhibited by borate and competitively by γ-glutamyl derivatives. With the p-nitroanilide as substrate it exhibits a K_m of 14.3 mM whereas the K_i values with glutathione and γ-glutamyl-S-methyl-L-cysteine as inhibitors are 0.20 and 2.14 mM respectively. From chromatography of the reaction products and from kinetic considerations it is concluded that the purified enzyme is a transpeptidase (E.C.2.3.2.1) whereas crude onion extract may, in addition, possess a true γ-glutamyl hydrolase. It is suggested that such enzymes may play a role in the disappearance of peptides in post-dormant onions and may be useful evoking the full flavor potential of onion.

POST-IRRADIATION INACTIVATION OF HORSERADISH PEROXIDASE. B.J. MACRIS & P. MARKAKIS. *J. Food Sci.* **36,** 812–815 (1971)–Horseradish peroxidase in aqueous solution was exposed to gamma radiation in the dose range of 0–24 Krad and its activity measured immediately after irradiation and during the following 24 hr. Inactivation of varying degree was observed as a result of irradiation and the post-irradiation storage. Higher levels of dose, post-irradiation temperature, dilution of the enzyme or pH (9.0) resulted in greater post-irradiation inactivation. Irradiating the enzyme frozen, mixing it with glycerol or diluting it after irradiation prevented or diminished the inactivation. A mechanism for the post-irradiation of this enzyme is proposed.

ODOR THRESHOLD LEVELS OF PYRAZINE COMPOUNDS AND ASSESSMENT OF THEIR ROLE IN THE FLAVOR OF ROASTED FOODS. P.E. KOEHLER, M.E. MASON & G.V. ODELL. *J. Food Sci.* **36,** 816–818 (1971)–Ten pyrazines, purified by gas-liquid chromatography, were subjected to sensory evaluation studies in both aqueous and lipid media. Both the odor-detection threshold levels and a subjective evaluation of the odor of the compounds were obtained. The concentration of alkylpyrazine compounds in roasted peanuts, coffee and potato chips was determined quantitatively. This information, coupled with the knowledge of the odor-detection threshold levels, allows an assessment of the probable significance of the pyrazines in the aroma of these food products.

MINOR CONSTITUENTS OF WHISKY FUSEL OILS. 1. Basic, Phenolic and Lactonic Compounds. K. NISHIMURA & M. MASUDA. *J. Food Sci.* **36,** 819–822 (1971)–Basic, phenolic and lactonic fractions of the fusel oils obtained from Japanese and Scotch whiskies were analyzed mainly by means of gas-liquid chromatography coupled with mass spectrometry. α-Picoline, γ-picoline, 2,4-lutidine, 2,5-lutidine, 3,5-lutidine, α-ethyl pyridine, γ-ethyl pyridine, α-isopropyl pyridine, 2-methyl-5-ethyl pyridine, quinoline, 2-methyl quinoline, 6-methyl quinoline, 2-ethyl pyrazine, 2,5-dimethyl pyrazine, 2,3-dimethyl pyrazine, 2,5-diethyl pyrazine, 2-methyl-5-ethyl pyrazine, trimethyl pyrazine, 2,5-dimethyl-3-ethyl pyrazine, tetramethyl pyrazine, guaiacol, 4-methyl guaiacol, 6-methyl guaiacol, 4-ethyl guaiacol, phenol, o-cresol, p-cresol, o-ethyl phenol, p-ethyl phenol, o-isopropyl phenol, 2,6-xylenol, eugenol, o-hydroxy acetophenone, 2-hydroxy-5-methyl acetophenone, γ-nonalactone, cis- and trans-3-methyl-4-hydroxy caprylic acid γ-lactone and five furan compounds were found in Japanese or Scotch whiskies. Origins of these compounds are discussed.

ISOLATION AND CHEMICAL PROPERTIES OF CAPSANTHIN AND DERIVATIVES. T. PHILIP & F.J. FRANCIS. *J. Food Sci.* **36,** 823–827 (1971)–A solvent system for thin layer chromatography on silica gel was developed to separate the six classes of carotenoid pigments in paprika. The isolation, R_f values, visible, infra-red and NMR spectra of capsanthin and its derivatives (capsanthin, capsanthol, 3-ketokryptocapsone, 3-keto-beta-apo-8′-carotenal, capsanthin dilaurate, capsanthin (trimethyl silyl) diether) are presented. NMR and mass spectra of capsanthin are also given as well as beta-carotene and canthaxanthin for comparison.

CHANGES IN CARBOHYDRATES OF ALFALFA DURING ARTIFICIAL DRYING. B. Lj. MILIĆ & M. N. VLAHOVIĆ. *J. Food Sci.* **36,** 828–830 (1971)–Various temperatures and durations of artificial drying of alfalfa caused marked changes in seven of ten examined constituents. There were significantly less monosaccharides, disaccharides, starch and hemicellulose in the samples of artificially-dried herbage tissue. The contents of free galacturonic acid and cellulose were higher in the herbage tissue at the highest drying temperatures and at a moisture level above 4% for the former, and below 7% for the latter. The content of pectin, lignin and methoxyls changed very little during artificial drying. The main factors affecting changes in constituents measured in herbage tissue were temperature and duration of temperature treatment.

SPOILAGE UNITS OF RADURISED BOMBAY DUCK (Harpodon nehereus). U.S. KUMTA, M.D. ALUR, S.S. MAVINKURVE & N.F. LEWIS. *J. Food Sci.* **36,** 831–832 (1971)–The usefulness of Spencer and Baines' equation as a parameter for defining spoilage units of radurised fishery products was examined and found sufficiently accurate for computing shelf life taking a sensory score of five as a critical level of spoilage. Evidence on the suppression of spoilage units in Bombay duck (*Harpodon nehereus*) exposed to a radurisation dose of 0.1 Mrad is reported.

IMPACT OF LOW DOSES OF GAMMA RADIATION AND STORAGE ON THE MICROFLORA OF GROUND RED MEAT. N.P. TIWARI & R.B. MAXCY. *J. Food Sci.* **36,** 833–834 (1971)–Fresh ground beef and fresh pork sausage obtained from retail stores had an average total microbial count of 3.5×10^7/g and 3.1×10^7/g, respectively. Ground beef

ABSTRACTS:
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from a commercial central processing operation had less than 1% as many microorganisms and the effect of irradiation on this product was studied. By irradiation with low doses of gamma radiation, there was a major improvement in the microbial quality as judged by total, coliform, enterococcal and staphylococcal counts. On storage for 6 days at 5°C, the ground beef irradiated with 68 Krad contained only 0.2% of the total count of the control unirradiated samples. The combination of irradiation and storage at 2°C up to 9 days limited the count in the commissary product to less than that obtained in the best of the fresh samples from commercial retail stores. The total count of the irradiated product did not reach the range of unacceptability until 14 days. The potential usefulness of low doses of gamma radiation in public health protection is apparent.

RAPID DETERMINATION OF WATER IN MEAT BY THE SAPONIFICATION REACTION. R.L. GLASS & P.B. ADDIS. *J. Food Sci.* **36,** 835–836 (1971)–A method is described which permits rapid (< 30 min) determination of water in meat yet is convenient and inexpensive. Good agreement was obtained between the new method and conventional methods for cooked and uncooked light and dark turkey meat, ham, jowl fat, uncooked sausage emulsion and frankfurters. The method involves homogenization of meat in anhydrous methanol followed by reacting an aliquot of the extract with a water sensitive reagent and titration of the reaction mixture with standard HCl as previously described.

PHOSPHOLIPID CONCENTRATION ESTIMATED FROM TOTAL MUSCLE LIPID. A.M. CAMPBELL & L.T. HARRILL. *J. Food Sci.* **36,** 837 (1971)–The inverse relationship between total muscle lipid concentration and phospholipid concentration of the total lipid was linear when a log-log plot was made of the data from 67 diverse samples. The regression equation presented should have some predictive value.

LYSOSOMAL ENZYME ACTIVATION AND PROTEOLYSIS OF BOVINE MUSCLE. K. ONO. *J. Food Sci.* **36,** 838–839 (1971)–Biochemical evidence is presented to show that lysosomes are present in bovine semitendinosus muscle. Apparently, maximum activation of lysosomal enzymes occurs within 4 days postmortem. Significant increases in amino acid concentrations coupled with the increasing trend in β-galactosidase activity suggest that catheptic activity also increases.

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APPLIED SCIENCE and ENGINEERING

KAYE FUNK and MARY E. ZABIK
Dept. of Food Science & Human Nutrition, Michigan State University, East Lansing, MI 48823

COMPARISON OF FROZEN, FOAM-SPRAY-DRIED, FREEZE-DRIED AND SPRAY-DRIED EGGS
8. Coagulation Patterns of Slurries Prepared with Milk and Whole Eggs, Yolks or Albumen

SUMMARY—Coagulation patterns of slurries prepared with milk and frozen, foam-spray-, freeze- and spray-dried whole eggs, yolks and albumen were studied. All slurries were heated and stirred at constant rates using a Brabender Visco/Amylo/Graph. Ranked in order of initial gelation temperatures were slurries prepared with frozen, freeze-, spray- and foam-spray-dried whole eggs. Slurries prepared with frozen yolks or albumen showed initial gelation at lower temperatures than those prepared with dried yolks or albumen. Viscosity data indicate detrimental effects of drying to gelation properties of whole eggs, yolks and albumen.

INTRODUCTION

USE OF dehydrated eggs offers convenience, minimum storage space requirements as well as reduced transportation and storage costs to food service systems. To study the effect of foam-spray, freeze- and spray-drying on the physical, chemical and functional properties of eggs in comparison to those of frozen eggs, an extensive investigation was initiated (Zabik, 1968; Zabik and Figa, 1968; Wolfe and Zabik, 1968; Funk et al., 1969; Janek and Downs, 1969; Downs et al., 1970).

The primary purpose of this study was to compare the coagulation patterns of slurries prepared with milk and frozen, foam-spray-, freeze- and spray-dried whole eggs, yolks and albumen. These simple systems eliminated effects of additional ingredients on the coagulating ability of the egg proteins in solution but provided the salt necessary for coagulation (Lowe, 1955). Because protein coagulation is influenced by pH (Lowe, 1955), the effect of an adjusted pH on the coagulation patterns of whole eggs was investigated. For this purpose, the pH was adjusted to 6.7, thus preventing possible damage to milk proteins.

EXPERIMENTAL

PROCUREMENT, processing, packaging and storage of whole eggs and yolks containing corn sirup solids and albumen were as outlined by Zabik and Figa (1968). Dried whole milk, purchased in nitrogen-packed No. 10 cans, was weighed into 53.4g portions, sealed in polyethylene pouches and refrigerated at 4–5°C until needed for slurry preparation. 445 ml of distilled water were used for milk reconstitution. Quantities of whole eggs or egg components as well as amounts of distilled water used for reconstitution of dried eggs are shown in Table 1. All distilled water was adjusted to pH 7.0 using 0.1N NaOH before quantities were measured.

Slurry preparation

For slurries prepared with defrosted whole eggs and albumen, dried milk was reconstituted by blending with water for 2 min in the bowl of a Hobart mixer, model K4, set at a speed of 120 rpm. Whole eggs or albumen were then added and mixing was continued for 3 min. Using the same speed, defrosted yolks were blended with 100 ml of water for 30 sec and after scraping the bowl, blending was continued for an additional 30 sec. The bowl was then covered with Saran and refrigerated for 1 hr before the yolks and water were mixed for three additional 30 sec periods, scraping the bowl between each mixing period. Milk was reconstituted as above with the remaining quantity of water and then blended with the egg-water mixture for 2 min.

To prepare slurries using dried egg components, the egg and milk solids were blended for 30 sec at a speed of 130 rpm. After the speed of the mixer had been changed to 87, 120 and 70 rpm for whole eggs, yolks and albumen, respectively, one-third of the water was added. Two 30 sec mixing periods were used and the bowl was scraped between each. After the two remaining portions of water had been added in the same manner, the mixture was blended for 2 min. All slurries were strained at the conclusion of mixing to insure complete reconstitution of the egg components and milk.

pH and adjustments

The pH of all raw and cooked slurries was determined using a Beckman Zeromatic pH meter. For the whole egg-milk slurries with an adjusted pH, 75 ml of water was withheld in the above mixing procedures. Using a magnetic stirrer, 0.1N HCl was slowly and continually added until the pH of the mixture was lowered to 6.7. After the remaining water less the milliliters of HCl had been added, the slurry was mixed for 1 min.

Cooking

500 ml of each slurry was poured into the bowl of a Brabender Visco/Amylo/Graph, model VA-V. Using a 125 cmg sensitivity cartridge, 50 rpm and 35°C setting, the slurry was heated at a rapid rate until a temperature of 50°C was reached. Thereafter, all slurries were programmed to heat at a constant rate of 1.5°C

Table 1—Quantities of whole eggs, yolks or albumen used to prepare slurries

	Egg component			Water for reconstitution		
Egg process	Whole (g)	Yolk (g)	Albumen (g)	Whole (ml)	Yolk (ml)	Albumen (ml)
Frozen	96	72	120	--	--	--
Foam-spray-dried[1]	30.0	37.4	13.9	66	35	106
Freeze-dried[1]	29.8	36.3	13.3	66	36	107
Spray-dried[1]	30.5	37.5	14.1	66	35	106

[1]Quantities of egg components and water based on moisture content of processed eggs (AOAC, 1955).

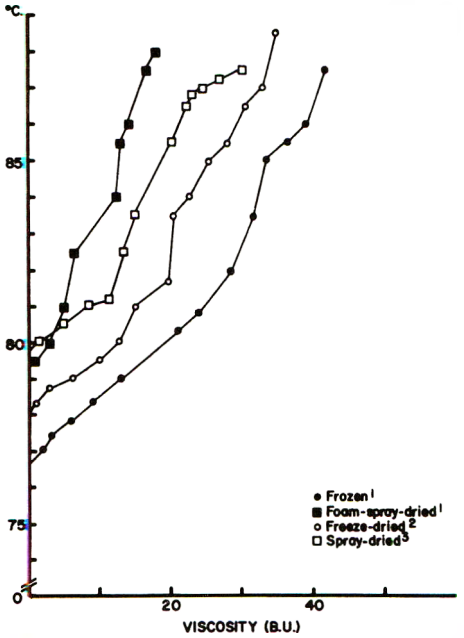

Fig. 1–Temperatures (°C) and viscosities (B.U.) of whole egg-milk slurries plotted at ½-min intervals. ([1]Average of two replications; [2]Average of three replications; [3]Average of four replications.)

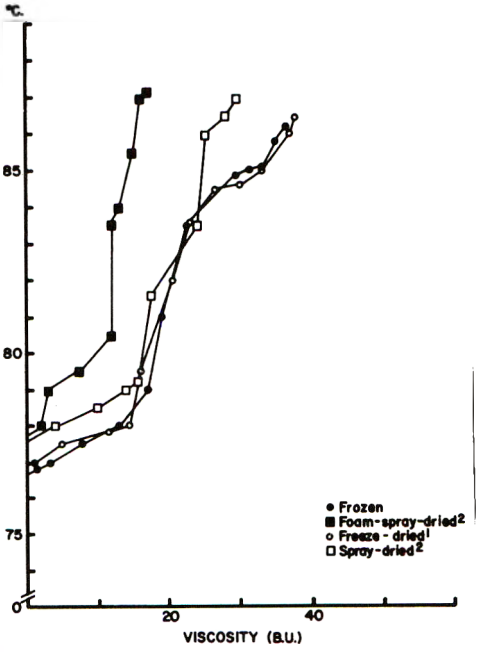

Fig. 2–Temperatures (°C) and viscosities (B.U.) of whole egg-milk slurries with an adjusted pH plotted at ½-min intervals. ([1]Average of two replications; [2]Average of three replications.)

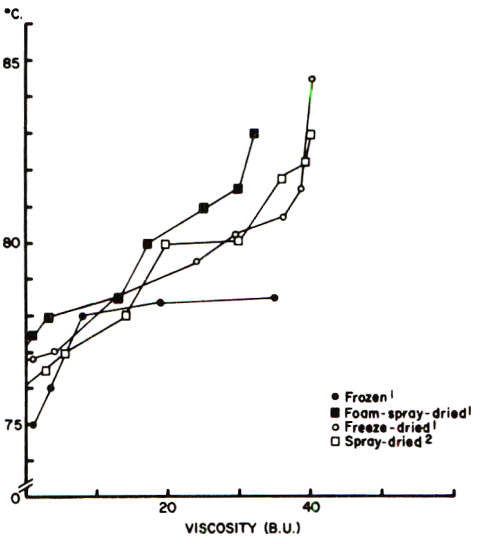

Fig. 3–Temperatures (°C) and viscosities (B.U.) of albumen-milk slurries plotted at ½-min intervals. ([1]Average of two replications; [2]Average of three replications.)

per min. During heating, the temperature was continuously recorded using a 4-in. immersion length iron-constantan potentiometer lead from a Brown Electronic high speed multiple-point recorder which had been adjusted to continuously record the temperature at only one point. When readings reached 20 Brabender units (B.U.) and at 10 B.U. intervals thereafter until curdling appeared, 10 ml samples of the slurry were pipetted from the mixture with no interruption to the heating and stirring cycle. The sample was delivered from the pipette into a 4-in. watch glass and any curdling was noted.

The recorded Brabender units and temperatures were averaged at ½ min intervals for each process type of whole egg, yolk and albumen. The data were plotted after the initial increase in viscosity was noted, using the appropriate coding for each egg process to indicate the ½ min time intervals throughout the remainder of the cooking process thereby permitting a three-dimensional collation of the data.

RESULTS & DISCUSSION

Whole egg-milk slurries

An average pH value of 6.9 was recorded for both uncooked and cooked slurries prepared with the four types of eggs. In contradiction, other studies (Funk et al., 1969; Zabik and Figa, 1968; Miller et al., 1959) have shown custards became more alkaline during baking.

Time-temperature data recorded during the first 6 min of the heating cycle or prior to a temperature of 50°C showed very rapid rates of temperature rise. For the next 20, 22, 21 and 22 min for slurries containing frozen, foam-spray-, freeze- and spray-dried eggs, respectively, temperatures rose at the programmed rate. Thereafter, time-temperature relationships were apparently dependent on protein coagulation (Fig. 1).

In slurries containing frozen eggs, initial gelation was noted at an average temperature of 76.5°C whereas the endothermic process of protein coagulation was most apparent between the average temperatures of 76.5–80°C and 85.5–86.0°C; however, the continued increase in the viscosity of the slurry during cooking indicated protein coagulation continued between 79.0 and 85.5°C. Samples of the slurry removed at 80 B.U. were curdled thereby suggesting excessive protein coagulation occurred between 85.5–86.0°C.

Slurries containing freeze-dried eggs showed initial gelation at an average temperature of 78.5°C with protein coagulation most apparent between the average temperatures of 78.5–80.0°C. In slurries containing foam-spray- and spray-dried eggs initial gelation was apparent at average temperatures of 79.5 and 80.0°C, respectively. For the spray-dried egg slurries protein coagulation was most apparent between average temperatures of 80.0–81°C and 86.5–87.5°C while foam-spray-dried egg slurries showed greatest protein coagulation between average temperatures of 79.5–81.0°C (Fig. 1).

In contrast, Funk et al. (1969) noted initial gelation at 74°C in baked custards containing eggs processed by the same methods, milk, sugar and salt, while the endothermic process of protein coagulation was most apparent between 74–82°C. In a study of custard sauces prepared with the same types of processed eggs, milk, sugar and cornstarch, Downs et al. (1970) noted initial gelation

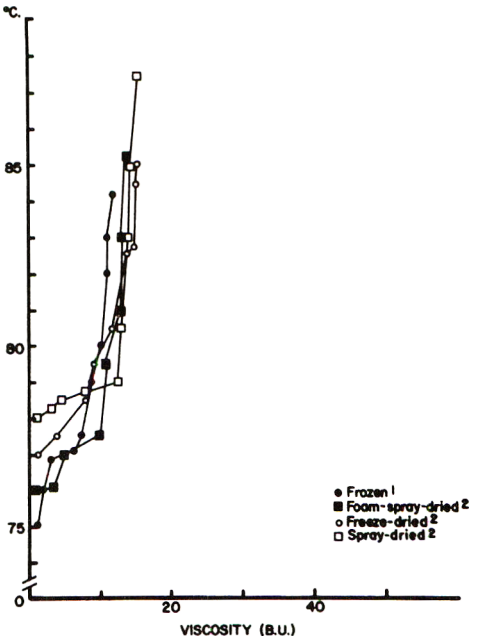

Fig. 4–Temperatures (°C) and viscosities (B.U.) of yolk-milk slurries plotted at ½-min intervals. ([1]Average of three replications; [2]Average of four replications.)

at 70°C while the greatest amount of thickening took place between 82–84°C. However, direct comparisons of gelation temperatures cannot be made because, according to Lowe (1955), the temperature at which gelation starts varies with the ingredients as well as the rate of heating.

Ranked in order of decreasing viscosities were slurries containing frozen, freeze-, spray- and foam-spray-dried eggs. These data, showing detrimental effects of processing to the gelation properties of dried whole egg, are in agreement with data reported by Zabik and Figa (1968) who ranked baked whole egg-milk slurries in the same order for decreasing gel strength. However, Zabik and Brown (1969) reported electrophoretic patterns indicated less distinct protein bands for spray-dried eggs than those processed by the other methods. In contradiction to this study, Downs et al. (1970) noted custard sauces prepared with freeze-dried eggs were more viscous than sauces prepared with frozen eggs. Wolfe and Zabik (1968) reported baked custards prepared with freeze-dried eggs were firmer ($P < 0.05$) than baked slurries prepared with the same freeze-dried eggs but with sugar omitted. Sugar, in the normal custard ratio of 2 tablespoons per cup of milk, did not influence the gel strengths produced by the other types of eggs. Thus the effect of sugar on the gel strength of custards prepared with freeze-dried whole eggs contributes to the differences reported for these two systems.

Whole egg-milk slurries with an adjusted pH

Prior to cooking, the slurries prepared with the four types of eggs each had a pH value 6.7. During cooking the pH changed very little. Hence, values of 6.8 for slurries prepared with frozen eggs and 6.75 for slurries prepared with the three types of dried eggs were recorded.

Time-temperature relationships recorded prior to gelation followed the same pattern as outlined for slurries with no pH adjustment. Programmed heating times of 20, 21, 20.5 and 21 min were recorded for frozen, foam-spray, freeze- and spray-dried eggs, respectively, before the initial gelation was noted at temperatures slightly lower than those observed for slurries with no pH adjustment.

When the pH of the slurries was adjusted, initial gelation was noted at the same average temperature of 76.5°C for frozen egg slurries while the increase in pH lowered gelation temperatures for slurries containing the dried eggs approximately 1°C. Average initial gelation temperatures of 77, 78 and 77.5°C were observed for slurries prepared with foam-spray-, freeze- and spray-dried eggs, respectively (Fig. 2).

Protein coagulation occurred at an increased rate when the pH was adjusted; for example, 2 min after initial coagulation was noted, frozen egg slurries with an adjusted pH showed a viscosity of 34 B.U.'s while slurries with an unadjusted pH showed a viscosity of 26 B.U.'s. However, the maximum viscosity attained was the same for both slurries. Slurries containing dried eggs demonstrated similar patterns in the initial phase of the cooking period; however, the maximum viscosity of pH adjusted freeze-dried egg slurries exceeded that of pH unadjusted freeze-dried egg slurries (Fig. 2).

Zabik and Figa (1968) reported slurries containing frozen, foam-spray-, freeze- and spray-dried eggs with the pH adjusted to 6.6 were less firm than similar slurries at an unadjusted pH of 7.0. The difference in firmness was attributed to the peptizing action of the acid on the egg proteins.

Albumen-milk slurries

Prior to cooking, pH values of 6.6, 6.75, 6.7 and 6.7 were recorded for slurries prepared with frozen, foam-spray-, freeze- and spray-dried albumen, respectively. Cooked slurries prepared with frozen and foam-spray-dried albumen showed pH values of 6.7 and 6.8, respectively, while the other values remained unchanged. Initial gelation was noted 18.5, 20, 19.5 and 20 min after programmed heating began for slurries containing frozen, foam-spray-, freeze- and spray-dried albumen, respectively, while initial gelation occurred at average temperatures of 75, 77, 77 and 76.5°C for the slurries containing albumen processed by the respective methods. The endothermic process of protein coagulation was most apparent in the frozen albumen slurries between average temperatures of 78 and 78.5°C while foam-spray-, freeze- and spray-dried albumen slurries exhibited greatest gelation between average temperatures of 78.0–81.5°C, 77.0–80.5°C and 77.0–82°C, respectively (Fig. 3).

These data indicate the drying processes had a detrimental effect on the gelation properties of the albumen. In a study of the whipping properties of pasteurized albumen, Garibaldi et al. (1968) concluded heat denaturation of the ovomucin-lyozyme electrostatic complex was responsible for increased whip times for pasteurized albumens. The extent of damage reported in their study was a function of heating time and pH.

Ranked in order of decreasing viscosity 5 min after initial gelation was noted were slurries containing frozen, spray-, freeze- and foam-spray-dried albumen. Zabik (1968) reported similar results for baked slurries containing albumen processed by the same method although similar values were reported for gel strengths of the baked slurries containing frozen and freeze-dried albumen.

Yolk-milk slurries

Cooking had no effect on the pH of all yolk-milk slurries. Values of 6.6 were recorded before and after cooking. The slurries were heated for 19.5, 20, 20.5 and 21 min at the programmed heating rate of 1.5°C for each minute before the initial gelation was noted at average temperatures of 75, 76, 77 and 78°C for frozen, foam-spray-, freeze- and spray-dried yolk slurries, respectively. Gelation was most apparent between average temperatures of 76.5–77.5°C, 76.0–77.5°C, 77.0–78.5°C and 78.0–79.0°C for slurries containing frozen, foam-spray-, freeze- and spray-dried yolks, respectively, thus indicating protein coagulation occurred at a rapid rate and within a short temperature range (Fig. 4).

Ranked in order of decreasing viscosity were slurries containing freeze-, spray-, foam-spray-dried and frozen yolks although differences were slight. In contrast, Zabik (1968) reported slurries containing foam-spray-dried yolks exhibited greater gel strength than slurries containing spray-, freeze-dried and frozen yolks. According to Zabik and Brown (1969) electrophoretic patterns of spray-dried yolks exhibited the least distinct bands and reduced mobility.

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Michigan Agricultural Experiment Station Journal Article No. 5165.

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ACTION OF EMULSIFIERS IN ICE CREAM UTILIZING THE HLB CONCEPT

SUMMARY—Experimental results show that the level of milk solids-not-fat normally used in ice creams, alone, had sufficient emulsifying capacity to emulsify the fat present in a standard ice cream mix. The added chemical emulsifier, regardless of its HLB, contributed little to the stability of the mix. This was true even where butteroil was used as a source of fat. Fat deemulsification during freezing increased with the HLB of the emulsifier while the dryness showed no meaningful trend. The meaning of the terms dryness, stiffness, melt resistance, and stand-up quality has been clarified.

INTRODUCTION

EARLIER THEORIES of emulsifier action in ice cream were based on the knowledge of simple oil-in-water emulsion systems. Thus the emulsifiers were thought to control the surface phenomena that favor a stable fat dispersion and decrease the tendency for fat particles to coalesce (Snyder, 1949). Another theory for the action of emulsifiers was one in which the emulsifiers were thought to be adsorbed at the air-serum interface making possible a fine, stable air cell, associated with a "dry" appearing product (Keeney and Josephson, 1958). These theories were assumptions not based on experimental work with ice cream systems. To transpose these simple theories to a complex emulsion system such as ice cream is erroneous since ice cream consists of other major constituents such as milk solids-not-fat (MSNF), sucrose and stabilizer gums. It is a well known fact that MSNF have emulsifying properties and thus may contribute to the total emulsification requirements of ice cream.

Counter to the theories mentioned above, later evidence points to the fact that emulsifiers may actually deemulsify the fat emulsion, and this deemulsification is suggested as being responsible for dryness in ice cream (Kloser and Keeney, 1959). The once popular belief that emulsifiers are necessary to prevent "churning" in ice creams made with butter or butteroil as a source of fat, is no longer valid (Kloser and Keeney, 1959).

A new concept about emulsifiers was developed by Griffin (1949) in which a specific emulsifier HLB (Hydrophile-Lipophile-Balance) is required to produce a particular type of emulsion. This theory has been shown to hold true with all nonionic emulsifiers.

Simply stated, in the HLB system, each emulsifier is assigned a numerical value which is called its HLB. The number indicates, essentially, the percentage weight of the hydrophilic portion of the emulsifier molecule divided by 5. The whole HLB scale therefore ranges from 0–20.

In this paper emulsifier action in ice cream was investigated utilizing the HLB concept. Physical effects of emulsifiers in ice cream such as fat deemulsification, dryness, and stiffness were studied in relation to the emulsifiers and their HLB.

EXPERIMENTAL

Required HLB of an emulsion system

The optimum emulsifier HLB necessary to make a stable emulsion, under a given set of conditions, is called the required HLB of that particular emulsion and can be determined experimentally for many emulsion systems. The method used was based on the one described by Atlas Chemical Industries, Inc. (undated). In this procedure an arbitrary constant concentration (0.1% in this experiment) of emulsifiers with increasing HLB's was added to each of a series of emulsions consisting of 12 parts butteroil and 62 parts water. This is the ratio of oil to water in a standard ice cream mix. The emulsions were heated to pasteurization temperature (160°F), homogenized and held at 40°F for 24 hr before determining their stability with the emulsion stability test. The emulsifier HLB which produces the maximum emulsion stability is called the "required HLB" of that particular emulsion system. The HLB's used in this procedure ranged from 2 to 18.

In order to determine the minimum concentration of emulsifier (of the required HLB) necessary to yield maximum stability, a series of test emulsions were made as above with increasing concentrations of the emulsifier having the required HLB. After heating, homogenizing and holding at 40°F for 24 hr, the emulsions were tested for their stability.

Preparing the mix

All the standard mixes were prepared in 1-gal batches using the following formula:

Fat	12 parts
Milk solids-not-fat	11 parts
Sucrose	15 parts
Water	62 parts

The required quantities of MSNF and sucrose were weighed and thoroughly mixed. The mixture was stirred into the weighed amount of water and stored at 40°F for 24 hr to attain complete solubility and hydration. The samples were pasteurized at 160°F for 30 min. The emulsifiers were added when the temperature reached 160°F. The fat (pure anhydrous milkfat) was melted, heated separately to the pasteurization temperature and stirred into the aqueous portion of the mix just before homogenizing. The samples were homogenized at the pasteurization temperature in a Manton-Gaulin two-stage homogenizer using a pressure of 2000 psi on the first stage valve and 500 psi on the second. During the homogenizing process the mix in the hopper was stirred constantly in order to avoid "oiling-off" of the fat. The samples were then cooled in cold running water and stored at 40°F for 24 hr before freezing.

Freezing the mix

The mix was frozen in a model 5P Electro Freeze soft-serve ice cream freezer. This particular model has an automatic temperature control and is capable of maintaining a constant temperature of 19 ± 1°F.

The procedure consisted of freezing a trial mix until a temperature of 22°F was reached and it was then drawn off. After an initial rinsing of the freezer barrel with the test mix, a half gallon of the test mix was introduced into the freezer. After each freezing trial the freezer barrel was washed out in order to eliminate carry-over from sample to sample. The time required for a test mix (at 40°F) to reach 22°F was about 3 min. The constant temperature of 19°F was reached between 6–9 min. The ice cream was packaged in round pint paper cartons.

Determining the dryness of ice cream

"Dryness" is the absence of a wet, soft or slack appearance. A dry product is dull in appearance and less glossy, whereas a wet and slack-appearing product is highly glossy. This was the criterion of this test. The gloss was measured by means of a Gardner Gloss Meter (model GG-9040 P-5 60°). Samples were collected from the freezer at the desired time intervals into shallow cups, the top surface leveled off with a knife held so that its flat surface was perpendicular to the plane of the cup, and the gloss reading taken immediately. The whole operation was completed in a few seconds, and the same speed was maintained with all samples. This was very important since the gloss value changes rapidly with time.

A dry product being less glossy gives a lower reading. Thus, a decrease in gloss value indicates an increase in dryness. Usually a gloss reading over 15 indicates a wet appearing product, and a reading below 10 an extremely dry product, with the readings between these values representing varying degrees of dryness.

Emulsion stability test

Principle. In emulsion stability studies, the increase in particle size with time is important since it results from flocculation and coalescence, that is, deemulsification. An increase in particle size with time, other conditions being the same, is thus an indication of instability.

The time necessary for the particle size to reach a maximum (thermodynamic equilibrium) in an "unstable" emulsion system may range from a fraction of a second to days or months. On the other hand, a decrease in parti-

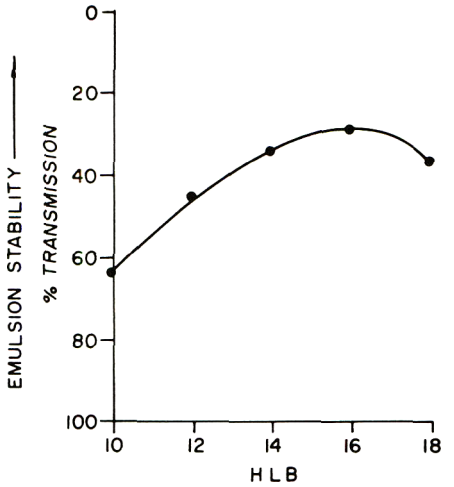

Fig. 1—Effect of emulsifier HLB (0.1% concentration) on the stability of an oil-in-water emulsion possessing the same ratio as a standard ice cream mix.

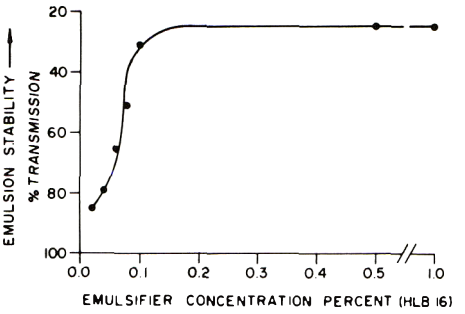

Fig. 2—Effect of emulsifier concentration on the stability of an oil-in-water emulsion possessing the same ratio as a standard ice cream mix.

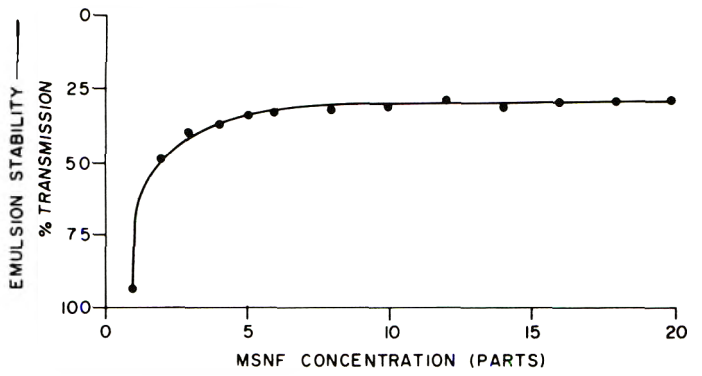

Fig. 3—Effect of MSNF concentration on the stability of an oil-in-water emulsion possessing the same ratio as a standard ice cream mix.

cle size indicates an increase in stability.

From such a relationship between particle size and stability, a method which could measure a change in particle size would, in effect, measure a change in stability. Light transmission is one of the optical methods which has been found suitable for this purpose. The data obtained by the light transmission method were substantiated by a direct microscopic examination of the emulsions.

Procedure. The procedure for preparing samples for light transmission measurement was a slight modification of the test described by Keeney and Josephson (1958) for determining the extent of fat deemulsification in ice cream. The freshly prepared emulsions were stored for 24 hr at 40°F and the test was performed as follows: a 10.0g sample was weighed into a 200 ml volumetric flask and diluted to the mark. 2 ml of this dilution were further diluted to 50 ml to yield a 1/500 dilution. This final dilution of the emulsion was then measured for percent light transmission at a wavelength of 540 nm in a Coleman Junior Spectrophotometer. Distilled water was used as a blank. The percent light transmission is directly proportional to the particle size; hence, a greater percent transmission, other conditions being equal, indicates instability. Conversely, the lower the percent transmission, the greater is the stability.

Direct microscopic examination of the emulsions

Direct microscopic examination of the emulsions was made mainly to substantiate data obtained by the light transmission method.

A hanging drop slide of a 1/20 dilution of the sample was prepared and examined using an oil immersion objective. The actual measurement of the particle size was accomplished with a calibrated ruled micrometer disc placed in the microscope eye piece. The ranges of particle sizes reported were obtained from examining at least ten different fields.

Determining fat deemulsification in ice cream

The method of Keeney and Josephson (1958) was followed for determining the extent of fat deemulsification. Their method is based on the principle that during the process of freezing a mix in an ice cream freezer, fat is removed from the finely emulsified state existing in the unfrozen mix; the extent of this change is correlated to a measurable increase in light transmission. Thus, the percent transmission of light has been shown to be directly proportional to the extent of fat deemulsification.

The procedure for this test was the same as described for the emulsion stability test, except that the ice cream samples were thawed and thoroughly stirred before performing the test.

Meltdown test

The object of this test was to determine the rate of melting of ice cream samples at ambient temperature.

The procedure consisted of transferring pint samples which had been tempered in an ice cream cabinet at 5–7°F from their original containers onto an aluminum wire-mesh screen (252 pores per sq in.) by peeling off the container material. This method of transferring the samples was preferable to the conventional method of using a dipper which could introduce several errors. The screen holding the sample was placed on a beaker, and the amount of melted ice cream which had dripped through in a period of 30 min was weighed and reported as grams of ice cream melted. The melting was done at a temperature of approximately 70°F.

The greater the amount of melted ice cream, the faster the rate of meltdown. The samples used for the meltdown test were those collected after 12 min in the freezer.

RESULTS

Required HLB of a standard ice cream mix

Ice cream mix, an emulsion system, is not a simple oil-in-water emulsion, consisting of other major constituents such as MSNF, sucrose and stabilizer gums. In determining the required HLB of such a system it is necessary to know how much each of these constituents contributes to the stability of the basic mix emulsion. However, the required HLB of the oil-in-water portion of the mix must be determined first.

The required HLB of the basic emulsion (12 parts fat, 62 parts water) was determined and results are presented in Figures 1 and 2. Results in Figure 1 show that an HLB of 16 is optimum for producing maximum stability. Results presented in Figure 2 indicate that an emulsifier (HLB 16) concentration of approximately 0.2% provides maximum stability. A microscopic examination of the emulsions corresponding to the horizontal part of the curve showed no clumps or clusters, and all the droplets were in the size range of 1–3μ in diameter. The emulsions corresponding to the ascending part of the curve produced heavy oiling-off immediately after homogenizing with droplets in the size range of 15–30μ.

Stabilizers are used in ice cream to produce smoothness in body and texture, retard or reduce ice crystal growth during storage and provide resistance to melting. They do not function as emulsifiers so they were not included in this study. Preliminary work showed that sucrose contributed nothing to the stability of the mix emulsion. Sucrose was included in the mixes to facilitate easy dispersion of MSNF into the water.

Increasing concentrations of MSNF ranging from 1–20 parts were added to a system containing 62 parts water, 12

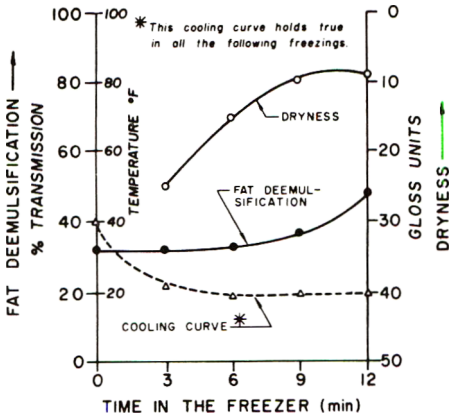

Fig. 4–Relationship between fat deemulsification and dryness during freezing of a standard ice cream mix.

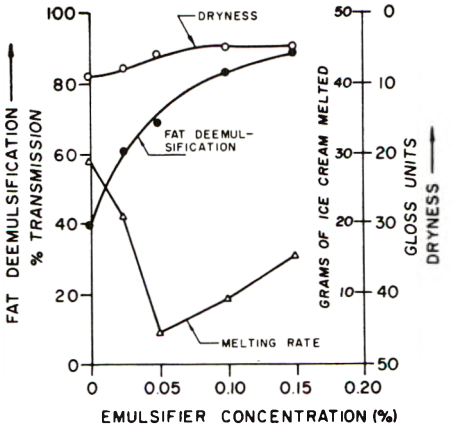

Fig. 5–Effect of emulsifier concentration (constant HLB 16) on fat deemulsification, dryness, and melting rate of ice cream at the end of 12 min of freezing.

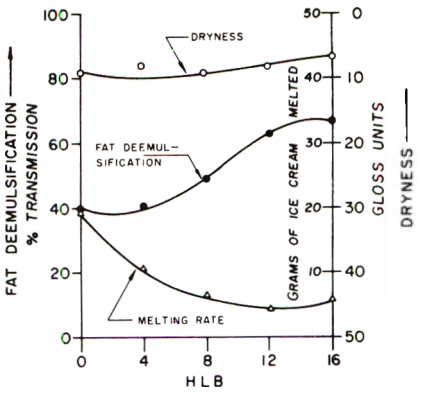

Fig. 6–Effect of emulsifier HLB (constant concentration, 0.05%) on fat deemulsification, dryness and melting rate of ice cream at the end of 12 min of freezing.

parts butteroil and 15 parts sugar. The results are presented in Figure 3. The overall shape of the curve is similar to that obtained with a chemical emulsifier in that when the concentration of MSNF required to produce maximum emulsion stability was reached, there was no change in stability with increasing concentrations of MSNF. The microscopic examination of the emulsions also revealed similar information to that in Figure 2. Here, the samples corresponding to the horizontal part of the curve did not show any fat clumps or clusters, and all the emulsion particles were in the size range of $1-3\mu$. This shows that an MSNF concentration of about 6 parts, alone, can produce an emulsion stability equal to one produced experimentally with a chemical emulsifier of HLB 16. A standard ice cream mix contains about 11 parts of MSNF which is thus more than adequate so far as emulsification needs are concerned.

Originally it was thought that the MSNF might supply only a part of the emulsification requirement, in which case an added chemical emulsifier would be needed to adjust the difference. From the above results it appears that the need for an added chemical emulsifier might not exist so far as the emulsification of the mix is concerned. Experiments were made showing the effect of the concentration and HLB of an added chemical emulsifier was superfluous so far as the emulsion stability of an ice cream mix was concerned.

Effect of emulsifier concentration (constant HLB) on fat deemulsification, dryness, and melting rate of ice cream

In this experiment a standard mix containing no emulsifier was made and frozen. Fat deemulsification and dryness were determined at intervals during freezing. Data in Figure 4 indicate the effect of freezing on fat deemulsification and dryness of this mix containing no emulsifier. While both fat deemulsification and dryness increased with time, their rates, however were not the same.

A series of standard ice cream mixes were made to which increasing concentrations of emulsifier (HLB 16) were added. They were frozen and data recorded on fat deemulsification, dryness and melting rate.

Figure 5 contains data showing the effect of emulsifier concentration on fat deemulsification, dryness, and melting rate at the end of the freezing operation (12 min). There is a steady increase in fat deemulsification with an increase in emulsifier concentration. The effect of emulsifier concentration on dryness, however, was not very distinct, because all the samples including the control attained a gloss value below 10, which is considered extremely dry. The rate of melting decreased with emulsifier concentration up to a point after which it tended to increase. A close visual examination of the samples corresponding to this increase showed "butter granules" and "wheying-off."

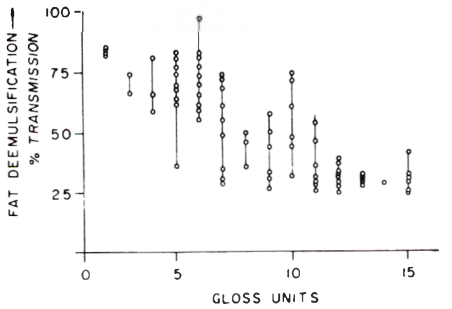

Fig. 7–Range of fat deemulsification values for each gloss value.

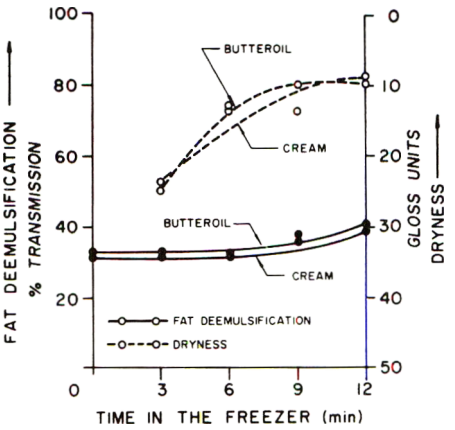

Fig. 8–Comparison of mixes made with cream and butteroil as sources of fat with respect to fat deemulsification and dryness during freezing.

Effect of emulsifier HLB (constant concentration) on fat deemulsification, dryness and melting rate of ice cream

Results in Figure 6 show the effect of increasing the HLB of the emulsifier on fat deemulsification, dryness and melting rate at the end of the freezing operation (12 min). The effects appear to be similar to those of the emulsifier concentration study (Fig. 5); that is, there was an increase in fat deemulsification with an increase in the HLB of the emulsifier, but the dryness was not affected appreciably. There was also a decrease in the melting rate as the HLB of the emulsifier increased.

Relationship between fat deemulsification and dryness

It has been assumed that emulsifiers

Table 1–List of HLB values and their corresponding emulsifier composition

HLB	Composition
2	8% Span[a] 80, 92% Span 85
4	88% Span 80, 12% Span 85
6	83% Span 80, 17% Tween[b] 80
8	65% Span 80, 35% Tween 80
10	46% Span 80, 54% Tween 80
12	28% Span 80, 72% Tween 80
14	9% Span 80, 91% Tween 80
16	60% Tween 20[c], 40% Tween 80[d]
18	100% Tween 20[e]

[a]"Spans" are sorbitan esters of fatty acids.
[b]"Tweens" are polyoxyethylene sorbitan esters of fatty acids.
[c]"20" series are laurate esters.
[d]"80" series are oleate esters.
[e]Contains more oxyethylene groupings than Tween 20.

Table 2–Effect of increasing the concentration of the emulsifier at a constant HLB (16) on dryness in a fat-free ice cream

Sample	Time in the freezer (min)				
	3	6	9	12	15
	Gloss values				
Control (0.00% emulsifier)	24	6	6	6	9
0.025% conc	22	6	6	6	6
0.05% conc	25	7	6	6	6
0.10% conc	22	7	6	7	7

Table 3–Effect of increasing the HLB of the emulsifier at a constant concentration (0.05%) on dryness in a fat-free ice cream system

Sample	Time in the freezer (min)				
	3	6	9	12	15
	Gloss values				
Control	24	9	7	7	8
HLB 2	22	9	8	9	7
HLB 6	17	8	10	10	6
HLB 10	17	6	7	9	6
HLB 14	20	7	8	8	8
HLB 16	18	9	8	8	8

promote dryness by actually deemulsifying the fat emulsion. Fat deemulsification has been considered necessary for proper dryness in ice cream. In Figure 7 fat deemulsification and dryness data are plotted against each other in order to show the range of fat deemulsification values for each gloss value. Many factors apparently affect dryness. However, the data suggest that even though fat deemulsification and dryness are associated, they may not be directly related; there may be factors other than fat deemulsification which contribute to dryness.

If fat deemulsification is not responsible for dryness, it should be possible to get dryness in the absence of fat deemulsification or perhaps even a fat phase. Therefore, a study of dryness in a fat-free ice cream system was made. Tables 2 and 3 contain results showing the effect of emulsifier concentration and HLB on the dryness of a fat-free ice cream system. From these data it may be seen that extreme dryness was obtained even in the total absence of fat which suggests that a fat phase and fat deemulsification are not required for dryness. It is also apparent that the emulsifier HLB or concentration had little or no effect on dryness.

Contribution of the fat globule membrane material to the emulsification of the mix

In order to study the contribution of the fat globule membrane material to the emulsification of the mix, two mixes were prepared, one with butteroil as a source of fat and the other with 50% cream as a source of fat and fat globule membrane material. Microscopic examination of the emulsions showed no difference, and the emulsion droplet size in both cases was in the range of $1-3\mu$.

In Figure 8 a comparison of fat deemulsification and dryness during freezing of these mixes is shown. As may be seen, the curves run in close proximity, indicating that the phospholipids in the fat globule membrane material had no appreciable effect on the rate of deemulsification or on dryness of the ice cream.

DISCUSSION

IT HAS BEEN shown that MSNF alone provide the total emulsification requirements of the ice cream mix, and the addition of a chemical emulsifier, regardless of its HLB or concentration, contributed little to the stability of the mix.

This observation brings up an interesting point, the fallacy of the earlier belief regarding the theory of emulsifier action in ice cream. It was believed that when a normal ice cream mix containing fresh cream was homogenized, the enormous increase in surface area of the fat globules which resulted could not be resurfaced by the limited amount of "natural emulsifier" (fat globule membrane material). The added emulsifier was believed to supply this deficit. It was also believed that the need for an added emulsifier was even greater when butter or butteroil was used as a source of fat since these sources are devoid of fat globule membrane material. It was shown in this study that the contribution of the fat globule membrane material to the emulsification of the mix was practically negligible compared to the contribution of the MSNF. The importance of MSNF in ice cream emulsification is thus demonstrated.

The results in this study also confirm those of Keeney and Josephson (1962) in which emulsifiers were not found to produce any difference in ice cream emulsion stability.

During freezing of the mix, with emulsifier concentration remaining constant, fat deemulsification increased with the HLB of the emulsifier. The coefficient of correlation (r) between HLB of the emulsifier and fat deemulsification was found to be +0.87, indicating a high positive correlation.

The above relationship is very informative since it explains the basis for variations in action between different types of ice cream emulsifiers hitherto unexplained. Thus "polys" (which are known to produce greater fat deemulsification in ice cream than the mono- and di-glycerides) have an HLB over 10 (15 for Tween 80, which is commonly used) whereas the mono- and di-glycerides have an HLB around 3. Egg yolk solids and buttermilk solids were found to enhance fat deemulsification in ice cream (Kloser and Keeney, 1959). This appears reasonable since lecithin, the active constituent of these materials, is known to be highly hydrophilic. The HLB-fat deemulsification relationship also explains the observation that polys are 20 times more powerful in producing fat deemulsification than glyceryl mono-stearate (Klotzek, 1965). The present work thus enables one to explain why ice cream emulsifiers function so differently even though they all belong to the same class of nonionic emulsifiers.

In a simple oil-in-water emulsion, the fact that the emulsifier molecule is oriented at the oil-water interface is not sufficient to stabilize the emulsion particle. Instead, the stability of the emulsion is dependent on the relative strengths (number) of the hydrophilic and lipophilic groupings of the emulsifier used, that is, the HLB of the emulsifier.

Thus, at a lower emulsifier HLB, since the hydrophilic groupings are weaker, the bare fat globule is weakly associated with the aqueous phase and hence the emulsion is unstable. At a higher emulsifier HLB, since the hydrophilic groupings are stronger, the bare fat globule is strongly associated with the aqueous phase and hence the emulsion is stable. However, if the HLB of the emulsifier used is extremely high, the hydrophilic ends would be so strong as to make a weak orientation of the lipophilic ends into the lipid

phase. In such circumstances, there would be instability. This was evident from the results which indicated that there was a decrease in stability when an HLB of 16 was exceeded.

The mechanism described above, however, is unlikely in an ice cream mix in view of the strong denatured protein film enveloping the newly formed fat globules (Walker, 1960). This protein film tends to make the fat globules hydrophilic, and the whole system behaves as a fat-free system so far as the emulsifier action is concerned. Under these circumstances, therefore, the emulsifier molecule, regardless of its HLB, is situated remotely from the oil-water interface in the serum. This idea supports the findings of Keeney (1962) that the emulsifier content was higher in the serum portion, than in the fat portion, of melted ice cream.

It has long been known that the emulsifiers belonging to the group called polys (HLB 15) are better whipping agents than the emulsifiers of the mono-di-glyceride type (HLB 3). Based on this fact, a probable mechanism for an increase in fat deemulsification with an increase in emulsifier HLB is suggested. Due to increased whipping, a closer packing of the fat globules results in their eventual coalescence. The net effect being chains of coalesced fat globules surrounding the air cells in the ice cream. This condition has been observed in this study and has also been reported by Durham et al. (1962).

It has been generally believed that emulsifiers were necessary to produce a "dry" ice cream, and by so doing, they invariably caused fat deemulsification. This led to the further belief that emulsifiers promote dryness by deemulsifying the fat emulsion and that fat deemulsification is essential for dryness.

In this study there was no evidence to support this theory since samples with no emulsifier produced extreme dryness. Furthermore, extreme dryness could be obtained even in the absence of a fat phase and such dryness was not affected by the presence of an emulsifier.

Dryness apparently is only one of the desirable properties looked for in ice cream. Other qualities such as melt resistance, "stand up" quality, and stiffness are of equal importance. John and Sherman (1962) have shown a correlation between fat deemulsification and melt resistance, which was confirmed in this study.

In the present work dryness was not always related to good melt resistance; conversely, good melt resistance was invariably accompanied by a low-gloss value. This observation is particularly interesting since hitherto it has been generally believed that a dry ice cream has a good melt resistance quality. Apparently, this is not always true.

Results of the present work suggest there is an error in relating gloss readings to melt resistance since the gloss meter only indicates the surface gloss of the samples and does not differentiate between samples having varying degrees of fat deemulsification which is essential for stiffness and melt resistance.

The term "controlled fat deemulsification" is more in order than just fat deemulsification as being essential for structure, stiffness and melt resistance. The results showed that melt resistance increased with fat deemulsification only up to a point. Beyond this point there were visible signs of "churned" fat as butter granules and wheying-off was observed. Thus, there is an optimum point in the degree of fat deemulsification for good structure and melt resistance.

The role of the emulsifier therefore lies in producing a controlled deemulsification which is influenced by the HLB of the emulsifier and its concentration. Any desired level of fat deemulsification can be achieved by making use of the HLB-concentration relationship.

Since the HLB's that cause the least and greatest amount of deemulsification are 2 and 16, respectively, a compromise to achieve good melt resistance with the least amount of fat deemulsification would mean selecting an emulsifier of approximately HLB 8. Interestingly enough, some commercial blends of ice cream emulsifiers which claim to produce optimum results have an HLB ranging from 4.50–7.80.

The knowledge gained by the use of the HLB concept of emulsifier action in ice cream thus enables one to select an emulsifier to meet individual production problems, and eliminates the laborious and rather uncertain trial-and-error method of selecting the proper emulsifier blend for the purpose.

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STABILITY OF MILK FAT-WATER EMULSIONS CONTAINING SINGLE AND BINARY EMULSIFIERS

SUMMARY—The stability of 25% milk fat-water emulsions containing sorbitan-fatty acid emulsifiers were compared to determine the influence of single vs. binary emulsifier systems, and saturated vs. unsaturated emulsifier side chains. These variables were compared at emulsifier HLB values of 9.5–10.0 and at usage levels of 0.5–2.0% of the fat. There were no statistical differences ($P > 0.05$) between single and binary emulsifier mixtures or between emulsifiers with saturated or unsaturated side chains. There also was no significant difference ($P > 0.05$) between emulsifier HLB numbers of 9.5 and 10.0. However, as the amount of emulsifier in the system was increased the stability of the emulsion increased ($P < 0.01$).

INTRODUCTION

THE SELECTION of emulsifiers for a particular food involves several factors, one of which is the emulsifier's fat-water solubility ratio (Atlas, 1962; Baker et al., 1966, Becher, 1965). This ratio, which can be expressed as hydrophile-lipophile balance (HLB), must be matched with the fat-water ratio in the food for optimum emulsion stability (Titus et al., 1968). The optimum amount of emulsifier needed to stabilize a food emulsion is related to the HLB value of the emulsifier and can only be defined when the HLB is known (Baker et al., 1966; Becher, 1965; Mickle et al., 1971; Titus et al., 1968).

Several workers have reported that a mixture of two or more emulsifiers will produce a more stable system than will any of the component emulsifiers used separately (Atlas, 1962; Buddemeyer et al., 1962; MacDonald and Bly, 1966). The degree of saturation in the fatty acid side chain of an emulsifier also has been reported to influence emulsion stability (Atlas, 1962; Knightly and Klis, 1965). Since various workers have indicated that emulsion stability results from a number of factors all acting at the same time, it seemed desirable to investigate the importance of single vs. binary emulsifier systems as these factors were related to emulsifier HLB and usage level.

MATERIALS & METHODS

A SINGLE LOT of anhydrous milk fat having a titratable acidity of < 0.1 meq/ml was used in this work. Single lots of the following emulsifiers (donated by Atlas Chemical Industries) were used: sorbitan monostearate (Span 60), polyoxyethylene sorbitan monostearate (Tween 60 and Tween 61) and polyoxyethylene sorbitan monooleate (Tween 81). Binary mixtures of these emulsifiers were prepared by combining the two component emulsifiers in proportions necessary to give the desired HLB number according to the manufacturer's directions (Atlas, 1962). Sufficient stock of each binary mixture was prepared at one time to conduct the entire study. The emulsifier HLB values reported in this paper were obtained using the gas-liquid chromatography procedure of Mickle et al. (1971).

Model systems containing 25% fat plus emulsifier, and 75% water were prepared using techniques described previously (Titus et al., 1968). The amount of fat which separated from these emulsions after 6 hr quiescent incubation at 37°C was measured and expressed as a stability index (Titus et al., 1968). Using this measurement, a maximum index of 100 would indicate no fat separation while an index of 0 would indicate separation of the emulsion into fat and water layers.

The first experiment was designed to detect differences caused by small changes in HLB values (< 0.5 unit) as compared to the effects of emulsifier level and single vs. binary emulsifier systems. To test this, six different emulsifiers were used at levels of 0.5, 1.0, 1.5 and 2.0% (of the fat) making a total of 24 fat-water systems per trial. Each system was duplicated within the trial, then the entire trial was repeated making a total of 96 analyses. A second experiment was designed to confirm the finding of the first, using two emulsifier systems, each at the same four levels as before. These eight fat-water systems were replicated six times. The third experiment was designed to test the difference between saturated and unsaturated fatty-acid side chains in binary emulsifier mixtures. Two emulsifiers were used at four levels and each fat-water system was replicated six times. The data from all three trials were analyzed statistically with analysis of variance techniques.

RESULTS & DISCUSSION

IN THE FIRST experiment the mean square values for the variation among duplicate analyses was larger in trial 2 than in trial 1 (Tables 1 and 2). The stability analyses in trial 1 were performed by only one person while two people worked on the second trial. Accordingly, the data for each trial were analyzed separately. However, these single analyses resulted in the same statistical differences as when the two trials were combined. Thus, only the combined analysis of variance (AOV) is shown in this manuscript. To avoid any misinterpretation of results, the interaction terms which included the variance between trials were used to test for differences caused by the experimental variables.

The only statistically significant differ-

Table 1—Stability indices for 25% milk fat-water emulsions containing various emulsifier systems

		Trial 1				Trial 2			
	Emulsifier	Emulsifier level (% of fat)							
HLB	system	0.5	1.0	1.5	2.0	0.5	1.0	1.5	2.0
9.5	T 61[a]	37	54	59	64	44	53	62	52
		39	55	65	66	66	60	54	76
	S 60[b]+T 60[c]	43	58	59	65	44	57	59	64
		49	62	56	71	63	57	63	63
9.7	T 61+T 81[d]	33	49	63	69	42	56	59	62
		42	59	61	69	59	53	59	66
	S 60+T 60	40	54	41	67	62	40	57	63
		63	41	61	60	36	47	54	57
10.0	T 81	39	32	59	69	45	59	57	69
		33	41	54	57	47	49	55	78
	S 60+T 60	44	64	63	61	51	61	57	64
		40	55	66	66	61	57	63	70

[a]Tween 61 (polyoxyethylene sorbitan monostearate).
[b]Span 60 (sorbitan monostearate).
[c]Tween 60 (polyoxyethylene sorbitan monostearate).
[d]Tween 81 (polyoxyethylene sorbitan monooleate).

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Table 2–Analysis of variance of stability indices for 25% milk fat-water emulsions containing various emulsifier systems

Source	df	MS	F
Total	95		
Trial (A)	1	189.84	
Usage level (B)	3	1,504.62[b]	10.77[c]
A × B Interaction	3	139.68[a]	2.73
Emulsifier (C)	5	116.41[b]	2.17
(HLB within C)	(2)	93.17[b]	1.74
A × C Interaction	5	53.60[a]	1.05
B × C Interaction	15	51.19[b]	1.43
A × B × C Interaction	15	35.63[a]	0.69
Error (duplicates)	48	51.02	
Trial 1	24	39.40	
Trial 2	24	62.75	

[a]Tested by error (duplicates).
[b]Tested by corresponding interaction.
[c]$P < 0.01$.

Table 3–Stability indices for 25% milk fat-water emulsions containing single or binary emulsifier systems

Emulsifier system[a]	Emulsifier level (% of fat)							
	0.5		1.0		1.5		2.0	
	62	61	67	68	52	71	73	62
T 61	60	58	60	64	50	64	72	74
	58	65	60	62	79	60	75	77
	63	61	60	68	60	60	80	66
S 60+T 60	58	64	66	66	62	67	76	65
	55	62	62	71	60	70	65	67

[a]See Table 1 for description of emulsifiers. HLB = 9.5 ± 0.4.

Table 4–Stability indices for 25% milk fat-water emulsions with emulsifier systems having a monostearate or monooleate side chain

Emulsifier system	Emulsifier level (% of fat)							
	0.5		1.0		1.5		2.0	
	36	35	56	62	63	61	67	67
S 60+T 60[a]	41	40	56	55	60	53	68	69
	41	36	55	59	62	62	65	70
	33	34	59	56	59	61	66	66
S 80+T 80[b]	39	34	59	60	57	60	68	60
	–	40	57	52	60	58	65	69

[a]Span 60 (sorbitan monostearate) plus Tween 60 (polyoxyethylene sorbitan monostearate) HLB 10.0 ± 0.4.
[b]Span 80 (sorbitan monooleate) plus Tween 80 (polyoxyethylene sorbitan monooleate) HLB 10.0 ± 0.4.

ence in this experiment was the increased stability of the fat-water systems caused by increasing the amount of emulsifier ($P < 0.01$). No statistical differences ($P > 0.05$) were found between HLB values of 9.5, 9.7 and 10.0. When one remembers that duplicate HLB measurements have been observed to differ by as much as 0.4 units (Mickle et al., 1971), the comparison between emulsifier systems becomes more meaningful. The HLB measurement technique used in the work could not be expected to distinguish between emulsifiers whose HLB values differed by only 0.2 or 0.3 units, as those in Table 1 (9.5–9.7 or 9.7–10.0). In addition this HLB measurement could not be expected to distinguish one of these emulsifiers from the average value of the other two (i.e., 10.0 from 9.6–the average of 9.5 and 9.7). This latter type of comparison is inherent in AOV statistical analysis; thus it is not surprising that for statistical purposes there was no difference between the HLB of any emulsifier used in this work. It is rather surprising, though, that there were no statistically significant differences ($P > 0.05$) between the systems containing single emulsifiers, as compared to those which contained binary emulsifier mixtures, or between emulsifiers with saturated side chains when compared to those with unsaturated side chains.

Statistical analyses of the data from the second experiment (Table 3) confirmed the results of the previous experiment in that there was no statistically significant difference ($P > 0.05$) in the stability of fat-water systems containing single emulsifiers as compared to systems containing a mixture of two emulsifiers. In this experiment all the emulsifiers had a saturated (stearate) fatty acid side chain and the HLB values of both the mixture and the single emulsifier were the same (10.0 ± 0.4).

In the third experiment (Table 4) there was no difference ($P > 0.05$) between the stability of fat-water systems containing emulsifiers with saturated fatty acid side chains (stearate) as opposed to those with unsaturated side chains (oleate). Of the variables measured, the most important consideration in emulsion stability was the amount of emulsifier in the system. Small changes in relative fat-water solubility (changes in HLB numbers of 9.5–10.0) were relatively unimportant. However, larger changes in HLB numbers (2.0 or more) have been shown to cause a change in emulsion stability (Baker et al., 1966; Titus et al., 1968). In this work, a single emulsifier was just as effective as a mixture of two emulsifiers at the same HLB and usage level. In addition, emulsifiers containing saturated fatty acids had an identical influence on emulsion stability as did emulsifiers of the same HLB containing unsaturated fatty acids. This is not to say that multiple emulsifier systems and the degree of emulsifier saturation will not influence the stability of an emulsion. The individual components of emulsifier mixtures often have widely different HLB values. The HLB of the mixture, though, will be the algebraic average of these component values (Becher, 1965). Thus, it is quite possible that a mixture's average HLB will differ by several units from that of any component. In such a case, it is possible that the change in HLB alone could account for the change in emulsion stability attributed to the mixture. There are, of course, other possible explanations for the improved emulsifying properties of mixtures (i.e., changes in molecular arrangements, etc.). However, if one controls the HLB as was done in this work, the effect of mixtures is so small that for most purposes there is no need to consider saturation or the number of emulsifier components in the system.

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EXTRACTABILITY OF COCONUT PROTEINS

SUMMARY—Studies were made on nitrogen (protein) solubility of coconut meal in aqueous media over a range of pH's. The point of minimum solubility was found at pH 3.9 and nitrogen solubility increased toward the acidic and basic sides. Coconut proteins displayed a markedly different pH-nitrogen solubility profile in salt solutions, showing minimal solubility at acidic pH's (pH ≤ 3), a sharp rise of solubility and a maximum at neutrality. Coconut meal prepared from fresh coconuts with and without testa removed, from parings and from a sample of sun-dried copra showed comparable solubility characteristics. However, meal prepared from commercial desiccated coconut showed low solubility, even under acid and alkaline conditions (pH 2 and 10). The most efficient solvent-to-meal ratio was found at 20:1 (v:w) for a single extraction, and a two-step 15:1 process for multiple extractions. Extractions using salt solutions show that the efficiency of extraction is greatly dependent upon pH; concentrations ≥ 0.25M effect maximum extraction. Osborne classification studies of coconut meal indicate that 90% of the proteins would be classified as albumins and globulins.

INTRODUCTION

COCONUTS are an indigenous product and a major article of commerce in many countries of the world which are suffering from protein deficiency. A conservative estimate, probably low, of world coconut production in the early 1960's gives 5.6 million tons a year. This is equivalent to more than 200,000 tons of crude protein, only a minor proportion of which is presently being utilized for human food consumption (Orr and Adair, 1967; PAG, 1965; Teply, 1967).

The major proportion of coconuts is converted to coconut oil and coconut meal via copra. Commercial processes for extracting coconut oil commonly utilize expellers at temperatures which denature proteins. Furthermore, prevailing world practice in handling coconuts for the production of copra are not conducive to further utilization of the coconut meal for human consumption.

These studies were undertaken as a necessary preliminary step to the study of basic properties of coconut proteins in connection with the larger objective of finding methods for the better utilization of coconuts as protein-food sources. To insure that the present work was being conducted on good quality coconut protein, care was taken to prepare coconut meal by processes not expected to denature proteins.

Previous studies on coconut meal have commonly used meal prepared from commercial coconut oil processing (Chelliah and Baptist, 1969) or from sun-dried copra (Butterworth and Fox, 1963), or have made use of meats dehydrated by the application of heat (Sison et al., 1968). Studies by Chandrasekaran and King (1967) suggest that enzymatic processes may be required for the efficient extraction of coconut protein (from a control value of 50–60% to 85%); however, they do not indicate how their starting coconut flour has been prepared, except that it was supplied by the Central Food Technological Research Institute, Mysore, India.

EXPERIMENTAL

Preparation of coconut meal

The coconuts used were food-grade fresh coconuts obtained from the wholesale market in Houston, Texas. Indications are the coconuts came from Central America. As is common with commercial coconuts, these were received dehusked. The meats (kernel) are enclosed in a hard shell (endocarp). Between the shell and the kernel is a thin, brown seed coat (testa) which adheres firmly to the kernel. In the manufacture of desiccated coconut, the testa is removed by paring.

Coconut meal was prepared from the fresh coconuts by procedures that would not be expected to denature the proteins. The nuts were cracked and the coconut water discarded. The meats were removed from the shell, washed with water and then sliced into smaller fragments using an Urschel mill (cutting head 2-J-030510B or 2-K-020060B). The shredded meats (which included the testa) were dried under vacuum at 40°C for 24 hr to a moisture content of less than 2%. The dried meats were further comminuted by means of the Urschel mill, this time using a finer cutting head (2-K-020035C or 2-K-010030B), prior to defatting by several extractions with hexane. Residual solvent was removed with dry air.

Typical analyses of coconut meal prepared in this fashion are given in Table 1. Moisture, ash, oil and crude fiber analyses were made by standard AOCS (1969) procedures. Table 1 also gives typical analyses of fresh coconut meats and desiccated coconut meats involved in the preparation of coconut meal. It is to be noted that the moisture content of the dried meats equilibrated to a level of 7–10% on standing at ambient temperature and humidity.

Extraction of protein

Extraction of coconut proteins from coconut meal was carried out at room temperature, for at least 30 min, using a solvent:meal ratio of 20:1 (v:w), unless otherwise specified. The predetermined pH of extraction was obtained by addition of 0.5N NaOH or 0.5N HCl solution; the pH was rechecked and readjusted after 30 min of stirring. Sample size was 2.0g and the volume of solution was brought to 40 ml after the final pH adjustment. After centrifugation at 4300 × G (Sorvall RC2-B Refrigerated Centrifuge) for 20 min, the supernatant extracts were filtered through Whatman Filter Paper No. 1 to remove flocculent materials. A 20 ml aliquot of each extract was taken for nitrogen analysis. Nitrogen was determined either by macro-Kjel-

Table 1—Coconut meal: Proximate analyses

Sample	Moisture and volatile material %	Oil %	N %	Protein (%N × 6.25) %	Ash %	Crude fiber %
Fresh meats	47.0					
Desiccated meats[a]	1.8	73.0				
	1.0	71.1				
	1.5	69.3				
Coconut meal A[b]	9.9	1.1	3.6	22.2	5.2	7.5
Coconut meal B[b]	7.2	4.8	3.8	23.9	3.9	5.9
Coconut meal C[b]	5.1	0.4	3.8	23.8	4.4	7.6

[a]These are analyses of different lots dehydrated in the freeze drier.
[b]Coconut meals A, B and C are different samples, prepared from three lots of fresh coconuts obtained at different times.

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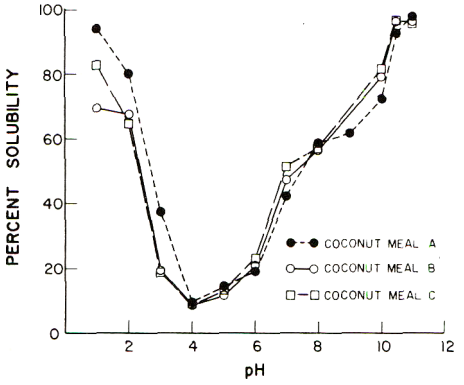

Fig. 1–Protein solubility profiles of coconut meals prepared from three lots of fresh coconuts obtained at different times (see Table 1).

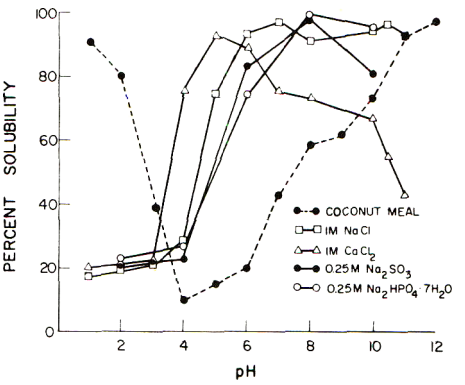

Fig. 2–Protein solubility profiles of coconut meal in several different salt solutions at varied pH's adjusted by the addition of 0.5N NaOH or 0.5N HCl.

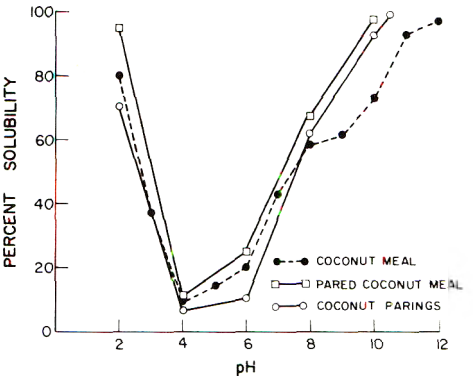

Fig. 3–Protein solubility profiles of a desiccated coconut and a specially prepared copra.

dahl (AOAC, 1965; AOCS, 1969) or micro-Kjeldahl methods (Aminco, 1959; Miller and Houghton, 1945). In most cases, the data shown represent duplicate analyses, using two samples of meal.

RESULTS & DISCUSSION

pH-Nitrogen solubility profiles

Studies were made on nitrogen (calculated as protein using the customary 6.25 factor) solubility or extractability of three coconut meals in aqueous media over a range of pH's. The data shown in Figure 1 are for extracted meals prepared from three different lots of coconuts. The solubility profile for coconut meal C represents the average of four replications; the 95% confidence limits for a single mean is ± 2.95%.

The point of least solubility was further refined by extractions at pH's 3.0, 3.3, 3.6, 3.9, 4.2, 4.5, 4.8 and 5.0. Minimum solubility was obtained at pH 3.9. [Peters (1960) found maximum precipitation at pH 3.9. Sison et al. (1968) reported the best pH for protein precipitation in a citrate-phosphate buffer at pH 3.4–3.5. Chelliah and Baptist (1969) precipitate coconut protein at pH 4.5.]

Nitrogen solubility increased with increasing acidity and alkalinity. The inflection in the curves near pH 8 suggests that there may be two main protein fractions: one soluble at pH 8–8.5 and the other at pH 10.5–11.

The solubility profile of coconut meal is strikingly different in salt solutions (Fig. 2). In 1M NaCl solution, there is rather low solubility on the acid side, up to pH 4. This is followed by a steep rise in solubility to over 90% at pH 6 and more alkaline conditions. Solubility in 1M $CaCl_2$ displays a similar low solubility at acidic pH's, followed by high solubility near pH 5–6. An unexplained decrease in solubility at more alkaline pH's is also observed.

Similarly, coconut proteins show low solubility at lower pH's for 0.25M Na_2SO_3 and 0.25M dibasic sodium phosphate. The pH-solubility profile rises steeply beginning at pH 4 and reaches a maximum of almost total solubility at pH 8. A decrease of solubility at more alkaline conditions follows.

The unique solubility characteristics of coconut proteins in salt solution have been used to prepare an interesting isolate by extraction in 1M NaCl at pH 7 followed by precipitation at pH 2. The preparation and characteristics of this and other isolates prepared by extractions at pH 2, 8, and 10.5 are the subject of another manuscript now in preparation.

The classical Osborne classification of proteins (Lund and Sandstrom, 1943), as applied to coconut meal, confirms the solubility data obtained in aqueous and salt media. Table 2 indicates that over 90% of the proteins in coconut meal would be classified as albumins and globulins. The sodium tungstate test indicates

Table 2–Classification of the proteins of coconut meal according to solubility[a]

Fractions	Extraction conditions	% of Total N
Albumin	CO_2-free water, pH 6.7	30.6
Globulin	1M NaCl solution, pH 7.0	61.9
Prolamine	70% aqueous ethanol	1.1
Glutelin	0.2% NaOH solution	4.7
Insoluble residue	– – –	1.8
Nonprotein nitrogen	12% sodium tungstate to precipitate proteins	0.1

[a]Osborne classification.

Table 3–Pared coconut meal and coconut parings: Proximate analyses

Sample	Moisture and volatile material %	Oil %	N %	Protein (%N × 6.25) %	Ash %	Crude fiber %
Fresh pared meats	47					
Fresh testa	35					
Desiccated pared meats	1.1	73.5				
Desiccated testa	2.2	54.7				
Pared meal	10.6	5.2	3.6	22.5	4.2	5.6
Defatted testa (meal)	8.9	3.6	3.2	20.0	2.9	12.1

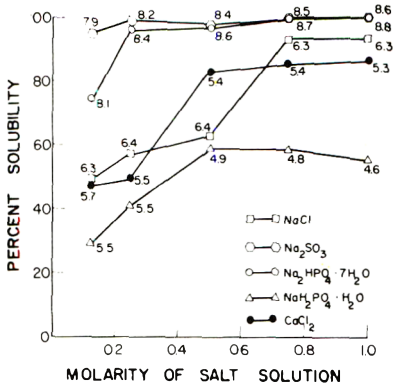

Fig. 4–Solubility of the proteins of coconut meal in different concentrations of several salts. (Data next to solubility points indicate resulting pH's.)

that there is very little nonprotein nitrogen in coconut meal.

The solubility curves (Fig. 1 and 2) were made on coconut meal which included the testa. In addition, some coconut meal was prepared with the testa removed by manual paring. The parings were also dried and defatted and the solubility profile of the proteins determined. Table 3 summarizes some properties of coconut meal without the testa, and also of the coconut parings. The pared coconut meal gave a slightly higher protein analysis than the parings (22.5% protein compared to 20.0%; 22.8% protein compared to 17.7% in another determination). The solubility curves of pared coconut meal and of the parings are comparable to those of coconut meal with the parings.

Solubility profiles of the proteins in meals prepared from commercial coconut products have been obtained. Figure 3 gives the solubility profile for coconut meal obtained by defatting commercial desiccated coconut and sun-dried copra prepared under a "heat tent." The meal from sun-dried copra gave a pH-solubility profile comparable to the laboratory-prepared coconut meal. That from desiccated coconut, however, displayed rather low solubility at both acidic and basic pH's. The very low solubility (26%, compared to about 70% in the laboratory-prepared meal) at pH 8 is also striking.

The behavior of commercial desiccated coconut is very puzzling, inasmuch as the product is perfectly white. Preliminary work done at Texas A&M University (to be reported elsewhere) has indicated that browning might be a useful indication of incipient denaturation, as shown by loss of solubility. This, however, does not seem to be the case with commercial desiccated coconut, which, though pure white, has rather poor solubility.

Efficiency of protein recovery

Protein solubility or extractability is not the only factor governing efficiency of protein extraction. Other variables such as length of time of extraction meal:solvent ratio, temperature of extraction and the effect of added salts are also very important.

Table 4 gives data obtained by varying the meal:solvent ratios, while extracting at pH 8 in aqueous solution and pH 7 in 1M NaCl (conditions chosen as representative for aqueous and salt extraction). The sample size for these extractions was 10g. After centrifugation and gravity filtration, the supernatant volume was measured. Under these conditions, three to five times the weight of coconut meal (i.e., 30–50g) was absorbed by the meal, and was not recovered as part of the separable solution. The amount not separable under practical conditions would vary depending upon the efficiency of the separation techniques used. The data in Table 4 indicate that a solvent:meal ratio of 20:1 (v:w) would be the most efficient, for single extraction.

Table 5 gives efficiency of protein

Table 4 Effect of solvent:meal ratio on extraction

Solvent:meal ratio (ml:g)	At pH 8		At pH 7, 1M NaCl	
	% N recovered[a]	% N extracted[a]	% N recovered[a]	% N extracted[a]
5:1	11.9	60.9	17.1	95.1
7.5:1	25.8	62.3	38.3	101.0
10:1	32.2	60.1	53.6	97.4
15:1	40.8	61.2	63.3	94.0
20:1	47.1	63.9	75.0	97.4
25:1	46.6	59.2	77.9	91.7
30:1	49.4	62.4	83.6	100.3

[a]The percent nitrogen extracted (or soluble) is based on the total nitrogen present in the coconut meal sample and the total volume of solvent used for extraction. The percent nitrogen recovered refers to that nitrogen in the separable supernatant extract, excluding the amount of extract (solvent) absorbed by the meal. The difference between the percent nitrogen extracted (dissolved) and recovered represents the nitrogen in solution in the absorbed solvent. Percent nitrogen recovered is the more useful data for process development purposes.

Table 5 Effect of successive extractions with different solvent:meal ratios

Solvent:meal ratio (ml:g)	Extraction	At pH 2 %N recovered[a]	At pH 8 %N recovered[a]
10:1	First	45.8	33.7
	Second	17.7	12.5
	Third	7.1	8.2
	TOTAL	70.6	54.4
15:1	First	65.5	40.7
	Second	17.9	13.3
	TOTAL	83.4	54.0
	(Third)	5.9	9.5
20:1	First	61.3	43.6
	Second	14.1	14.8
	TOTAL	75.4	58.4

[a]Based on actual recovery of extract.

Table 6–Effect of time on extraction

Time (min)	At pH 8 %N extracted[a]	At pH 7, 1M NaCl %N extracted[a]
5	58.6	96.9
15	59.6	99.5
30	58.4	99.0
45	59.3	98.6
60	60.5	103.0
90	65.4	104.0

[a]Based on total solvent used.

Table 7–Effect of temperature on extraction[a]

Temperature (°C)	%N extracted[b]
4	49.0
10	57.7
15	61.5
29	71.6
52	96.1
75	92.3

[a]Solvent: 0.2M phosphate buffer, pH 7.0.
[b]Based on total solvent used.

extraction and recovery with multiple extractions, using 10:1, 15:1 and 20:1 ratios. From these data it appears that a two-step 15:1 extraction may be most efficient. Though using one step less than a triple 10:1 extraction, the overall yield of the two-step 15:1 is almost as high. Compared to the two-step 20:1 extraction, it uses almost a third less solvent, with only a small loss of efficiency of protein recovery. The most practical extraction conditions would be selected on the basis of total process economics.

Other factors affecting protein solubility

In determining the effect of length of time of extraction on nitrogen solubility, it usually took at least 5 min to attain the desired pH, and then 15 min for centrifugation. Thus, the "time" variable employed was artificially set as actual stirring time after the desired pH had been attained. Table 6 indicates that maximum nitrogen solubility is attained in 15 min or less.

Table 7 summarizes the effect of temperature on nitrogen solubility. A temperature increase to about 50°C increases nitrogen solubility. The decrease observed at 75° may be due to partial coagulation of protein at the higher temperature. A similar decrease in solubility at higher temperatures has also been reported in studies on sunflower seed proteins in the same laboratory (Gheyasuddin et al., 1970).

The effect of various added salts on nitrogen solubility has also been studied. From Figure 2, giving the solubility curves of coconut proteins under varying pH's for different salt solutions, it is evident that these salt solutions, except toward the acid end of the pH scale, are rather effective solvents for coconut meal.

Figure 4 summarizes the effect on nitrogen solubility of several salts at various concentrations. The tests were run using the salts without adjusting the pH. In Figure 4, data next to the solubility points indicate the resulting pH at each salt concentration.

Concentrations at 0.25M or higher of dibasic sodium phosphate or sodium sulfite extract most of the available nitrogen in coconut meal. NaCl is most efficient at concentrations of 0.75M or higher. $CaCl_2$ solutions, at 0.5M or higher concentrations, effect over 80% extraction. Monobasic sodium phosphate, however, reaches a maximum extraction of a little less than 60% of the available nitrogen, at concentrations of 0.5M or higher. This difference in solubility could probably be explained in part by noting the resulting pH's: both Na_2SO_3 and Na_2HPO_4 attain pH's on the basic side of neutral, in which coconut protein is very soluble (as seen in Figure 2) while NaH_2PO_4 results in pH's between 4.6–5.5. Perhaps by coincidence, $CaCl_2$ and NaCl effect pH's at or near the highest points in their pH-solubility profiles (see Fig. 2).

It should be noted that the very high solubility of coconut proteins in dibasic sodium phosphate and sodium sulfite is in marked contrast to the rather poor solubility of sunflower seed proteins under similar conditions, in work done in the same laboratory (Gheyasuddin et al., 1970). This confirms the need to study each plant seed protein system individually and makes generalizations impossible prior to actual experimental work.

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AN ENZYMATIC PROCESS FOR A PROTEIN-CONTAINING BEVERAGE BASED ON SOYBEAN PROTEIN AND LEMON JUICE

SUMMARY—An acidic enzymatic process for a bland soybean protein hydrolysate is described. Cooked suspensions (5.0%) of isolated soybean protein are mixed with small amounts of an acid-protease preparation from Trametes sanguinea, *adjusted to pH 3.3–3.5 with concentrated lemon juice and incubated at 50°C for 8–10 hr. After stopping the proteolysis by heat treatment, the clear supernatant is separated from any insoluble residue by centrifugation. A solubilized nitrogen recovery of 88–90% is obtained. All nitrogenous substances in the hydrolysate are 5% trichloroacetic acid-soluble, and the dehydrated hydrolysate easily reconstitutes to a clear solution with cold water. After being diluted about twice and sweetened with sugar, the hydrolysate constitutes a clear, slightly yellowish lemonade-like flavored beverage. The data on chemical analysis, gel-filtration and rheological behavior of the hydrolysate are also described and discussed.*

INTRODUCTION

ALTHOUGH soy milk is nutritious, it contains many kinds of unpleasant flavor components which seem to inhibit its world-wide acceptance. It appears quite difficult to eliminate completely these undesirable factors, particularly the minor flavor constituents, from soybean or its products. For instance, it is said some unpleasant soybean flavor substances still remain in isolated soy protein, Arai et al. (1970a).

Recently, Fujimaki et al. (1968a), Arai et al. (1970c) and Noguchi et al. (1970) suggested these flavor components might be avoided by treating isolated soy proteins with microbial acid proteases. According to their reports, undesirable flavors, including bitterness, frequently observed in the soy protein hydrolysates obtained using other proteases, Murray and Baker (1952), Ichikawa et al. (1959), Fujimaki et al. (1968b; 1970); Yamashita et al. (1969) and Arai et al. (1970b), could not be recognized in the hydrolysates.

A recipe for a citrus-flavored "whey-soy drink" has been presented by USDA scientists, in which a special stabilizer was applied to prevent the soy protein from settling out, Anon. (1970).

Our experience indicates that one can obtain a bland-tasting enzymatically modified soy protein soluble at pH 4.6 by treating commercial isolated soy protein with an acid-protease preparation from a higher fungus, *Trametes sanguinea*, Tomoda (1964) and Yasumatsu et al. (1966). This paper deals with a process for a lemon-flavored soy protein hydrolysate which is suitable as a beverage base.

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MATERIALS & METHODS

Isolated soy protein

A commercial neutral isolated soybean protein in the sodium form, Promine D (Lot No. 4640) was supplied by Central Soya, Chicago, Ill. Chemical analysis of this product indicated the following composition: moisture 5.13%, crude protein (Kjeldahl N × 6.25) 92.7%, dispersible protein (as percent of crude protein) 82.5%, formol nitrogen, Hawk et al. (1954), 0.523% and total carbohydrates, Dubois et al. (1956), 2.83% (as glucose). Use of a protein isolate reduces substances such as stachyose in the hydrolysate. Dispersible protein equals that in suspension passing through an Agway milk filter pad No. 87-0440 (Agway Inc., Syracuse, N.Y.).

Concentrated lemon juice

Lemon juice was used as a convenient acidifying agent with desirable organoleptic qualities. A canned pasteurized concentrate (No. 2309) was supplied by Sunkist Growers, Ontario, Calif. Chemical analysis of the material indicated: total solids, Hughes and Maunsell (1934), 41.1g/100 ml; crude protein 2.69g/100 ml; formol nitrogen 0.15g/100 ml; total carbohydrates 11.9g/100 ml (as glucose); titratable acid, Yokoyama (1965), 488 meq/100 ml (equivalent to 31.3g/100 ml anhydrous citric acid) and pH = 2.40.

Comparing these data with those obtained on fresh lemon juice, Birdsall et al. (1961) and Joseph et al. (1961), the degree of concentration of this product was assumed to be 5.5–5.9 times fresh lemon juice.

Source of enzyme

A commercial acid-protease preparation, A-12, from *T. sanguinea* was obtained from Takeda Chemical Industries, Ltd., Osaka, Japan. According to Hagiwara's method A (1954), 1g of the enzyme preparation showed $1{,}170 \times 10^{-3}$ and 912×10^{-3} PU at pH 2.5 and 3.5, respectively. In this method, 1 PU corresponds to 1 meq tyrosine liberated at 50°C per minute from milk casein.

Enzymatic procedure

A typical example of the enzymatic procedures is summarized in Figure 1. A suspension of 5% (w/v) Promine D was autoclaved at 121°C for 4 min, Liang and Yang (1963), Hackler et al. (1965) and Lo et al. (1968). The purpose was to denature the protein, so that it could be efficiently hydrolyzed by the acid protease. After cooling, a calculated amount of the enzyme was added and the mixture adjusted to pH 3.3–3.5 by means of dropwise addition of the concentrated lemon juice with vigorous stirring. Adding the enzyme before the lemon juice enhances its homogeneous distribution into the mixture. The mixture was then incubated at 50°C and samplings made at appropriate intervals. During the enzymatic reaction, the pH changes, usually less than 0.30, were considered to have only slight effects on digestion. The reaction was stopped by placing the mixture in a boiling-water bath for 5 min. After cooling the mixture with running tap-water, the insoluble residue was eliminated by centrifugation and the supernatant thus obtained used for chemical analysis and organoleptic tests. An advantage of carrying out the enzyme reaction under acidic conditions is the low probability of putrefaction during the incubation.

Gel filtration

The supernatant of the hydrolysate (15 ml) was freeze dried and brought up to 10 ml with acetate buffer solution of ionic strength 1.082, pH 2.75. A 3-ml portion was chromatographed on a Sephadex G-25 (fine) column (1.5 by 107 cm) with the same buffer solution at the rate of 28.0 ml per hr. Fractions (5 ml) were collected and the absorbancy at 280 mμ measured. The absorbancy derived from the enzyme and the lemon juice was estimated separately under the same conditions. Gel-filtration analysis of Promine D was precluded because of a tendency to gel.

Measurement of viscosity

A viscometer, Epprecht Rheomat – 15 (Centraves AG, Zurich, Switzerland) was employed for rheological investigations. The apparent viscosities of the mixture of materials and the hydrolysate were estimated at 30°C under several different shear stresses with measuring systems MS-A and MS-O, respectively.

RESULTS & DISCUSSION

THE RELATIONSHIP between the amounts of the concentrated lemon juice added to Promine D suspension and pH changes of the mixture is shown in Figure 2. To adjust a 100-ml suspension of 5% (w/v) Promine D to pH 3.34, addition of 5.36 ml of the concentrated lemon juice was required. Although the optimum pH for the digestion of soybean protein with the acid-protease from *T. sanguinea* lies at pH 2.5–3.0, Yasumatsu et al. (1966), it appears more practical to carry out the

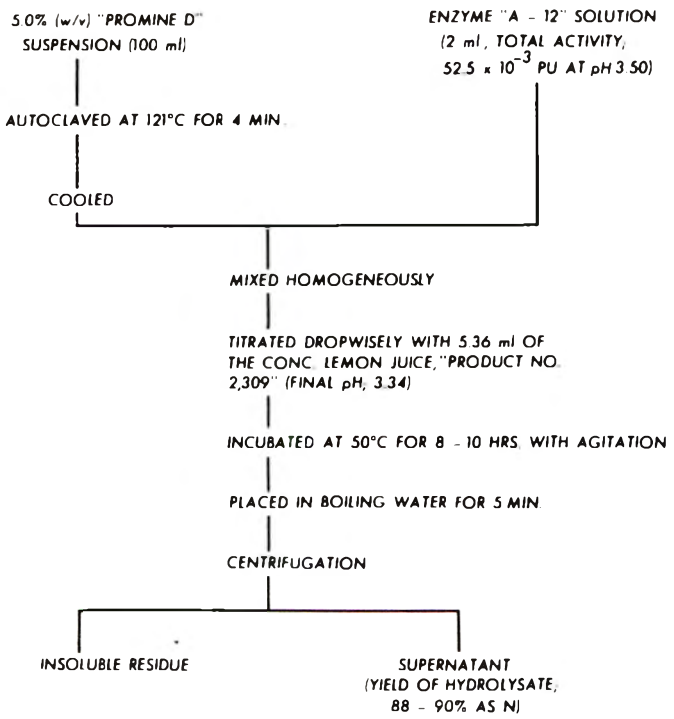

Fig. 1—A typical process for enzymatic digestion of Promine D.

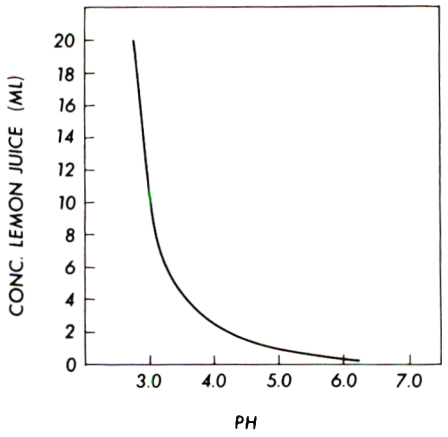

Fig. 2—Relationship between volume of concentrated lemon juice added to 5% (w/v) Promine D suspension and pH change of mixture.

(Volume of the concentrated lemon juice is expressed in terms of ml per 100 ml Promine D suspension.)

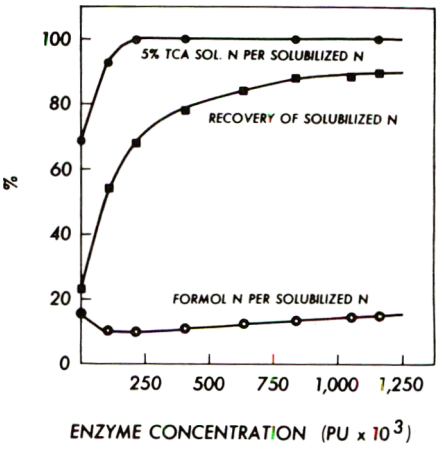

Fig. 3—Relationship between enzyme concentration and proteolysis of Promine D.

[Incubation period: 8 hr. Enzyme (A-12) concentration is expressed in terms of PU at pH 3.5 per 100g Promine D.]

reaction at pH 3.3—3.5, because a great amount of lemon juice is needed to decrease the pH of the reaction mixture below 3.30.

In general, as the enzyme concentration increased, so did the yield of the soluble nitrogen in the hydrolysate. Judging from Figure 3, however, an enzyme level of 1,000—1,200 x 10^{-3} PU (at pH 3.5) seemed to be almost enough for the maximum digestion of 100g Promine D. Under these conditions, the soluble nitrogen in the hydrolysate indicated about an 88% yield. For calculation of nitrogen recovery, nitrogen contents of the enzyme and lemon juice were subtracted from that of the appropriate hydrolysate. Use of higher concentrations of the enzyme resulted in a greater relative increase of formol nitrogen than of soluble nitrogen recovery. The existence of excess amounts of formol nitrogen in the hydrolysate gives a complicated meaty flavor to the hydrolysate.

Almost 100% of the soluble nitrogen was also 5% (w/v) trichloroacetic acid (TCA)-soluble nitrogen. The formol nitrogen was below 14.5% of the solubilized nitrogen at 1,050 x 10^{-3} PU (at pH 3.5) enzyme concentration level per 100g Promine D.

Enzymatic reaction usually proceeded for 8 hr. Although there seemed to be a slight increase in soluble nitrogen after 10 hr, the increase was very little, Figure 4. Furthermore, the long-time incubation often introduced a peptone-like aroma in the hydrolysate.

The hydrolysates might be expected to be of high nutritional quality, since it has been found that hydrolysates of soy protein with acid proteases are equal to the original protein in PER values, Sotokawa (1968). It is possible for the Maillard browning reaction to take place in the hydrolysis mixture, due to the presence of sugars and free amino groups; however, the low pH serves to slow down the reaction. Although the dry, lyophilized hydrolysate did not show any appreciable browning during 3 months of storage at 20°C, exposure to adverse temperature conditions should be avoided, to minimize the sugar-amino reaction and consequent loss in nutritional value.

Gel filtration of the hydrolysate indicated that the soluble fraction consisted mostly of relatively large-size peptides, together with a small amount of small-size peptides of amino acids, Figure 5. This agrees with the hypothesis of Verma and McCalla (1966), that an enzymatic proteolysis under acidic conditions is apt to induce formation of large quantities of polypeptides as intermediates in the reaction. This might be the reason the enzymatic hydrolysis obtained in the present method was almost entirely free from unpleasant flavors, including bitterness and meat-like taste. As a matter of fact, besides the flavor derived from lemon juice, the hydrolysate tasted almost bland. Much lower values of the percent formol nitrogen, based on total solubilized nitrogen, of the hydrolysate than that of the usual enzymatic proteolysis also confirm the above result and assumption.

As a result of the enzymatic treatment, viscosity of the isolated soy protein suspension decreased significantly, Figure 6. Furthermore, the rheological characteristic was dramatically changed from that of non-Newtonian, thixotropic fluid to that of a typical Newtonian solution.

The hydrolysate possessed a high buffer capacity; dilution of the hydrolysate caused almost no pH change (Fig. 7).

The hydrolysate diluted to 2% protein has aroma and flavor characteristics close to those of lemons. Its low viscosity was similar to that expected with a lemon-juice drink. Addition of sugar results in a

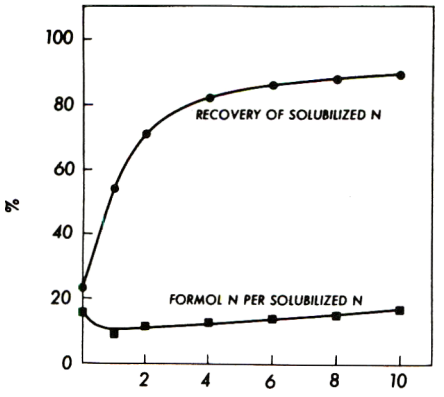

Fig. 4—Time course of enzymatic (A-12) proteolysis of Promine D.

(Enzyme concentration: 1,050 × 10^{-3} PU at pH 3.5 per 100g Promine D.)

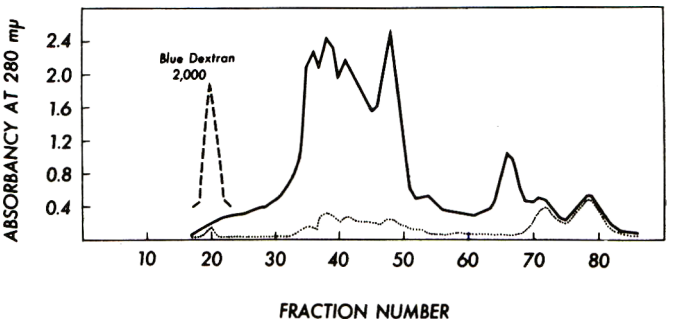

Fig. 5—Gel-filtration profile of enzymatic hydrolysate of Promine D.

(Solid line and dotted line indicate the absorbancy of the supernatant of the hydrolysate, and that derived from the enzyme and lemon juice, respectively.)

beverage similar to lemonade in organoleptic qualities. Such a beverage illustrates the possibilities of increasing the types of protein-containing drinks available to the public.

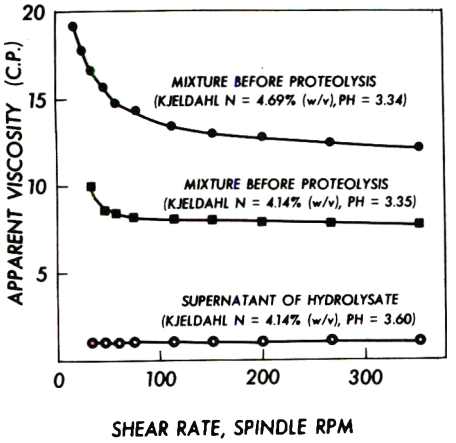

Fig. 6—Rheograms of Promine D—lemon juice mixture and its enzymatic hydrolysate.

(Enzyme reaction was carried out in the way shown in Figure 1.)

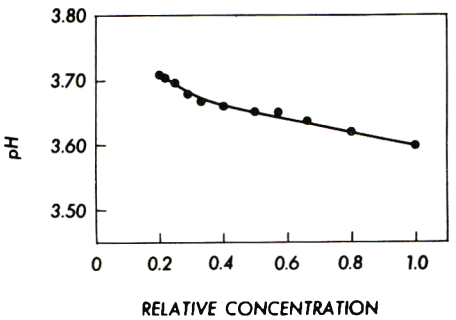

Fig. 7—Effect of dilution on pH change of enzymatic hydrolysate of Promine D.

This study has dealt with the use of a specific combination of protein, enzyme and acidulant. One might expect that other similar materials would also be satisfactory and could have advantages of price and availability in particular situations.

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STORAGE STABILITY OF CSM: ALTERNATE FORMULATIONS FOR CORN-SOY-MILK

SUMMARY—Corn-soy-milk (CSM), a high-protein food supplement for children, contains pregelatinized corn meal, soy flour, nonfat dry milk, vitamins and minerals. To increase the choice of possible ingredients, several formulations were studied. Flavor and chemical tests after storage at 77, 100, and 120°F were made on experimental samples containing corn meals and soy flours, dry whey, increased levels of nonfat dry milk, sucrose and dextrose hydrate. Stability was adequate for all formulations tested except those containing dextrose hydrate or unprocessed whole corn meal. At storage temperatures of 100°F or above, substantial losses in available lysine occurred in the samples containing dextrose hydrate.

INTRODUCTION

BLENDED Food Product, Child Food Supplement, Formula No. 2, was developed to supplement the diets of recently weaned infants and preschoolers in areas of the world that need greater amounts of dietary protein. The product is referred to as CSM from the initials of corn, soy, and milk—the main ingredients (Senti, 1969). Over a billion pounds of CSM has been donated to more than 100 countries by the U.S. Government.

Formulations for CSM were suggested by the American Corn Miller's Federation based on guidelines developed by USDA in cooperation with the Agency for International Development and the National Institutes of Health. The original specifications (USDA, 1966) required 10% maximum moisture, 19% minimum protein and 2% minimum fat, dry basis. In the current specifications (USDA, 1969), protein and moisture requirements remain unchanged while the minimum fat content has been increased to 6%. In addition, cooked and uncooked consistency, granulation (sieve analysis) and density are specified. Consistency of the cooked and uncooked product (Bookwalter et al., 1968b) at prescribed concentrations provides a check on the extent of precooking of the corn meal (Anderson et al., 1969).

The original formulation for CSM is a stable food blend (Bookwalter et al., 1968a) suitable for supplying immediate worldwide needs for a dietary supplement. The storage stability of alternative formulations was tested to expand the choice of raw materials, to improve the product and to decrease costs. It was reported from the field that sweetener was added during preparation of gruels to improve palatability. This prompted consideration to include sweetener at the time of manufacture. The specific objective was to determine the effect of various ingredients on storage stability characteristics. Changes in flavor were measured by taste panel evaluation; in fat stability, by free fatty acids and peroxide values; and in protein stability, by available lysine. Storage stability tests were made to determine the effect of different corn meals, different soy flours, increased amounts of nonfat dry milk (NFDM), dry whey (DW) and sweetener solids.

MATERIALS & METHODS

Ingredients

Supplies (Table 1) were obtained commercially for preparation of the experimental CSM blends, except unprocessed whole corn meal which was prepared with laboratory milling equipment. Partially gelatinized low-fat corn meal, toasted soy flours, spray process NFDM, mineral premix, and vitamin premix with and without butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT), complied with specifications as outlined in Announcement PS-GR-16 (USDA, 1966). The full-fat soy flour, soybean oil containing BHA and BHT and partially gelatinized corn meal containing 4% processed ground germ complied with specifications of Announcement CSM-1 (USDA, 1969). The partially gelatinized high-fat corn meal was a commercial preparation made for this special study and the degree of cooking was similar to that of the low-fat processed corn meal. The specially expelled, toasted soy flour, DW, sucrose, dextrose hydrate and sodium chloride conformed to commercial standards of identity. Analyses of the corn meals, soy flours and dairy ingredients are listed in Table 1.

Formulations

The CSM blends were prepared according to the CSM standard formula given in Table 2 (USDA, 1966). To test the effect of various ingredients, substitutions were made in this formula. Only one ingredient, such as the corn meal, was varied at a time, with the remaining ingredients held constant. The analyses of the alternate formulations are given in Table 3.

The effect of different corn meals (Test A) was compared by formulating CSM mixes with 68% processed low-fat, 68% processed high-fat and 68% uncooked whole corn meal.

CSM containing 68% processed corn meal with 4% added processed ground corn germ (Test B) was prepared commercially. The ground germ was the major source of fat in the Test B formulation. Defatted, toasted soy flour replaced the 25.0% defatted, toasted, refatted soy flour, with BHA and BHT added with the vitamin premix instead of with the soybean oil.

The effect of different soy flours (Test C)

Table 1—Analyses of ingredients for CSM[a] blends

Ingredients	Moisture %	Protein[b,c] %	Fat[b] %	Fiber[b] %
Corn meals:				
Processed low-fat	11.8	8.6	0.8	0.5
Processed low-fat with 4% ground germ	8.5	10.3	3.3	1.4
Processed high-fat	10.7	8.7	3.5	0.9
Unprocessed whole corn	11.0	10.1	4.4	1.9
Soy flours:				
Defatted, toasted, refatted[d]	5.0	50.4	6.9	3.1
Defatted, toasted	8.5	54.5	1.1	3.2
Special expelled, toasted	7.5	52.8	6.0	3.1
Full-fat, processed	5.8	42.5	23.1	2.6
Dairy products:				
Nonfat dry milk	3.2	37.1	0.8	–
Dry whey	4.3	12.5	0.7	–

[a]CSM = corn-soy-milk.
[b]Dry basis.
[c]% Nitrogen × 6.25.
[d]6% Refined soybean oil containing 2.5 mg butylated hydroxy anisole (BHA) and 2.5 mg butylated hydroxy toluene (BHT) per 100g of formulated product.

[1]USDA Biometrical Services, ARS, stationed at the Northern Laboratory.

was compared by formulating CSM mixes with 25.0% defatted, toasted, refatted soy flour; 25.0% special expelled soy flour; and a blend of 18.0% defatted, toasted and 7.0% full-fat, processed soy flours. The vitamin premix containing BHA and BHT was utilized in the formulations containing either specially expelled or full-fat soy flours.

In Test D, experimental blends were prepared to compare 5% DW with 5, 10 and 15% NFDM on an equivalent fat and protein basis.

Ingredients–Test D	Whey, 5%	Nonfat dry milk 5%	10%	15%
Corn meal, processed low fat	66.0	68.0	66.5	65.0
Soy flour, defatted, toasted, refatted, BHA, BHT	27.0	25.0	21.3	17.6
Nonfat dry milk	—	5.0	10.0	15.0
Dry whey	5.0	—	—	—
Soybean oil, refined stabilized, BHA, BHT	—	—	0.2	0.4
Mineral premix	1.9	1.9	1.9	1.9
Vitamin premix	0.1	0.1	0.1	0.1
	100.0	100.0	100.0	100.0

In Test E, dextrose hydrate was compared with sucrose in CSM blends. Initial taste panel tests had indicated equivalent sweetness in blends containing 1.3% sodium chloride and either 20% dextrose hydrate or 9.2% sucrose. These formulations were compared on an equivalent sweetness, protein and fat basis as follows:

Ingredients–Test E	Dextrose hydrate, 20%	Sucrose, 9.2%
Dextrose hydrate	20.0	—
Sucrose	—	9.2
Corn meal, processed low fat	44.7	57.5
Soy flour, defatted, toasted, refatted, BHA, BHT	27.0	25.0
Nonfat dry milk	5.0	5.0
Sodium chloride	1.3	1.3
Mineral premix	1.9	1.9
Vitamin premix	0.1	0.1
	100.0	100.0

A 10-gal stainless-steel drum equipped with internal baffles and a mechanical roller effectively blended the experimental mixtures. All formulations were tumbled at 60 rpm for 1 hr.

Packaging

The experimental samples were packaged in 4-, 8-, and 32-oz glass containers with foil-lined closures. Such containers maintained moisture at a constant level during storage.

Storage conditions

The experimental samples were stored in temperature-controlled cabinets at 100 and 120°F and at approximately 77°F room temperature. Reference samples were stored at 0°F.

Organoleptic tests

Withdrawals from storage were made at approximately the following intervals: at 120°F after 0, 14, 28, and 56 days; at 100°F after 90 and 182 days; and at 77°F after 182 and 365 days. The CSM food blends were evaluated as gruels (10% solids) made by stirring the dry mix into boiling water and cooking for 1 min. Coded, randomized samples were presented to an experienced 18-member taste panel who rated their flavor using the 10-point score sheet shown in Figure 1.

Statistical evaluations were made on the experimental data after completion of each taste panel series. Analysis of variance, regression analysis (Snedecor, 1959), and Duncan's multiple range test (Duncan, 1955) were used to evaluate differences. Statistical significance is at the 5% probability of error level or lower wherever the term "significant" is used in this paper. The standard error of the mean flavor score was approximately 0.24. The least significant difference for comparing two mean flavor scores was 0.7.

Analytical tests

Samples were withdrawn from storage for analysis at intervals similar to those followed for organoleptic tests.

Peroxide values and free fatty acids of the pentane-hexane extracted fat were determined by standard procedures (AOCS, 1967). Available lysine was assayed by a method based on the reaction with dinitrofluorobenzene (Baliga et al., 1959). Moisture analyses were made on a Brabender moisture tester (120°C, 60 min). Other analyses were determined by standard procedures (AACC, 1962).

RESULTS & DISCUSSION

Corn meals

Effects of processed low-fat, processed high-fat and unprocessed corn meal (Test A) on the flavor stability of CSM blended food are plotted in Figure 2. Each flavor stability curve was constructed from the average panel scores for each sample withdrawn during the storage period and this same procedure was followed for subsequent flavor stability results in this manuscript. At 0 day's storage, the flavor scores of CSM containing either processed low-fat corn meal or unprocessed whole corn meal were significantly higher than those of CSM containing processed high-fat corn meal. At the three storage temperatures, CSM containing unprocessed whole corn meal deteriorated in

Description	Score	Sample 1		Sample 2		Sample 3	
		Odor	Flavor	Odor	Flavor	Odor	Flavor
Excellent	10						
Very Good	9						
Good	8						
Less Desirable	7						
	6						
Objectionable	5						
	4						
Unpleasant	3						
	2						
Repulsive	1						

Fig. 1—Score sheet for organoleptic evaluation of blended food mixes.

Table 2—Composition of CSM blended food (USDA, 1966)

Ingredient	CSM, %
Corn meal, processed, low-fat	68.0
Soy flour, defatted, toasted, refatted, BHA and BHT[a]	25.0
Nonfat dry milk	5.0
Mineral premix	1.9
Vitamin premix[b]	0.1
	100.0

[a]Refatted with 6% refined soybean oil containing antioxidants to adjust 100g final formulated product with 2.5 mg BHA and 2.5 mg BHT.

[b]BHA and BHT are added with vitamin premix if minimum fat is reached by other means.

Table 3—Analyses of experimental CSM blends

Test	CSM blends[a] formula variables	Moisture %	Protein[a,b] %	Fat[a] %	Fiber[a] %
A	Corn meal, processed low-fat	9.8	21.5	2.5	1.2
	Corn meal, processed high-fat	9.6	20.7	4.1	1.7
	Whole corn meal, uncooked	9.9	21.1	4.8	2.2
B	Corn meal, processed low-fat with 4% ground germ	7.5	23.0	2.8	2.0
C	Soy flour, defatted, toasted, refatted	10.3	20.7	2.6	1.0
	Soy flour, special expelled, toasted	10.2	20.8	2.3	0.8
	Soy flour, full fat, processed and defatted, toasted	10.3	21.1	2.6	0.8
D	5% Nonfat dry milk	9.2	20.4	2.4	1.1
	10% Nonfat dry milk	8.6	20.6	2.5	1.1
	15% Nonfat dry milk	9.1	21.1	2.6	1.0
	5% Dry whey	8.8	21.1	2.4	1.3
E	20% Dextrose hydrate	9.5	19.8	2.7	1.2
	9.2% Sucrose	8.8	19.3	2.6	1.2

[a]Dry basis.
[b]% Nitrogen × 6.25.

Table 4—Analyses of stored CSM samples containing three different corn meals (Test A)

Corn meals	Storage Days	Storage Temp °F	Peroxide value meq/kg fat	Free fatty acids % oleic	Available lysine % of protein
Processed low-fat	28	0	3.3	2.2	4.5
Processed high-fat	28	0	4.3	3.0	5.3
Unprocessed whole corn	28	0	1.4	4.9	4.9
Processed low-fat	28	120	2.0	3.7	4.0
Processed high-fat	28	120	1.6	4.0	4.5
Unprocessed whole corn	28	120	1.0	33.7	3.9
Processed low-fat	182	100	2.6	8.2	3.8
Processed high-fat	182	100	2.0	8.2	4.3
Unprocessed whole corn	182	100	2.2	71.0	4.0
Processed low-fat	365	77	2.9	4.0	4.1
Processed high-fat	365	77	2.9	4.9	4.3
Unprocessed whole corn	365	77	2.2	48.4	4.5

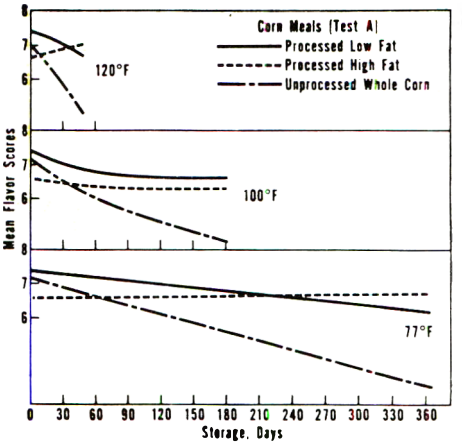

Fig. 2—Corn-soy-milk (CSM) flavor stability: comparison of processed low-fat, processed high-fat and unprocessed whole corn meals in the formulations.

Table 5—Analyses of stored CSM samples containing processed corn meal with 4% processed ground germ (Test B)

Storage Days	Storage Temp °F	Peroxide value meq/kg fat	Free fatty acids % oleic	Available lysine % of protein
56	0	1.4	2.7	4.6
90	0	1.4	3.0	4.7
56	120	2.0	3.9	4.3
90	100	2.3	3.7	4.6
182	100	2.6	4.6	4.4
365	0	4.5	3.4	4.7
365	77	6.9	3.6	4.6

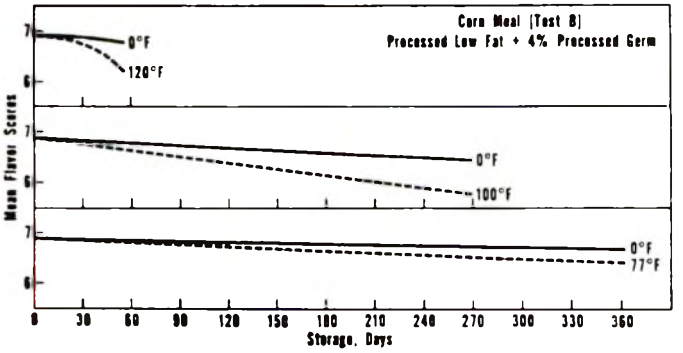

Fig. 3—CSM flavor stability: effect in the formulations of processed low-fat corn meal containing 4% processed ground germ.

flavor at a significantly higher rate than CSM containing either low-fat corn meal or high-fat corn meal. No significant differences were found in flavor scores of CSM made with either processed low-fat or processed high-fat corn meals. CSM made with low-fat corn meal received higher initial scores, but differences decreased with increased storage time. Flavor scores were in a satisfactory range (above 6) in the samples containing either processed high-fat or processed low-fat corn meal. A marked increase in free fatty acids (Table 4) occurred in the CSM samples containing unprocessed whole corn meal. This increase in free fatty acids is indicative of high lipase activity and illustrates the need for adequate heat treatment. CSM made with either processed low-fat or processed high-fat corn meals was much lower in free fatty acids, because the heat processing had destroyed or reduced enzyme activity. Peroxide and available lysine values (Table 4) did not differ substantially for CSM containing any of the processed corn meals.

The flavor stability curves for CSM commercially formulated with processed corn meal containing 4% processed ground germ (Test B) are shown in Figure 3. Flavor evaluations were made between 0°F reference samples and samples stored at 77, 100 and 120°F. At 77 and 120°F all flavor scores fell in a satisfactory range (above 6). At 100°F there was a significant decrease in flavor scores with a final average panel score of 5.8 after storage for 270 days. Analytical results (Table 5) are similar for test samples stored at 0°F, as well as at elevated temperatures, and indicate satisfactory stability in a CSM blend containing processed corn germ.

Soy flours

Flavor stability characteristics of CSM blends containing defatted, toasted, refatted; specially expelled, toasted; and full-fat, processed plus defatted, toasted soy flours (Test C) are compared in Figure 4. Initial taste panel evaluations were made after 7 days' storage at 120°F, 14 days at 100°F and after 90 days at 77°F. At the three storage conditions, flavor scores were satisfactory. At 120°F there was a significant decrease in flavor scores, whereas changes at 77 and 100°F were not great enough to be statistically significant. After storage at 120°F, CSM containing full-fat, processed plus defatted, toasted soy flours scored significantly higher than CSM containing specially expelled, toasted soy flour. At 77 and 100°F, flavor differences were not significant. Analytical values on the effect of soy flours are shown in Table 6. Available lysine content remained about the same after storage regardless of the soy flour used. Peroxide values and free fatty acids were slightly higher in CSM formulations containing defatted, toasted, refatted soy flour than those of the other two formulations. All formulations appear satisfactory.

Dairy products

The effect on flavor stability of substituting 5% DW for NFDM and increased amounts, up to 15%, of NFDM (Test D) in CSM is illustrated in Figure 5. At 0 day's storage, the flavor scores of CSM

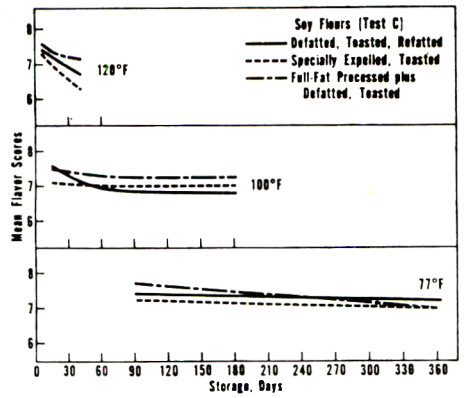

Fig. 4—CSM flavor stability: comparison of defatted, toasted, refatted; specially expelled, toasted; and full-fat, processed plus defatted, toasted soy flours in the formulations.

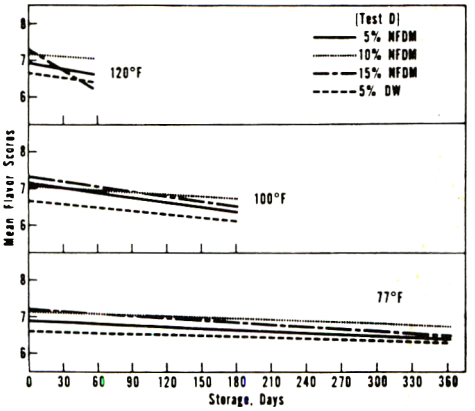

Fig. 5—CSM flavor stability at increased levels of nonfat dry milk (NFDM) and dry whey (DW).

containing either 10 or 15% NFDM were significantly higher than those of CSM containing 5% of either NFDM or DW. At the three storage temperatures there was a significant decrease in flavor scores during the storage period. At 120°F, flavor scores of the CSM sample containing 15% NFDM decreased at a slightly higher rate than the other formulations. The differences between products were not significant, although the samples containing 5% DW received slightly lower scores and those containing 10% NFDM received slightly higher scores. Flavor scores maintained satisfactory levels (above 6) at the three storage conditions. No substantial changes were detected analytically (Table 7) that could be attributed to the presence of DW or increased amounts of NFDM in the CSM food blends.

Sweetener solids

The effect of added sweetener solids, 20% dextrose hydrate and 9.2% sucrose, is plotted in Figure 6. At the outset of Test E, the taste panel detected the flavor change caused by the addition of sweetener solids. Their flavor scores were 1 to 1½ units lower than usual with unsweetened CSM. At 120°F storage, CSM samples containing 20% dextrose hydrate declined at a significantly greater rate than either the CSM sample containing 9.2% sucrose or the 20% dextrose hydrate CSM sample stored at 0°F. After 56 days' storage, flavor scores of the 20% dextrose hydrate CSM sample stored at 0°F were significantly higher than either the 20% dextrose hydrate or 9.2% sucrose CSM blend stored at 120°F. The sample of CSM containing 20% dextrose hydrate gradually developed a caramel flavor and received the lowest flavor scores. At

Table 6—Analyses of stored CSM samples containing defatted, toasted, refatted; special expelled, toasted; and full-fat, processed plus defatted, toasted soy flours (Test C)

Soy flours	Storage Days	Storage Temp °F	Peroxide value meq/kg fat	Free fatty acids % oleic	Available lysine % of protein
Defatted, toasted, refatted	42	0	5.2	1.5	4.6
Special expelled, toasted	42	0	1.3	1.5	5.0
Full-fat, processed and defatted, toasted	42	0	1.8	1.4	5.1
Defatted, toasted, refatted	42	120	2.0	3.9	3.7
Special expelled, toasted	42	120	1.2	2.2	4.0
Full-fat, processed and defatted, toasted	42	120	1.0	2.1	4.0
Defatted, toasted, refatted	182	100	2.6	6.2	3.9
Special expelled, toasted	182	100	1.0	2.1	4.2
Full-fat, processed and defatted, toasted	182	100	1.0	1.9	4.2
Defatted, toasted, refatted	365	77	5.1	5.1	4.3
Special expelled, toasted	365	77	4.1	2.7	4.4
Full-fat, processed and defatted, toasted	365	77	2.9	2.4	4.3

Table 7—Analyses of stored CSM samples containing 5, 10 and 15% nonfat dry milk (NFDM) and dry whey (DW) (Test D)

Dairy product	Storage Days	Storage Temp °F	Peroxide value meq/kg fat	Free fatty acids % oleic	Available lysine % of protein
5% NFDM	0	0	4.0	1.8	4.6
10% NFDM	0	0	2.3	1.6	4.6
15% NFDM	0	0	2.9	1.5	4.8
5% DW	0	0	2.7	1.6	4.7
5% NFDM	56	120	1.3	1.2	4.0
10% NFDM	56	120	0.8	2.7	3.5
15% NFDM	56	120	1.0	2.4	3.5
5% DW	56	120	1.5	2.5	3.7
5% NFDM	182	100	7.4	2.6	4.0
10% NFDM	182	100	4.7	2.4	3.8
15% NFDM	182	100	4.3	2.4	3.7
5% DW	182	100	4.5	2.8	4.1
5% NFDM	365	77	1.4	2.5	4.1
10% NFDM	365	77	1.4	2.5	4.6
15% NFDM	365	77	1.6	2.1	4.3
5% DW	365	77	2.2	2.6	4.4

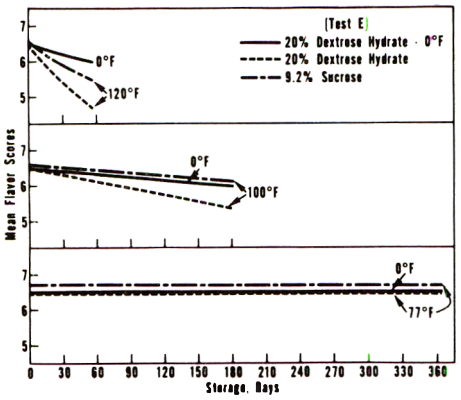

Fig. 6–CSM flavor stability: comparison of dextrose hydrate and sucrose.

Table 8–Analyses of stored CSM samples containing 9.2% sucrose and 20% dextrose hydrate (Test E)

Sucrose and dextrose hydrate variables	Storage Days	Storage Temp °F	Peroxide value meq/kg fat	Free fatty acids % oleic	Available lysine % of protein
9.2% Sucrose	56	0	2.2	1.2	4.5
20% Dextrose hydrate	56	0	2.6	1.1	4.6
9.2% Sucrose	56	120	4.1	3.1	3.2
20% Dextrose hydrate	56	120	2.9	2.9	1.1
9.2% Sucrose	182	100	5.5	4.7	3.6
20% Dextrose hydrate	182	100	5.9	4.7	2.8
9.2% Sucrose	365	77	6.7	2.9	3.6
20% Dextrose hydrate	365	77	4.8	2.9	3.8

100°F storage, no flavor changes occurred that were statistically significant. Caramel flavors reported for the stored CSM samples containing 20% dextrose hydrate resulted in decreased flavor scores, but the reported flavor differences were not of the magnitude to be significant. At 77°F storage, no significant flavor changes were detected, and no important differences were found in analytical values (Table 8). At 100 and 120°F, no substantial differences in peroxide values or free fatty acids occurred, but available lysine was lower in the CSM samples containing 20% dextrose hydrate than with 9.2% sucrose. The samples containing dextrose hydrate had become caramel colored and caked during storage at 100 and 120°F.

CONCLUSIONS

CSM FORMULATIONS can easily include several additional or alternate ingredients. Processed high-fat corn meal or processed low-fat corn meal containing 4% processed ground germ can be used in addition to processed low-fat corn meal. Unprocessed whole corn meal produces unsatisfactory stability characteristics. Specially expelled, toasted soy flour and a blend of full-fat with defatted, toasted soy flour are satisfactory in CSM formulas as well as defatted, toasted, refatted soy flour. Dry whey could be substituted for 5% nonfat dry milk in CSM. Also, the formulation might increase the amount of nonfat dry milk up to 15%. Sucrose is preferable to dextrose hydrate in supplying sweetener solids to blended food mixes.

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STORAGE STABILITY OF CSM: INCREASING FAT TO 6% IN CORN-SOY-MILK BLENDS

SUMMARY—CSM is a high-protein food supplement for infants and preschool children; it consists of partially gelatinized corn meal, toasted soy flour and nonfat dry milk and is fortified with vitamins and minerals. The original CSM formulation contained only 2% fat. Storage stability characteristics were determined for CSM blends containing as much as 6% fat. The fat sources tested were corn germ, full-fat soy flour, refined soybean oil, expeller crude corn oil and a combination of high-fat corn meal with full-fat soy flour. Tests for changes in flavor, free fatty acids, peroxide values and available lysine were made on blends stored at 120°F for 56 days, 100°F for 6 months and 77°F for 1 year. Higher fat content was associated with improved palatability. Flavor scores declined at about the same rate under all test conditions. In experimental blends containing crude corn oil, palatability varied with the particular sample tested. One sample of crude corn oil was satisfactory while another caused off-flavors in the blends.

INTRODUCTION

GUIDELINES for the composition and nutrient content of formulated food products such as CSM (corn-soy-milk) were issued by the Department of Agriculture's Committee on Food Processing in Developing Nations, Senti et al. (1967). The guidelines per 100g blended food mix included energy requirements of 350 kcal, 18–22g protein, 2g fat and 1g linoleic acid. It was recognized that a fat content greater than 2% would be desirable for an infant food, to improve the caloric density and facilitate better metabolism of fat-soluble vitamins. Fat content in the original CSM specifications, USDA (1966), was kept low purposely to minimize any possible rancidity problem. Storage tests have shown that the original formulation (Table 1) containing only 2% fat is stable for at least a year at 77°F, Bookwalter et al. (1968). Further storage tests have shown similar stability for experimental CSM blends containing corn germ to meet the 2% fat requirement, Bookwalter et al. (1971).

Additional storage stability tests were carried out on experimental CSM blends containing 5.6–8.2% fat. The fat sources were corn germ, full-fat soy flour, refined soybean oil and crude corn oil. The experimental results justified a change in specifications, USDA (1969), as shown in Table 1. The latest formulation requires a minimum of 6% fat and has a higher caloric density than the original one. The effect of increased fat levels on storage stability of experimental CSM blends was measured in three ways: change in flavor by taste panel evaluation, change in fat stability by the amounts of free fatty acids and peroxide values and change in protein stability by the amount of available lysine.

Table 1—Composition of CSM (corn-soy-milk) blended food

Ingredients	Formulation: 2% Fat (USDA, 1966) (%)	Formulation: 6% Fat[a] (USDA, 1969) (%)
Corn meal, low-fat, processed	68.0	63.8[b]
Soy flour, defatted, toasted, refatted	25.0[c]	
Soy flour, defatted, toasted		24.2[d]
Nonfat dry milk	5.0	5.0
Soybean oil, refined, BHA and BHT		5.0[e]
Mineral premix	1.9	1.9
Vitamin premix	0.1[e]	0.1[e]
	100.0	100.0

[a]Optical ingredients to obtain the 6% minimum fat content: refined soybean oil, corn germ and full-fat soy flour.
[b]May include corn germ (maximum 10% of final food blend).
[c]Refatted with 6% soybean oil containing antioxidants to adjust 100g final formulated product with 2.5 mg butylated hydroxy anisole (BHA) and 2.5 mg butylated hydroxy toluene (BHT).
[d]Optional replacement with full-fat soy flour (minimum 24% of final food blend, fat-free basis).
[e]BHA and BHT may be added either with the vitamin premix or to the refined soybean oil if this option is used.

MATERIALS & METHODS

Ingredients

All experimental CSM blends were prepared mainly from ingredients obtained commercially. They are listed, with their analyses, in Table 2. Partially gelatinized low-fat corn meal, toasted soy flours, spray process nonfat dry milk, mineral premix and vitamin premix with and without butylated hydroxy anisole (BHA) and butylated hydroxy toluene (BHT) complied with specifications as outlined in Announcement PS-GR-16, USDA (1966). The full-fat soy flour and soybean oil containing BHA and BHT complied with specifications of Announcement CSM-1, USDA (1969). The partially gelatinized high-fat corn meal and the corn germ were special commercial preparations made for this study and the degree of cooking was similar to that of the low-fat corn meal. The extrusion-cooked full-fat soy flour was processed as described by Mustakas et al. (1970) to meet specifications, USDA (1969). The comparative analyses and flavor characteristics of two different commercial samples of crude expeller process corn oils, identified as I and II, and of refined soybean oil are listed in Table 3.

A 10-gal stainless steel drum equipped with internal baffles and revolving on a mechanical roller was used to blend all the experimental mixtures. All formulations were tumbled for 1 hr at 60 rpm.

Table 2—Analyses of ingredients for CSM formulations

Ingredients	Moisture (%)	Protein[a,b] (%)	Fat[a] (%)	Fiber[a] (%)
Corn products				
Meal, low-fat, processed	10.5	8.6	0.9	0.6
Meal, high-fat, processed	10.7	8.7	3.5	0.9
Germ, processed	10.0	17.8	22.2	4.4
Soy flours				
Defatted, toasted, refatted[c]	7.0	49.5	6.5	3.2
Defatted, toasted	8.5	54.4	1.1	3.2
Full-fat, cooked	5.8	42.5	23.1	2.6
Full-fat, extrusion-cooked	3.4	42.5	23.3	1.8
Nonfat dry milk	3.2	37.1	0.8	—

[a]Dry basis.
[b]Percent nitrogen × 6.25.
[c]6% Refined soybean oil added.

Ingredients	Test X–formula variables: Original CSM formula	Refined soybean oil	High-fat corn meal plus full-fat soy flour
	(%)		
Corn meal, low-fat	68.0	65.2	...
Corn meal, high-fat	...	...	68.0
Soy flour, defatted, toasted, refatted, BHA and BHT	25.0	...	...
Soy flour, defatted, toasted	...	23.9	11.0
Soy flour, full-fat	...	...	14.0
Soybean oil, refined, BHA and BHT	...	4.2	...
Nonfat dry milk	5.0	4.7	5.0
Mineral premix	1.9	1.9	1.9
Vitamin premix	0.1	0.1	...
Vitamin-BHA and BHT premix	...	...	0.1
	100.0	100.0	100.0
Total fat, dry basis	2.4	5.6	5.7

Ingredients	Test Z–formula variables: Original CSM formula	Full-fat soy flour	Refined soybean oil	Corn germ	Full-fat soy flour extruded
	(%)				
Corn meal, low-fat	68.0	63.7	63.9	49.5	63.7
Corn germ	...	...	...	26.0	...
Soy flour, defatted, toasted, refatted, BHA and BHT	25.0	...	...	...	...
Soy flour, defatted, toasted	...	4.3	24.2	17.5	4.3
Soy flour, full-fat	...	25.0	...	...	...
Soy flour, full-fat, extrusion-cooked	...	...	...	...	25.0
Soybean oil, refined, BHA and BHT	...	...	4.9	...	...
Nonfat dry milk	5.0	5.0	5.0	5.0	5.0
Mineral premix	1.9	1.9	1.9	1.9	1.9
Vitamin premix	0.1	...	0.1	...	...
Vitamin-BHA and BHT premix	...	0.1	...	0.1	0.1
	100.0	100.0	100.0	100.0	100.0
Total fat, dry basis	2.4	6.5	6.5	8.2	6.5

Storage tests

The CSM blends were prepared according to the original CSM formula shown in Table 1, USDA (1966), except where indicated. To accommodate the higher fat levels in the experimental samples, formula changes were made for comparisons on an approximately equivalent protein basis. All formulations contained 2.5 mg BHA and 2.5 mg BHT per 100g final formulated product added with the oils or vitamin premix.

All experimental samples were packaged in 4-, 8- and 32-oz glass containers with foil-lined closures. The container held moisture at a constant level during the tests.

All experimental samples were stored in temperature-controlled cabinets at 100 and 120°F and at approximately 77°F room temperature. Reference samples were stored at 0°F.

Test X. Two blends prepared for comparison with the original CSM formula had higher fat. One contained additional soybean oil; the other, high-fat corn meal and full-fat soy flour.

Test Y. CSM blends with a little more than 6% fat content were formulated with refined soybean oil and with corn oils I and II. The formulation was the same as that given in Table 1 for CSM (6% fat), except for the addition of the respective crude corn oils instead of refined soybean oil.

Test Z. Fat content above 6% was attained in CSM blends by addition of four different fat sources.

Organoleptic tests

Withdrawals from storage were made at 120°F after 0, 14, 28 and 56 days; at 100°F after 90 and 182 days and at 77°F after 182 and 365 days. The blends were evaluated as gruels (10% solids) made by stirring the dry mix into boiling water and cooking for 1 min. Coded, randomized samples were presented to an experienced 18-member taste panel who rated for flavor using the 10-point score sheet described by Bookwalter et al. (1968; 1971). A score of 10 described the sample as Excellent, a score of 9 Very good, 8 Good, etc. Scores of 6 and above were considered acceptable. Objectionable flavors were assigned scores of 5 and below. The primary objective of the panel was to determine difference or change in flavor.

Statistical evaluations were made on the experimental data after completion of each taste panel series. Analysis of variance, regression analysis, Snedecor (1959), and Duncan's multiple range test, Duncan (1955), were used to examine results. Statistical significance is at the 5% probability of error level wherever the term Significant appears in this paper. The standard error of the mean flavor score was approximately 0.24. A least significant difference (0.95 probability level) for comparing two mean flavor scores was 0.7.

Analytical tests

Samples for analysis were withdrawn from storage at intervals similar to those followed for organoleptic tests.

Peroxide values, free fatty acids of the pentane-hexane extracted fat and other fat assays were determined by standard procedures, AOCS (1967). Available lysine was measured by a method based on the reaction with dinitrofluorobenzene, Baliga et al. (1959). Moisture analyses were made on a Brabender moisture tester (120°C, 60 min). Other analyses were determined by standard procedures, AACC (1962).

RESULTS & DISCUSSION

THE ANALYSES of experimental blends X, Y and Z are listed in Table 4.

The effect on flavor stability of CSM (Test X) from increased fat, either by adding more refined soybean oil or combining high-fat corn meal and full-fat soy flour, is shown in Figure 1. Each flavor stability curve was constructed from the average panel scores for each sample

Table 3–Analyses and flavor characteristics of a refined soybean oil compared to two crude corn oils

Analytical and flavor tests	Refined soybean oil	Crude corn oil I	Crude corn oil II
Refractive index (40°C)	1.4672	1.4673	1.4678
Specific gravity (25°C)	0.916	0.916	0.917
Iodine number	129.8	121.8	118.0
Saponification equivalent	294.0	296.8	297.3
Free fatty acids (% oleic)	<0.1	1.6	0.8
Peroxide value (meq/kg fat)	0.0	1.4	0.0
Phosphatides (%)	0.03	0.86	1.88
Predominant flavor	Bland, soy	Burnt, corny	Burnt, peanut
Flavor score (10-point scale)	7.2	2.8	1.9

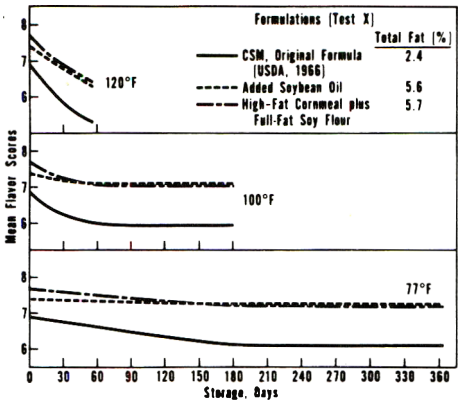

Fig. 1—Comparison of the flavor stability of corn-soy-milk (CSM) containing 2.4% fat with formulations having more fat either by adding refined soybean oil or by combining high-fat corn meal with full-fat soy flour.

withdrawn during the storage period. Higher fat content was associated with improved palatability. Mean flavor scores declined at essentially the same rate under all test conditions. At all three storage temperatures, the mean flavor scores of the two high-fat formulations remained significantly higher than those of the 2.4% fat formulation. Flavor stabilities of the 5.6 and 5.7% fat formulations were satisfactory at the three storage conditions.

In Table 5 are shown the analyses of stored samples comparing CSM at 2.4% fat with higher fat levels (Test X) attained by addition of refined soybean oil and high-fat corn meal plus full-fat soy flour. Comparison of values for fatty acids and available lysine at the three storage temperatures indicates slightly better stability in the higher fat formulations. Peroxide values were similar in the CSM containing 2.4% fat and in the formulation containing additional soybean oil. Peroxide values were slightly lower in the experimental CSM sample containing high-fat corn meal and full-fat soy flour. Analytical results indicate adequate stability for experimental CSM blends containing high fat levels.

Flavor stability curves are plotted in Figure 2 for CSM with approximately 6% fat by addition of refined soybean oil and crude corn oil samples I and II (Test Y). CSM with oil II scored significantly lower than the two other products at the three storage temperatures. Flavor scores for CSM containing either refined soybean oil or oil I were not significantly different. The strong burnt, peanut flavor of expeller-processed corn oil II (Table 3) was carried over to the CSM blend and received unsatisfactory flavor scores (below 6). The expeller-processed corn oil I (Table 3) had a burnt, corny flavor and was assigned a score of 2.8 as compared to 7.2 for the sample of refined soybean oil, but this burnt, corny flavor was not carried over to the CSM blend. The level of phosphatides in oil II was approximately double that of oil I and may be associated with the poorer flavor scores for experimental blends containing oil II. Analyses of stored CSM samples containing refined soybean oil and crude corn oils are given in Table 6.

Analytical values were similar except for the higher free fatty acids in the CSM samples containing crude corn oil. This difference in value was expected, since the base corn oils (Table 3) were higher in free fatty acids than the sample of refined

Table 4—Analyses of experimental CSM blends containing 2.4–8.2% fat

Test	CSM blends formula variables	Moisture	Protein[a,b]	Fat[a]	Fiber[a]
		(%)			
X	Original formula[c]	10.3	21.1	2.4	1.1
	Soy flour, defatted, toasted; refined soybean oil	9.8	21.0	5.6	0.9
	Corn meal, high-fat; soy flour, full-fat	10.2	20.4	5.7	1.2
Y	Refined soybean oil	8.9	21.9	6.2	0.8
	Crude corn oil I (expeller process)	9.4	21.9	6.1	0.8
	Crude corn oil II (expeller process)	9.1	21.9	6.1	0.7
Z	Original formula[c]	9.8	21.2	2.4	1.1
	Soy flours: full-fat and defatted, toasted	8.9	21.3	6.5	0.8
	Soy flour, defatted, toasted; refined soybean oil	9.4	21.3	6.5	0.9
	Corn germ; soy flour, defatted, toasted	8.9	21.1	8.2	1.5
	Soy flours: full-fat, extruded and defatted, toasted	9.1	21.6	6.6	0.7

[a]Dry basis.
[b]Percent nitrogen × 6.25.
[c]USDA, 1966.

Table 5—Comparison of stored CSM at 2.4% fat with samples containing higher fat levels

CSM formula variables (Test X)	Total fat in blends[a] (%)	Storage		Peroxide value (meq/kg fat)	Free fatty acids (% oleic)	Available lysine (% of protein)
		Days	Temp (°F)			
Original formula[b]	2.4	56	0	3.5	1.8	5.0
Refined soybean oil	5.6	56	0	2.3	0.7	4.6
High-fat corn meal plus full-fat soy flour	5.7	56	0	1.6	2.0	4.9
Original formula	2.4	56	120	2.3	5.2	4.2
Refined soybean oil	5.6	56	120	2.5	1.3	4.6
High-fat corn meal plus full-fat soy flour	5.7	56	120	1.0	2.8	4.6
Original formula	2.4	188	100	5.3	8.4	3.6
Refined soybean oil	5.6	188	100	4.6	1.5	3.9
High-fat corn meal plus full-fat soy flour	5.7	188	100	2.6	3.0	4.4
Original formula	2.4	365	77	6.5	5.3	4.0
Refined soybean oil	5.6	365	77	6.9	1.0	4.4
High-fat corn meal plus full-fat soy flour	5.7	365	77	1.6	2.5	4.4

[a]Dry basis.
[b]USDA, 1966.

soybean oil. These data indicate adequate stability for CSM containing either refined soybean oil or crude corn oils, but inherent off-flavors in the crude expeller corn oils eliminate them from further consideration until development of adequate analytical screening procedures for specifications. Preliminary tests indicate satisfactory storage stability for CSM containing refined corn oil. Refined corn oil would be more costly than crude oil.

In Table 7 is shown the effect on flavor stability of increased fat levels in CSM (Test Z) by using additional soybean oil, commercial full-fat soy flour, laboratory-extruded full-fat soy flour and corn germ. After storage at 120°F for 56 days, all scores were in the acceptable range, except the original formula CSM, which was significantly lower after storage. The mean flavor scores of the samples containing higher fat levels did not change significantly during storage. Initial scores of CSM containing either extruded full-fat soy flour or corn germ were significantly lower than the original formula CSM, but all three had similar scores after storage. Analyses of stored samples comparing CSM at 2.4% fat with higher fat levels attained by addition of full-fat soy flour, refined soybean oil, corn germ and extruded full-fat soy flour are listed in Table 8. Analytical values are similar both before and after storage and indicate

Table 6—Analyses of stored CSM samples containing refined soybean oil and two crude corn oils

CSM formula variables (Test Y)	Total fat in blends[a] (%)	Storage		Peroxide value (meq/kg fat)	Free fatty acids (% oleic)	Available lysine (% of protein)
		Days	Temp (°F)			
Soybean oil[b]	6.2	56	0	6.1	0.6	4.6
Corn oil	6.1	56	0	3.5	1.8	4.7
Corn oil II	6.1	56	0	4.3	1.0	4.8
Soybean oil	6.2	56	120	0.6	2.0	3.9
Corn oil I	6.1	56	120	1.4	3.1	4.0
Corn oil II	6.1	56	120	0.6	2.6	3.9
Soybean oil	6.2	190	100	1.0	2.7	4.5
Corn oil I	6.1	190	100	1.2	3.4	4.5
Corn oil II	6.1	190	100	1.2	3.0	4.5
Soybean oil	6.2	365	77	1.0	0.6	4.6
Corn oil I	6.1	365	77	1.0	2.8	4.5
Corn oil II	6.1	365	77	1.0	2.0	4.5

[a]Dry basis.
[b]USDA, 1969.

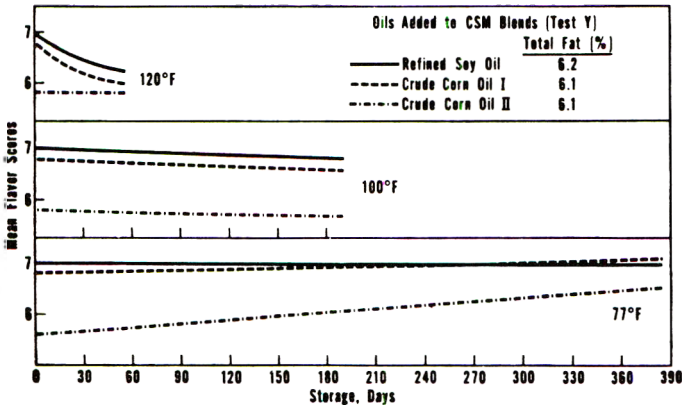

Fig. 2—Comparison of the flavor stability of CSM having two crude corn oils with a formulation containing refined soybean oil at approximately 6% fat.

Table 7—Flavor stability of stored samples comparing CSM at 2.4% fat with samples containing higher fat levels

CSM formula variables (Test Z)	Total fat in blends[a] (%)	Mean flavor scores[b] Days at 120°F	
		0	56
Original formula[c]	2.4	7.1v	5.9y[d]
Full-fat soy flour	6.5	6.8t,u,v	6.7z
Refined soybean oil	6.5	7.0u,v	6.4y,z
Corn germ	8.2	6.1t,u	6.3y,z
Full-fat soy flour, extruded	6.6	6.0t	6.0y

[a]Dry basis.
[b]Scores with no common letter, either (y—z) or (t—u—v), differ significantly (95% level).
[c]USDA, 1966.
[d]Significant (95% level) flavor decline with storage time.

Table 8—Comparison of stored CSM at 2.4% fat with samples containing higher fat levels

CSM formula variables (Test Z)	Total fat in blends[a] (%)	0 Days			56 Days at 120°F		
		Peroxide value (meq/kg fat)	Free fatty acids (% oleic)	Available lysine (% of protein)	Peroxide value (meq/kg fat)	Free fatty acids (% oleic)	Available lysine (% of protein)
Original formula[b]	2.4	2.1	1.8	4.5	2.5	3.4	3.8
Full-fat soy flour	6.5	1.0	0.7	4.8	1.0	1.3	4.3
Refined soybean oil	6.5	1.5	0.6	4.7	2.5	1.1	4.0
Corn germ	8.2	1.5	0.8	4.8	2.7	1.6	4.2
Full-fat soy flour, extruded	6.6	0.8	0.8	4.8	0.8	1.5	4.3

[a]Dry basis.
[b]USDA, 1966.

adequate stability for all formulations. After storage, the formulations containing higher fat levels were slightly higher in available lysine and slightly lower in free fatty acids than the CSM with 2.4% fat.

CONCLUSIONS

THE STORAGE STABILITY characteristics of corn-soy-milk blends containing about 6% fat are similar or improved when compared with those at about 2% fat. To increase the fat content, satisfactory results were obtained by using either refined soybean oil, corn germ, laboratory-extruded or commercial full-fat soy flour or by combining processed high-fat corn meal and commercial full-fat soy flour. Damage to flavor can result if an unsuitable expeller crude corn oil is used and, therefore, this oil appears to be an unreliable source of increased fat in CSM-type blended foods.

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BLANCHING OF WHITE POTATOES BY MICROWAVE ENERGY FOLLOWED BY BOILING WATER

SUMMARY—Small white potatoes were heated with microwave energy followed by boiling water to determine the penetration of heat, inactivation of peroxidase and firmness of the potatoes. Treatments consisted of heating potatoes with microwave energy for 0.5, 1, 1.5 and 2 min followed by boiling water for 0, 1, 2, 3, 4 and 5 min. Temperature measurements were made at depths of 0.75 and 1.5 cm using tubers with a mean radius of 1.95 cm. Peroxidase inactivation was measured along the radius of a slice removed from the equatorial region. Firmness was determined by the ALLO-Kramer shear press. Potatoes, when heated by microwave energy, became hot first at the core with a heat gradient developing toward the periphery. Boiling water produced a heat gradient from the periphery toward the core. Consequently, the tissue located about midway of the radius was subjected to the least amount of heat. The minimum time required to completely inactivate peroxidase and the firmness values of the potato tissue at the time of enzymatic inactivation were as follows: 1.5 min microwaves and 3 min boiling water, 119 lb shear force; and 2 min microwaves and 2 min boiling water, 124 lb shear force. Peroxidase was not completely destroyed when the potatoes were subjected to energy for 1 min or less followed by heating in boiling water up to 5 min.

INTRODUCTION

BLANCHING OF POTATOES is preliminary to frozen storage or canning. Blanching expels occluded gases and heats the potatoes sufficiently to inactivate certain enzyme systems (Talburt and Smith, 1967). The conventional hot water or steam blanching methods are relatively slow and result in a substantial loss of some fresh potato characteristics. As an alternative, microwave energy may be used to blanch various food items. Within certain restrictions, heat will penetrate to the center of the food in a relatively short time. Heating may be accomplished without contact with water or steam (Copson, 1962; Proctor and Goldblith, 1948).

Peroxidase (E.C. 1.11.1.7) is one of the most heat-stable enzymes in plants (Reed, 1966). Inactivation of this enzyme is often used to determine when the vegetable has been satisfactorily blanched. Collins and McCarty (1969) found that in tubers with a mean radius of 2.27 cm, peroxidase was inactivated in 13 min by boiling water and in 4.7 min by microwaves when the potatoes were submerged.

Textural changes of the potato tissue are caused by heat. When the temperature of a potato is raised above 122°F, starch granules start to swell and begin to gelatinize at 147–160°F. This process results in the rounding off of cells and in cell separation; sogginess of the tissue may ensue (Roberts and Proctor, 1955). Upon prolonged heating, the hemicellulose and cellulose components undergo some breakdown (Reeve, 1954).

This study was conducted to determine the effect of microwave energy and hot water combination treatments on penetration of heat, inactivation of peroxidase and firmness of the potatoes.

EXPERIMENTAL

FRESH SMALL WHITE potatoes (Katahdin variety), obtained from Winter Garden Freezer Co., Bells, Tenn. and grown in experimental plots, were utilized for this investigation and graded for uniformity of size; those used had a mean radius of 1.95 cm and a mean weight of 29g. The potatoes were not peeled before heating.

Five potatoes at a time were placed in a Tappan electronic range (Model R-2000, 2450 MHz, 1 kw) on a fiberglass tray. The arrangement of the potatoes on the tray was consistent throughout the study. Periods of heating were 0.5, 1, 1.5 and 2 min by microwave energy followed by 0, 1, 2, 3, 4 and 5 min in boiling water. The experimental design permitted 24 treatments and the treatments were replicated three times.

Immediately after each heating treatment, four of the five potatoes were cooled in tap water. These potatoes were used later for peroxidase assay and firmness measurement. The remaining potato was used for determination of temperature.

Temperature measurements were made at depths of 0.75 and 1.5 cm. An implant thermocouple was inserted into the tuber and the temperature was read from a potentiometer equipped with a temperature read-out scale. The temperature was measured at three loci on the equatorial area of the potato and temperature readings were obtained at only one depth from a given tuber. Therefore, to provide a sufficient number of potatoes for measurement (four tubers per each depth), additional potatoes were heated under conditions similar to those previously described.

For peroxidase inactivation assay, four potatoes from a sample of five were cut longitudinally through the central region to obtain a slice about 4–5 mm thick. Active peroxidase was detected by flooding the surface of the slice with 0.05% solutions of guaiacol and hydrogen peroxide. Peroxidase reacted with these substances to produce a reddish-brown coloration. The portion of the disk (periphery and core) which remained colorless was used to indicate where inactivation of the enzyme had occurred. Measurements were made in each quadrant of the slice.

An ALLO-Kramer shear press was used to determine the force required to shear samples of potatoes after the heat treatments. The 1,000-lb capacity proving ring was employed and a 28-sec thrust was used. A sample consisted of 50g of diced tissue. Two samples were prepared for each group of four tubers. With the exception of the slice removed for peroxidase measurement, the whole tuber (plus peel) was used in preparing the sample.

RESULTS & DISCUSSION

TEMPERATURES of the potato tissue at a depth of 0.75 cm, which resulted from heating with microwaves followed by boiling water, are presented in Figure 1. Temperatures for the 1.5-cm depth are shown in Figure 2. When the potatoes were heated with microwaves, they became hot first at the core with a heat gradient developing toward the periphery. This is evident from observation of the difference in temperature of the tissue at depths of 0.75 and 1.5 cm. For instance, at the 1.5-cm depth (Fig. 2) the temperature after 0.5 min of microwave treatment was about 188°F, whereas the

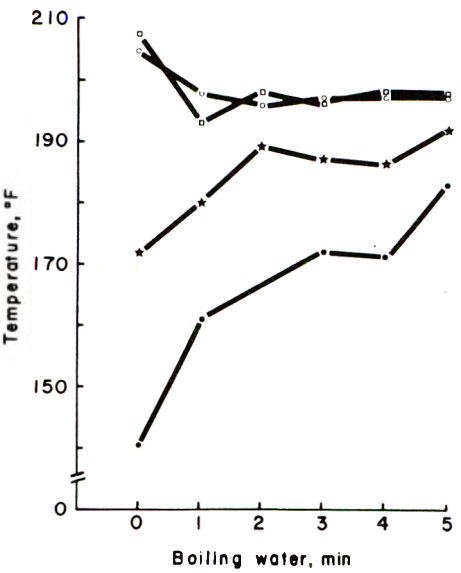

Fig. 1—Temperature at 0.75-cm depth for potatoes heated by microwave energy followed by boiling water. Potato mean radius, 1.95 cm. Minutes microwave treatment: —●—, 0.5; —★—, 1; —□—, 1.5; —○—, 2.

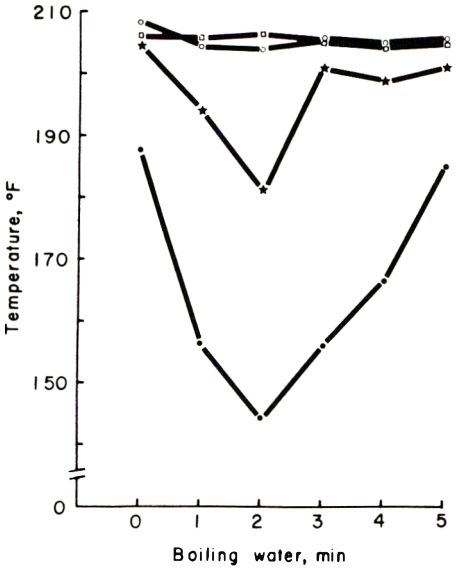

Fig. 2–Temperature at 1.5-cm depth for potatoes heated by microwave energy followed by boiling water. Potato mean radius, 1.95 cm. Minutes microwave treatment: –●–, 0.5; –★–, 1; –□–, 1.5; –○–, 2.

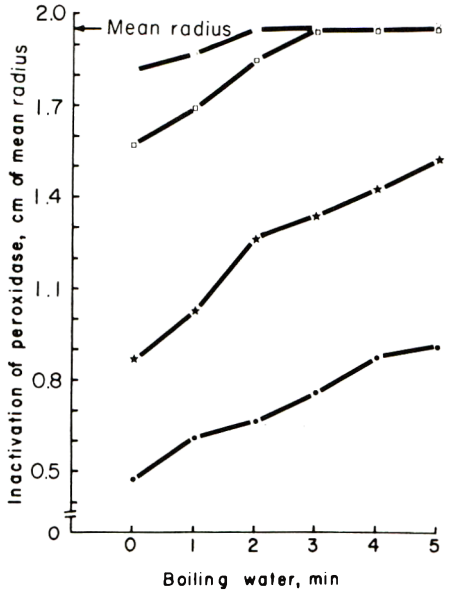

Fig. 3–Inactivation of peroxidase in potatoes heated by microwave energy followed by boiling water. Potato mean radius, 1.95 cm. Minutes microwave treatment: –●–, 0.5; –★–, 1; –□–, 1.5; –○–, 2.

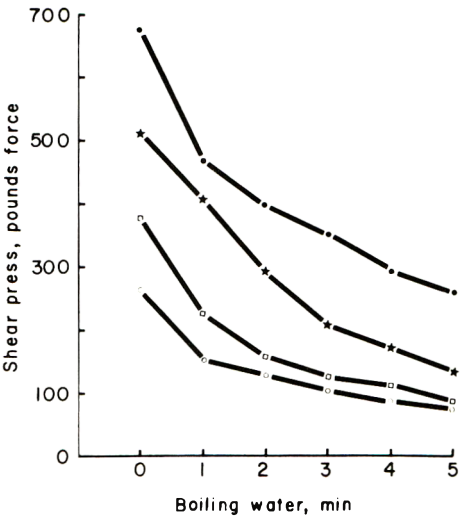

Fig. 4–Firmness of potatoes heated by microwave energy followed by boiling water. Potato mean radius, 1.95 cm. Minutes microwave treatment: –●–, 0.5; –★–, 1; –□–, 1.5; –○–, 2.

temperature at 0.75 cm (Fig. 1) was 130°F. A similar difference in temperature was found after 1 min of microwave treatment; however, the temperature values were higher after 1 min.

Temperature at the 1.5-cm depth dropped during the interim in which the potatoes were transferred from the microwave oven after 0.5- and 1-min treatment to boiling water and held up to 2 min. This was because the temperature was higher at the depth of 1.5 cm than it was in the more peripheral tissue. During the interim of transfer, some of the heat present at the inner depth dissipated in the direction of the periphery. This phenomenon was not observed in potatoes subjected to 1.5 and 2 min of microwave heating, indicating that the temperature had reached a maximum and was fairly uniform across the radius of the tuber. There was one exception concerning the uniformity of heat. At the 0.75-cm depth there was a small decrease in temperature after 1.5 min of microwaves similar to that observed at 1.5 cm. However, after 2 min in boiling water the temperature had risen to a stable level.

The peripheral tissue of potatoes which received a relatively short duration of microwave treatment (0.5 and 1 min) was heated primarily by the boiling water. Thus, two heat gradients were established before the temperature was elevated to the maximum level across the radius, one from the core and one from the periphery. Consequently, the tissue located about midway between these points was subjected to the least amount of heat.

There were two zones where the enzyme was inactivated before complete inactivation. These zones, developed initially at the core and periphery, were the consequence of increases in temperature produced by the two sources of heat. The total portion of the radius in which peroxidase was inactivated under the different heating treatments is shown in Figure 3. The heating times required to inactivate peroxidase completely across the mean radius were estimated as follows: 1.5 min microwaves and 3 min boiling water; and 2 min microwaves and 2 min boiling water. Peroxidase was not inactivated completely by microwave energy of 1 min or less with subsequent treatments in boiling water for 5 min.

The potatoes became softer as the application of heat was extended (Fig. 4). The softening trends appeared to be similar among the different durations of microwave application. Shear values at the minimum time required for complete inactivation of peroxidase were estimated as follows: 119 lb force after 1.5 min microwaves and 3 min boiling water; and 124 lb force after 2 min microwaves and 2 min boiling water. The potatoes that received these treatments were partially cooked; therefore, processors of frozen whole potatoes may not desire to heat them to this extent. Since peroxidase was not inactivated completely by microwave heating for 1 min or less, comparable shear values for these treatments could not be presented.

Results of this study indicate that microwave energy coupled with hot-water treatments would shorten the time required for blanching when compared to hot water alone. This combination should contribute also to a more uniform texture throughout the tubers, because with hot water the outer tissues would be almost cooked by the time sufficient heat reached the core.

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SPECIFIC GRAVITY DETERMINATION WITH A UNIVERSAL TESTING MACHINE

SUMMARY—A rapid, precise technique for determining specific gravity of fruits and vegetables was developed. The method utilized a universal testing machine. The effects of crosshead speed, choice of standard density fluid and weight measurements were studied and optimized. A standard sphere of known volume and weight was used to compare this method with other techniques now used. In addition to using the standard sphere, tomatoes were studied to evaluate the efficiency of this method. Calculation of specific gravity involved determining fruit volume and weight. Other volume measurement techniques investigated proved inaccurate or too time-consuming. An Instron universal testing machine was used in measuring the buoyancy force of a fruit submerged in a standard density fluid. The buoyancy force is directly related to the fruit volume. Error analysis showed relatively low sensitivity to errors in weight measurement, but was influenced mainly by errors in determining the fluid density. This error was minimized by use of high-density fluids; thus, saturated salt brines and high-density organic fluids were used for the experimental work.

INTRODUCTION

SPECIFIC GRAVITY is one of the basic physical properties of a horticultural commodity having important applications. Various techniques have been employed for determining specific gravity. Malcom et al. (1956) used flotation in brines of varying densities with peas. Kattan et al. (1968) used the same method but with ethanol solutions for sorting tomato fruit.

Volume by water displacement was used by Nettles (1950) in determining the specific gravity of tomatoes. Sorenson (1955) determined specific gravity of tomato fruit from the weight of the fruit in air and in ethanol. This same technique of direct weighing in air and in a fluid of lesser density than the fruit was used by Strietelmeier (1959).

Recent research in our laboratory on the rate-of-rise principle indicated a need for determining specific gravity of tomato fruit rapidly and very precisely. A survey of techniques available showed that none sufficiently met this need. Some, such as water displacement and direct weighing in air and fluid, lacked the needed precision. Others, such as a specific gravity column and an air pycnometer for measuring porosity (Day, 1964), were too time-consuming for testing large numbers of samples.

The buoyancy force exerted by a fluid on a submerged fruit is directly related to the volume of the fruit by Archimedes' principle:

$F_B = V\rho_F$ [1]

F_B = Buoyancy forces
V = Submerged volume of the fruit
ρ_F = Density of the fluid

A technique for measuring buoyancy force precisely would first require determination of fruit volume, from which specific gravity could be calculated by the following:

$S.G. = (W/V) / \rho_W$ [2]

S.G. = Specific gravity of the fruit
W = Fruit weight
V = Fruit volume
ρ_W = Density of water

An Instron universal testing machine has the facility to rapidly and precisely measure buoyancy force. This paper reports development of a technique for determining specific gravity of fruits and vegetables with a universal testing machine.

EXPERIMENTAL

Apparatus

A floor-model Instron universal testing

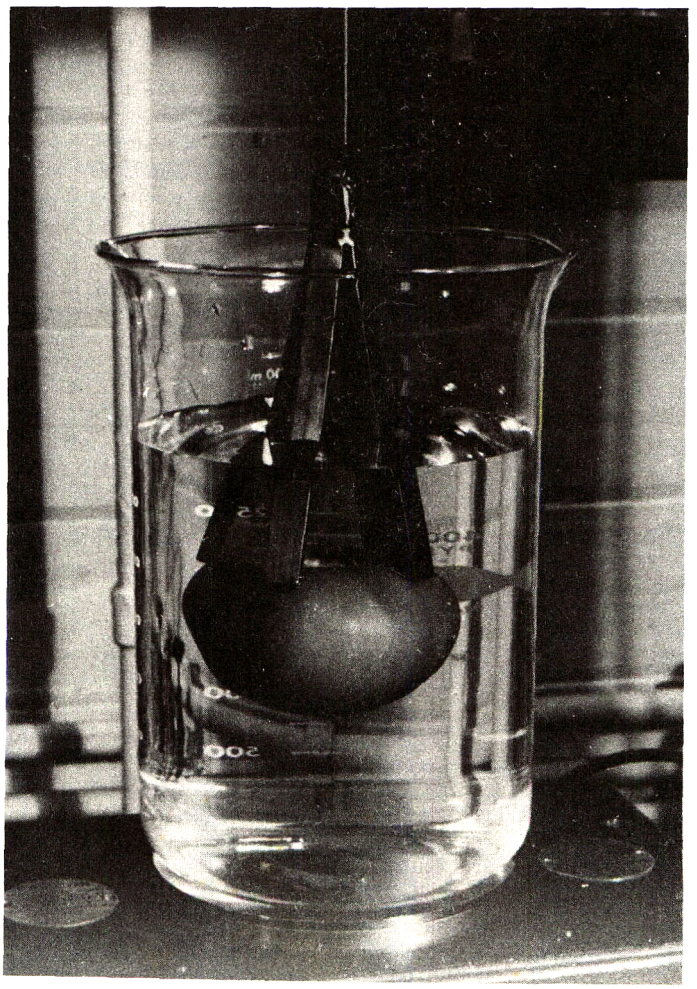

Fig. 1—Submersion apparatus.

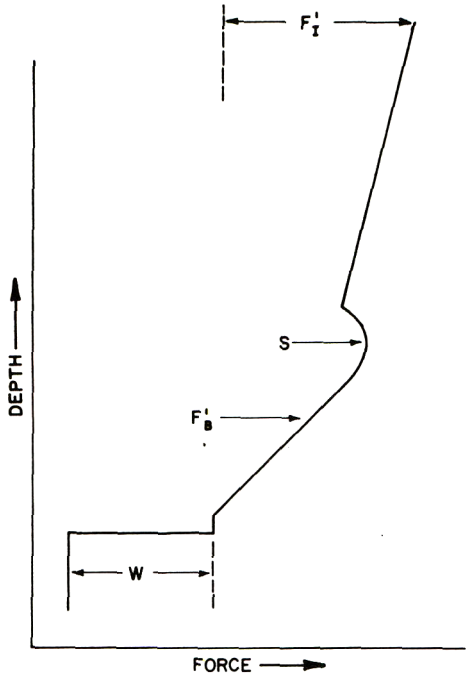

Fig. 2–Typical curve for a tomato fruit obtained by the Instron technique.

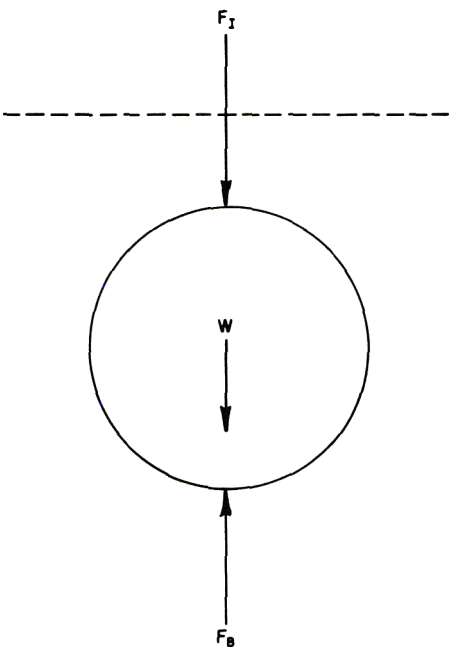

Fig. 3–Forces acting on the submerged fruit.

machine was equipped with a type "C" compression load cell. Calibration was 1 lb full scale on high sensitivity with a 10-in. chart. Chart speed was 10 ipm.

The submersion apparatus consisted of a large container approximately 6 in. in diameter containing the fluid. It was placed on the load cell plate and a submersion device attached beneath the crosshead. This device was made of four flat metal prongs which diverged downward (Fig. 1).

Standard volume spheres

To provide information on precision of the technique and allow comparisons between methods, a series of standard volume spheres was obtained. These precision-ground polyethylene spheres had a diameter of 2.500 ± 0.002 in.

Fluid

Saturated salt brines and high-density organic fluids, such as carbon tetrachloride, were used for the experimental work.

Crosshead speed

Speeds used were 2.5, 5, 10 and 20 ipm.

Error analysis

Results were subjected to error analysis according to standard techniques, Standard Handbook for Electrical Engineers (1949).

Procedure

The Instron testing machine was calibrated for 1 lb full scale on high sensitivity with the 10-step suppression control activated. The container with approximately ¾-gal fluid was placed on the load cell plate and the 10-step suppression used to suppress the container and fluid weight to obtain maximum available chart space for testing and still maintain only 1 lb full scale on the chart.

The tomato after weighing was placed in the fluid, stem-up. As the crosshead moved down, the submersion prongs contacted the fruit and forced it beneath the surface. The preset gauge length automatically stopped submersion of the prongs at the same position each time. A gauge length of approximately 4 in. was used to allow complete submersion of all sizes of fruit. To provide replications on the same fruit, the Instron can be re-cycled automatically if desired.

RESULTS & DISCUSSION

A TYPICAL CURVE obtained when testing with tomato fruit is shown in Figure 2. The portion of the curve W is due to the increased force on the compression cell from the weight of the fruit placed in the fluid. As the prongs contact the fruit and begin its submersion, an increasing buoyancy force F_B' is recorded, as more fruit volume is continually being submerged.

With fruits such as tomato there is an area of the curve denoted by "S" where there is a temporary apparent volume indication that is greater than true volume. This is an effect of the fruit's shape. With tomatoes, just before complete submersion, the shoulders around the stem cavity temporarily prevent the fluid from flowing into the stem cavity, even though the cavity is actually below surface level of the fluid. This results in an indicated volume greater than its true volume until the point of complete submersion is achieved. After the fruit is totally submerged, the continued increase in buoyancy force is due to the continually increasing volume of the prongs being submerged to the preset depth. The force, F_I', at this depth minus the force of the prong device submerged by itself to the preset depth (to correct for the volume of the prongs on the buoyancy force) is denoted as the Instron force, F_I.

Forces acting upon the fruit in the static condition after the preset depth has been reached are shown in Figure 3. From this it is shown that:

$F_B = W + F_I$
F_B = Buoyancy force on the fruit
W = Fruit weight
F_I = Instron force measured [3]

and from Equation [1]

$$V\rho_F = W + F_I \quad [4]$$

Thus:

$$V = (W + F_I) / \rho_F \quad [5]$$

and from Equation [2]

$$S.G. = (W\rho_F) / (W + F_I)\rho_W \quad [6]$$

Equation [6] is the general equation for determining specific gravity with the Instron universal testing machine.

While the general equation is sufficient for most work requiring a value for specific gravity, there is an error introduced in the technique which can be corrected for, if very high precision is required. The correction for the volume of prongs on buoyancy force as described before (to obtain F_I) is slightly smaller than the actual value. While the prongs always submerge to the same relative vertical position in reference to the machine as controlled by the preset gauge length, the height of fluid on the prong device varies due to displaced fluid from the volume of the fruit in the container. As fruit volume increases, the additional height of fluid on the prong device increases. For an average-sized tomato and a 6-in.-diameter container the additional height of fluid on the prong device over that when no fruit is in the fluid will be about 0.3 in.

The corrected equation has the form:

$V' = V - VK$
V' = Corrected volume
V = Apparent volume (from Equation [5])
K = Correction factor [7]

The correction factor is defined as follows:

$$K = (4LX) / (\pi D^2) \quad [8]$$

where

L = Linear relationship of force per unit length of prong device submerged

Table 1–Precision in measured variables and influence on specific gravity by the Instron technique

	δ (lb)	Δ	% Error
W	0.001	0.00144	——
F_I	0.002	0.00383	——
ρ_F	0.0002/in.^{3}	0.00316	——
S.G.	——	0.0052	± 0.57

Table 2–Most probable errors in determining specific gravity of a standard sphere by several techniques

	Error	% Error
Standard sphere measurement	0.0034	± 0.37
Instron	0.0052	± 0.57
Air pycnometer	0.0349	± 3.83

X = Relationship of indicated volume/unit force measured, equal to unit Force/ρ_F

D = Diameter of container for fluid

As shown later in the error analysis section, this corrected value used for determining specific gravity has a very high degree of precision.

For there to be a measurable buoyancy force while using this technique, all fruit must float. The greater the density of the fluid used, the larger the buoyancy force and the easier it can be measured. Also, the error analyses indicated smaller errors when using as high a density fluid as possible. These factors indicated the need for using high-density fluids; thus, saturated salt brines and high-density organic fluids such as carbon tetrachloride were used.

As crosshead speed increased, the amount of horizontal "chatter" increased in the pen recording the force curve. There was no increased precision in measuring F_I from the smoother curves of slower speeds as compared to higher speeds, except at the highest speed of 20 ipm. Thus 10 ipm, which still had the high precision of the slower speeds plus reduced testing time, was selected for the standard speed in testing.

The error analysis of a function, for form of

$$R = f(x,y,z) \qquad [9]$$

where the dependent variable, R, is a function of the several independent variables x, y and z, is described in the Standard Handbook for Electrical Engineers (1949). The most probable error, Δ, in the dependent variable, R, is a combined effect of the errors in R due to each of the independent variables and can be expressed as:

$$\Delta = \sqrt{(\Delta x)^2 + (\Delta y)^2 + (\Delta z)^2}$$

The effect on R by error in the independent variable x is

$$\Delta_x = \frac{\partial R}{\partial x} \delta_x$$

where δ_x is the precision of measuring the magnitude of the variable, x. Similarly, Δ_y and Δ_z can be determined.

An error analysis of the specific gravity measurement was made using the independent variable included in Equation [6]. Table 1 shows a summary of the error analysis of the Instron technique. This shows that the technique is relatively insensitive to errors in weighing the fruit, since precision to the nearest 0.001 lb (approximately 0.5g) is needed. The errors experienced in determining specific gravity by using the variables F_I and ρ_F are of the same magnitude, but the measurement of ρ_F requires precision to the fourth decimal place, while only to the third decimal place is required for F_I. Thus, the technique is most sensitive to errors in determining ρ_F.

Comparisons of most probable errors and per cent error are shown in Table 2. An error is realized when determining the specific gravity by direct calculation of the standard sphere because of errors in the measurement of its diameter and weight. There is relatively little difference between the error of determining the specific gravity of the standard volume spheres directly and the error of determining their specific gravity with the Instron. The error in determining the specific gravity of the sphere by an air pycnometer, Day (1964), one of the more precise methods available, was approximately seven times that of the Instron technique.

CONCLUSIONS

THE TECHNIQUE of utilizing the Instron universal testing machine can be a rapid and precise method of determining specific gravity of fruits and vegetables. The general equation for determining specific gravity by this method is:

$$S.G. = (W\rho_F) / (W + F_I)\rho_w$$

Error analysis showed relative insensitivity to weight measurement errors, while being influenced mainly by error in determining ρ_F. The precision realized by this method appears to be much better than that of formerly available methods for measuring specific gravity of large numbers of samples.

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COMPUTER EVALUATION OF IRRADIATION PROCESSES IN CYLINDRICAL CONTAINERS WITH GAMMA SOURCES

SUMMARY—A numerical technique is presented for evaluating food irradiation processes using cylindrical gamma sources. Dose rate distributions obtained by dividing a container into finite concentric rings permit the use of a nonuniform initial spore load distribution and the determination of both number and distribution of survivors. The double integral giving dose rates was computed using several methods. The computations gave survivors significantly different from those obtained by using approximations given in literature. Accurate evaluations require utilization of dose rate distributions in radial and axial directions.

INTRODUCTION

IRRADIATION processes were originally evaluated by determining the lethal effect of the dose received by a single point in the container. This may involve nonuniform processing of the food. At sterilization doses the organoleptic properties of foods are affected making it important that overprocessing be avoided; under processing constitutes a health hazard.

The same degree of microbiological destruction is usually maintained for irradiation and thermal processes (Charm et al., 1954) although they should be evaluated with respect to the most resistant organism for the process. The technique of integrating lethality of heat at all points in the container, for conduction-heated foods, is well established (Stumbo, 1965; Teixeira et al., 1969). Charm et al. (1954) attempted a similar approach for irradiation processes by recognizing that the point, line or surface of minimum dose is not the single factor on which to base an irradiation process. For lethality calculations, they approximated the average dose rates along the radius to a parabola, but neglected the distribution along the container length. Dose inhomogeneity is found to be much less in a cylindrical-source geometry than in a plane-source geometry (Brynjolfsson, 1960).

Charm et al. (1954) in addition to neglecting the dose rate distribution along the container length, did not obtain the distribution of survivors. A numerical technique is presented here for evaluation of irradiation processes using dose rate distributions along both the radius and length of the container. This technique permits using a nonuniform initial spore load distribution and gives the survivor distribution too. Therefore, the method is useful in evaluating combination treatments, where the food container is subjected to consecutive processes of heat and radiation.

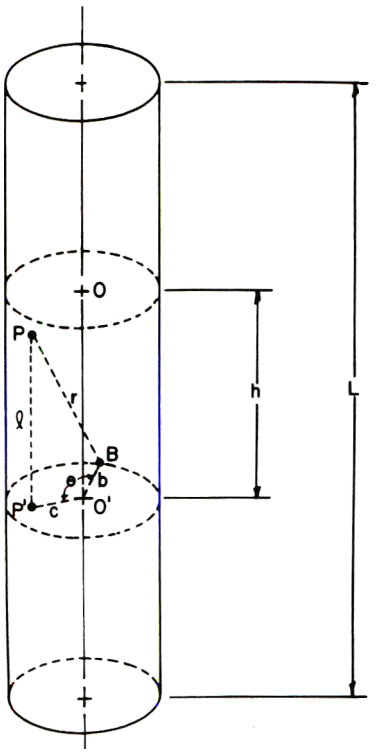

Fig. 1—Hypothetical source-container configuration.

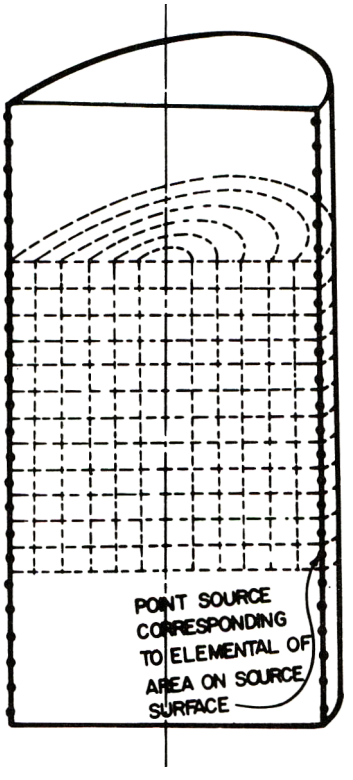

Fig. 2—Elemental volumes and point sources for numerical techniques.

PROCEDURE

CONSIDER a hollow cylindrical gamma source of as small a radius as possible and with all its activity painted on the inside wall (Dillon et al., 1954). Further, consider the food container to be of the same radius as the source (Charm et al., 1954). Thus, the maximum obtainable intensity from a given source geometry would be incident on the most efficient absorber. Figure 1 shows the hypothetical source-container configuration. In this analysis, classical target theory is assumed to hold and the radiation received as the container approaches and leaves the source is neglected (Charm et al., 1954).

This idealized situation is valid for purposes of demonstrating a technique which is capable of utilizing dose rate distributions and nonuniform spore loads to give the integrated lethality and the survivor distribution. A sequel paper indicates the use of this approach in studying combination processes (Purohit et al., 1971). Once the technique is established in principle, programs to simulate actual source and absorber configurations and movements may be readily developed.

The dose rate received by any point P of the food container, placed inside a cylindrical source, is given as,

$$D(P) = \frac{2\,KC_A\,b}{4\,\Pi} \int_0^{L} \int_0^{\Pi} \frac{e^{ur}}{r^2}\, d\theta\, dl \qquad [1]$$

where

$$r = \sqrt{l^2 + c^2 + b^2 - 2bc\cos\theta}$$

The food container was imagined to be divided into volume elements appearing as layers of concentric rings having rectangular cross-sections. P was taken to be at the center of any such volume element and both the dose rate and spore concentration at P were considered to be representative of the differential volume surrounding it. Similarly, the source wall was imagined to be divided into surface elements, with the radiation activity concentrated at the centers as point sources. Figure 2 shows the finite difference grid of both the container and source.

For a given container-source geometry, source strength and absorption coefficient of

Table 1—Data used in sample calculations

b = 4.36 cm	L = 11.58 cm
h = 11.58 cm	C_A = 5.05 curies/cm^2
μ = 0.074 cm^{-1}	t = 30,000 sec
Di = 139,600 rads	
Initial spore load per container = 10	

food, equation 1 was programmed for solution on a CDC 3600 computer.

Each point P was assigned an initial spore concentration, representing the volume element surrounding it. The survivor distribution was determined using the equation,

$$n(P) = no(P)\, e^{-[D(P)/Di] \cdot t} \qquad [2]$$

The total number of survivors in the container was then obtained by the equation,

$$\int_0^v n(P)\, dv = \int_0^b \int_0^h no(P) \cdot e^{-[D(P)/Di] \cdot t}\, dh\, db \qquad [3]$$

Table 1 gives the values of the variables used in the sample calculations for this study.

RESULTS & DISCUSSION

THE RADIATION intensity at the center of the vertical axis of a cylindrical source attains an optimum (maximum) at a finite length of the source (Dillon et al., 1954). Figure 3 demonstrates such maxima for points lying in a plane intersecting the vertical axis at its center and perpendicular to it. However, Figure 3 also shows that, for points lying in the planes containing the top (and bottom) of food containers, the dose rate increases with an increasing L/b ratio.

Referring to Figure 4, observe that neglecting the dose rate distribution in the axial direction is inaccurate. The extent to which this approximation influences survivors is obvious from Table 2. Figure 4 also shows that the "cold-spot" is at the top (and bottom) of the vertical axis of the container and not at the geometrical center.

Table 2 briefly summarizes the comparison studies undertaken. It indicates a wide variation in the number of survivors obtained by using several methods of computations and some approximations suggested in the literature. Except in rows 2, 3 and 8, integration was by Simpson's Rule. The combined technique of integration, of row 2, was used to handle combination treatments, wherein a one-to-one correspondence of volume elements is important. The technique used to handle the thermal process (Teixeira et al., 1969) uses the rectangular rule only. This extension to combination processes appears in a sequel paper (Purohit et al., 1971).

Table 2—Summary of comparison studies

Method of computation	Spores surviving process
1. $\int_0^{\Pi}\int_0^{L}$ By Simpson's Rule	
(A) L = h = 2.66b	2.91×10^{-8}
(B) L = 3h = 8b	4.36×10^{-15}
2. $\int_0^{\Pi}$ By Simpson's and $\int_0^{L}$ by Rectangular Rule	
(A) L = h	9.23×10^{-9}
(B) L = 3h	3.68×10^{-15}
3. $\int_0^{\Pi}\int_0^{L}$ By Rectangular Rule, (L = h)	8.25×10^{-9}
4. Radial distribution only, (L = h)	3.54×10^{-10}
5. Avg of dose at (b, h/2) and (0, h/2) to all elemental volumes, (L = h)	6.93×10^{-19}
6. Dose at (0, h/2) to all elemental volumes, (L = h)	1.92×10^{-10}
7. Dose at (0, 0) to all elemental volumes, (L = h)	4.93×10^{-6}
8. Quote of Charm et al. (1954) (L = 3h)	2.30×10^{-10}

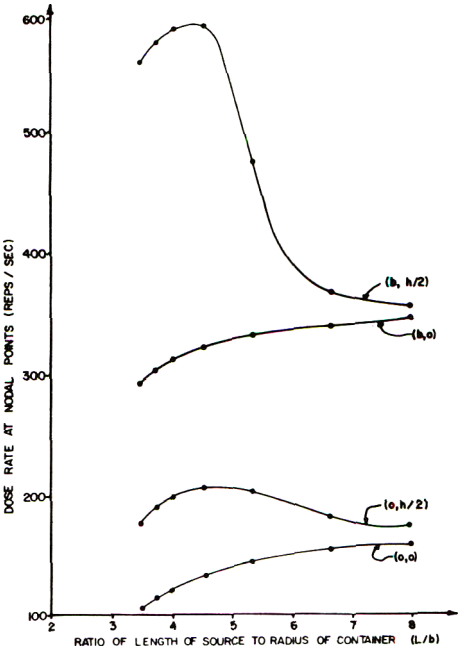

Fig. 3—Dose rate extremes as a function of length of source.

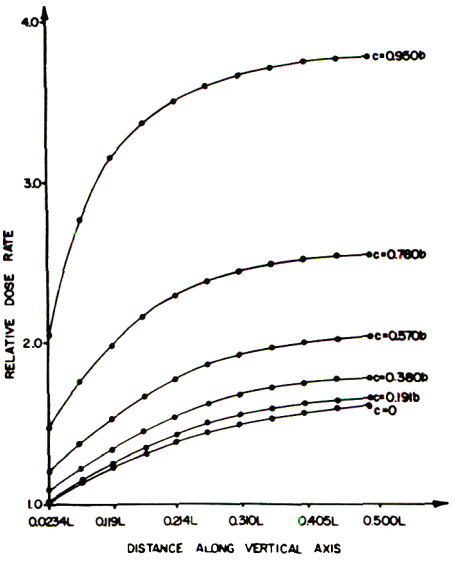

Fig. 4—Relative dose-rate distribution in container.

CONCLUSIONS

LETHALITY calculations can be made for irradiation processes inside cylindrical sources, using actual dose-rate distributions in cylindrical food containers. Use of numerical methods make the technique suitable for studying combination processes where heat treatment follows irradiation and vice versa.

NOMENCLATURE

b	=	radius of food container, cm
c	=	distance P' to center of plane, cm
C_A	=	source strength in curies per cm^2
Di	=	37% inactivation dose of organism, rads
D(P)	=	dose rate received by any point P in rads per sec
h	=	height of food container, cm
K	=	conversion factor to convert aver-

age energy of a fission product source from curies to rads per sec

l = distance between the planes under consideration, or 00′, in cm

L = length of source, cm

$n_o(P)$ = initial spore concentration at P

$n(P)$ = survivor concentration at P

P = any point in food container (absorber)

P′ = projection of P on any plane in container

t = exposure time, sec

v = volume of food container, cm^3

μ = absorption coefficient of food material, cm^{-1}

θ = angle P′0′B, where B is any one of the point sources on source wall, where activity of surface elements is assumed to be concentrated

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THEORETICAL EVALUATION OF COMBINED IRRADIATION AND THERMAL PROCESSES IN CYLINDRICAL CONTAINERS WITH GAMMA SOURCES

SUMMARY—A digital computer technique is presented for studying combined effects of heat and radiation in food sterilization, using steam retorts and cylindrical gamma sources. Temperature and dose rate distributions in cylindrical containers are computed using numerical methods. Distributions due to preliminary treatments—survivor and degree of spore sensitization—are inputs to subsequent treatment. The heat-radiation sequence was studied using data available, which indicates no synergism. This additive combination served as control to demonstrate synergism for radiation-heat sequence. Synergy, as influenced by irradiation exposure time, source strength, thermal process time and retort temperature was studied.

INTRODUCTION

RELATIVELY MILD heat treatments inactivate enzymes and *Cl. botulinum*, whereas, large radiation doses are necessary, resulting in off-flavors, off-odors and degraded texture. Low radiation doses inactivate thermophiles, whereas severe heat treatments are required, causing overcooking and loss of nutrients. A combined process of milder heat and radiation treatments could not only provide the required lethality, but also allow higher nutrient retention. Synergism, due to the combination, may make such a process even more effective. On the industrial scale, irradiation and thermal processing could then be carried out independently at different places at different times (Kempe, 1960).

Preirradiation, using ionizing radiations, has been found to sensitize bacterial spores to subsequent heat treatment. Synergism has been observed for spores of TA3814, PA3679, FS2253 (Morgan and Reed, 1954), *Cl. botulinum* 62A, *Cl. botulinum* 213B (Kempe, 1955), *B. cercus*, PA3679 in ham solids and puree (Kan et al., 1957) and *R. stolinfier* (Sommer et al., 1967). Cooking time of products of high starch content is reduced by preirradiation (Huber et al., 1953). Heat sensitization of spores by radiation is equally effective in raw cooked ground beef (Kempe, 1960). *Cl. botulinum* spores irradiated in the liquid state are highly heat sensitive and preirradiation in the frozen state results in a smaller loss of heat resistance (Grecz et al., 1967). Sensitization of microorganisms to heat following irradiation by ultraviolet and even visible light has been cited in literature (Kempe, 1960).

Some investigators, however, found heating just prior to irradiation to be more effective than the radiation-heat sequence. Moderate short-duration preliminary heating has been found to increase the radiosensitivity of R_1 strain *Micrococcus radiodurans* (Duggan et al., 1963), *Monilinia fructicola*, *Cladosporium herbarm* (Sommer et al., 1967) and various species of mold normally found on damp kernels of maize (Poisson and Cahagnier, 1969). Stehlik and Kaindl (1966), working with yeast cells, reported a simultaneous heat and radiation treatment as the most effective combination; less effective was the heat-radiation sequence, and the radiation-heat sequence was least effective.

For conduction-heated foods in cylindrical containers, Teixeira et al. (1969) developed a digital computer technique to obtain the integrated bacterial lethality due to temperature distributions over process time. Purohit et al. (1971) presented a numerical technique for evaluating irradiation processes by integrating lethal effects of dose distributions in cylindrical containers.

Presented here in this study is a method using the computer techniques of Teixeira et al. (1969) and Purohit et al. (1971) for studying various combination processes of heat and radiation treatment.

PROCEDURE

LITERATURE reviewed contains very few reports on radio-sensitization of bacterial spores by preheating, and almost no data are available. Heat-sensitization by preirradiation is well documented, although rate data are far from complete, especially for degradation of quality factors. This work studies combination processes in cylindrical food containers using steam retorts and cylindrical gamma sources. The numerical technique employed computes the integrated lethality effects of dose distributions and temperature distribution histories in the container.

Reports of thermal sensitization of bacterial spores by preirradiation have been cited earlier. What is also needed is a relationship, indicating the effect of preirradiation doses and temperature of heating on this sensitization. Decrease in thermal resistance due to preirradiation doses is almost independent of temperature of heating (Kan et al., 1957; Stehlik and Kaindl, 1966). For simultaneous treatment, synergism was observed at different temperatures (Stehlik and Kaindl, 1966; Sommer et al., 1967). Between 196°C and 25°C thermal sensitization caused by increments in preirradiation doses was D_{10} = 1 min at 99°C per 0.1 Mrad, while sensitization due to solid-liquid transition was another 12 min (Grecz et al., 1967).

After preliminary calculations, equation 1 was considered adequate to demonstrate the ability of the technique to evaluate combined processes. Sensitization was assumed to be independent of temperature of heating. Data for two organisms considered are given in Table 1.

$$D_x = D_o [(D_i)^x / D_o] \quad [1]$$

Numerical techniques available for evaluating thermal (Teixeira et al., 1969) and irradiation (Purohit et al., 1971) processes were used as such and so their assumptions and characteristics hold. Being coupled together, however, they had to be compatible in regard to container and grid size. Spore concentration and preirradiation dose received by each differential volume element had to be stored in memory. D values of surviving spores, in each differential volume element, after the preirradiation exposure, had to be computed using equation 1 and stored. Therefore, not only does the thermal process have a nonuniform initial spore load distribution, but it also has a nonuniform distribution of D values.

Effects of combination processes on *Cl. botulinum* and PA3679 were studied. Both the radiation-heat and the heat-radiation sequence were studied using data of Table 1. Variations in exposure time and source strength during radiation treatment were studied. For the heat treatment, variations in process time and retort temperature were studied.

RESULTS & DISCUSSION

RESULTS obtained are summarized in

Table 1—"D" values used in sample calculations

	Thermal D_{10} in min at 250°F		Radiation D_{10} in Mrad	
Organism	Without preirradiation	With 1.0 Mrad preirradiation	Without preheating	With preheating
Cl. botulinum	0.14	0.07	0.33	0.33
PA3679	0.44	0.07	0.20	0.20

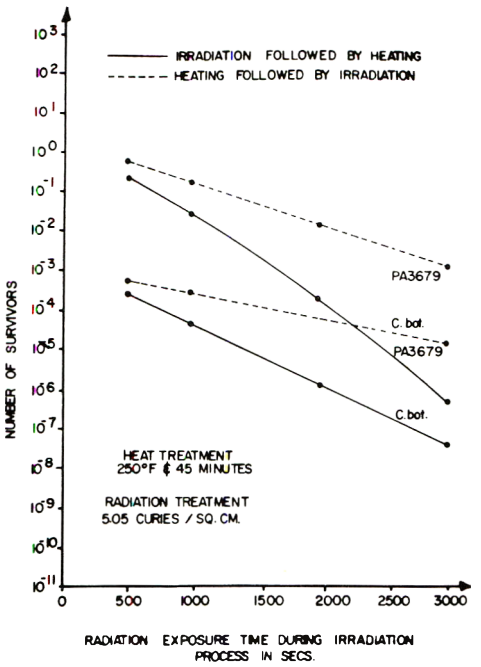

Fig. 1–Effects of varying radiation exposure time in a combined process.

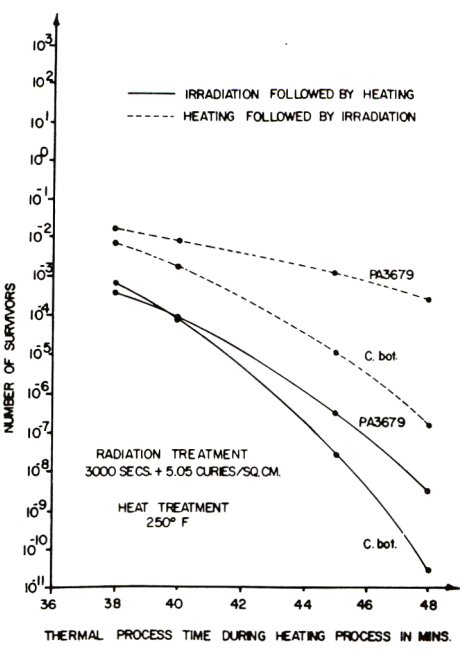

Fig. 2–Effects of varying thermal process time in a combined process.

Figures 1 and 2. Dotted lines represent a heat-radiation sequence and as Table 1 indicates, there is no synergism for such a combination. However, computations were made to demonstrate the technique, should data on radio-sensitization by preheating be available. The results were plotted to show the purely additive effect of two individual treatments. They are useful for comparison with results for a radiation-heat sequence, shown by solid lines, where synergism does in fact occur.

In Figures 1 and 2 the heat treatment of the combination is indicated by the retort temperature and thermal process time, while the radiation treatment of the combination is qualified by the source strength and radiation exposure time. The initial bacterial contamination was taken to be 1000 spores per container of radius 4.36 cm and height 11.58 cm.

Figure 1 gives the probable number of survivors for a thermal process with a retort temperature of 250°F and a process time of 45 min, for abscissae values of zero. The positive direction of the abscissae indicates increasing levels of pre- or post-irradiation treatments for the combined process.

Similarly, Figure 2 gives the probable number of survivors for an irradiation process with a source strength of 5.05 curies per sq cm and a radiation exposure of 3000 sec for abscissae values of zero. The positive direction of the abscissae indicates increasing levels of pre- or post-heating treatments for the combined process.

Varying the exposure time during the radiation treatment, using a source strength of 5.05 curies per sq cm, affected survivors for the combined process as shown in Figure 1. Similar results for variations in source strength, for a 3000 sec radiation exposure, were obtained. For very high levels of preirradiation, PA3679 is sensitized to the extent that its heat resistance becomes lower than that of *Cl. botulinum* receiving the same level of preirradiation.

Figure 2 shows the effect of varying the process time, with a retort at 250°F during the heat treatment. The effects of changes in retort temperature, for a 45-min thermal process, on the survivors of the combined process are similar. In Figure 2 the fixed level of the preirradiation treatment given to the spores (3000 sec exposure with a source strength of 5.05 curies per sq cm) not being very high, the survivors of PA3679 exceed those of *Cl. botulinum*, even though synergism for PA3679 is greater.

CONCLUSIONS

NUMERICAL techniques enable study of combination processes of heat and radiation. Effects of magnitude and sequence of individual treatments on synergism can be studied. Bacterial spores were shown to be synergistically inactivated in a radiation-heat sequence, using available data. To effectively evaluate a combination process, however, kinetics of degradation of quality factors would be needed to ascertain if they are also being synergistically degraded. Simultaneous application of heat and radiation is reported to be highly synergistic, but available data are incomplete. Ultimate value of any food processing technique depends on its economics and its effects on flavor, color, texture and nutrients.

NOMENCLATURE

D_0 = D value of organism at 250°F, where the preirradiation dose is zero. Conventional 90% reduction time at 250°F.

D_1 = D value of organism at 250°F, preirradiated with 1.0 Mrad. Sensitized 90% reduction time at 250°F for 1.0 Mrad preirradiation.

D_X = D value of organism at 250°F, preirradiated with X Mrad. Sensitized 90% reduction time at 250°F for any dose X in Mrads.

X = dose in Mrad received by spores in any differential volume element during preirradiation.

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EFFECT OF RADURIZATION ON SHEAR RESISTANCE AND FRAGMENTATION OF CHICKEN MUSCLE

SUMMARY—Excised pectoralis muscles of chicken received radurizing doses of γ-radiation at varying times post-mortem to determine the effect on shear resistance, myofibrillar fragmentation (F-ratio) and pH. Irradiation doses between 0.1 and 0.3 Mrad produced decreases in F-ratio and increases in shear resistance. The magnitude of the changes was directly proportional to the duration of postmortem aging prior to irradiation. Muscle pH subsequent to irradiation between 2 and 12 hr post-mortem was not significantly affected by any of the dose-time treatment combinations. Shear resistance and F-ratio changes were strongly correlated on an average basis (r = +.857; n = 6) but not on an individual sample basis.

INTRODUCTION

PRESERVATION of poultry by irradiation has been hindered by development of undesirable textural properties. Textural changes produced in irradiation-sterilized meats have usually been described as a softening or tenderization, Cain et al. (1958), Pearson et al. (1958; 1960) and Coleby et al. (1961). Hanson et al. (1963) found that raw, sterilized (4.5 Mrads) chicken that had been frozen and thawed developed a mushy, disintegrated texture after 3 months of storage at 21 or 38°C. Pre-irradiation heat inactivation of enzymes did not prevent textural changes.

Stadelman and Wise (1961) studied the influence of precooking before freezing of 24-hr-aged chicken fryer breasts, on the subsequent effects of sterilizing doses of radiation on texture. Irradiation produced a slight but significant ($P \leqslant 0.05$) increase in shear resistance (throughout this paper the terms "shear resistance" and "toughness" are used interchangeably) of uncooked and partially cooked meats, but cooked meat showed a negligible increase. De Fremery and Pool (1960) also reported that raw chicken muscle given a sterilizing dose of irradiation before passing through rigor mortis was tougher than unirradiated muscle. Muscle irradiated 25 hr post-mortem was not significantly different from unirradiated.

Organoleptic tests of aged, irradiation-pasteurized (0.1 and 0.5 Mrep) chicken meat by McGill et al. (1959) indicated no difference in texture between irradiated and unirradiated samples after 4 days of storage. However, MacLeod et al. (1969) reported that expert panelists were able to detect textural differences between irradiated (0.46 Mrad) and unirradiated chicken stored at 1.1 or 6.7°C for 0–3 wk. De Fremery and Pool (1960) reported that as the rate of onset of rigor mortis increased (measured by breakdown of ATP or glycogen, decline in pH or change in extensibility) the subsequently cooked meat became increasingly tougher. Irradiated (2 Mrep) muscles passed into rigor earlier (judged by tactile observations) and required greater shear force than did unirradiated muscle.

Takahashi et al. (1967) subjected chicken muscle to a standardized blending treatment and found a tendency for myofibrils to break into small fragments of one to four sarcomeres with increasing time post-mortem. It was assumed that the susceptibility to fragmentation had a direct relationship with the tenderness of chicken meat. However, Sayre (1970), using muscles treated to alter the glycolytic rate, concluded that fragmentation pattern does not provide a reliable index of tenderness.

The objectives of the studies reported herein were 1) to obtain quantitative data on the textural properties of radurized chicken muscle by measuring shear resistance and fragmentation ratio, and 2) to determine relationships between these parameters.

EXPERIMENTAL

Source of muscles

Chickens used in Experiment 1 were Single Comb White Leghorn and in Experiment 2, commercial chicken fryers (ca., 8.5 wk) obtained directly off the line at a commercial processing plant. Slaughter with an electric knife was followed by a 70-sec scald at 65°C, spin picking and evisceration. Carcasses were

Table 1—Means and results of paired "t"-tests for F-ratio and shear force—Experiment 1

Treatment	Mean values: Irrad.	Mean values: Cont.	$\bar{d}$[a]	"t" Value	"t" Prob.
	F-ratio				
0.005 Mrad					
6 hr	.470	.480	−.010	−0.267	0.787
12 hr	.607	.563	+.044	2.819	0.037*
29 hr	.718	.674	+.044	1.370	0.228
0.1 Mrad					
6 hr	.448	.480	−.032	−0.689	0.526
12 hr	.481	.531	−.050	−1.126	0.312
29 hr	.546	.667	−.121	−4.205	0.009**
0.2 Mrad					
6 hr	.404	.443	−.039	−0.996	0.367
12 hr	.467	.513	−.046	−1.373	0.227
29 hr	.534	.625	−.091	−2.628	0.046*
	Shear force[b]				
0.005 Mrad					
Anterior	18.62	19.50	−.88	−0.690	0.525
Posterior	23.13	22.84	+.29	0.138	0.864
0.1 Mrad					
Anterior	20.16	14.91	+5.25	2.863	0.035*
Posterior	28.36	21.91	+6.45	2.712	0.042*
0.2 Mrad					
Anterior	25.07	20.30	+4.77	2.576	0.049*
Posterior	28.00	25.99	+2.01	1.400	0.220

*$P \leqslant 0.05$.
**$P \leqslant 0.01$.
[a]Mean difference (irradiated minus control).
[b]lb force/gram sample.

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Table 2–Simple correlation coefficients among shear resistance, F-ratio and pH of unirradiated muscles– Experiment 1

	(hr)	Anterior shear	Posterior shear	F_{29}	F_{12}	F_6	pH 24	pH 10	pH 5.5	Means	Standard deviation
ΔpH[a]		.638	.717	−.632	−.827	−.771	.195	−.731	.795	2.92%	3.43
pH	5.5	.554	.491	−.379	−.592	−.575	.403	−.168		5.85	0.14
pH	10	−.424	−.613	.599	.680	.600	.138			5.67	0.13
pH	24	.012	−.075	−.140	−.365	−.102				5.74	0.08
F	6	−.383	−.581	.805	.814					0.47	0.11
F	12	−.423	−.514	.710						0.54	0.11
F	29	−.347	−.614							0.65	0.06
Post. shear		.805								24.1 lb/g	6.42
Ant. shear										18.2 lb/g	4.66

[a]ΔpH = Change in pH between 5.5 and 10 hr as a percent of pH at 5.5 hr.
r(.05)(17) = .456. r(.01)(17) = .575.

then placed in crushed ice for transport to the laboratory, where they were stored near 0°C until required. By random selection the pectoralis muscles from one side of each bird were excised and assigned to an irradiated treatment; muscles from the other side of the same bird were excised and used as controls. After excision, muscles were sealed in plastic bags to prevent desiccation and aged at 0 ± 1°C. Once excised, muscles were not restrained. Analyses of irradiation effects were conducted by paired-comparison of a treated muscle with its respective control and by analysis of variance.

Irradiation

A Gammacell 220 (Atomic Energy of Canada, Ltd.) containing Co 60 was used to irradiate the muscles at an approximate rate of 16,000 rads/minute. A lead attenuator was used to achieve the 5,000-rad dose. If irradiation time exceeded 10 min, samples were packed in ice.

pH determinations

5g pectoralis minor were blended at high speed with 50 ml of 0.005 M Na iodoacetate at 0°C for 1 min, Marsh (1952). The slurry was warmed to room temperature and pH measured with a semimicro combination electrode on an expanded scale pH meter.

Shear press

An Allo-Kramer Shear Press with recorder was used to shear pectoralis major muscles that had been clamped between two aluminum plates spaced ¼-in. apart and cooked for 30 min in boiling water, De Fremery and Pool (1960). In Experiment 1, two samples were cut, one from each of the anterior and posterior portions of the muscle so as to obtain a maximum size. The samples were weighed and sheared across the fibers with the multiple-blade cell. In Experiment 2, five 1.5-cm strips were cut from various areas of the muscle in such a way that the long axis of each strip was parallel to the fiber direction. Four of the strips were sheared twice and the fifth sheared once with the single-blade cell. The nine shear resistance determinations were averaged for analysis.

Fragmentation

Myofibers were mechanically fragmented by blending 1g of raw muscle from the posterior portion of p. major with 100 ml of 0°C, 0.08 M KCl for 1 min at medium speed in an Osterizer blender, Smith et al. (1969). From a drop of the suspension placed under a phase contrast microscope, photomicrographs (625×) of approximately 175 fibrils per sample were taken. The F-ratio was determined by calculating the proportion of the total number of fibrils found to contain four or fewer sarcomeres, Takahashi et al. (1967).

Table 3–Simple correlation coefficients between final F-ratio and shear resistance–Experiments 1 and 2

Experiment 1			Experiment 2		
Shear resistance measurement	n	r	Shear resistance measurement	n	r
Anterior (irr. and cont.)	36	−.272	Average (irr. and cont.)	72	−.358**
Posterior (irr. and cont.)	36	−.482**	Location K[a] (irr. and cont.)	72	−.263*
Anterior (cont. only)	18	−.347	Average (cont. only)	36	+.030
Posterior (cont. only)	18	−.614**	Location K (cont. only)	36	−.072
Anterior (irr. only)	18	−.172	Average (irr. only)	36	−.319
Posterior (irr. only)	18	−.367	Location K (irr. only)	36	−.113

*P ≤ 0.05. **P ≤ 0.01.
[a]Location K designates the muscle strip taken from a position adjacent to the sample selected for determination of F-ratio.

RESULTS

Experiment 1

Six fowls were assigned to each of three irradiation treatments, 0.005, 0.1 and 0.2 Mrads. Starting at 4 hr post-mortem, pectoralis muscles from both sides of the bird were excised and at 5 hr those from one side were irradiated. Tactile observation confirmed that the muscles were in rigor mortis at this time. pH slurries of p. minor were made at 5.5, 10 and 24 hr post-mortem, and F-ratios taken on p. major at 6, 12 and 29 hr post-mortem. The remaining p. major was cooked at 35 hr post-mortem and shear resistance of anterior and posterior samples determined.

Paired "t"-test analyses failed to reveal significant effects of irradiation treatment on pH values or changes in pH with time post-mortem.

The 0.1- and 0.2-Mrad doses did consistently reduce the F-ratio, although few paired comparison tests of F-ratio were significant.

Analysis of the control muscles showed that the posterior portion had a significantly higher ($P \leq .0001$) shear resistance than the anterior; 23.58 and 18.24 lb/g, respectively. Thus, paired comparison tests of shear resistance (Table 1) were conducted separately on the anterior and posterior portions.

Both irradiation doses increased the shear resistance of the cooked muscle and the increase was significant ($P \leq 0.05$) in all cases except the posterior portion given 0.2 Mrad.

Correlations of pH and ΔpH with shear resistance and F-ratio indicated that a high shear resistance (35 hr) and low F-ratio were associated with a high pH at 5.5 hr and a low pH at 10 hr. In addition, greater shear resistance and a lower F-ratio were found in samples with a high percentage decline in pH between 5.5 and 10 hr. The percentage decline in pH between 5.5 and 10 hr was positively and significantly related to pH at 5.5 hr. All of these relationships were particularly evident in control samples (Table 2) and the general pattern was similar in irradiated samples. These results appear to contradict previous findings that a rapid decline in postmortem pH is associated with high shear resistance, De Fremery and Pool (1960). However, our results might be explained by a differential shortening of muscles after excision at 4 hr post-mortem, the amount of shortening depending inversely upon the pH and degree of inextensibility established by that time, Marsh and Leet (1966).

Table 4—Means and results of paired "t"-tests of final F-ratios for irradiated and control muscles—Experiment 2

Dose (Mrad)	Irrad. time (hr)	Mean values Irrad.	Cont.	$\bar{d}$[a]	"t" Value	"t" Prob.
0.1	2	.622	.747	−.125	−4.220**	0.009
	5	.670	.754	−.084	−1.813	0.128
	12	.708	.760	−.052	−2.289	0.069
0.3	2	.610	.757	−.147	−4.766**	0.006
	5	.596	.716	−.120	−2.353	0.064
	12	.662	.784	−.122	−5.206**	0.004
0.1	Pooled	.667	.754	−.087	−4.360**	0.001
0.3	Pooled	.623	.752	−.129	−6.389**	0.001
Pooled	2	.616	.752	−.136	−6.587**	0.001
	5	.633	.735	−.102	−3.064*	0.011
	12	.685	.772	−.087	−4.641**	0.001

*P ≤ 0.05. **P ≤ 0.01.
[a]Mean difference (irradiated minus control).

Table 5—Means and results of paired "t"-tests of shear resistance for irradiated and control muscles—Experiment 2

Dose (Mrad)	Time (hr)	Mean shear Irrad.	Cont.	$\bar{d}$[a]	% Toughening	"t" Value	"t" Prob.
		lb force					
0.1	2	14.21	10.75	3.46	32.2	2.691*	0.024
	5	12.72	10.05	2.67	26.6	3.457**	0.007
	12	11.64	10.34	1.30	12.6	1.363	0.204
0.3	2	14.70	11.06	3.64	32.9	3.979**	0.003
	5	12.45	9.74	2.71	27.8	2.702*	0.024
	12	13.66	11.35	2.31	20.4	2.590*	0.028
0.1	Pooled	12.86	10.38	2.48	23.9	4.161**	0.001
0.3	Pooled	13.60	10.72	2.88	24.8	5.420**	0.001
Pooled	2	14.45	10.90	3.55	32.4	4.619**	0.001
	5	12.59	9.90	2.69	27.2	4.367**	0.001
	12	12.65	10.84	1.81	16.6	2.793*	0.011

*P ≤ 0.05. **P ≤ 0.01.
[a]Mean difference (irradiated minus control).

The shear resistance of anterior and posterior samples from the p. major were strongly correlated. Correlation coefficients for control and irradiated tissues singly (n = 18) and in combination (n = 36) were + .805, + .798 and + .798, respectively.

The shear value for the posterior portion of p. major adjacent to the location sampled for determination of F-ratios was significantly ($P \leq 0.01$) correlated with the final F-ratio, except when the irradiated samples were considered alone (Table 3). Anterior shear values were poorly correlated to final F-ratio in all cases.

Experiment 2

Six carcasses were assigned to each of six treatments consisting of all combinations of two irradiation doses (0.1 and 0.3 Mrad) and three times of application (2, 5 and 12 hr post-slaughter). The 2-, 5- and 12-hr irradiation times were selected to correspond nominally to the before, during and after rigor states, respectively. Tactile observation of the carcasses indicated that the carcasses at 2 and 12 hr post-mortem were relatively flexible compared to the carcasses at 5 hr, which exhibited the typical rigidity associated with the rigor state. Final pH values from p. minor and F-ratios from the posterior of p. major were obtained after storage at 0°C for 48 and 50 hr post-mortem, respectively. The average shear resistance of p. major from the nine shears per muscle was obtained from muscle cooked after 60 hr of storage at 0°C. To obtain more statistical certainty, four additional carcasses per treatment combination (24 carcasses) were included for shear resistance evaluation.

None of the dose-time treatment combinations had a significant effect on final pH.

The mean F-ratio of each of the treatment groups was lower than the mean of the corresponding control. With one exception, the differences were either significant ($P \leq 0.01$) or approached significance ($P \leq 0.07$) (Table 4). A paired comparison test of the pooled within-dose data indicated that mean F-ratios of the irradiated samples were significantly ($P \leq 0.001$) lower than the controls. When the two doses were combined for each application time, the mean F-ratio of the irradiated samples was significantly ($P \leq 0.011$) lower than the corresponding control in all three cases.

Each of the six irradiation treatments increased the shear resistance of muscles compared to their controls, although not significantly for 0.1 Mrad administered at 12 hr (Table 5). The toughening effect decreased as the postmortem holding time prior to irradiation increased. When the data were pooled by irradiation level and time of application, each treatment was found to have a significant toughening effect ($P \leq 0.011$).

Differences in shear resistance and F-value between the irradiated and control muscles of each carcass were subjected to a 2 x 3 factorial analysis of variance (dose x time of application), but the large variation in treatment effect among carcasses treated alike resulted in nonsignificance of differences between dose levels and among application times.

Correlations between F-ratio and shear resistance were significant only for the pooled data (Table 3). Correlations of pH with both average shear and F-ratio were consistently low and nonsignificant.

The mean changes ($\bar{d}$) in shear resistance and F-ratio between the irradiated muscles and the controls decreased as time of irradiation increased (Tables 4 and 5). The linearly decreasing change in shear resistance can be extrapolated to reach zero at approximately 24 hr post-mortem. The mean changes in shear resistance and F-ratio were significantly ($P \leq 0.05$) correlated ($r = + 0.857$; $n = 6$).

DISCUSSION

pH VALUES between 5.5 and 48 hr were not affected significantly by irradiation as early as 2 hr post-mortem. Earlier experiments, Whiting (1970), indicated that pH decline during the first 4 hr post-mortem was not affected by irradiation applied 30 min post-mortem. Thus, the toughening effect of irradiation does not seem to be mediated by hastening the onset of rigor mortis as reported by De Fremery and Pool (1960) for pre-rigor application of mechanical beating, postmortem temperature extremes and a sterilizing dose of electron irradiation. This conclusion is supported by the occurrence of toughening from irradiation at 5 and 12 hr post-mortem, i.e., after the onset of rigor. The extrapolation of the irradiation-induced increase in shear resistance to zero at 24 hr agrees with the reports of other workers, McGill et al. (1959), De Fremery and Pool (1960), Coleby et al. (1961) and Hanson et al. (1963), who irradiated samples after 24 or more hr of postmortem aging.

On the basis of these results and evidence in the literature, it seems reasonable to hypothesize that irradiation affects the extent of breakdown at the Z line and the Z line-I band junction associated with tenderization, Davey and Dickson (1970) and Fukazawa et al. (1969), and that the factors responsible for this breakdown, perhaps sarcoplasmic proteins, Fukazawa et al. (1969), are especially susceptible to radiation inhibition or destruction during early postmortem aging. This hypothesis would explain the minor effects on shear resistance from irradiation after 24 hr of aging reported by De Fremery and Pool (1960) and Stadelman and Wise (1961). The soft or mushy texture frequently reported in

aged, irradiation-sterilized raw meats may be attributed to enhancement of residual proteolytic activity by elevated storage temperatures.

Decreases in F-ratio after irradiation were consistent with increases in shear resistance. However, the wide variation among duplicates made it difficult to show significant responses to a treatment or significant correlation of F-ratios with shear resistance. Thus, although average treatment-induced changes in shear resistance and F-ratio are correlated, the F-ratio does not provide a consistently reliable index of tenderness on an individual-sample basis. Sayre (1970) similarly reported that changes in shear resistance are not consistently reflected by changes in fragmentation ratio. Furthermore, the selection of adjacent sample locations for determination of shear resistance and fragmentation ratio does not consistently increase the reliability.

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PALATABILITY OF BONELESS FRESH PORK HAM AND LEG OF LAMB PREPARED BY INTERRUPTED COOKING PRIOR TO FROZEN STORAGE

SUMMARY—Boneless fresh pork hams and legs of lamb were scored acceptable or above in palatability when prepared by the "roasteak" procedure, involving preroasting to 44°C, slicing and chilling or freezing for as long as 6 months before grilling. Significant differences in means for flavor and juiciness indicated that an antioxidant dip (sodium tripolyphosphate-sodium ascorbate) is advisable for pork roasteaks subjected to freezing, but no such advantage was noted for antioxidant treatment of lamb roasteaks. Although there were differences in tenderness due to muscle within roasteak slices, meat from both muscles of both species was acceptable to the panel in this attribute. Total cooking losses for the roasteak method were higher than for one-step roasting.

INTRODUCTION

IF INTERRUPTED COOKING procedures for meats can be used without sacrificing quality, convenience can be introduced by releasing some preparation time just before serving meals. Previous research, Korschgen et al. (1963; 1964), demonstrated than an interrupted cooking procedure, "roasteak," was suitable for large boneless cuts of beef such as shoulder clod and chuck roll roasts. This roasteak method involved preroasting the meat to an internal temperature of 44°C, slicing the roasts and grilling the slices just before serving. This procedure provides meat with the popular broiled flavor, but requires less grilling time. The process can be interrupted after preroasting by either chilling or freezing the meat. Palatability of frozen roasteak was protected for as long as 1 yr by dipping the slices of meat in an aqueous antioxidant solution of sodium tripolyphosphate and sodium ascorbate before freezing, Baldwin and Korschgen (1968).

Since the roasteak procedure was previously found to be suitable for beef, it was the purpose here to extend the study of the roasteak procedure to pork and lamb. Palatability of boneless fresh pork ham and leg of lamb prepared by this interrupted cooking procedure was investigated. The influence of freezing on palatability was determined for both species of meat by using the standard roasteak procedure as the control.

EXPERIMENTAL

Preparation of meat

Paired (from right and left sides of the carcass) fresh boneless hams and legs of lamb from eight animals of each species were kept in freezer storage (−21 ± 2°C) until tested (approximately 10 days). Prior to cooking, the roasts were placed in a refrigerator (5°C) for 2 days and attained mean internal temperatures of −2 and −3°C, respectively, for fresh hams and legs of lamb. Average weights were 5.8 kg for fresh hams and 3.6 kg for legs of lamb.

The meat was cooked uncovered to an internal temperature of 44°C in an oven heated to 149°C. Temperatures of meat and ovens were measured with iron-constantan thermocouples connected to a recording potentiometer. Times required to reach 44°C were 42 min/kg for pork (range 31–53 min/kg) and 46 min/kg for lamb (range 33–55 min/kg). After removal from the oven, the meat was allowed to stand at room temperature while the internal temperature of the meat continued to rise. For fresh hams, the maximum mean internal temperature was 60°C (range 55–63°C). The final average temperature attained in lamb roasts was 56°C (range 52–61°C). This rise in temperature was comparable to that found in previous research on shoulder clods, chuck rolls and bottom rounds of beef, with raw weights averaging 4.5, 5.4 and 6.0 kg, respectively, Korschgen et al. (1963; 1964).

Meat was weighed before and after cooking. From these data and from weights of drippings, percent evaporation and drip losses were calculated.

The preroasted meat was refrigerated (5°C) overnight, weighed and sliced 11/16-in. thick. An average of 13 slices, or roasteaks was obtained from both pork and lamb roasts. Average weights for slices were approximately 1 lb for pork and 8 oz for lamb. Slices from one of a pair of roasts, randomly chosen, were used for

Table 1—Mean panel scores[a] for palatability of boneless fresh pork hams prepared by the roasteak procedure

Treatment	Muscle[b]	Length of frozen storage: 0	1 Day	1 Month	3 Months	6 Months
				Flavor		
Frozen-antioxidant-dipped	T		6.5	7.5	6.6	7.0
	M		6.4	7.0	6.8	6.4
Frozen-untreated	T		6.3	7.2	6.5	6.3
	M		6.5	6.9	6.8	6.3
Control	T	6.8	7.6	7.1	6.8	
	M	6.5	6.6	7.3	7.2	
				Tenderness		
Frozen-antioxidant-dipped	T		7.0	7.3	7.0	6.9
	M		6.0	6.6	6.4	6.0
Frozen-untreated	T		6.5	7.6	7.0	7.0
	M		5.6	6.3	6.5	6.0
Control	T	7.8	8.0	7.3	7.1	
	M	6.8	5.7	6.6	6.7	
				Juiciness		
Frozen-antioxidant-dipped	T		6.7	7.5	6.9	6.8
	M		6.2	7.1	6.2	5.8
Frozen-untreated	T		6.3	7.3	6.4	6.5
	M		5.9	6.9	6.2	5.9
Control	T	7.2	7.8	7.2	7.0	
	M	6.8	6.9	7.0	6.8	
				General acceptability		
Frozen-antioxidant-dipped	T		6.8	7.5	6.7	6.9
	M		6.2	7.0	6.5	6.1
Frozen-untreated	T		6.5	7.3	6.5	6.4
	M		6.1	6.8	6.5	6.0
Control	T	7.1	7.8	7.3	7.0	
	M	6.7	6.3	7.1	7.2	

[a]Scale: 9, Like extremely, to 1, Dislike extremely; n = 24. No significant ($P < .05$) differences among means for flavor and for juiciness. $LSD_{.05}$ for tenderness, .64 for general acceptability, .58.
[b]T = Semitendinosus. M = Semimembranosus.

Table 2—Mean panel scores[a] over all storage periods for palatability of boneless fresh pork hams and legs of lamb prepared by the roasteak procedure

Treatment	Flavor	Tender-ness	Juici-ness	General accepta-bility
	Fresh pork ham			
Frozen-antioxidant-dipped	6.8	6.7	6.7	6.7
Frozen-untreated	6.6	6.6	6.4	6.5
Control	7.0	7.0	7.1	7.1
$LSD_{.05}$	0.21	0.23	0.20	0.21
	Leg of lamb			
Frozen-antioxidant-dipped	6.9	6.5	6.4	6.7
Frozen-untreated	6.8	6.4	6.5	6.5
Control	7.3	6.8	7.1	7.1
$LSD_{.05}$	0.21	0.27	0.22	0.21

[a]Scale: 9, Like extremely, to 1, Dislike extremely; n = 192.

Table 3—Analyses of variance of panel scores for boneless fresh pork hams prepared by the roasteak procedure

Source of variation	Degrees of freedom	Mean square: Flavor	Tenderness	Juiciness	General acceptability
Judges (J)	5	12.11**	22.16**	17.90**	13.00**
Storage period (SP)	3	11.81**	2.86	19.66**	9.81**
Treatment (T)	2	7.35**	11.36**	23.31**	13.84**
Muscle (M)	1	2.46	125.63**	35.50**	27.13**
SP × T	6	1.45	4.53**	1.39	2.38*
SP × M	3	3.54*	4.57*	0.59	3.50*
T × M	2	1.16	1.00	0.94	0.42
SP × T × M	6	1.34	2.54	1.17	1.99
Error	547	1.16	1.27	1.01	1.05
Coefficient of variation (%)		15.88	16.76	14.97	15.19

*P < .05; **P < .01.

the roasteak frozen samples. Slices from the other roast of that pair were designated as control samples and were not subjected to freezing after preroasting. From the roast assigned to freezing, two adjacent slices, treated alike, were assigned for subjective and objective evaluation. Treatments were assigned randomly to the sets of adjacent slices. Details for handling the treated and control samples were as follows: 1) Frozen-untreated: Slices packaged in polyester bags (Scotchpak bags and sealer, Kapak Industries, Inc., Minneapolis, Minn.) and kept in frozen storage (−34 ± 2°C) for 1 day, 1, 3 and 6 months. 2) Frozen-antioxidant-dipped: Slices immersed for 30 sec in antioxidant solution (10g sodium tripolyphosphate and 2.7g sodium ascorbate brought to 1-liter volume with water) and drained 10 sec before packaging and handling as just described. 3) Control (zero storage): Slices subjected neither to freezing nor to antioxidant treatment after preroasting.

The experimental design requires two replications within each pair of roasts from an animal. Slices from paired roasts from two animals (four replications) were assigned to each storage period (1 day, 1, 3 and 6 months). Control roasts were kept frozen in the raw state until they were prepared for evaluation for the designated storage period. Even though paired roasts were randomly assigned to storage period, animal variation was confounded with storage period in this experimental design.

It has been suggested that raw lamb and pork chops maintain high quality through 4 months of storage at −18°C, USDA (1967; 1969). For cooked meats, a maximum of 3 months of storage was suggested for this temperature. Our longest storage (−21 ± 2°C) period (6 months) was chosen to exceed the above recommendations, and 1 and 3 months evaluations were chosen as appropriate monitoring periods. The 1-day testing period was included to assess the effects of freezing with a minimum of storage influence.

Preparation of fresh pork ham roasteak for taste panel

2 Days of testing were required to complete evaluation of two replicates from each of the two muscles (semitendinosus and semimembranosus) within a slice of pork. The order of preparing and serving samples for evaluation was randomized. Slices of meat were weighed before and after grilling and losses due to grilling calculated. Frozen pork slices were placed on an electric grill (171°C) and cooked for 11 min on one side and 7 min on the other side. To obtain the same internal temperature (68°C), control pork slices were grilled at 204°C for 4 min on one side then 3 min on the other side.

Portions of meat, approximately 1.25-cm-square, were cut from the semitendinosus and from the semimembranosus muscles for sensory evaluation. Each judge received a cube of meat from the same position within each muscle from each roasteak. A warm-up sample from a freshly prepared roasteak was served at the beginning of each tasting session.

The meat was approximately 61°C when served to the panel in randomly coded, preheated 50-ml beakers capped with aluminum foil. The beakers were preheated in a sand bath improvised from a 20-cm cake pan. The sand bath functioned both as a means for keeping the samples warm and as a tray for transporting the beakers to the serving windows.

Preparation of lamb roasteak for taste panel

The control slices of lamb were grilled at 191°C for 4 min on each side. To achieve the same internal temperature (69°C), the frozen slices were grilled at 163°C for 10 min on one side and then 8 min on the other side. All other preparation of lamb was the same as for fresh ham roasteak.

Sensory evaluation

5 Days of training were conducted for seven experienced judges before collection of data for the first storage period. 1 Day of training simulating panel procedures preceded the data-collecting sessions for the 1-, 3- and 6-month storage periods. Meat was judged for flavor, tenderness, juiciness and general acceptability on a 9-point hedonic scale from 9, Like extremely, 5, Neither like nor dislike to 1, Dislike extremely.

Objective testing

Samples for shearing were taken from slices of meat adjacent to those used for sensory testing, and prepared the same as those slices judged by the panel. Slices were grilled individually and cored and sheared immediately. Two and four 1-in. cores from the semitendinosus and semimembranosus muscles, respectively, from two treated and two control pork slices from each animal were tested with the Warner-Bratzler shear. For lamb, one and two 2.54-cm cores for shearing were cut from the semitendinosus and semimembranosus muscles, respectively, from two treated and from four control slices from each animal.

Statistical analyses

In the analyses of variance, judges were considered the testing instrument. Consequently, sums of squares for all interactions involving judges were included in the error term. Data for controls (zero storage) were included with each storage period. To locate significant differences among means, the test for least significant difference, Steel and Torrie (1960), was applied.

RESULTS & DISCUSSION

Cooking losses

The mean loss due to preroasting the fresh pork hams was 22%. 13% of this loss resulted from evaporation and 9%

from drip. Average losses occurring during grilling of the control, frozen-untreated and frozen-antioxidant-dipped pork slices were 17, 25 and 23%, respectively. The mean percent loss due to grilling the control pork slices was significantly (LSD, $P < .05$) less than the mean percent losses for slices subjected to freezing. Preroasting and grilling losses are not precisely additive, because the percent loss for preroasting was calculated on the basis of weight of the whole roast, and only a portion of the roasts was sliced for roasteak. Also, as previously pointed out, different times and temperatures were required for grilling control and frozen slices to achieve the same internal temperature. However, if the total cooking losses for the roasteak method are approximated by adding preroasting and grilling losses, they exceed those for one-step roasting recorded in the literature. According to weights, for raw and cooked boned fresh ham butts reported by Carlin et al. (1968), total cooking losses ranged from 33–34%, and higher losses were associated with higher internal temperature.

The difference in grilling losses between frozen slices with and without antioxidant treatment was not significant. Interest in phosphates has not been confined to their antioxidant property but has extended to their influence on palatability and retention of juices, Tims and Watts (1958), Rust (1963) and Miller and Harrison (1965). However, methods of application and concentration of phosphates were not comparable to the dipping procedure utilized in this study, and similar results should not be expected.

The mean preroasting loss for lamb, 16%, was lower than for pork and was similar in amount to that reported for beef, Korschgen et al. (1963) and Baldwin and Korschgen (1968). The mean grilling losses for control lamb slices, 17%, was significantly lower (LSD, $P < .01$) than the 25% for frozen-untreated slices and the 28% for the frozen-antioxidant-dipped slices. When total cooking losses for lamb are estimated by adding preroasting and grilling losses for this roasteak procedure, they exceed the 17.3–25.9% losses reported by Paul et al.

Table 4—Mean panel scores[a] for palatability of boneless legs of lamb prepared by the roasteak procedure

Treatment	Muscle[b]	Length of frozen storage: 0	1 Day	0	1 Month	0	3 Months	0	6 Months
				Flavor					
Frozen-antioxidant-dipped	T		7.3		6.8		6.9		7.0
	M		7.2		6.6		6.7		6.6
Frozen-untreated	T		6.9		6.8		6.6		6.5
	M		7.2		6.6		6.9		6.6
Control	T	7.1		7.5		7.5		7.2	
	M	7.2		7.1		7.3		7.4	
				Tenderness					
Frozen-antioxidant-dipped	T		6.5		6.8		6.8		6.7
	M		6.0		6.3		6.4		6.2
Frozen-untreated	T		5.0		6.4		6.8		7.1
	M		6.9		6.2		7.0		5.6
Control	T	7.3		7.2		7.0		7.5	
	M	6.0		6.6		6.5		6.5	
				Juiciness					
Frozen-antioxidant-dipped	T		6.8		6.4		6.4		6.6
	M		6.5		6.4		5.9		6.5
Frozen-untreated	T		6.3		6.1		6.0		6.3
	M		7.3		6.5		6.6		6.5
Control	T	7.3		7.2		6.9		6.8	
	M	6.8		7.0		7.5		7.0	
				General acceptability					
Frozen-antioxidant-dipped	T		7.1		6.8		6.8		6.8
	M		6.7		6.5		6.5		6.5
Frozen-untreated	T		6.2		6.6		6.4		6.5
	M		7.3		6.3		6.7		6.2
Control	T	7.2		7.3		7.3		7.3	
	M	6.8		6.9		7.1		7.3	

[a]Scale: 9, Like extremely, to 1, Dislike extremely; n = 24. No significant ($P < .05$) differences among means within attributes.
[b]T = Semitendinosus. M = Semimembranosus.

Table 5—Analyses of variance of panel scores for boneless legs of lamb prepared by the roasteak procedure

Source of variation	Degrees of freedom	Mean square: Flavor	Tenderness	Juiciness	General acceptability
Judges (J)	5	33.60**	20.13**	27.26**	31.79**
Storage period (SP)	3	2.48	5.02*	2.22	0.64
Treatment (T)	2	14.94**	10.45**	24.19**	20.38**
Muscle (M)	1	0.44	25.00**	2.38	1.78
SP × T	6	1.81	1.92	1.94	0.93
SP × M	3	0.78	6.89**	0.29	1.26
T × M	2	1.69	10.41**	6.36**	3.44**
SP × T × M	6	0.44	8.67**	2.40	2.30
Error	547	1.10	1.79	1.21	1.11
Coefficient of variation (%)		15.04	20.42	16.57	15.56

*P < .05. **P < .01.

Table 6—Analyses of variance of Warner-Bratzler shear values for boneless fresh pork hams and legs of lamb prepared by the roasteak procedure

Source of variation	Pork: Degrees of freedom	Pork: Mean square	Lamb: Degrees of freedom	Lamb: Mean square
Storage period (SP)	3	39.65*	3	7.59
Muscle (M)	1	54.38	1	125.02**
Treatment (T)	1	33.75	1	74.27*
SP × M	3	6.78	3	0.28
SP × T	3	10.01	3	26.18
M × T	1	23.01	1	0.00
SP × M × T	3	11.06	3	0.95
Error	176	14.64	128	15.71
Coefficient of variation (%)		33.88		33.70

*P < .05. **P < .01.

(1964a; 1964b) for roast leg of lamb.

Palatability of pork roasteak

Mean panel scores for flavor, tenderness, juiciness and general acceptability for all treatments of pork roasteak indicated that the meat was acceptable, or liked, by the panel throughout the entire study. Mean scores ranged from 6.3–7.6 for flavor; 5.6–8.0 for tenderness; 5.8–7.8 for juiciness and 6.0–7.8 for general acceptability (Table 1).

The mean panel score for flavor of the control pork samples was significantly higher ($P < .05$) than that for the frozen-untreated slices. However, the mean score for the flavor of the antioxidant-dipped samples was not significantly different from the mean scores for either the control or frozen-untreated roasteaks (Table 2).

The significant effect of storage ($P < .05$) on flavor scores for fresh ham roasteak was due to a higher mean value for this attribute at the 1-month evaluation than at the other periods and was not related to a decrease in means as storage time increased. Although the analysis of variance of panel scores for flavor of fresh ham revealed a significant interaction ($P < .05$) between storage period and muscle (SP x M), there was no consistent trend in mean scores for either the semitendinosus or semimembranosus muscle to be better than the other (Tables 1, 3).

Mean tenderness scores for the semitendinosus muscle of fresh ham roasteaks were significantly higher ($P < .05$) than those for the semimembranosus muscle at each storage period. Also, there was a significant ($P < .01$) effect for the interaction of storage period and treatment (SP x T), but control pork samples were rated significantly more tender than frozen-untreated samples only for the 1-day evaluation period (Tables 1, 2 and 3).

In the analysis of variance, there were significant ($P < .01$) differences in juiciness of pork roasteaks due to storage periods, treatment and muscle. There is no logical explanation for the significantly ($P < .05$) higher means for juiciness of pork samples tested after 1 month of storage than for those evaluated at the other storage periods. Over all storage periods, the mean score for juiciness of control samples was significantly higher ($P < .05$) than for frozen roasteaks. Of the frozen slices, the antioxidant-dipped samples were rated significantly higher ($P < .05$) in juiciness than the untreated samples (Tables 1, 2 and 3). As previously mentioned, the antioxidant dip is not comparable to the techniques of phosphate application reported in the literature, Tims and Watts (1958), Rust (1963) and Miller and Harrison (1965). The apparent retention of juiciness indicated by panel scores in the antioxidant-dipped slices may or may not have been related to the phosphate in the dip.

The means for the semitendinosus muscle of pork were always higher for general acceptability than those for the semimembranosus muscle. However, this difference was significant ($P < .05$) only for the first two evaluation periods. Examination of the mean scores revealed a nearly constant quality of general acceptability for the control pork samples throughout the entire study. Mean scores for general acceptability of control slices were significantly higher ($P < .05$) than means for frozen samples at 3 and 6 months' evaluations (Tables 1, 2 and 3).

Palatability of lamb roasteak

Mean panel scores for flavor, tenderness, juiciness and general acceptability of lamb roasteak for all storage periods were above the acceptable level. Means ranged from 6.5–7.5 for flavor, 5.0–7.5 for tenderness, 5.9–7.5 for juiciness and 6.2–7.3 for general acceptability (Table 4).

In the analysis of variance there were significant ($P < .01$) main effects for treatment for all attributes of lamb roasteak. Over all storage periods, mean scores for the control slices were significantly higher ($P < .05$) than for frozen slices. The semitendinosus muscle of lamb was more tender than the semimembranosus muscle at all storage periods, but this difference was significant only at the 6-month evaluation period. The control samples of lamb taken from the semitendinosus muscle were rated significantly more tender than samples of this muscle subjected to freezing. Mean scores for flavor, juiciness and general acceptability did not decline significantly as storage time lengthened for lamb roasteaks (Tables 2, 4 and 5).

Warner-Bratzler shear values

According to the analysis of variance of Warner-Bratzler shear values for pork roasteaks, there was a significant ($P < .05$) effect of storage. Mean shear values tended toward a linear increase as storage time progressed. Mean shear values for the semimembranosus muscle (5.3 kg/2.54-cm core) were higher than the semitendinosus muscle (4.8 kg/2.54-cm core) of pork roasteaks, but the difference was not significant. However, as previously mentioned, panel scores for tenderness indicated a significant ($P < .05$) difference between the roasteaks from the two muscles (Tables 3 and 6). The correlation coefficient (−.29) between mean scores for tenderness and means for shear values of pork roasteaks was not statistically significant.

Warner-Bratzler shear values were significantly ($P < .05$) lower for control lamb roasteaks (5.1 kg/2.54-cm core) than for slices subjected to freezing (5.8 kg/2.54-cm core). There was no difference due to storage. As shown in Table 6, mean shear values were significantly lower ($P < .01$) for the semitendinosus muscle (4.7 kg/2.54-cm core) than for the semimembranosus muscle (5.6 kg/2.54-cm core). The correlation coefficient between mean panel scores for tenderness and mean shear values of lamb roasteaks was −.47 ($P < .05$).

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AN EMULSION METHOD FOR RAPID DETERMINATION OF FAT IN RAW MEATS

SUMMARY—There is a need for a rapid, safe, precise and practical method for determining fat in raw meats, while insuring sample representativeness. The method developed consists of preparing an emulsion from the meat to be analyzed, and purposely breaking down a portion by heating under alkaline conditions to separate the fat which is measured in graduated Babcock bottles. A good correlation was found between this method taking about 10 min and Salwin's method (Babcock technique) taking about 20–25 min as well as the official ether extraction method taking about 24 hr. The new method seems applicable to samples varying between 4 and 40% fat. It also permits the analysis of large samples of almost any desirable or practical size by analyzing a homogeneous aliquot of a prepared emulsion. It also applies directly to the analysis of meat emulsions prepared in the manufacture of sausage products, for quality control purposes.

INTRODUCTION

CONSUMER protection and meat trading among various firms often require that the lean or fat contents of meat be known as quickly as possible because commercial value is based on lean content. In the preparation of most processed meat products, the functional properties of the meat ingredients are largely dependent upon their lean content which also influences the organoleptic quality of the finished product. The lean contents supply the protein which allows to satisfy the maximum permissible ratio of moisture to protein in these products. Consequently, for reasons of economy and quality, lean or fat determinations of the meat ingredients and of the resulting emulsions are important in quality control. These considerations clearly demonstrate the need for a rapid, precise, safe, practical and simple method of measuring fat in meat products.

Conscious of this need, some research workers devoted their efforts to finding such a method by improving or accelerating available methods.

An ultrasonic apparatus designed to measure the total fat content of a live animal was described by Cutler (1962). The Madigan Co. (Anonymous, 1961) developed a method called Anyl-Ray for measuring fat in meat; it is based on the principle that the penetration of gamma rays is proportional to the fat content. This method is rapid but the advantage is partially nullified by the high cost and special type of equipment required. Variability in the packing technique as well as the presence of salt (such as in processed products) would interfere with the results.

In 1965, Goss described an indirect method of measuring fat in meat called "Juice Extraction by Indirect Heating." Samples of 15–25g of ground meat are heated for 2–3 min and an accuracy of 1½% is claimed. Bellis et al. (1967) were also able to measure the fat content in meat by means of thermal extraction. This method is relatively rapid (15 min) and requires a relatively large sample (2 oz or 56.7g). The results showed a highly significant correlation (1% level) with the official AOAC (1965) method, over the range covered 14–29%.

Other methods of a basically chemical nature have been developed for measuring fat in meat. They present some advantages and disadvantages. A few seem to be rapid and simple but have a major disadvantage: the sample used is usually too small to be sufficiently representative of the material analyzed. In 1954, Kelley et al. modified the classical Babcock method for fat determination in milk by using a mixture of sulfuric and acetic acids, instead of sulfuric acid alone. Salwin et al. (1955) replaced sulfuric acid in the Babcock method with an equal mixture of glacial acetic acid and 60% perchloric acid which completely digests proteins, cereals and spices thus liberating a clear column of liquid fat easy to read. According to the authors, results agreed closely (within 0.5%) with the AOAC (1965) ether extraction method. The method of Salwin et al. (1955) was generally found satisfactory, except it took about 20–25 min and presented an explosion danger.

Windham (1957) compared the above described method with the AOAC method and a new technique called the Steinlight method which requires 50–75g samples and about 30–40 min. He concluded that all three methods should be acceptable for plant control, market survey and screening use. Anderson et al. (1962) described a method called the Banco test based on the principle of the "detergent test" used for determining fat in milk and milk products. The accuracy of the results seemed quite satisfactory but the method requires considerable handling; a small sample (9g) is used and it takes about 30 min. Whalen (1966) also modified the Babcock method: a quick digestion with hot HCl, followed by a solution of the protein hydrolysis products in hot dimethyl sulfoxide. The mean variation from the Mojonnier method was 0.5%. The size of the sample (9g) is still a limiting factor and dimethyl sulfoxide is a strong skin irritant. Using some of the Babcock equipment or some alternate material, Moreau et al. (1971) developed a new rapid, safe and simple test using samples of 18g in most instances. In this test meat is directly heated in an alkaline medium and the liquid fat that separates is measured. The test takes about 7 min. Its results agree very closely with the method of Salwin et al. (1955) and with the AOAC ether extraction method.

Methods for determining simultaneously both fat and moisture contents in meat have been studied. Davis et al. (1966) obtained significant correlation coefficients with official methods for determining fat and moisture contents. Their method consists of distillation of moisture and removal of fat using n-butyl ether. It takes from 2–2½ hr and the sample size (10g) is relatively small. Ben-Gera and Norris (1968) accomplished the same determinations directly by means of infrared spectrophotometry. The results were satisfactory since the standard error for fat content was ± 2.12 and for moisture within ± 1.4%. This method still needs improvement, according to the authors, and evidently requires expensive equipment.

From this review, it appears that a precise, rapid, safe, practical and simple method for measuring fat in meat products is lacking. The method described in this article, taking about 10 min, seems to satisfy these requirements with samples varying from 4 to about 40% fat content.

MATERIALS & METHODS

Apparatus

The apparatus employed consists of: a commercial-type meat grinder equipped with a head plate having holes 1/8 in. diam; a multi-mix homogenizer, Model MM-1, of Lourdes; a macro homogenizer, Model 45, of Virtis; a constant temperature water bath maintained at 99°C; a pH-meter; and a Babcock cream bottle (9g capacity) graduated to 50% (Kimax brand, U.S. government standard).

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Table 1–Comparative fat contents obtained with three methods of fat analysis in raw beef

New suggested method		Salwin's modified Babcock method		Goldfisch ether extraction method	
Mean % Fat[a]	Standard deviation (±)	Mean % Fat[a]	Standard deviation (±)	Mean % Fat[a]	Standard deviation (±)
5.9	0.36	5.8	0.36	4.9	0.61
8.3	0.17	6.8	0.25	7.3	0.50
7.6	0.40	7.7	0.29	7.7	0.12
9.0	0.76	9.9	0.00	8.7	0.87
8.5	0.50	9.4	0.16	8.8	0.75
10.1	0.12	10.2	0.17	10.6	0.14
11.3	0.69	12.6	0.29	12.7	0.35
13.2	0.46	14.0	0.28	13.1	0.15
13.7	0.50	13.2	0.45	13.9	0.23
16.9	0.87	16.9	0.29	15.2	0.31
16.1	1.15	15.7	0.15	16.3	0.12
17.9	1.15	15.5	0.29	16.8	0.25
18.1	1.04	18.2	1.33	17.5	0.70
21.4	1.17	19.6	0.23	19.6	0.49
21.1	0.93	20.5	0.76	19.7	0.35
18.6	1.26	20.5	0.17	20.3	0.40
19.1	1.29	20.7	0.15	20.6	0.42
26.2	1.25	25.3	1.21	23.3	0.30
21.2	1.25	23.1	0.31	23.4	0.64
24.0	0.29	22.6	0.29	23.9	0.42
25.5	2.90	24.0	0.29	24.0	0.40
22.6	1.01	23.3	0.50	24.7	0.57
26.6	0.75	26.2	0.29	25.2	0.75
24.7	1.32	25.9	0.61	26.7	0.47
24.7	0.46	25.7	0.17	26.8	0.81
27.5	0.00	27.8	0.72	27.9	0.12
23.0	1.15	28.9	0.45	28.9	0.10
32.4	1.04	30.0	0.36	29.5	2.05
27.2	1.62	29.5	0.96	29.9	1.27
29.7	0.46	31.2	0.36	31.3	0.40
31.2	1.25	33.5	0.82	31.9	0.57
32.2	2.11	32.7	0.45	32.1	0.70
33.9	3.00	34.1	0.45	34.1	0.83
36.8	3.88	34.9	0.23	34.3	0.69
34.9	3.75	36.5	0.45	36.6	0.50
33.7	2.50	37.2	0.40	37.2	0.00
41.1	1.15	38.3	0.52	38.8	0.40
39.1	4.01	40.2	0.29	39.6	0.68
44.7	3.46	39.9	0.50	40.2	1.36
36.2	7.62	41.0	0.25	40.5	0.46
41.0	1.32	42.2	0.45	41.7	0.55
39.1	1.89	41.5	0.29	42.6	0.56
47.8	1.44	41.7	0.81	42.8	0.36
50.5	1.15	44.7	0.91	44.8	0.85
47.9	1.93	45.7	0.71	44.8	0.68
54.3	4.52	46.8	0.23	45.5	0.78
Average					
26.45	±1.55	26.34	±0.43	26.23	±0.53

[a]Each reported percentage is the mean of triplicate analysis on a meat sample.

Reagents

The following reagents were employed: NaCl aqueous solution (3g per 100 ml); Tween 80, practical grade; anhydrous $CaCl_2$ (no. 4 mesh, previously ground in a mortar); NaOH aqueous solution (20g per 100 ml); NaOH in pellets; Antifoam A spray (Dow Corning); NaCl in solid form; and NaOH and HCl 0.1N aqueous solutions.

Methods

Experiments were carried out with samples of raw meat with fat contents ranging from 4 to about 50%. The meat used consisted mostly of beef, but pork, veal and mutton were also tested. The wide range of fat contents was achieved by using lean cuts, commercial ground meat and samples with high-fat content which were often obtained by blending fatty portions of the carcass (flanks and plates) with meat portions of medium fat content. The samples were homogenized by repeated mincings in a meat grinder. The minced meat was divided into three portions for analysis by the new method and with two other popular methods: Salwin's modified Babcock method (Salwin et al., 1955) and by the standard ether extraction method (AOAC, 1965) using a Goldfisch apparatus. In each method, the analyses were run in triplicate.

In the new method, 50g of meat were weighed in a Mason jar of 470 ml capacity and 200g of cold NaCl solution added. The pH of the meat-brine mixture was adjusted to 6.0 by the addition of a few drops of NaOH or HCl solutions. The jar was held in an ice-water bath to maintain a temperature of 6–7°C in order to favor emulsion formation. The emulsion was prepared by stirring the above material in a multi-mix homogenizer operating at maximum speed (16,000 rpm) for 2 min. An emulsion aliquot was collected with an appropriate spoon from the bottom of the container. This location seemed to yield the most reliable and consistent results. With meat assumed to contain up to 20% fat, the size of the emulsion sample thus taken was 45g. An error of up to 5% assumed fat content above 20% seemed allowable. With meat assumed to contain more than 20% fat, an 18g sample of emulsion was used in combination with 27g of water. The readings were subsequently corrected for this dilution by the factor 5/2 in the expression of the results.

The emulsion sample, and water if needed, were then placed in a 250 ml beaker. A quantity of 0.6 ml of Tween 80 emulsifier was mixed into the emulsion sample by means of a Virtis homogenizer, operating at low speed (100–200 rpm) for 30 sec. Quantities of 1g of anhydrous $CaCl_2$, 8g of NaOH and 0.5g of Antifoam A spray were added to the emulsion while stirring with a glass rod. The mixture was then digested by heating over a gas burner for 1–2 min.

A quantity of 5g of NaCl was added and the resulting blend poured into 9g 50% Babcock bottles subsequently held in a water bath at 99°C. The beaker was rinsed with a hot concentrated solution of NaOH which was used to complete the filling of the bottle to the 50 graduation. The bottles were allowed to remain in the water bath for 2 min and then at room temperature for 1 min. The fat then appeared as a yellowish-white translucent column clearly separated from the aqueous phase. The reading was made by measuring the graduations between the bottom of the meniscus at the top of the fat column and the border between the fat and the aqueous phase with a compass. The percentage of fat was given directly by the reading. If the emulsion had been diluted, the pertinent correction factor was applied.

Occasionally in samples with high-fat content, a small dark yellow layer with a fleecy appearance was visible in the fat layer. It could easily be separated from the fat layer for reading purposes, and thus was excluded from the reported values. The various chemicals were added for the following reasons: $CaCl_2$ seemed to help in separating the protein from the fat portion; NaOH for breaking down the emulsion; Tween 80, although an emulsifying agent, helped in the separation of fat from the aqueous phase; and NaCl (solid) for increasing the density of the aqueous medium, thus helping the separation of fat from the aqueous phase.

Table 2–Comparative fat contents obtained with three methods of fat analysis

Kind of meat	New suggested method Mean % fat	Salwin's modified Babcock method Mean % fat	Goldfisch ether extraction method Mean % fat
Veal	4.2	3.6	3.8
	2.6	2.8	3.9
	9.1	9.4	9.8
Pork	12.8	13.3	13.9
	23.1	23.6	25.1
	31.5	31.7	33.8
	43.2	43.6	43.4
Lamb	6.6	7.7	8.8
	14.5	13.5	13.3
	19.1	17.8	17.4
	22.0	20.1	19.6
	27.0	25.3	24.7
	41.4	41.4	42.5
Mutton	13.7	12.3	12.0
	14.1	13.7	14.9
	20.3	21.0	21.0
	25.5	22.2	22.6
	30.7	28.9	29.6
	31.6	29.4	30.0

[a]Each reported percentage is the mean of three analysis replications on a meat sample.

Table 3–Comparative results in fat content obtained with two methods of fat analysis in raw sausage emulsion

Salwin's modified Babcock method Mean % fat[a]	New suggested method Mean % fat[a]
23.1	22.2
	23.1
	22.3
22.5	23.0

[a]Each reported percentage represents the mean of three analyses on a meat emulsion sample.

The validity of the new method for determining the fat content of a raw emulsion used in the preparation of sausage products, such as wieners and bologna, was determined. Samples of sausage raw emulsion were obtained from an industrial production line and analyzed for fat content by the new method for comparison with Salwin's modified Babcock method. In the new method, the emulsion formation stage was evidently unnecessary. The other steps were the same as previously described except for the following changes: the holding period in the water bath at 99°C was increased to 2½ min and the resting period after the water bath was increased to 2 min.

RESULTS

The comparative fat contents obtained with raw beef by the new method, Salwin's modified Babcock method and the Goldfisch ether extraction method are grouped in Table 1. Paired "t" tests showed there was no significant difference ($P < .01$) between the means of the various methods.

The results of Table 1 are presented in Figures 1 and 2 in the form of graphs and equations showing the relationship between the new method and Salwin's modified Babcock method and the Goldfisch ether extraction method, respectively. A highly significant correlation was obtained between the new method and the other two methods. The agreement seemed to be better with meat containing less than 40% fat, than with fattier meats. Above 40% fat content, the standard deviation between readings in the new method increased and agreement with the other methods decreased gradually. At these high levels of fat content, the emulsion likely breaks down very quickly, even before the sampling takes place and the aliquot used might then lack homogeneity. The two usual methods seem to agree very well, as already stated by Windham (1957).

A comparison of the data in Table 2 shows that the new method seems to be valid also for other types of raw meat, such as fresh pork and veal, frozen lamb and mutton.

Results of tests with raw sausage emulsion by the new method agreed with those obtained with Salwin's modified Babcock method (Table 3).

DISCUSSION

A rapid method (10 min) was designed and evaluated for measuring fat in raw meat and meat products. The method is inexpensive, safe and rather simple while being sufficiently accurate between 4 and 40% fat contents. This fat level is sufficient for most industrial and commercial applications. Results showed a significant correlation with those obtained from the lengthy standard ether extraction method (20–24 hr) and from the relatively quick (20–25 min) modified Babcock method described by Salwin et al. (1955). Results of the new method seemed slightly less precise than those derived from the two other methods; however, other advantages compensate for this disadvantage. The principle of the new method depends upon analysis of an emulsion, a portion of which is used and subsequently broken down purposely in order to separate the fat portion which is measured in Babcock bottles. The preparation of the emulsion permits analysis of large meat samples of almost any desirable or practical size; a homogeneous aliquot of the emulsion is used for the test. Since meats are natural-

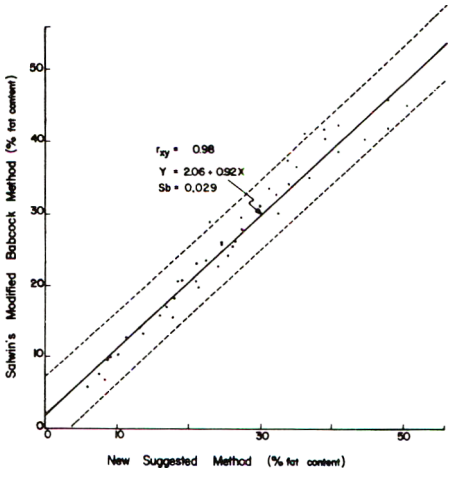

Fig. 1–Relation between the new suggested method and Salwin's modified Babcock method, obtained with raw beef meat. Regression line and 95% confidence interval.

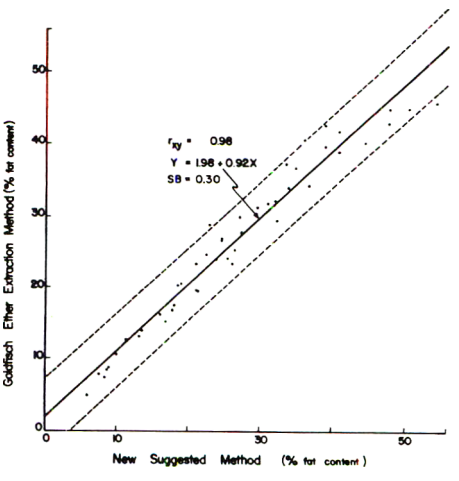

Fig. 2–Relation between the new suggested method and the Goldfisch ether extraction method, obtained with raw beef meat. Regression line and 95% confidence interval.

ly variable in fat content and often available in large lots, this feature may be advantageous for control purposes.

In plant quality control in the manufacture of sausage products, the fat determinations can be run directly on emulsions. It is highly probable that modifications and improvements can be made on the suggested method with regards to specific conditions and situations. The total time required for the test could likely be shortened using previously prepared materials and by using volumetric measures instead of weighing.

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VACUUM PACKAGING OF LAMB. 1. Microbial Considerations

SUMMARY—Paired loins from 148 lamb carcasses were utilized to determine the subsequent case-life of chops from loins that had been stored in vacuum packages. Bacterial samples which were obtained from the surface of the longissimus muscle were used to evaluate the retail case-life of loin chops. Longer periods of storage in vacuum packages were associated with higher initial psychrotrophic counts for subsequent loin chops. Comparisons between vacuum packaged loins that were stored at 0° C vs. 7°C indicated that storage temperatures of 7°C were more favorable for psychrotrophic bacteria growth and thus resulted in decreased subsequent case-life for chops from these loins. 8 days appears to be a reasonable maximum period for the storage of loins in vacuum packages. Fresh chops that were fabricated 8 days postmortem and displayed immediately exhibited an advantage of 1¼ days of increased case-life in comparison to those chops from loins which were vacuum packaged 8 days postmortem, stored under vacuum for 8 days, fabricated and subsequently displayed. The average case-life of fresh chops was 3½ days when stored in a retail case at 0° C. The results of the present study indicate that vacuum packaging of lamb loins may be feasible; however, unless low storage temperatures are maintained and the storage interval is short, the retail case-life of chops will be reduced.

INTRODUCTION

MICROBIAL spoilage of meat is one of the major problems facing the meat industry. Vast quantities of meat products are damaged or destroyed each year as a result of microbial spoilage. Such losses must be absorbed by packers and retailers and are reflected in higher prices for meat products to the consumer.

Numerous studies (Rogers and McCleskey, 1957; Stringer and Naumann, 1966) have enumerated those factors associated with microbial spoilage of meat. The report of Barlow and Kitchell (1966) reveals that bacterial spoilage in lamb stored in vacuum packages occurs much more rapidly than in beef and attributes this species difference to microbial influences. However, few studies have evaluated the factors affecting the retail case-life of lamb chops or those associated with the vacuum packaging of fresh lamb. Correspondingly, this study was designed (1) to evaluate vacuum packaging as a method for extending the storage life of lamb loins and its effects on the subsequent case-life of chops and (2) to determine the effect of storage of loins under vacuum at 0°C vs. 7°C on the subsequent case-life of chops.

EXPERIMENTAL

148 LAMB CARCASSES that were selected from a commercial processing plant, provided loins for the two experiments reported in this study. These carcasses were transported approximately 190 mi and fabricated on the 8th day postmortem. Right and left loins were randomly assigned to storage and temperature treatments following completion of carcass fabrication. One loin from each carcass was cut into chops (2.54 cm), placed in plastic trays, wrapped in oxygen-permeable film and displayed in a retail case at 0° C. The opposite loin from each lamb was packaged in polyvinylidene chloride bags under 15-in. vacuum and assigned to a specific storage and temperature treatment (dependent upon yield grade, quality grade and experiment). Following storage, the vacuum-packaged loins were fabricated and displayed in the manner previously described for fresh loins.

Experiment 1

Loins from 70 carcasses (ten U.S. Choice carcasses representative of each of the yield grades 1 through 5 and ten U.S. Good carcasses representative of each of the yield grades 1 and 2) were utilized for this experiment. Storage time and temperature treatments consisted of the following: (a) 8 days at 0°C; (b) 16 days at 0°C; (c) 24 days at 0°C; (d) 32 days at 0°C; (e) 40 days at 0°C; (f) 8 days at 7°C; (g) 16 days at 7°C; (h) 24 days at 7°C; (i) 32 days at 7°C and (j) 40 days at 7°C. Individual chops from each loin were removed from the retail case after 1, 5 and 9 days of display to determine bacterial counts.

Experiment 2

Loins were removed from 78 lamb carcasses (12 U.S. Choice carcasses representative of each of the yield grades 1 through 5, 12 U.S. Good carcasses representing yield grade 2, and 6 U.S. Good carcasses representing yield grade 1) and utilized for this experiment. The storage time and temperature treatments which were studied are as follows: (a) 8 days at 0° C;(b) 12 days at 0°C; (c) 16 days at 0°C; (d) 8 days at 7°C; (e) 12 days at 7°C and (f) 16 days at 7°C. Bacterial

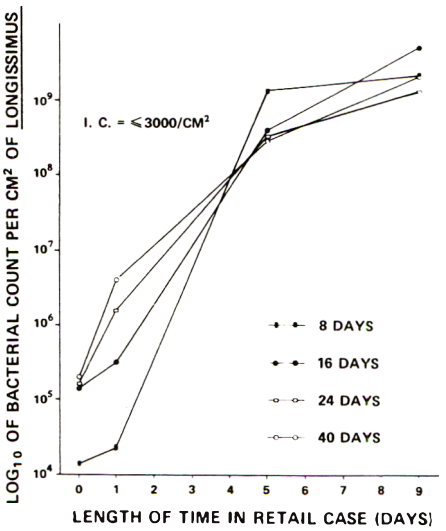

Fig. 1—Bacterial counts in the retail case for lamb chops from loins stored under vacuum for 8, 16, 24 or 40 days at 0° C.

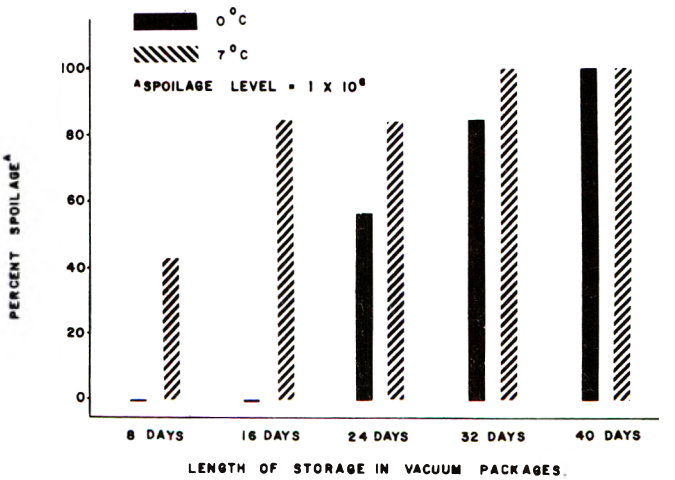

Fig. 2—Effects of temperature and length of storage in vacuum packages on spoilage levels for lamb loin chops after 1 day in the retail case.

counts were taken from individual chops from each loin after 0, 2, 3 and 4 days of display in the retail case.

Sterile aluminum templates (1 cm^2) and cotton swabs were used to collect bacterial samples from the longissimus muscle of the loin chops. Appropriate dilutions were prepared from dilution blanks containing sterile phosphate buffer. The total psychrotrophic counts were determined from spread plates of standard plate count agar incubated at 3°C for 7 days. Chops were considered spoiled from a microbiological standpoint when the psychrotrophic counts reached or exceeded 1×10^6 per cm^2 which is consistent with, but slightly lower than, the microbial standards proposed by Le Fevre (1917), Stringer (1963) and Weinzirl and Newton (1914a, b) for hamburger steak, prepackaged beef and hamburger, respectively (1×10^6 per gram). In the present study, mean values were calculated for psychrotrophic counts observed on sampling days, dependent upon the specific experiment. and were reported as psychrotrophic bacteria per cm^2.

RESULTS & DISCUSSION

Experiment 1

Bacterial growth patterns for lamb chops from loins that had been stored for various periods of time after vacuum packaging are presented in Figure 1.

These data reveal that storage periods of 16 days or longer resulted in a ten-fold increase in initial psychrotrophic counts compared to that for loins which were stored 8 days under vacuum. This finding is in agreement with the reports of Rogers and McCleskey (1957) and Halleck et al. (1958). Barlow and Kitchell (1966) observed that the microflora which developed on vacuum-packaged lamb chops consisted largely of facultative anaerobes resembling *Microbacterium thermosphactum*. Loins stored under vacuum would provide a near optimal environment for the growth of such bacteria, which would explain the higher initial psychrotrophic counts observed for chops taken from loins which were stored in vacuum packages.

Data from the present study suggest an advantage of approximately ¾, 1½ and 1¾ days in subsequent case-life for chops from vacuum packaged loins that were stored 8 days at 0°C, when compared with chops from vacuum packaged loins that were stored 16, 24 or 40 days, respectively. High percentages of the chops from loins that were stored for 24 or 40 days reached the designated spoilage level after approximately 20 hr in the retail display case. The rate of increase in bacterial numbers after 1 day in the retail case was greatest for chops from loins that had been stored for 8 days after vacuum packaging.

Differences in storage temperature, as well as in length of time in storage for wholesale loins, resulted in pronounced effects on the retail case-life of loin chops (Fig. 2).

Comparisons of percent spoilage after one day in the retail case for chops from vacuum-packaged loins that were stored for 8, 16, 24 or 32 days at 0°C were lower than for those chops from loins stored under vacuum at 7°C for the same periods. These results are in agreement with those of Halleck et al. (1958), Scott (1957), Ayres (1960) and Gould (1963). None of the chops from loins that had been stored in vacuum packages at 0°C for 8 or 16 days was considered spoiled (spoilage = 1×10^6 bacteria/cm^2) after 1 day in the retail case; while the chops from vacuum-packaged loins stored at 7°C exhibited percentages of 43% and 85% spoilage after 8 and 16 days of storage, respectively. These data indicate that vacuum packaging does not compensate for improper refrigeration temperatures during storage.

Loins that had been stored in vacuum packages for 24, 32 or 40 days exhibited high levels of spoilage at both 0°C and 7°C which suggests that the optimal time for storage of vacuum packaged lamb loins is less than 24 days.

Experiment 2

The data from Experiment 1 indicated that spoilage levels (1×10^6) of bacteria were attained after 1–5 days in the retail case and that storage periods exceeding 16 days were not feasible from a microbiological standpoint. Correspondingly, in Experiment 2, microbiological samples were obtained after 0, 2, 3 and 4 days in the retail case and storage periods of 8, 12 and 16 days were evaluated.

Bacterial growth patterns for chops from loins that had been stored under vacuum conditions for periods of 8 or 12 days at either 0°C or 7°C indicated that both length and temperature of storage affected the retail case-life of chops (Fig. 3).

Chops from loins that had been stored under vacuum for 8 days at 0°C or 7°C reached the designated level of microbial spoilage after 2¼ and 1½ days in the retail case, respectively. Those from loins that were stored for 12 days at either 0°C or 7°C had surpassed the spoilage level upon completion of the storage interval. Chops from loins stored for 8 days at 0°C exhibited the lowest bacterial counts at the end of the storage period and displayed ¾ day longer subsequent case-life than those from loins stored at 7°C for the same time period. Higher storage temperatures and longer periods of storage enhanced the growth of psychrotrophic bacteria, which is in general agreement with the previous findings of Rogers and McCleskey (1957), Halleck et al. (1958) and Barlow and Kitchell (1966).

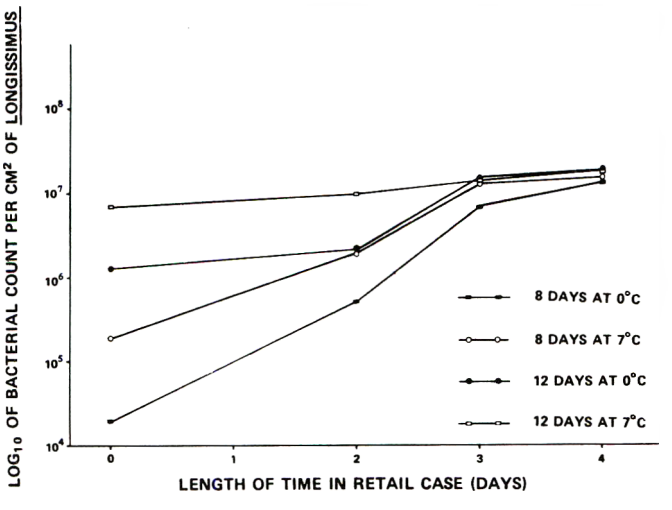

Fig. 3—Bacterial counts for lamb chops from loins stored under vacuum for 8 or 12 days at either 0° C or 7° C.

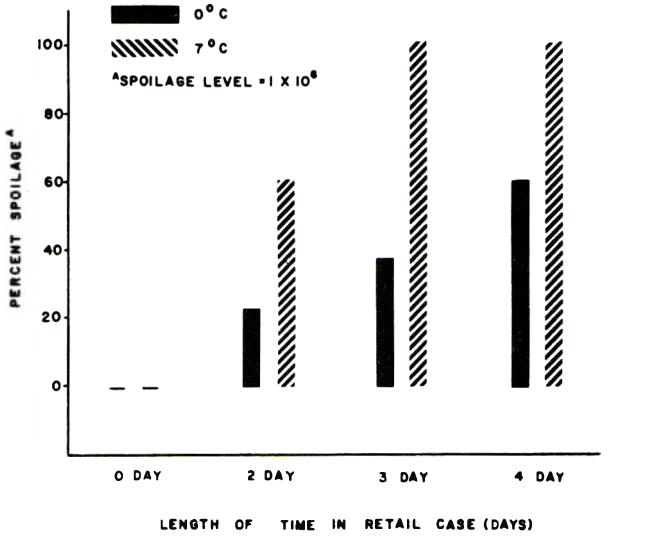

Fig. 4—Effects of storage temperature on the case-life of lamb chops from loins stored 8 days under vacuum.

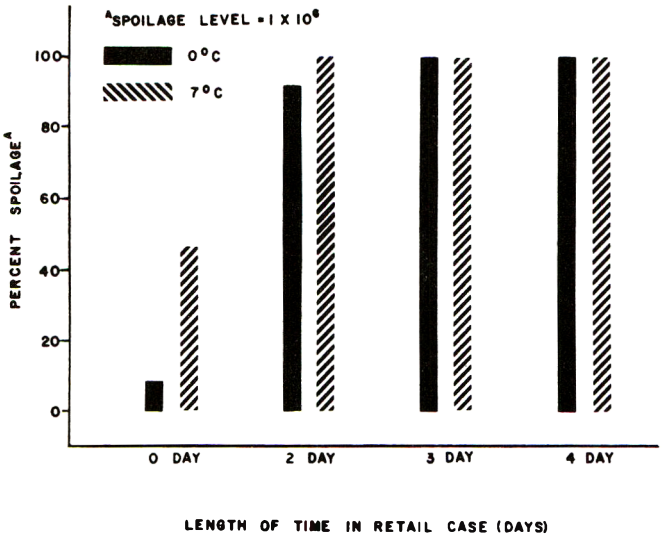

Fig. 5 Effects of storage temperature on the case-life of lamb chops from loins stored 12 days under vacuum.

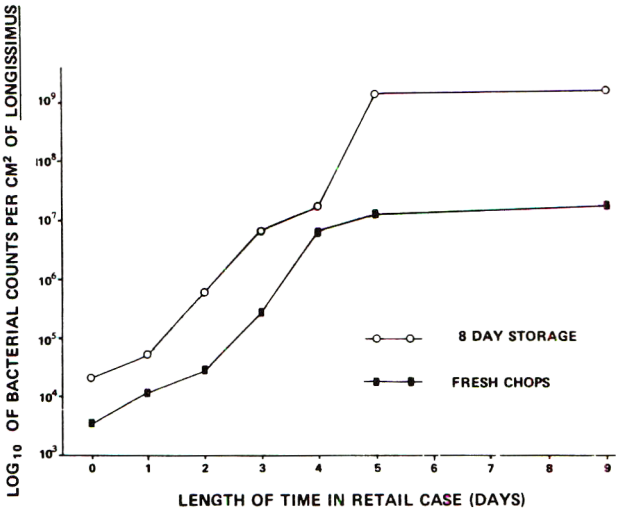

Fig. 6—Comparison of bacterial counts for fresh chops and chops from loins stored for 8 days after vacuum packaging.

Levels of spoilage for chops from loins that were stored for 8 days at 0°C or 7°C and subsequently displayed in the retail case are presented in Figure 4.

Bacterial counts for chops from loins stored for 8 days at 0°C and 7°C did not exceed the designated spoilage level on the initial day in the retail case. However, after 2, 3 and 4 days of retail display, chops from loins stored at 0°C exhibited 22, 38 and 62 percents of spoilage, respectively. Chops from loins stored at 7°C evidenced percents of spoilage of 62, 100 and 100 at these same intervals of retail display.

The subsequent case-life of chops from vacuum packaged loins that were stored 12 days at 0°C vs. 7°C is presented in Figure 5.

Bacterial counts for chops from loins stored for 12 days at 0°C vs. 7°C revealed spoilage levels of 9 and 47%, respectively, on the initial day of retail display. By the second day essentially all of the chops exhibited psychrotrophic counts in excess of the designated spoilage level (1×10^6 bacteria/cm^2). Obviously, the subsequent retail case-life of chops is adversely affected if vacuum-packaged loins are stored for periods in excess of 8 days and/or at temperatures approaching 7°C. Vacuum packaging does not compensate for the detrimental effects of high storage temperatures or for extended periods of storage.

The subsequent case-life of chops from loins that were stored for 8 days in vacuum packages is compared with that for chops from fresh loins in Figure 6.

Fresh chops (fabricated 8 days postmortem and displayed immediately) exhibited lower psychrotrophic counts than chops from vacuum-packaged loins (vacuum packaged 8 days postmortem, stored 8 days under vacuum, fabricated and subsequently displayed) throughout the display period in the retail case. Lower psychrotrophic counts were associated with longer case-life (1¼ days) for the fresh chops. The average case-life for fresh chops approached 3½ days while acceptable case-life for the chops from vacuum packaged loins was approximately 2 days.

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EFFECT OF MARBLING AND OTHER VARIABLES ON CASE LIFE OF NEW YORK STEAKS

SUMMARY—Paired short loins from carcasses representing five degrees of marbling, slight through slightly abundant, were randomly assigned one from each pair to air and vacuum aging in a 35° F cooler before cutting into New York steaks and prepackaging for display. Half of the packaged steaks were held in a dark cooler at 35 ± 2° F for 24 hr before being placed in a lighted, open-top display case; the other half was put immediately into the display case. Color description and color desirability of all steaks were evaluated daily by a three-member panel. Case life was terminated when a visible spot resulting from formation of metmyoglobin appeared on the surface of the meat. Vacuum-aged short loins had a higher yield of trimmed retail cuts than air-aged short loins. There was no difference in case shrink attributable to type of aging. The steaks held in a dark cooler for 24 hr had 23.8 hr less case life than those put immediately into the lighted display case. Degree of marbling had a significant positive linear effect on color description scores at 24 and 48 hr post-cutting, but did not affect color desirability score. Marbling had a significant curvilinear effect on case life with slight (the least) and slightly abundant (the greatest) amounts having the shortest case life.

INTRODUCTION

FRESH MEAT is a relatively perishable product. This is especially true of those characteristics of bloom and color that create eye appeal at the retail level. The length of time within which fresh meat will maintain its desirable appearance, along with wholesomeness, is referred to as case life. Factors which affect the case life of meat have been studied quite extensively. Effects of light intensity and source, type of film used in packaging, bacterial contamination and storage temperature have been at least partially elucidated.

Several workers, Kraft and Ayers (1954), Rikert et al. (1957) and Ramsbottom et al. (1951), have demonstrated that both the source and intensity of light have a marked effect on the case life of meat. However, Kraft and Ayers (1954) found that the intensity of soft white fluorescent light did not affect case life. The type of film used in different applications for wrapping meat for display in self-service cases has been extensively studied and continuously improved. Numerous workers, Kraft and Ayers (1952; 1954), Pirko and Ayers (1957), Ball et al. (1957) and Marriott et al. (1957), have shown that the type of film used in packaging has an effect on case life. The inverse relationship which exists between microbial contamination and case life has been amply demonstrated, Rikert et al. (1957) and Kraft and Ayers (1955; 1954). Rikert et al. (1957) have also shown that the case life of fresh meat is affected by display temperature, freezing and amount of external fat.

Marbling is one of the major factors in quality grading of beef, USDA (1965), and has been demonstrated, Wellington and Stouffer (1959), Field et al. (1966), Alsmeyer et al. (1959), Cover et al. (1956) and Romans et al. (1965), to have a relationship to organoleptic scores, especially tenderness and juiciness.

An ever-increasing percentage of fresh beef is being marketed as primal or saw-ready cuts which are vacuum packed at either the packing plant or breaking house before distribution to retail outlets. There is a dearth of information available concerning the effect of either marbling or type of aging (vacuum vs. air) on case life of fresh beef. Case life and eye appeal are both important factors in marketing fresh beef. This study was designed to determine the effects of marbling, type of aging and predisplay storage on color description, color desirability and case life of prepackaged New York steaks.

MATERIALS & METHODS

BEEF CARCASSES of as nearly the same maturity (A– and A) as possible and representing five degrees of marbling (slight, small, modest, moderate and slightly abundant) were selected 48 hr post-mortem at a commercial packing plant. The paired short loins taken from these carcasses were vacuum-packed in Cry-o-vac bags at the packing plant, and transported to the Oregon State University Meat Science Laboratory as soon as possible (about 1.5 hr).

Immediately upon arrival, one short loin from each pair was randomly chosen and removed from the Cry-o-vac bag, weighed and the loin eye area and back fat measured. The short loins to be aged in vacuum were also weighed using the weight of bag as a tare.

Table 1—Properties of Resinite RMF-61-Hy[a,b]

Properties	Units	Values	Test reference
Water vapor Transimission rate	g/100 sq in. per 24/hr/ at 100° F 95% RH	20	ASTM E 96-53 method E
Gas transimission Rate	cc/100 sq in. per 24/hr/ atm		Linde permeability cell
O_2		2,000–2,500	Model CS-135
CO_2		16,000–18,000	23° C at 1 atm

[a]Furnished by Borden Chemical, 1 Clark St., North Andover, MA 01845.
[b]Not a complete listing.

Table 2—Experimental design for case life study

Method of aging	Time lapse between wrapping and display (hr)	Degree of marbling				
		Slight	Small	Modest	Moderate	Slightly abundant
		(No. of steaks per replication)				
Air	1	2	2	2	2	2
	24	2	2	2	2	2
Vacuum	1	2	2	2	2	2
	24	2	2	2	2	2

[1]Dept. of Animal Science, [2]Dept. of Agricultural Economics; [3]Dept. of Food Science & Technology; [4]Dept. of Statistics.

Table 3—Scale used by panel for color and desirability evaluations

Score	Color description	Color desirability
6	Bright	Desirable
5	Slightly bright	Moderately desirable
4	Slightly dull	Slightly desirable
3	Dull	Slightly undesirable
2	Greyish	Moderately undesirable
1	Greenish	Undesirable

Table 4—Display case life[a] for each degree of marbling by time in case

Time into case after wrapping (hr)	Degree of marbling				
	Slight	Small	Modest	Moderate	Slightly abundant
1	121.1	130.3	130.3	128.1	120.5
24	95.7	100.8	103.7	110.7	100.2

[a]Mean case life in hours.

All short loins were then held for an additional 8 days, for a total of 10 days of aging, at approximately 35° F.

At the end of the aging period, all short loins were weighed and processed into New York and tenderloin steaks, lean trimmings, fat and bone. The New York strip was cut into 1-in.-thick steaks and assigned as follows, beginning at the anterior end of the longissimus dorsi muscle: 1) discarded; 2) chemical analysis (moisture, ether extract and pH); 3–6) case life study; 7) organoleptic evaluation of the fresh steaks. The anatomic location of these steaks begins at the middle of the 12th thoracic vertebra and continues through the 2nd lumbar vertebra.

The four steaks from each short loin used in the case life study were weighed individually before packaging, packaged in Resinite RMF-61-Hy (see Table 1) on a flat cardboard base and assigned at random to the two predisplay case treatments: 1) immediately into display case (approximately 1 hr after wrapping) and 2) held 24 hr in a dark cooler at 35°F before display case exposure (retail back-up cooler).

To reduce bacterial contamination and eliminate variation associated with different lengths of time exposed to higher temperatures, all cutting, weighing and packaging were conducted under sanitary conditions in a walk-in cooler held at approximately 35° F.

The data were analyzed statistically with a 2 × 2 × 5 factorial (Table 2) arrangement replicated six times. Since this model is a fixed-effect model, the error term was used to test all other effects. The dependent variables, case life, and the first- and second-day color and desirability scores, were analyzed in this manner.

To see what effect the degrees of marbling had on the dependent variables, the response curves were examined for that factor. This was accomplished by examining the four orthogonal trend components associated with the marbling factor.

A Star SM-101 Spot Merchandiser display case was used for the case life portion of this study. A small electric fan was placed in the bottom of the case and the pitch of the blades carefully adjusted so that a uniform temperature was maintained at all exposure sites. The mean temperatures were: shelf level 32 ± 2° F, highest level of exposed meat 34 ± 3° F. The steaks were placed in a slightly shingled (minimum overlap) position on a plastic grid shelf 13.5 cm from the top of the case.

The case was placed in a room lighted continuously by fluorescent lights (GE-F40CW) and the available outside light from the north and west outside windows. The case was positioned so that the meat never received direct sunlight. The foot-candles of incident light at the display surface were: 1) sunny day, 175 ft-c; 2) cloudy day, 150 ft-c; 3) night, 135 ft-c.

Color descriptions and desirability evaluations were made daily by a trained, three-member panel using the scale of Marriott et al. (1967) as presented in Table 3.

Percent moisture, percent ether extract and pH were determined on the longissimus dorsi muscle from each loin, AOAC (1965).

Case life was measured from the time the steaks were placed in the display case until the appearance of a brown spot resulting from formation of metmyoglobin. As each steak reached the end of display life, it was removed from the display case and the terminal weight recorded.

RESULTS & DISCUSSION

THE AIR-AGED short loins shrank .32 lb more than the vacuum-aged loins and yielded .11 lb less New York steaks and .07 lb less tenderloin steaks while producing .13 lb more lean trim. These differences are the result of drying and discoloration of the air-aged short loins during aging. The shrink in the vacuum-packed short loins was negligible and there was no discoloration. There was no significant difference in display case shrink, case life, color description score or desirability scores associated with type of aging. No steak was evaluated as undesirable on color evaluation prior to the visible appearance of metmyoglobin.

The mean case life by degrees of marbling and time into the case post-cutting is presented in Table 4. The 23.8-hr mean difference (significant at $P < .01$) in case life between the two predisplay treatments indicates that the oxidative process responsible for the production of metmyoglobin was not delayed by holding the steaks in a dark cooler.

Steaks held in the cooler had a slight but significantly ($P < .05$) higher color score at 24 hr post-cutting (Fig. 1) when they were put on display. This difference disappeared in the next 24 hr. This result differs from that reported by Marriott et al. (1967). Perhaps the extreme care used in temperature and sanitation control during processing in this experiment pro-

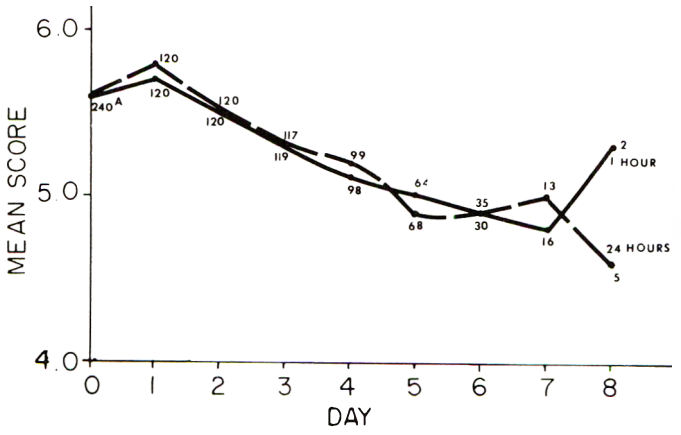

Fig. 1—Daily mean color description score by time into display case.
[a]*Total number of observations per day.*

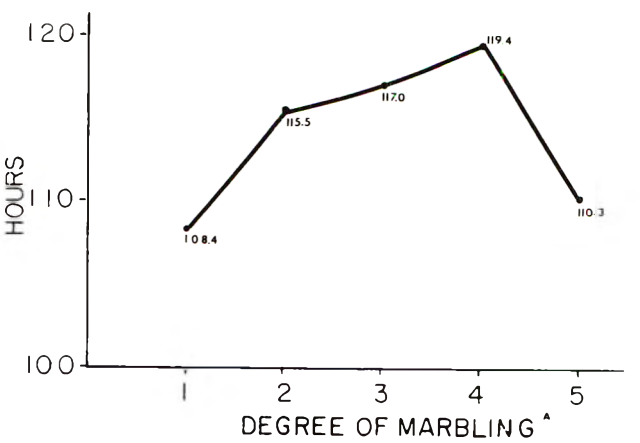

Fig. 2—Average display case life by degree of marbling.
[a]*1—Slight; 2—small; 3—modest; 4—moderate; 5—slightly abundant.*

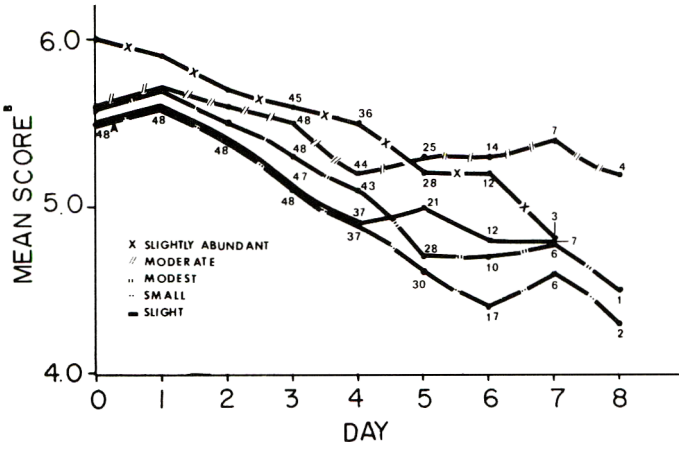

Fig. 3—Daily mean color description score by degree of marbling.
[a]*Total observations per day.*
[b]*N = 48 for all points not otherwise noted.*

duced sample cuts with lower initial bacterial counts, causing a difference in color degradation patterns between the two studies.

The degrees of marbling had a significant ($P < .05$) curvilinear effect (Fig. 2) on the case life of fresh steaks, with slight and slightly abundant amounts of marbling being the low points of the curve. These degrees of marbling are typical of USDA Good and Prime beef, respectively. The three other degrees of marbling are typical of USDA Choice beef. Further research will be necessary to elucidate these apparently anomalous results. Fortunately, the longer case life was associated with the degrees of marbling typical of USDA Choice beef, which is the most popular grade of beef sold in self-service retail meatmarkets in this country.

Marbling had a significant ($P < .01$) linear effect on color description scores at 24 and 48 hr, with a positive relationship. Although the effects were not tested after 48 hr, because of a biased sample size resulting from removal of some steaks, the relationship was maintained through day four as illustrated in Figure 3.

Marbling did not have a significant effect on color desirability scores.

As shown in Table 5, marbling was significantly correlated ($r = -.771$, $P < .01$) to percent ether extract in the longissimus dorsi muscle. This is in agreement with results reported by McBee et al. (1968) and confirms that degree of marbling is a good estimate of the amount of intermuscular fat in the longissimus dorsi muscle.

The reasonably high correlation ($r = -.886$, $P < .01$) between percent moisture and percent ether extract illustrates the interrelationship of the parts to the whole, especially where the ratio of moisture to fat-free dry matter is relatively constant for a given muscle from animals of the same age.

Table 5—Correlation coefficients for proximate analysis and marbling

Variable 1	Variable 2 (%)	R value
Marbling	Water	−.724**
Marbling	Ether extract	0.771**
% H_2O	Ether extract	−.886**

**$P < .01$.

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QUALITY TRAITS ASSOCIATED WITH CONSUMER PREFERENCE FOR BEEF

SUMMARY—Consumer preferences for beef attributes are not closely related to grade. From a consumer viewpoint, however, they should be if grades are used. Such relationships will be found and quantified through research relating consumer preferences to beef variables. In this study, consumer preferences were related to selected beef characteristics, including indicators of tenderness, juiciness and flavor. Steaks from top rounds of 176 steers were divided into three or four classes for each characteristic and evaluated in pairs by a 950-family consumer panel. Paired comparisons were used and analysis made to obtain maximum-likelihood estimates of the probability that a given class would receive top rating when ranked. Probability sets were calculated for each characteristic and were found quite similar. The sets were approximately as follows: .30, .26, .23, .21 for four classes and .40, .32, .28 for three. The order from highest to lowest was usually that expected by food scientists. When ether extract and shear were combined in analysis, the set was almost identical to the above for four classes. Descriptive grades are suggested by these results, for there is a probability of from .60 to .70 that the consumer in this study will give top ranking to a class of steak other than what present grades and meat scientists consider "best." The major application of this research is in the development of hypotheses for future research. These hypotheses will be concerned with how discerning consumers are, how consumer tastes differ, what characteristic—or group of characteristics—can best be used to describe beef and whether grading can be meaningful if done by carcass vs. by muscle, cut or some grouping of muscles or cuts.

INTRODUCTION

IT IS OFTEN desirable to relate characteristics of a raw or cooked food with consumer preferences. It may be done using an hedonic scale or by paired comparisons as described by Bradley and Terry (1952). Reported here are procedures used and some results of research using the top round (semimembranosus and adductor) muscle and a consumer panel in Roanoke, Virginia. The objective was to relate characteristics of the carcass or top round, or both, to consumer preferences. The relationships should be useful in evaluating grades and suggesting possible changes in them. The methodology offers many possibilities in the various areas of food science.

The basic assumptions in this research are that top round varies meaningfully in eating quality from carcass to carcass, that consumers can detect these differences, that consumers are homogeneous in terms of what they desire in beef and that selected characteristics of beef can be related to consumer preferences. The top round was selected because it offers a wider range among carcass attributes than other muscles commonly used for steak.

EXPERIMENTAL

TOP ROUNDS were collected from the left sides of 176 steer carcasses evenly divided by thirds of a grade from average Standard to top Choice. All carcasses were obtained from federally inspected plants in Virginia, were in the B maturity category according to USDA grades prior to 1965, weighed from 450–650 lb, and nearly all aged 7 days prior to selection. Carcasses selected should represent most of the beef and provide about as much variation among carcasses as one would expect to find within the block beef supply.

Data were collected on a wide range of both carcass and muscle variables. Marbling score, maturity, conformation, estimated percent kidney fat and carcass grade were furnished by a representative of the USDA grading service. Loin-eye area and fat thickness over the 12th rib were determined by the method described by Bray (1963). The percent yield of the carcass was estimated by the equation developed by Murphey et al. (1960).

One day after selection, each top round was double-wrapped in freezer paper, identified and quick frozen at −20°F. After freezing, the anterior and posterior face of each was trimmed to facilitate slicing. All muscles were trimmed to a width of 10 in. Depending upon the length, eight or nine slices ¾-in. thick were obtained. All external fat was removed. Each slice was halved, yielding 16–18 steaks per top round. All steaks were 5 in. wide, ¾-in. thick and 4.5–5.5 in. long. Steaks were numbered 1, 2, . . . 9, anterior to posterior on each side, individually wrapped and vacuum sealed with identification in clear 9- by 16-in. Cry-o-vac bags and returned to the freezer in the frozen state. The beef was held in frozen storage until compositional analysis was completed for each round on slice 4, left side. Ether extract and protein content were determined using procedures set forth by the AOAC. Moisture was determined by lyophilization.

The right side of the fourth slice was used for laboratory panel evaluation. Steaks were thawed at room temperature, placed 4 in. below the heating element in a conventional oven, broiled 7 min, turned and broiled an additional 6 min. A trained panel of eight judges scored each steak for tenderness, juiciness and number of chews required to thoroughly masticate. Each judge was served a 1-in. core from the steak. Each core was halved so that juiciness and tenderness could be determined on one half, number of chews on the other. To minimize the effect of within-muscle variation, cores were taken from a similar position in each steak. Warner-Bratzler shear scores were obtained for each steak using ½-in. cooked cores. KTG tenderness scores were obtained by averaging the values obtained on two 1-in. cooked cores, Kelly et al. (1965).

Ether extract was selected as the variable for pairing steaks for consumer panel evaluation, because it has been associated with both tenderness and flavor. Muscles were ranked from high to low ether extract. Six classes containing 28 muscles each were selected on a basis of judgment, since about one-sixth of the muscles ranked fairly high in ether extract and one-sixth ranked low. Steaks were paired within and among groups to ensure the comparison of all possible combinations by taking all steaks from a given position, randomly arranging them within class and then pairing for all possible class combinations. The same procedure was followed for all steak positions with random arrangement within class for each steak position. A metal ring was inserted at random in one of the steaks in each pair to serve as identification for the consumer panel.

Two thousand and five hundred families, stratified by median house value–house value was used as an indicator of income–within block, were selected in Roanoke, Virginia. A random sample of blocks was selected from each stratum so as to include a distribution of families among the strata in the sample approximately equal to the distribution of families for the city. Questionnaires were sent the occupants of each housing unit (a housing unit included all individual houses and each individual apartment in a multiple-family unit). The questionnaires sought information concerning family composition, age, steak-eating habits and willingness to cooperate in the study. There were 1,637 completed questionnaires returned. There were also 226 returned because of vacant houses or apartments. Of the returned questionnaires, 184 indicated they did not wish to participate in the study. In addition, all families were eliminated from the panel if they did not serve steak at least once a month. A large number of those in the lower house-value groups were eliminated for this reason. All households not including both husband and wife were also eliminated, leaving 950 families for the consumer panel. No actual tabulation of nonrespondents was made, but approximately 10% were contacted personally. They were mostly in low-value areas. Reasons given for nonparticipa-

Table 1—Population and panel distribution by house value group, Roanoke, Virginia[a]

House value group	Median value (000) ($)	Percent of city population	Percent of panel
1	5–6	12.5	4.6
2	6–7	5.3	4.1
3	7–8	13.0	6.8
4	8–10	12.0	7.6
5	10–12	16.6	15.1
6	12–14	20.0	29.0
7	14–17	9.6	19.8
8	Over 17	8.8	13.0

[a]Source of data from which these figures were calculated: Office of City Planning, Roanoke, Virginia.

Table 2—Consumer preference for top round steaks as related to laboratory panel scores for tenderness[a]

Comparison		Consumers preferring				No. with no preference	Percent
A	B	A	%	B	%		
I	I	62	41.9	50	33.8	36	24.3
I	II	150	44.4	130	38.5	58	17.1
I	III	199	48.2	130	31.5	84	20.3
I	IV	47	40.9	36	31.3	32	27.8
II	II	69	43.1	48	30.0	43	26.9
II	III	227	47.7	168	35.3	81	17.0
II	IV	61	46.6	46	35.1	24	18.3
III	III	171	47.2	111	30.7	80	22.1
III	IV	59	37.1	66	41.5	34	21.4
IV	IV	3	37.5	2	25.0	3	37.5

[a]Tenderness was rated 1 for very tender to 5 for tough. Classes were: I = Less than 2.39 (tender). II = 2.4–2.99. III = 3.00–3.69. IV = Over 3.70 (tough).

Table 3—Consumer preference for top round steaks in relation to carcass grade[a]

Comparison		Consumers preferring				No. with no preference	Percent
A	B	A	%	B	%		
I	I	78	40.8	61	31.9	52	27.2
I	II	220	42.2	199	38.2	102	19.6
I	III	140	30.1	232	49.9	93	20.0
II	II	137	42.9	116	36.4	66	20.7
II	III	237	35.7	307	46.2	120	18.1
III	III	125	37.1	140	41.5	72	21.4

[a]I—U.S. Standard. II—U.S. Good. III—U.S. Choice.

tion in the personal interview were: they did not wish to cooperate, did not eat steak or were out of town when the questionnaires were mailed.

For the above reasons the final panel selected was more heavily weighted towards the upper property value groups than was the total population (Table 1).

Since carcass grades are for those using the product, the final distribution of the panel did not cause concern. These are the people who buy and eat steak and whose preferences should be reflected in the grading system.

The following procedure was followed to distribute steaks and collect data. Families were notified of date and time of delivery 1 wk in advance. Steaks were distributed randomly, one pair to each family (i.e., no attempt was made to give a particular consumer a specific pair of steaks). Consumer and steak identification were recorded as the steaks were distributed. Directions were delivered with the steaks along with a self-addressed post-card on which the consumer recorded preference, cooking method, brand of tenderizer if used and degree of doneness. Both husband and wife were asked to evaluate the pair and record the preference for each (or no preference, if they were indifferent between the two). The post-cards were to be mailed after the steaks had been eaten and evaluated. The steaks were without cost to the panel members. Returns were obtained for 89.3% of the steaks distributed.

The statistical procedure is an adaptation of the method of analysis developed by Bradley and Terry (1952) for experiments involving paired comparisons and illustrated further by Bradley (1954). A priori assumptions were to pool all data for analyses, make statistical tests at the 5% level for a Type I error, consider indications of no preference as half indicating a preference for one class and half a preference for the other class of the pair under consideration, and probability independence between classes. The statistics presented as a result of the procedure are maximum-likelihood estimates of the probability that a given class will obtain top rating when ranked by the consumer panel. The null hypothesis tested for the overall analysis was that all probabilities were equal.

In this procedure a sum of rankings is obtained assigning a value of 1 for a sample judged superior and 2 for the other member of a pair. For indications of no preference in this study, half were assigned 1 and half 2. A set of all possible combinations of sums of ranks is obtained with the probability of the chance occurrence of each. Maximum-likelihood estimates are then obtained using repeated approximations in an iterative procedure. From the sums of ranks and the maximum-likelihood estimates, a test statistic is calculated for an overall test of significance. Values of Z were calculated for all possible pairs of classes and significance between them determined in a table for areas of the standard normal curve.

RESULTS

CONSUMER PREFERENCES were tabulated for each variable as presented in Table 2 for tenderness. Tenderness scores were divided into four classes. These are defined below the table. Class I includes muscles evaluated as tender by the laboratory panel, and class IV contains those that were tough. Using the four classes, 10 combinations were possible for comparison. Referring to Table 2, when steaks from muscles evaluated as tender (class I) were paired with muscles considered tough (IV), 47 panel members (or 40.9%) preferred the steaks from class I, 36 (31.3%) the steaks from IV and 32 (27.8%) indicated "no preference." Both husbands' and wives' preferences are included.

Generally, the majority of the consumer panel preferred steaks evaluated as more tender by the laboratory panel. One exception was in the comparison III–IV, although the difference was not large. Panel tenderness scores do not explain the differences in preference for the within-class comparisons I–I, II–II and III–III. (Within-class comparisons were always made because steaks in class I might be in II, III, or IV for some other characteristic.) One would have expected little or no difference between the number choosing A and B in these classes unless the explanation is in some other variable.

There was a slight bias for the ringed steak among the consumer panel. For all respondents, 44% selected the ringed, 36% the other and 20% indicated no preference. There was no difference between husbands and wives in amount of bias. Where within-class comparisons (I–I, II–II, etc.) were made, the bias was not really different than for the total (43% ringed, 35% not ringed and 22% no preference). Since rings were randomly placed, the bias effect should be cancelled for between-class comparisons—the only comparisons used in the statistical analysis.

Table 3 presents the relationship between consumer preference and USDA carcass grade. The data are presented both to show preferences and to explain statistical results.

Tabulations for other variables are not presented here because the paired comparison analysis is more significant. (See Table 4.) Probabilities indicate that pro-

Table 4—Statistical results from comparing consumer preferences with selected variables[a]

Variable	Probabilities			
Tenderness[b]	(3) .208	(4) .226	(2) .267	(1) .293
Juiciness[c]	(3) .213	(1) .227	(2) .262	(4) .301
No. chews[d]	(3) .277	(2) .338	(1) .392	
W-B shear[e]	(3) .273	(2) .335	(1) .394	
KTG[f]	(3) .214	(4) .231	(2) .263	(1) .293
Ether extract[g]	(1) .306	(2) .310	(3) .383	
Carcass grade[h]	(1) .298	(2) .309	(3) .400	
Yield grade[i]	(4) .206	(2) .228	(3) .256	(1) .314
Carcass conformation[j]	(4) .216	(3) .226	(2) .258	(1) .299
Marbling score[k]	(1) .285	(2) .316	(3) .398	
Protein dry[l]	(4) .206	(3) .233	(2) .262	(1) .301
Moisture[m]	(4) .223	(2) .224	(3) .247	(1) .308

[a]The probabilities represent the proportion of the population expected to prefer above all other classes the class indicated in parentheses beside it. There is no statistical difference at the 5% level between probabilities underlined.

[b] 1 – Less than 2.39 (tender).
2 – 2.40 to 2.99.
3 – 3.00 to 3.69.
4 – 3.70 and over (tough).

[c] 1 – 2.29 or under (juicy).
2 – 2.30–2.99.
3 – 3.00–3.59.
4 – 3.60 or over (dry).

[d] 1 – Required 23 chews or less.
2 – Required 24–29 chews.
3 – Required 30 or more chews.

[e] 1 – Under 8.0 lb (more tender).
2 – 8.0–10.9 lb.
3 – Over 10.9 lb (less tender).

[f] 1 – Under 24.0 sq in. (tender) (square inches are proportional to the amount of work required to masticate a 1-in. core of meat).
2 – 24.0–29.9 sq in.
3 – 30.0–35.9 sq in.
4 – Over 35.9 sq in. (tough).

[g] 1 – 11.7% or less.
2 – 11.8–16.7%.
3 – 16.8% and over.

[h] 1 – U.S. Standard.
2 – U.S. Good.
3 – U.S. Choice.

[i] 1 – 43.3–47.9% (low yield).
2 – 48.0–48.9%.
3 – 49.0–50.9%.
4 – 51.0–52.9% (high yield).

[j] 1 – Top Choice–Avg Choice.
2 – Low Choice–Top Good.
3 – Avg Good–Low Good.
4 – Top Standard–Low Standard.

[k] 1 – Devoid, P. Devoid, Traces.
2 – Slight, Small, Modest.
3 – Moderate, Sl. Abundant, Mod. Abundant

[l] 1 – 73.9% or less.
2 – 74.0–79.9%.
3 – 80.0–83.9%.
4 – 84.0% or more.

[m] 1 – 69.9% or less.
2 – 70.0–71.9%.
3 – 72.0–73.9%.
4 – 74.0% or over.

portion of the population expected to prefer a given class over all other classes. For carcass grade these were: Standard .30, Good .31 and Choice .40. At the 5% level, there was no statistical difference between the proportion preferring Good and Standard, but Choice was significantly above those two. These results suggest that when a population similar to the one from which the panel was selected is asked to indicate preferences for round steak they themselves prepared, about 30% will rank Standard as their first choice, about 30% Good and about 40% Choice. Notice that in Table 3 more actually preferred Standard than Good when the choice was between steaks from the two grades. The technique used, however, evaluates all comparisons at once, to give Good a slightly higher probability than Standard, even though not significantly so.

For all variables, preference for the different classes is quite evenly distributed. Not more than 40% prefer the most preferred group in any case, leaving the majority preferring something else. If consumers really can discriminate, then their tastes must vary.

Some variables appear to offer greater possibilities than others for describing relationships between variable levels and preferences. These are number of chews, W-B shear, ether extract, carcass grade and marbling score. Of these, only carcass grade and marbling score were closely related to each other (r = .96); therefore, each of the other three must be important to consumers (or related to other variables which are). Some combination of these should be more useful than any one alone.

To explore the possibility that two characteristics considered together might be more closely associated with preference than one, ether extract and W-B shear were used. The classes were defined in order of expected preference as hypothesized by the authors and are presented in Table 5 along with the results. In each case the class expected to be most preferred, was. Even with the class I–IV comparison between juicy, tender steaks and relatively dry, tough ones panel members were not as much in favor of class I as expected.

Table 5—Consumer preference for top round steaks in relation to the combination of characteristics, ether extract and W-B shear[a]

Comparison		Consumers preferring		No. with no preference
A	B	A	B	
I	II	125	116	52
I	III	113	92	71
I	IV	175	101	87
II	III	60	50	28
II	IV	148	101	79
III	IV	92	74	27

[a]Classes were: I, high ether extract and low shear. II, low ether extract and low shear. III, high ether extract and high shear. IV, low ether extract and low shear.

Table 6—Distribution of consumer panel under 50 years old, by method of round steak preparation, Roanoke, Virginia[a]

	Pan fry (%)	Broil (%)
All steaks	51	49
Tenderized	20	29
Well done	70	56
Not well done	30	34
Not tenderized	80	71
Well done	78	45
Not well done	22	55

[a]As an example of how this table should be read, 51% of all steaks were pan-fried. 80% of those pan-fried were not tenderized. Of those not tenderized and pan-fried, 78% were cooked well done.

The following probabilities were obtained for the four classes:

(4) .199 (3) .237 (2) .272 (1) .292

Differences are not statistically significant except between class I and IV.

Since consumers were permitted to prepare their steaks as they wished, there were a number of variables not accounted for in the above analysis. It was considered important that preparation method be up to the consumer, so that evaluation would reflect his tastes as applied to the way he prefers his beef. Such variation in cooking method, degree of doneness and use of tenderizer (see Table 6) could cause questions concerning the analysis; therefore, further analysis, while not complete in any sense, was undertaken.

The analysis suggested that differences

tend to disappear when a tenderizer is used, when steaks were broiled not well done and when pan-fried well done. Analysis is not completed to determine preference patterns with these methods of preparation removed, but what has been done yielded still higher probabilities for the most preferred classes and lower ones for the least preferred.

DISCUSSION

SEVERAL RESEARCHERS have related panel reactions to grade. Fielder et al. (1963), using eight different cuts and four cooking methods with a laboratory panel, found very little association between grade and scores for tenderness, flavor and juiciness. Kiehl et al. (1958) found a closer relationship between grade and consumer preference but still suggested that leaner carcasses as acceptable as Choice exist, if only there were an accurate method to identify them. This is consistent with the analysis using grade in Table 4, and was expected. It was the reason for relating preferences to the other variables; for, if one cannot relate consumer preferences to grade, then research must be concentrated upon relating other variables to preferences if grades are to be improved.

With marbling score as closely related to grade ($r = .96$) as found in this research, relating preferences or other variables to marbling score will not contribute greatly to improved grades either; results are likely to be the same as for grade. This is confirmed by Breidenstein et al. (1968), who found no statistically significant relationship between marbling scores and general evaluations of 60 semimembranosus muscles by an 18-member, experienced panel. Others, including Field et al. (1966) and Seuss et al. (1966), found few statistically significant relationships between marbling score and tenderness, juiciness and flavor. The marbling, tenderness and juiciness probability sets in Table 4 tend to support the above, except that the highest marbling class has a significantly higher probability than the other classes.

Distribution of preferences among classes raises many questions. Is there some other characteristic which more adequately represents quality from the consumer's viewpoint? Will some combination of factors permit more adequate identification of quality beef? Are consumers quite discriminating but with tastes differing meaningfully from group to group? Are perhaps as many as 80–90% of all consumers unable to detect differences in top round as they prepare it, and the remainder quite discriminating and heavily in favor of the beef that present grades suggest is "best"? Are the tastes of the consumer panel typical? Are consumers less able to detect differences when evaluating other cuts of beef?

While the above questions are not answered, objectives of the project were achieved. Results do not support quality-ordered grade names, even though in all but one case the highest probabilities are for the class expected to be most preferred. The reason is that the majority still selected another class as better. This does not prove that we should not have quality-ordered grades. Perhaps we do not yet have the knowledge for a good, quality-ordered system. Actually, if consumers really are discriminating and have different tastes for the characteristics used in analysis, descriptive grades are suggested.

Paired comparisons as used here have wide application in testing additional hypotheses which must be tested if research is to lead to improved grades. The technique has similar application in many areas of food science.

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EFFECTS OF HETEROTROPHIC AND AUTOTROPHIC GROWTH CONDITIONS ON THE COMPOSITION OF Chlorella sorokiniana

SUMMARY—The effects of heterotrophic and autotrophic growth conditions on the composition of Chlorella *are discussed in relation to the potential food value of algae. Cells from the two culture modes differ appreciably in proximate analysis and elemental composition; however, fiber content and vitamin and amino acid composition are essentially unchanged. On this basis it appears that heterotrophic algae, which are less expensive to produce, can be used to examine the acute toxicity problem with bacteria-free cells. In addition, heterotrophic algae may also be used to predict results that would be obtained with autotrophic material in studies of processing techniques designed to improve protein quality and digestibility.*

INTRODUCTION

UNICELLULAR ALGAE are being considered for use in life-support systems for long-term manned space missions and as a means of alleviating world protein shortages, Miller and Ward (1966) and Oswald and Golueke (1968). In addition to providing a means of carbon dioxide removal and oxygen regeneration, the algal cells formed by photosynthesis are rich in protein and can theoretically serve as a nutritious foodstuff. Relatively large quantities of biomass are required for feeding trials and testing of processing methods, however, and studies in these areas have been limited in scope.

Production of algal cells for processing and feeding trials could be greatly facilitated by means of heterotrophic culture in the large fermenters widely used in the yeast and antibiotics industries. While algae cultured on organic substrates have a lower specific growth rate than those produced autotrophically with carbon dioxide and light, heterotrophy permits the use of much higher culture densities, and the cellular production rate is markedly enhanced.

An additional consideration is that most feeding trials with algae have been done with non-axenic cells, and it is questionable whether the adverse symptoms frequently observed, Powell et al. (1961) and Dam et al. (1965), are due to the algae per se or to the bacterial contaminants invariably present in autotrophic mass cultures. Clarification of this point requires nutritional studies with aseptically cultured algae; however, the axenic cultivation of autotrophic algae is a formidable task. Batch methods are entirely inadequate, and continuous culture equipment capable of yielding more than a few grams of pure algae per day does not exist. In contrast, heterotrophic culture of bacteria-free algae could be done with existing industrial fermenters available on a lease or contractual basis. Trials with sterile algae produced in such systems might reveal whether development of axenic techniques for large-scale autotrophic culture is advisable or warranted.

Use of heterotrophic cells, in studies to be the basis for the use of autotrophic cultures, must have the premise that the biomass composition produced from the two culture modes is the same. Therefore, the purpose of this study was to compare the composition of sterile-grown heterotrophic and autotrophic algae.

EXPERIMENTAL

THE ALGA used in the study was *Chlorella sorokiniana*, Shihira and Krauss (1965). The original slant was obtained from Professor Jack Myers of the University of Texas, and stock cultures were maintained on trypticase soy agar. As shown in Table 1, the culture media used for cellular production contained, respectively, nitrate, nitrate plus glucose, urea and urea plus glucose. This protocol permitted a comparison of cells grown autotrophically and heterotrophically with two nitrogen sources. The nitrate media included molybdenum and cobalt, required for nitrate reductase enzymes.

The cells were cultured in an illuminated shaker-incubator (New Brunswick Model R27)

Table 1—Media for the autotrophic and heterotrophic culture of Chlorella sorokiniana

Constituent (Millimoles/liter)	Autotrophic		Heterotrophic	
	Nitrate	Urea	Nitrate	Urea
Glucose			267	267
Urea		25		25
KNO_3	50		50	
KH_2PO_4	10	10	10	10
Na_2SO_4	5	5	5	5
$MgSO_4 \cdot 7\,H_2O$	1	1	1	1
EDTA-KOH	0.85	0.85	0.85	0.85
$CaCl_2$	0.50	0.50	0.50	0.50
$MnCl_2 \cdot 4\,H_2O$	0.20	0.20	0.20	0.20
$ZnSO_4 \cdot 7\,H_2O$	0.05	0.05	0.05	0.05
$FeSO_4 \cdot 7\,H_2O$	0.10	0.10	0.10	0.10
$CuSO_4 \cdot 5\,H_2O$	0.01	0.01	0.01	0.01
MoO_3 (85%)	0.01		0.01	
$Co(NO_3)_2 \cdot 6\,H_2O$	0.01		0.01	
pH	6.1	6.1	6.1	6.1

equipped with an overhead bank of eight fluorescent lamps (F24T12CW/HO) and a lower bank of ten lamps to provide illumination from beneath the culture flasks. The incubator was maintained at 38 ± 0.5°C and gassed continuously with air–4% carbon dioxide at a rate of approximately 4 liters per minute. The flasks were shaken at a rate of 100 1.5-in. reciprocal strokes per minute.

The cells were grown batch-wise in 1-liter Erlenmeyer flasks, each flask containing 500 ml of medium. A seed culture was prepared from an agar slant and subcultured in the appropriate medium throughout the experiment. Cultures were maintained through the exponential growth phase and into linear growth before harvest. With glucose media, a two-day growth period permitted a yield of approximately 2.6g dry cells/liter, whereas with autotrophic culture, a 3- to 4-day period was required for a yield of 1.0g/liter. Thus, to obtain 100g dry algae needed for chemical analysis, approximately 77 flasks of heterotrophic culture and 200 flasks of autotrophic culture were composited for each nitrogen source.

The flasks were cultured 16 at a time in the incubator-shaker. At the end of the incubation period, each flask was tested for sterility by streaking an aliquot of the suspension on trypticase soy or plate count agar. While the test plates were incubated in the dark at 37°C for 24 hr, the culture flasks were refrigerated at 3–4°C. Any flask showing a positive test for bacterial contaminants was discarded. Cells from the bacteria-free flasks were harvested by centrifugation with a Sorvall Model RC-2 refrigerated centrifuge. The supernatant culture fluid was discarded and the cells resuspended in 0.01 M sodium phosphate buffer (pH 7.5) and again centrifuged. The collected cells were transferred to aluminum pans, frozen, lyophilized and ground to pass a 40-mesh screen with a Wiley No. 2 mill.

Each of the four composited samples were assayed for proximate analysis, pigments, elemental composition, vitamins, minerals and caloric value. Nitrogen content was determined by the Dumas and micro-Kjeldahl methods, and carbon and hydrogen by the Pregl technique, Chapman and Pratt (1961). Proximate analysis, chlorophyll and vitamins were determined by the AOAC (1965) method. Amino acids were determined by microbiological assay using *Streptococcus faecalis*, *Lactobacillus plantarum* and *Pediococcus cerevisiae* as test organisms. Inorganic minerals were determined in part by standard methods, AOAC (1965), and in part by atomic absorption. Caloric value was assayed by combustion analysis in a Parr oxygen bomb calorimeter.

Table 2–Proximate analysis, caloric value and elemental analysis of autotrophic and heterotrophic Chlorella sorokiniana

	Autotrophic culture		Heterotrophic culture		
	Nitrate	Urea	Nitrate	Urea	Method of analysis
Proximate analysis (%)					
Protein	53.4	64.7	39.1	40.6	AOAC[a] 2.044
Fat	3.1	3.9	2.6	2.4	AOAC 22.033
Fiber	1.1	1.9	1.2	3.2	AOAC 22.038
Carbohydrate	29.6	15.6	49.2	45.9	By difference
Ash	8.2	8.9	5.0	4.5	AOAC 13.006
Moisture	4.6	5.0	2.9	3.4	AOAC 13.004
Caloric value (Kcal/g)	5.02	5.25	4.98	4.93	Bomb calorimeter
Elemental analysis (%)[b]					
Carbon	49.9	51.6	46.5	48.0	Pregl method
Hydrogen	7.1	7.1	6.8	6.8	Pregl method
Nitrogen	9.3	10.3	6.0	6.7	Kjeldahl method
Oxygen	25.1	21.5	35.6	33.8	By difference

[a]Association of Official Analytical Chemists, 1965.
[b]Corrected to moisture-free basis.

RESULTS

TABLE 2 SHOWS results of the proximate and elemental analyses as well as the calorimetric value of the cells produced with each type of medium. The major difference noted in the proximate analyses was that the heterotrophic algae were markedly higher in carbohydrate, largely at the expense of protein and ash. The differences were relatively similar with the nitrate and the urea-containing media. The elemental analyses for the heterotrophically grown cells show reduced nitrogen and increased oxygen content compared to autotrophic cells. These shifts in elemental composition support the observed changes in proximate analyses. The moderate decrease in caloric value with heterotrophy can also be accounted for by the increase in carbohydrate content.

Fiber content (Table 2) is a consideration in nutritional studies with algae because it is a measure, at least in part, of the density of the cellulose cell wall, a severe detriment to digestibility. Since fiber content is not greatly different, it appears likely that the elevated carbohydrate of the heterotrophic tissues represents storage starch. Heterotrophic cells are considerably enlarged, and storage

Table 3–Pigment and vitamin content of Chlorella sorokiniana grown under autotrophic and heterotrophic conditions on nitrate and urea-nitrogen

	Autotrophic		Heterotrophic		
	Nitrate	Urea	Nitrate	Urea	Method of analysis
Chlorophyll (%)	0.5	0.9	0.4	0.4	AOAC 6.097
Carotene (IU/100g)	44,000	34,000	31,000	25,000	AOAC 39.014–.017
Xanthophyll (μg/100g)	128	102	96	99	Chromatographic method (AOAC)
Vitamin D (USP Units/100g)	400	400	400	400	AOAC 39.116–.132 (rat bioassay)
Vitamin E (IU/100g)	0.5	5.1	5.0	1.2	Bro-Rasmussen and Hjarde (1957)
Thiamine (mg/100g)	0.6	0.6	0.6	0.1	AOAC 39.018–.028
Riboflavin (mg/100g)	2.2	3.7	2.8	2.6	AOAC 39.033–.035
Niacin (mg/100g)	21	26	19	13	AOAC 39.067–.071
Pantothenic Acid (mg/100g)	3.1	1.4	2.6	1.1	AOAC 39.075–.080
Vitamin B6 (mg/100g)	1.9	1.6	1.5	0.9	AVC[a] (1966, p. 209)
Vitamin B12 (μg/100g)	NMA[b]	NMA	NMA	NMA	AOAC 39.051–.058
Biotin (μg/100g)	120	170	71	102	AVC (1966, p. 245)
Folic Acid (mg/100g)	12.5	16.0	14.5	NMA	AOAC 39.059–.066
Vitamin C, total (mg/100g)	7.9	7.0	8.0	28.6	AVC (1966, p. 302)
Vitamin C, reduced (mg/100g)	1.1	2.7	1.2	5.4	AVC (1966, p. 320)

[a]Association of Vitamin Chemists (1966).
[b]No Measurable Amount.

Table 4—Amino acid composition[a] (% of protein) of Chlorella sorokiniana grown under autotrophic and heterotrophic conditions on nitrate and urea-nitrogen

	Autotrophic		Heterotrophic	
N-source	Nitrate	Urea	Nitrate	Urea
Alanine	6.1	7.0	7.0	2.4
Arginine	5.2	6.5	6.7	8.1
Aspartic Acid	8.0	7.2	7.4	9.0
Cystine	0.4	0.4	0.3	0.6
Glutamic Acid	NMA[b]	1.9	NMA	NMA
Glycine	4.8	4.7	4.3	6.0
Histidine	1.8	1.8	1.9	1.7
Isoleucine	4.5	6.0	5.2	5.2
Leucine	4.3	7.6	4.7	6.9
Lysine	5.9	6.0	7.2	7.1
Methionine	1.8	1.5	2.1	2.5
Phenylalanine	4.3	4.7	3.7	9.3
Proline	4.6	4.3	5.2	4.5
Serine	8.0	4.7	5.2	3.6
Threonine	3.8	3.7	3.5	3.1
Tryptophan	1.8	1.2	1.3	1.4
Tyrosine	1.1	2.8	3.4	2.3
Valine	7.9	5.9	5.0	7.1

[a] Assayed in accordance with AOAC 39.094—.107.
[b] No Measurable Amount.

Table 5—Mineral assays (ppm) of Chlorella sorokiniana grown under autotrophic and heterotrophic conditions on nitrate and urea-nitrogen

	Autotrophic		Heterotrophic		
	Nitrate	Urea	Nitrate	Urea	Method of analysis
Sodium	3,870	2,380	2,080	3,180	Atomic absorption
Potassium	1,700	11,700	1,300	3,890	Atomic absorption
Magnesium	1,510	6,060	800	1,760	Atomic absorption
Phosphorus	23,000	21,100	27,800	11,900	AOAC 22.074
Sulfur	310	1,090	680	690	AOAC 20.036
Calcium	910	2,080	420	590	Atomic absorption
Iron	107	26	103	120	AOAC 13.011
Manganese	<20	24	<20	20	AOAC 6.014
Zinc	28	167	30	6	Atomic absorption
Copper	36	35	16	2	Atomic absorption
Cobalt	0.15	0.09	0.03	0.15	Polarograph
Molybdenum	3.1	2.9	4.2	5.5	Thiocyanate-$SnCl_2$
Iodine	16.4	4.6	10.0	5.6	Specific ion electrode
Chloride	NMA[a]	1,400	NMA	1,300	Amperometric titration

[a] NMA—No Measurable Amount.

granules are readily apparent with microscopic examination.

Heterotrophy appears to have little effect on the vitamin content of *C. sorokiniana* (Table 3). The reduced amounts of chlorophyll, carotene and xanthophylls all reflect the reduced photosynthetic activity of glucose-cultured cells. The absence of vitamin B_{12} in *C. sorokiniana* supports previous evidence that *Chlorella* species neither synthesize nor require that cofactor, Droop (1966).

Amino acid composition shows little significant difference among the four sets of growth conditions (Table 4). The sulfur-containing amino acids, generally a limiting consideration in using algae as food, appear to be unaffected by heterotrophy. Lysine, low in many vegetable crops, is relatively abundant and shows relatively little difference with nitrogen or carbon source. The increased protein content of urea-grown algae over that cultured on nitrate (Table 2) probably reflects direct incorporation of the urea amine group into amino acids.

The mineral assays shown in Table 5 indicate only minor variations for most elements. The inorganic content of algae has been shown to vary considerably with concentration of the medium, Krauss (1953), a contributing factor in this study. Uptake of potassium and magnesium appears to have been more affected by nitrogen species than by energy source. In terms of nutritional support, the assays do indicate adequate amounts of all essential minerals, with the possible exception of sodium and calcium. Sodium is not considered essential for the growth of green algae and calcium is required only in trace amounts.

DISCUSSION

CHEMICAL ANALYSES indicate that *Chlorella* should be an excellent mammalian foodstuff or protein source with some supplementation, principally in the sulfur-containing amino acids. The assays reported here for autotrophically grown *C. sorokiniana* are in good agreement with analyses cited by Lubitz (1963a), Dam et al. (1965), Matthern and Koch (1964), Miller et al. (1969) and Richardson et al. (1969) for the same species grown under a variety of different conditions, including batch and continuous cultures. The composition of *C. sorokiniana* is also very similar to that of *C. pyrenoidosa*, Krauss (1962), as well as mixtures of *Chlorella ellipsoidea* and *Scenedesmus obliquus*, McDowell and Leveille (1963) and Powell et al. (1961).

Although heterotrophic culture of algae has been employed for many growth experiments, only limited chemical assay of the cellular product has been reported. Pruess et al. (1954) grew various species of *Chlorella* and other algae under aseptic conditions in large (100—1,000-gal) glass-lined fermentation tanks using a dextrose-rich medium supplemented with a 150-w projecter lamp directed into the tank through a sight glass at the top. Temperature of the culture was maintained at 20—24°C during batch runs of 142—280 hr. Although the primary purpose of the test was to obtain weight yields of algae, limited chemical analyses were performed on two batches of *C. pyrenoidosa*. Unfortunately, the two sets of assays show a wide discrepancy and are thus of limited usefulness. Compared to autotrophically grown *Chlorella*, the data do show a reduced level of protein (36 and 17%), and an elevated carbohydrate content (36.8 and 56.7%), confirmed in our experiments.

Autotrophically grown algae, principally species of *Chlorella* and *Scenedesmus*, have been tested for nutritional adequacy in rats, Lubitz (1963b) and Leveille et al. (1962); chicks, Combs (1952) and Leveille et al. (1962); rabbits, Tamiya (1961) and humans, Morimura and Tamiya (1954), Powell et al. (1961), Dam et al. (1965), Lee et al. (1967) and Waslien et al. (1970). In weight-gain studies with animals, algae have been found to be uniformly poor when fed unsupplemented as dried whole green cells. Part of the problem is acceptability and digestibility of the biomass and part is due to deficiencies in amino acids. *C. pyrenoidosa*, for example, is known to be deficient in methionine for chicks, Combs (1952), and in methionine and histidine for rats, Leveille et al. (1962). Deficiencies of other algae may be predicted from the chemical analysis of amino acid content.

More important, perhaps, is the poor acceptance and relatively low digestibility of unprocessed cells. Leveille et al. (1962) noted that the food consumption of rats and chicks receiving algae was much lower than that of control animals eating soybean or casein as the protein source. Decolorization of the algae with alcohol extraction has been found to improve acceptance, Tamura et al. (1958a), but the extracted cells without supplementation were still inferior to casein. A marked characteristic of all animal and human feeding trials has been a high fecal output with increased fecal nitrogen content, Lubitz (1963b), Leveille et al. (1962), Powell et al. (1961), Dam et al. (1965) and Waslien et al. (1970). Furthermore, electron microscopic examinations of fecal specimens from algae-fed humans and rats have provided evidence that the

cellulose cell membrane passes through the gut intact when either green or decolorized algae are consumed, Tamura et al. (1958b).

In human feeding studies, the strong, bitter taste of green algae has always posed a probelm to acceptance. Adverse reactions including incomplete digestion have been observed in humans eating as little as 30g/day of *Chlorella*, Hayami and Shino (1958). Powell et al. (1961) reported that human subjects could tolerate a mixture of *Chlorella* and *Scenedesmus* in amounts up to 100g/day, but developed acute toxicity symptoms when the level was increased to 200 and 500g/day. These symptoms included nausea, vomiting, lower abdominal cramping and bulky, hard stools, all disappearing shortly after the algae were discontinued. Dam et al. (1965) reported similar symptoms for humans eating 92g/day of whole green and ethanol-extracted *Scenedesmus*, although 90g/day of ethanol-extracted *Chlorella* was accepted without complaint when the algae were first fried in butter to further diminish the bitter taste.

From these observations, there is little question that additional processing will be needed before algae can be considered as anything more than a supplementary foodstuff. The pigmentation and bad taste of whole cells can be partially removed with alcohol extraction, but further breakdown of the cellular structure is needed to achieve better digestibility and protein utilization.

The essential question addressed by this work was whether heterotrophic algae could be used to study the nutritional adequacy of pure bacteria-free algae. Previous feeding studies have used autotrophic algae grown under nonsterile conditions. Vanderveen et al. (1962) have suggested that the acute toxicity symptoms, and perhaps the poor acceptability and digestibility, associated with algae, may be due to bacterial contaminants in the algal cultures. Certainly, a true evaluation of this factor can be made only with cells grown and processed under sterile conditions. Since no large-scale, autotrophic, continuous culture systems exist for producing bacteria-free algae, the use of commercial fermenters and heterotrophic conditions appears to be the only realistic and economic means available.

The data reported here suggest that heterotrophic algae may be satisfactory in certain types of nutritional studies but unjustifiable in others. For example, gross feeding of sterile heterotrophic cells to determine acute toxicity symptoms appears feasible. Since the fiber content of the two culture modes is very similar, heterotrophic algae should provide the same natural barrier to digestion and thus approximate the assimilatory quality of autotrophic cells. One complicating factor is chlorophyll content, which is essentially insoluble and not assimilated in the human tract. The differences in pigment concentrations encountered here occurred with illuminated "mixotrophic" cultures. It can be anticipated that the use of unilluminated stainless steel fermenters of commerce would yield somewhat lower pigment levels, although *Chlorella* species will maintain some pigment even in absolute darkness, Myers (1951). Care should be taken to provide as much light as possible to maximize the pigment content and thus more closely approximate this aspect of autotrophic algae.

Use of heterotrophic algae to simulate autotrophic cells in such nutritional parameters as weight gain, energy utilization and nitrogen balance does not appear warranted. The protein and carbohydrate concentrations of heterotrophic and autotrophic algae are substantially different, and cells from the two types of culture would be expected to yield significantly different results in these parameters. Although the elevated carbohydrate content of heterotrophic algae results in a reduced total caloric value, it essentially dilutes such non-nutritional materials as ash, chlorophyll and nucleic acid content, and would cause an increase in digestible calories.

On the other hand, studies of processing techniques intended to enhance cell wall digestibility, and the quality of the extracted or purified protein, would probably be unaffected by culture mode. Since major breakthroughs are needed in this area before serious consideration can be given to algae as foodstuff, use of heterotrophic growth conditions for production of algae as raw material for processing appears justified.

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TEMPERATURE CYCLING EFFECTS ON BACTERIAL GROWTH. 1. Pseudomonas fluorescens

SUMMARY—Although temperatures occurring in nature are constantly fluctuating, virtually all research involving temperature effects on organisms has been carried out at constant temperatures. An investigation was made to determine the effect mild cycling of temperature has on certain species of bacteria. The effect of constant and cycled-up or cycled-down temperatures was determined for Pseudomonas fluorescens. *A thermal gradient-bar proved to be a rapid, efficient method for determining these effects, affording a wide range of temperatures with only a few degrees difference between two consecutive temperatures. Cycling effects appeared to be dependent upon the organism and whether or not the temperature was above or below the optimum growth temperature. In general, the cycled-down growth responses appear to be greater than those for the cycled-up responses. At temperatures both above and below the optimum, the cycled-down organisms produced greater growth responses than the cycled-up organisms. The constant values are only a small amount greater than the cycled-down values.*

INTRODUCTION

TEMPERATURE is one of the most important single environmental factors known to affect the growth of microorganisms, yet the effects of the natural temperatures which continually fluctuate have remained virtually unassessed by researchers. Practically all the research has been carried out using constant temperature conditions. However, the natural environment for microorganisms is rarely constant, continually fluctuating either mildly or drastically.

For example, microorganisms present on foods kept under refrigeration or in frozen storage are continually subjected to mild temperature fluctuations. Since the temperature of a refrigerator or freezer is dependent upon a thermostat regulated by air currents, the microenvironment around a cell or colony changes as the air currents cycle. Thus, the temperature-dependent rate of growth of microorganisms is a function of several temperatures fluctuating between a maximum and a minimum.

The loading of a refrigerated truck or box car and shipping of food products offers another example of temperature fluctuation. Temperatures within a refrigerated box car have been reported to vary as much as 5–7°F above the thermostat settings of the car (Stewart and Harvey, 1967; Harris et al., 1967). Refrigerated trucks have a temperature variance of 6°F (Stewart and Harvey, 1967). Steam tables in restaurants are another source for temperature variance which might conceivably facilitate the growth of microorganisms. Hot and cold vending machines are excellent sources for temperature fluctuations arising from the repeated heating and/or cooling of the food.

Ng et al. (1962) showed that when cultures of *Escherichia coli* growing exponentially were rapidly changed in one step to a higher temperature, the culture immediately assumed a rate of growth normal for the higher temperature. Sudden shifts to a lower temperature, resulted in the establishment of growth rates characteristic of the lower temperature.

Ng et al. (1962) and Shaw and Ingraham (1965) showed that temperature shifts of 10–15°C made no difference in rates of growth of *E. coli*, but that a shift of 25°C resulted in a lag phase and an increase in unsaturated fatty acids in the cellular lipids.

May et al. (1964) and Evison and Rose (1965) demonstrated additional effects of one-step temperature experiments. They showed that one-step temperature increases resulted in increasing proportions of tetracycline-sensitive and penicillinase-negative cocci and in a rapid decline in the viability and rates of respiration of endogenous reserves and of exogenous glucose and pyruvate respectively.

Under the conditions employed in their experiments, Lark and Maaløe (1954) showed that when the temperature of a culture growing at 37°C is reduced to 25°C, the growth rate is characteristic of the 25°C. However, if the temperature of a culture growing exponentially at 25°C is raised to 37°C, a lag of approximately 20 min occurs and is followed by a period of increased rate of growth over that normally found at 37°C.

Powers et al. (1965) reported that faster growth occurred with *Aerobacter aerogenes* and *Staphylococcus aureus* when incubating under cyclic conditions from 40–80°F than when incubating at a constant temperature of 60°F. However, they reported that the age of the culture affected this response.

The primary objective of this study was to determine temperature cycling effects on the growth of *Pseudomonas fluorescens*.

EXPERIMENTAL

Growth of Pseudomonas fluorescens

A strain of *Pseudomonas fluorescens* was grown in tryptic soy broth (Difco) and incubated in a temperature-gradient bar which was similar in design to the one developed by Oppenheimer and Drost-Hansen (1960). The temperature-gradient bar used for this research consisted of a solid aluminum bar 83.3 × 10 × 10.2 cm, one end of which was heated with three Ungar soldering irons (37½ watts) while the opposite end was cooled by circulating ice water through the specially designed hollow end of the aluminum bar. The aluminum bar was drilled to accept 34 standard 13 × 100 mm test tubes in 90 mm deep sample wells arranged in two parallel rows along the length of the bar. The test tubes were checked for possible optical defects before using them for determining optical densities. A thermocouple well filled with mineral oil was located halfway between each of the 17 pairs of sample wells to accommodate thermocouple wires from a recording potentiometer for monitoring temperatures. In preliminary trials, a thermocouple was inserted into the media tubes in the sample wells and in the thermocouple wells halfway between the sample wells and the temperature recorded for a sufficient period of time to show that the temperatures of the three were the same. Therefore, the temperature in the thermocouple well was the same as that of the media in the sample wells, and during actual experimentation the temperature was monitored solely from the thermocouple well.

The thermal-gradient block was insulated with polystyrene foam and equipped with a Fenwall thermo-regulator to provide constant heat input.

The center well was set at the temperature corresponding to the approximate optimum growth temperature for the bacteria being investigated, resulting in a thermal gradient with temperatures above and below the optimum.

Each sample tube was filled with 7 ml of broth and inoculated with a 1% inoculum of an 18 hr culture.

Growth was followed spectrophotometrically by the use of a Spectronic 20 colorimeter (Bausch and Lomb, Inc.) at 640 mμ. Optical density was recorded for each of the sample tubes every hour for the first 12 hr, and every 2 hr thereafter for a total of 30 hr.

The cycled replications were made by moving the samples either up or down the thermal gradient every ½ hr and moving them back again the second half hour. Preliminary tests showed that it required approximately 12 min for the sample medium to attain the new temperature after being moved either up or down the thermal gradient. Therefore, the temperature cycle is best described as a square wave. Since culture aeration was necessary, the entire thermal-gradient block was mounted on an Eberback Shaker and rapidly agitated.

Calorimetry

To obtain values for the heat of combustion of tryptic soy broth, a Phillipson oxygen microbomb calorimeter (Gentry and Weigert Instruments) was used. The culture medium was centrifuged at 3500 × G for 20 min to separate the

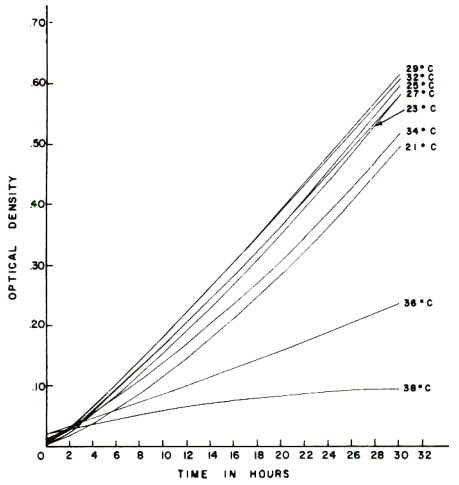

Fig. 1—Growth curves of Pseudomonas fluorescens at constant temperatures.

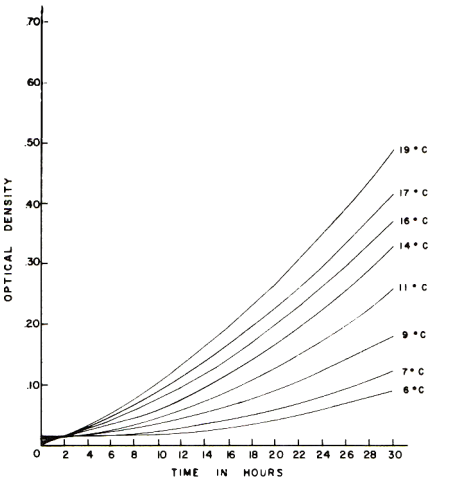

Fig. 2—Growth curves of Pseudomonas fluorescens at constant temperatures.

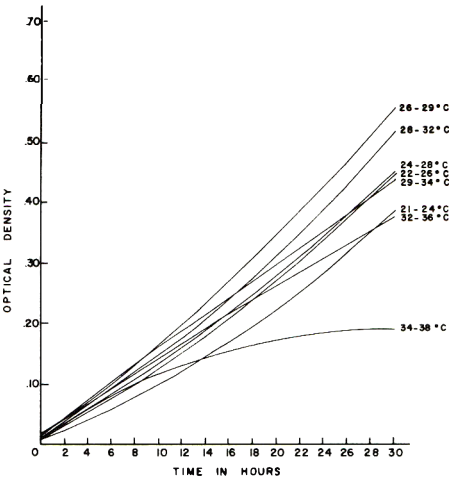

Fig. 3—Growth curves of Pseudomonas fluorescens at cycled-up temperatures.

broth from the cells. The sample was then prepared for combustion by lyophylization after which it was formed into a pellet. Each pellet weighed between 10–15 mg with weights recorded to the nearest 0.01 mg. Calculations for calories/mg were made according to the method proposed by Phillipson (1964).

Plate counts

Standard plate counts were made on TGE agar after 12 and 30 hr of incubation. Plate counts were made only during replications involving cycling of the temperature.

RESULTS & DISCUSSION

GROWTH CURVES for *Pseudomonas fluorescens* at temperatures ranging from 38–9°C with approximately 2°C intervals are shown in Figures 1 and 2. These and all subsequent growth curves represent the best fitting curves obtained from a regression analysis of the data. All curves are represented by an equation of the form $Y = a + bx + cx^2$, where y = optical density and x = time in hours. The optimum growth temperature using the thermal-gradient bar was in the range of 29–32°C with 29°C giving the best total growth response. The generation time or time required for a doubling of the optical density (Palumbo et al., 1967; Ingraham, 1958) was approximately 5 hr at this temperature. Palumbo et al. (1967) reported generation times of 4, 5 and 6 hr for *P. fluorescens* grown at 28°C with aeration, with shaking and with no aeration respectively. It is apparent that temperatures as high as 36°C and 38°C exerted a suppressive effect on the growth of *P. fluorescens*.

Figure 2 shows the completion of the family of curves representing growth obtained at 19–6°C inclusive. On the colder end of the thermal gradient bar, growth begins to decline markedly at 11°C, with temperatures of 7°C and 6°C affording very little growth at all.

Figures 3 and 4 show the growth responses of *P. fluorescens* at various cycled-up temperatures. The cycled-up temperature of 26–29°C gave the earliest and best growth response. It is interesting to note that 26–29°C represents a temperature from just below the optimum up to the optimum where the sample remained at 29°C for approximately 18 min; whereas, the temperature range of 28–32°C includes temperatures both above and below the optimum but gave a lesser growth response. This would be expected since in cycling from 28–32°C the sample merely passed through the optimum temperature and moved farther away from it. It can be seen from the graph that cycling-up above the optimum temperature, for example 28–32°C, gave a much greater growth response than cycling-up below the optimum such as at 24–28°C. Again a suppressive effect on growth is observed with temperatures as high as 34–38°C and as low as 16–19°C. As the average temperature decreases, the lag phase becomes

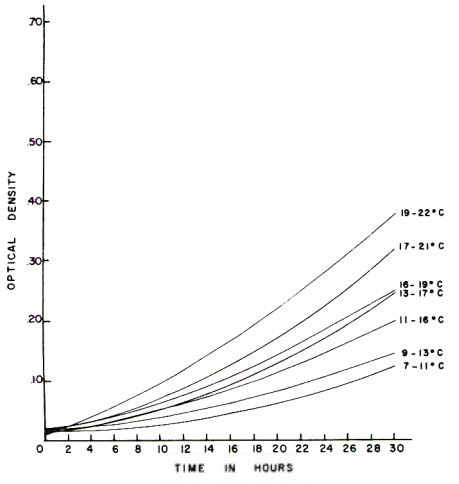

Fig. 4—Growth curves of Pseudomonas fluorescens at cycled-up temperatures.

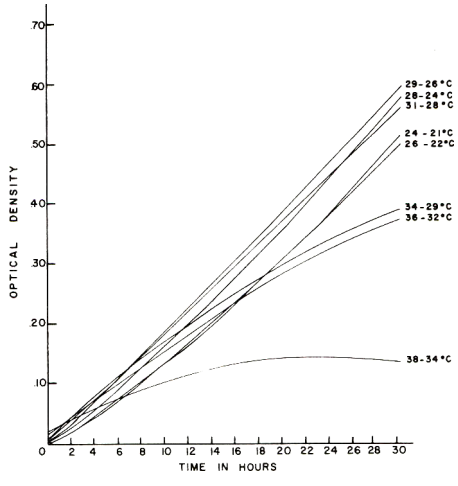

Fig. 5—Growth curves of Pseudomonas fluorescens at cycled-down temperatures.

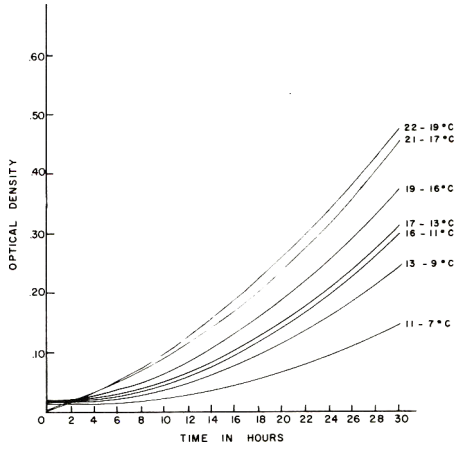

Fig. 6—Growth curves of Pseudomonas fluorescens at cycled-down temperatures.

Table 1—F values when comparing constant temperature growth curves to cycled-up temperature growth curves, constant to cycled-down temperature growth curves and cycled-up vs. cycled-down temperature growth curves

Constant Temp. (°C)	Cycled-up Temp. (°C)	Cycled-down Temp. (°C)	Constant vs. cycled-up F value	Constant vs. cycled-down F value	Cycled-up vs. cycled-down F values
36	34–38	38–34	2.3541	7.5855*	12.9452*
34	32–36	36–32	5.4494*	5.7562*	0.0971
32	29–34	34–29	16.2250*	49.2057*	1.2684
29	28–32	32–28	11.7186*	3.6316*	6.0866*
27	26–29	29–26	3.7026*	0.1507	5.0901*
25	22–26	26–22	31.0884*	13.2289*	4.0627*
23	21–24	24–21	58.9294*	6.2021*	24.3533*
21	19–22	22–19	12.4711*	0.7665	5.2120*
19	17–21	21–17	22.8524*	0.9539	30.3835*
17	16–19	19–16	69.1212*	6.8371*	115.3184*
16	13–17	17–13	15.2314*	4.8333*	19.7264*
14	11–16	16–11	19.2875*	9.8761	16.8771*
11	9–13	13–9	19.2167*	0.2610	33.3036*
9	7–11	11–7	6.6843*	1.8149	9.1630*

*Significant at the 5% level

longer below the optimum temperature and shorter above the optimum temperature.

Figures 5 and 6 show the growth responses of *P. fluorescens* at various cycled-down temperatures. Again the best growth response was obtained at 29–26°C, a temperature range including the optimum growth temperature and a temperature just below the optimum. Cycling-down from below the optimum, for example 28–24°C, gave a greater total growth response than cycling-down from above the optimum to just below the optimum such as at 32–28°C. A marked decrease in growth rate is observed at cycled-down temperatures beginning at 17–13°C.

Table 1 shows the F values when comparing the slopes of the constant temperature growth curves to the slopes of their corresponding cycled-up or cycled-down temperature growth curves, and when comparing the slopes of the cycled-up temperature growth curves to their corresponding cycled-down temperature growth curves. All but one of the constant vs. cycled-up slopes were significantly different at the 5% level, but only eight out of fourteen of the constant vs. cycled-down slopes were significantly different. This is further evidence that the cycled-down growth curves more nearly approached the magnitude and the duration of the constant temperature growth curves. Also, twelve out of fourteen of the cycled-up vs. cycled-down growth curves were significantly different.

Calorimetry

It is well established that the sum of the total energy of the bacterial cell and that used up by catabolism must equal the energy utilized from the medium. Mayberry et al. (1968) who were attempting to establish a common basis for predicting yields of dry weight of cells from various substrates used calorimetry. Determination of the heat of combustion of the cells and of the growth medium after incubation should give a measurement of growth and energy balance. The heat of combustion of the medium was determined as an indication of the energy utilized from the medium. The range in calories/mg medium left after 30 hr of constant temperature incubation was 3.37–3.68, while the unused medium contained 3.70 cal/mg. There was a failure to demonstrate any differences in the heat of combustion of the spent medium from the constant, cycled-up or cycled-down incubation flasks. Incubation periods of longer than 30 hr might have demonstrated a greater difference. Subsequent work by Howell et al. (1971) has shown that 48 hr is still not long enough to show a difference in caloric content of the medium. The caloric content of the cells appears to have a constant heat of combustion regardless of temperature or growth medium used which agrees with the work of Mayberry et al. (1968).

Plate counts

Total plate counts were made after 12 and 30 hr of incubation at cycled-up and cycled-down temperatures. The highest plate counts were obtained at a cycled-up temperature of 26–29°C after 12 hr of incubation and at 24–28°C after 30 hr of incubation. However, after 12 and 30 hr of incubation the highest plate counts were obtained at a cycled-down temperature of 29–26°C. In general, there was no significant difference in plate counts between cycling-up and cycling-down.

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EFFECT OF EDTA ON THE GERMINATION OF AND OUTGROWTH FROM SPORES OF Clostridium botulinum 62-A

SUMMARY—EDTA, in concentrations above 2.5 mM, was found inhibitory to germination of and outgrowth from spores of C. botulinum *Type A and to toxin production in a fish homogenate. Inhibitory action was influenced by pH of the medium in the range pH 6.5–8.1, the action increasing with pH. It was influenced by Mg and Ca concentrations in the medium, equimolar concentrations of added $CaCl_2$ or $MgCl_2$ completely erasing the growth inhibitory action. Initial spore concentration also influenced inhibitory efficacy—the higher the spore concentration, the higher the EDTA concentration required for inhibition. There was no evidence that EDTA, in any concentration used, promoted spore germination. Release of Ca, Mg and DPA from incubating spores was suppressed to varying extents by 5.0 and 10 mM EDTA.*

INTRODUCTION

IT IS WELL known that *Clostridium botulinum* is widely distributed in nature and its presence as a contaminant of most unprocessed foods may be expected. Three types (A, B and E) are of primary concern as causative agents of human botulism. These organisms will not grow and produce toxin in foods having pH values below about 4.5; therefore, they are of importance only in the low-acid foods—those with pH values above 4.5, of which there are a vast number. Most low-acid canned foods are given heat processes of sufficient severity to free them of spores of these organisms. However, there are a number of important food items which, for quality reasons, cannot be given such severe heat processes—especially, processes sufficiently severe to eliminate the more heat resistant spores of Types A and B. For these products, refrigeration and/or bacterial inhibitors must be depended upon to prevent growth of and toxin production by *C. botulinum*. Pasteurized cured hams and smoked fish items are good examples.

Types A and B will generally not grow at temperatures below about 10°C, but Type E may grow at temperatures as low as 4.5°C. Though refrigeration is a most effective growth inhibitor, perishable food items are vulnerable to temperature abuse from preparation to consumption. Historically, there are many cases of botulism traceable to such abuse. Therefore, there is a never ending search for acceptable inhibitors which will prevent growth of *C. botulinum* should temperature abuse occur.

Ethylenediaminetetraacetic acid (EDTA) has been shown to inhibit growth of a number of microbial species (Levin, 1967, and others). However, the authors failed to find any reference to efficacy of this compound as an inhibitor of *C. botulinum*. Therefore, this study was undertaken to determine its effect on germination of and outgrowth from spores of *C. botulinum* Type A. For the studies, pork infusion agar, polypeptone agar and polypeptone broth were chosen as representative of optimal growth media and fish homogenate as representative of low-acid food products.

EXPERIMENTAL

Materials

Test chemical. The tetra sodium salt of ethylenediaminetetraacetic acid was used.

Test organism. *Clostridium botulinum* Type A, strain 62 was used. That the organism was Type A was verified by mouse protection tests employing specific antitoxin. For the tests, the organism was cultured in polypeptone broth by inoculating with clean spores and incubating for 14 hr at 30°C. After incubation the culture was centrifuged and the supernatant used for mouse injection. All mice injected with heated supernatant, or injected with Type A antitoxin 30 min prior to injection of unheated supernatant, lived. All unprotected mice injected with unheated supernatant died within a period of 2–5 hr after injection. Symptoms in all mice that died were typical of botulism.

Medium for spore production. Beef heart-casein broth, prepared as described by Stumbo (1965), was employed.

Test media. Four different media were employed: pork infusion agar, polypeptone broth, polypeptone agar and fish homogenate. The pork infusion agar was prepared as described by Stumbo (1965). The polypeptone broth consisted of 50g polypeptone, 5g glucose and 0.5g sodium thioglycolate in 1 liter of 0.01M phosphate buffer. The polypeptone agar was prepared by adding 1.5% agar to the polypeptone broth. The fish homogenate was prepared from gutted white fish chubs (*Leucichthys hoyi*). A mixture of two parts distilled water and one part fish flesh (free of tails, fins and heads) was blended in a Waring Blendor for 5 min, then sterilized in the blender jar by autoclaving at 121°C for 20 min, cooled and blended again for 5 min. For use, all media were distributed in culture tubes in 20 ml quantities. The presterilized fish homogenate was transferred aseptically to sterile tubes. The other media were sterilized in the tubes after distribution.

Production and preparation of clean spores. A number of culture tubes, each containing 20 ml of beef heart-casein broth, were inoculated with mature spores. The inoculated tubes were heated at 80°C for 20 min to activate the spores, then incubated at 30°C. When outgrowth cells were judged to be in the logarithmic phase of growth, and before sporulation began, they were added to larger volumes (150 ml each) of medium and incubated at 30°C for mass spore production.

After sporulation was judged by microscopic examination as virtually maximal, the cultures were placed at 2°C for 24 hr to lyse remaining vegetative cells. The cultures were then filtered through layers of sterile cheesecloth and glass wool and the filtrates collected and centrifuged at 5000G for 20 min at 2°C. The supernatant was decanted and the pellet of spores resuspended in cold sterile distilled water, and again centrifuged. This procedure was repeated six times. After the final centrifugation the spores were resuspended in deionized sterile water and stored at 2°C. This stock spore suspension was employed throughout the study, in which case some of the suspension was stored for as long as 8 months before use. Periodic microscopic examination during this period revealed no evidence of "spontaneous" germination.

Preparation of test EDTA concentrations. A one molar stock solution of $Na_4EDTA \cdot 2H_2O$ was prepared in deionized water and sterilized by Millipore (0.45μ) filtration. Test concentrations were obtained by adding 0.1 ml of an appropriate dilution of this stock solution to 20 ml of medium so as to give the desired EDTA concentration in the medium.

Measurement of germination and outgrowth. When germination alone was being studied, its extent was measured by the loss of heat resistance method as described by Wynne and Foster (1948). When both germination and outgrowth were being studied they were followed by measuring change in optical density (O.D.) at 600 mM (Bonventre and Kempe, 1959) using a Spectronic 20 colorimeter.

Measurement of dipicolinic acid (DPA) released during germination. After an appropriate incubation (30°C) period, during which microscopic examination showed that most spores had germinated, the cultures were centrifuged at 5000G for 20 min and DPA concentrations in the germinated spores determined by the method of Jannsen et al. (1958). The same method was employed for determination of DPA in control spores. The difference between DPA content of incubated and control spores was expressed as DPA released.

Measurement of Ca and Mg released. Ca and Mg concentrations in control and incubated spores were measured using an Atomic Absorption Spectrophotometer (Perkin-Elmer). The procedures employed were adapted from those described by Perkin-Elmer (1968) which entailed ashing of the spores and treatment of the

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ash with lanthanum solutions for absorption measurement. For Ca, absorption was measured at 4226A°, and for Mg, at 2852A°. Ca and Mg concentrations were obtained by referring absorption readings to previously prepared standard curves. Amounts of Ca and Mg released were taken as the differences in contents of incubated and control spores.

Methods

Influence of EDTA on growth and toxin production in fish homogenate. Sterile fish homogenate, in culture tubes (20 ml per tube), containing varying concentrations of EDTA (0–7.5 mM per liter) was inoculated with spores of *C. botulinum* and incubated at 30°C. Each tube was inoculated with 100 spores. Anaerobiosis during incubation was maintained by overlaying the homogenate in each tube with about ½ in. of sterile sealing mixture (1 part paraffin, 1 part vaseline and 4 parts mineral oil). Ten replicate tubes were employed for each variable. During incubation, the tubes were observed for visible spoilage and after prescribed incubation periods the contents were tested for toxicity. These tests showed that only tubes judged as spoiled were toxic. For toxicity tests, the tubes of homogenate were removed from incubation, centrifuged and 0.25 ml quantities of supernatants injected intraperitoneally into male white mice weighing approximately 20g each. Results of this study are summarized in Table 1. It may be noted that growth and toxin production was completely inhibited by 5.0 and 7.5 mM of EDTA, though growth was delayed somewhat by 0.5 and 1.0 mM and considerably by 3.0 mM.

A second study was conducted to determine the effect of initial spore population on the ability of different concentrations of EDTA to inhibit growth of *C. botulinum* in fish homogenate. Again ten replicate tubes per variable, each containing 20 ml of homogenate, were employed and growth was judged by visual evidence of spoilage (gas production and fish flesh coagulation). Results of this study are summarized in Table 2. They demonstrate that initial spore concentration definitely influences the ability of a given EDTA concentration to inhibit growth; though 5.0 mM was adequate to inhibit growth from spore concentrations up to 500 per tube (25 per ml), 7.5 mM was required to completely inhibit growth from the higher spore concentrations.

Influence of EDTA on growth of C. botulinum in pork infusion and polypeptone agars. The experimental design and procedure used here were virtually the same as for the fish homogenate studies. Results were almost identical with those obtained when using fish homogenate as the substrate, except that slightly lower concentrations of EDTA were required for complete inhibition (Table 3). In this study complete inhibition was obtained with 5 mM of EDTA, even with an initial spore concentration of 50,000 per tube (2500 per ml). It should be noted that in this study gas production and diffusion of growth precluded making accurate colony counts though this was the original intention; therefore, only the number of tubes positive or negative for growth could be observed.

Effect of pH. In view of the observation that somewhat lower concentrations of EDTA were required to inhibit outgrowth from spores of *C. botulinum* in pork infusion and polypeptone agars than in fish homogenate, it was reasoned that perhaps pH difference might account, at least in part, for the results observed. (The pH of the agars was about 7.1, while the pH of the fish homogenate was about 6.6.) To test this hypothesis, the following studies were carried out.

Aliquots of sterile polypeptone broth were adjusted to pH values of 6.0, 6.5, 7.1, 7.5, 8.1 and 9.00. 4 ml quantities of each were then inoculated with 0.2 ml of stock spore suspension to give initial O.D. readings in the range of 0.40–0.45 at 600 nm. These were then incubated at 30°C and O.D. readings taken hourly until growth was near maximum. In all O.D. studies side arm tubes were employed so that O.D. readings could be taken periodically with minimum disturbance of incubating samples. Results of the study are graphically depicted in Figure 1. Taking reduction in O.D. as indicative of germination, it is evident that pH affected germination only slightly over the range pH 6.5–8.1, being completed somewhat sooner at pH 7.1. At pH 6.0 it was definitely retarded, while at pH 9.0 it was virtually blocked. Taking rate and extent of O.D. change after comple-

Table 1—Growth of C. botulinum in fish homogenate containing varying concentrations of EDTA and toxicity of samples after indicated incubation periods

Supernatant from	Inoculation	Observation		Mouse reaction within 4 days	
		Growth	Weeks incub.	#1	#2
Plain FH[a]	UI[b]	NG[d]	6	S[b]	S
Plain FH	I[c]	G[e]	1	D[g]	D
FH + 0.1 mM EDTA	UI	NG	6	S	S
FH + 0.1 mM EDTA	I	G	1	D	D
FH + 0.5 mM EDTA	UI	NG	6	S	S
FH + 0.5 mM EDTA	I	G	1–2	D	D
FH + 1.0 mM EDTA	UI	NG	6	S	S
FH + 1.0 mM EDTA	I	G	1–2	D	D
FH + 3.0 mM EDTA	UI	NG	6	S	S
FH + 3.0 mM EDTA	I	G	4–6	D	D
FH + 5.0 mM EDTA	UI	NG	6	S	S
FH + 5.0 mM EDTA	I	NG	6	S	S
FH + 7.5 mM EDTA	UI	NG	6	S	S
FH + 7.5 mM EDTA	I	NG	6	S	S

[a]FH = Fish Homogenate
[b]UI = Uninoculated
[c]I = Inoculated (100 spores per 20 ml)
[d]NG = No Growth
[e]G = Growth
[f]S = Survived
[g]D = Died

Table 2—Influence of initial spore population on the ability of different concentrations of EDTA to inhibit outgrowth of C. botulinum in fish homogenate

EDTA in mM	Inoculum in spores per tube containing 20 ml of homogenate											
	0		100		500		1,000		5,000		10,000	
	Observation time (wk)	No. spoiled tubes in ten	Observation time (wk)	No. spoiled tubes in ten	Observation time (wk)	No. spoiled tubes in ten	Observation time (wk)	No. spoiled tubes in ten	Observation time (wk)	No. spoiled tubes in ten	Observation time (wk)	No. spoiled tubes in ten
0	6	0	1	10	1	10	1	10	1	10	1	10
2.5	6	0	6	8	4	10	2	10	2	10	2	10
5.0	6	0	6	0	6	0	6	1	6	10	6	10
7.5	6	0	6	0	6	0	6	0	6	0	6	0
10.0	6	0	6	0	6	0	6	0	6	0	6	0

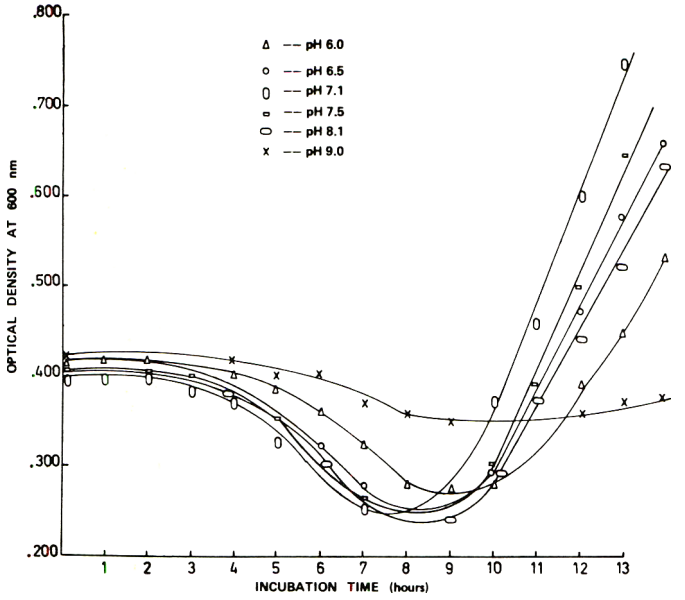

Fig. 1—The effect of pH on the germination of and outgrowth from spores of C. botulinum Type A in polypeptone broth.

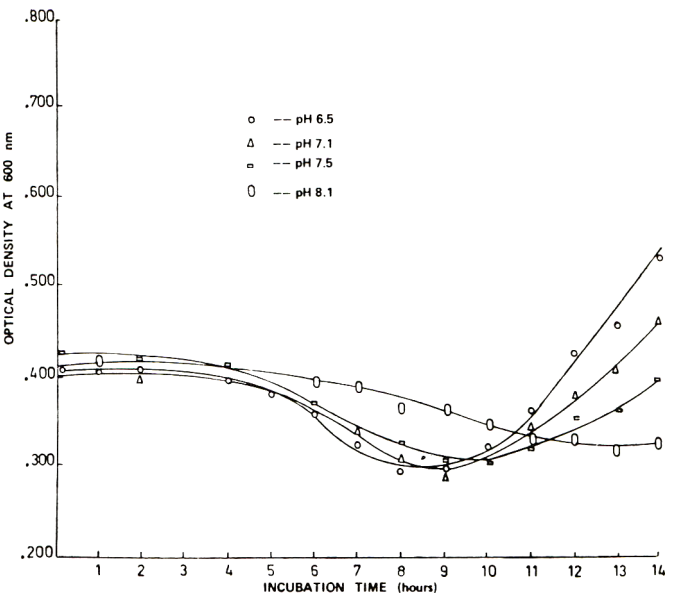

Fig. 2—The effect of pH on the germination of and outgrowth from spores of C. botulinum Type A in polypeptone broth with 3.0 mM EDTA.

tion of germination as a measure of growth, it is obvious that growth was best at pH 7.1 and next best at pH 7.5, At pH 6.0, it was substantially retarded, while at pH 9.0 it was entirely prevented.

A second similar study was carried out using polypeptone broth, to which 3 mM of EDTA per liter was added. Results of this study are graphically depicted in Figure 2. Comparing results depicted in Figures 1 and 2, three things are quite obvious: (1) EDTA in this concentration definitely decreased the extent of germination in the pH range of 6.5–8.1, virtually blocking it at pH 8.1, during the 14-hr incubation period; (2) it substantially reduced growth over the entire pH range, completely preventing growth at pH 8.1; and (3) the growth inhibitory ability of 3 mM of EDTA directly correlated with pH over the pH range of 6.5–8.1, increasing as pH of the medium increased.

Effect of added Ca and Mg on the ability of EDTA to inhibit growth. It was believed that another factor which might account, at least in part, for EDTA being less effective in fish homogenate than in agar could be the greater cation (particularly Ca^{++}) content of the fish homogenate—the fish were not deboned before blending. To test this hypothesis a study was carried out to determine the effect of added Ca and Mg on the inhibitory efficacy of EDTA.

The study was carried out using both pork infusion and polypeptone agars as culture media. Tubes of agar (10 per variable containing 20 ml of agar each), to which different concentrations of Ca or Mg, and 10 mM of EDTA were added, were each inoculated with approximately 500 activated spores and incubated at 30°C. It was originally intended to make colony counts after prescribed periods of incubation; however, spreading growth and gas production made accurate colony counting impossible. Therefore, growth was recorded as either positive or negative based on visible growth and/or gas production. Results obtained with added Ca are given in Table 4. Results obtained with added Mg were essentially identical.

Effect of EDTA concentration on germination. The method of Wynne and Foster (1948) employing loss of heat resistance as the criterion of germination, was used in this study. Polypeptone broth (pH 7.2), to which varying concentrations of EDTA were added, was used as the germination medium. Tubes of this broth (20 ml each) were inoculated so as to give about 400 spores per tube and heated at 75°C for 20 min to dispel dissolved oxygen and activate the spores for germination. All tubes were then incubated at 30°C. At given intervals four tubes were removed from each set and heated at 75°C for 20 min to inactivate germinated spores and vegetative cells that might have developed during incubation. Residual spore counts were made by subculturing in pork infusion agar and making colony counts after 3–4 days incubation at 30°C. Results of the study are graphically depicted in Figure 3. It is obvious that germination was only slightly affected by 2.5 mM EDTA, while it was substantially reduced by 5.0 mM and virtually prevented by 10 mM. The result with 5.0 mM is of particular interest since it was found earlier that this concentration completely inhibited outgrowth in polypeptone agar. The next study was conducted to shed further light on this point.

Table 3—Effect of EDTA on outgrowth from different spore concentrations of C. botulinum Type A in pork infusion (PI) and polypeptone (PP) agars

EDTA Conc. (mM)	Inoculum in spores per ml									
	0		50		500		5,000		50,000	
	PI agar + tubes[a]	PP agar + tubes[a]	PI agar + tubes[a]	PP agar + tubes[a]	PI agar + tubes[a]	PP agar + tubes[a]	PI agar + tubes[a]	PP agar + tubes[a]	PI agar + tubes[a]	PP agar + tubes[a]
0	0	0	10	10	10	10	10	10	10	10
4.0	0	0	0	0	0	0	0	3	2	10
5.0	0	0	0	0	0	0	0	0	0	0
7.5	0	0	0	0	0	0	0	0	0	0

[a]Number of tubes in ten positive for growth after 14 days' incubation at 30°C.

Table 4—The effect of different concentrations of $CaCl_2$ on growth of C. botulinum Type A in pork infusion and polypeptone agars containing 10.0 mM EDTA

Concentration of $CaCl_2$ (mM)	Growth in PI agar		Growth in PP agar	
	Incubation time (days)	No. positive tubes	Incubation time (days)	No. positive tubes
0	14	0	14	0
1.0	14	0	14	0
2.5	14	0[a]	14	0[a]
5.0	14	0[a]	14	7[a]
7.5	14	4[a]	2	10
10.0	3	10	2	10
15.0	3	10	2	10

[a]Observation continued until there was no change for 1 month in number of tubes positive or negative for growth.

Table 5—Effect of EDTA on release of DPA, Ca and Mg during incubation of C. botulinum Type A spores in polypeptone broth[a]

Incubation time (hours)	Percent released in broth containing								
	0 mM EDTA			5.0 mM EDTA			10.0 mM EDTA		
	Ca	Mg	DPA	Ca	Mg	DPA	Ca	Mg	DPA
3	11	5	32	13	6	30	8	–	13
6	61	32	72	45	21	52	10	7	16
9	83	46	96	50	28	61	15	8	31

[a]Concentrations of Ca, Mg and DPA in spores (control) were: Ca—5.4 μg/mg; Mg—0.9 μg/mg; and DPA—39.1 μg/mg on a dry weight basis.

Effect of EDTA concentration on germination and outgrowth as determined by changes in optical density. Again, polypeptone broth was used as the germination and outgrowth medium. Tubes containing 4 ml of broth, to which variable amounts of EDTA were added, were inoculated with 0.2 ml of stock spore suspension to give initial O.D. readings of about 0.40. The inoculated tubes were then incubated at 30°C and O.D. readings taken periodically until growth appeared to be near maximum. O.D. readings observed were plotted against corresponding incubation times to obtain the curves depicted in Figure 4. Again taking reduction in O.D. as indicative of germination, results of this study confirm the above study in which loss of heat resistance was taken as a measure of germination. It may be noted that 2.5 mM of EDTA decreased the extent of germination slightly, 5.0 mM decreased it appreciably, 7.5 mM almost blocked it and 10mM completely inhibited it. It is interesting that, though there was appreciable germination with 5.0 mM EDTA, outgrowth was completely inhibited. With 7.5 mM germination was slight and outgrowth was completely inhibited and with 10 mM both germination and outgrowth were completely inhibited.

Effect of EDTA on release of dipicolinic acid (DPA), Ca and Mg. Since the above described studies demonstrated that EDTA influences germination as well as outgrowth, and since it is well known that DPA and cation release is closely associated with spore germination, it was considered of interest to determine the influence of different concentrations of EDTA on such release.

Flasks of polypeptone broth (50 ml each) containing 0, 5.0 or 10.0 mM of EDTA per liter were each inoculated with 2.0 ml of stock spore suspension and incubated at 30°C. After 3, 6 and 9 hr incubation, flasks were removed and the cultures centrifuged at 5000G. Supernatants were discarded and the sediments suspended in deionized distilled water. These were centrifuged and the supernatants again discarded. DPA, Ca and Mg contents of the sediments and of control spores were determined by methods described above. Amounts released were taken as differences in contents of control and incubated spores. Results obtained are given in Table 5. (The data represent averages of duplicate determinations.) It may be noted that release of both DPA and Ca was substantially suppressed by 5.0 mM of EDTA and severely suppressed by 10 mM. The effect on Mg release was not clearcut. The effect on DPA and Ca release appears compatible with observed effects of different concentrations of EDTA on germination of spores of *C. botulinum* Type A, strain 62.

DISCUSSION

THE FOREGOING studies demonstrated that a 5 mM concentration of EDTA in fish homogenate (1 part fish flesh, 2 parts distilled water) completely inhibited both growth of and toxin production by *C. botulinum* Type A, strain 62, when the sterile fish homogenate was inoculated with 5 spores per ml. (In all cases tested, where there was visibly detectable growth there was toxin production—and only then.) Lower concentrations failed to

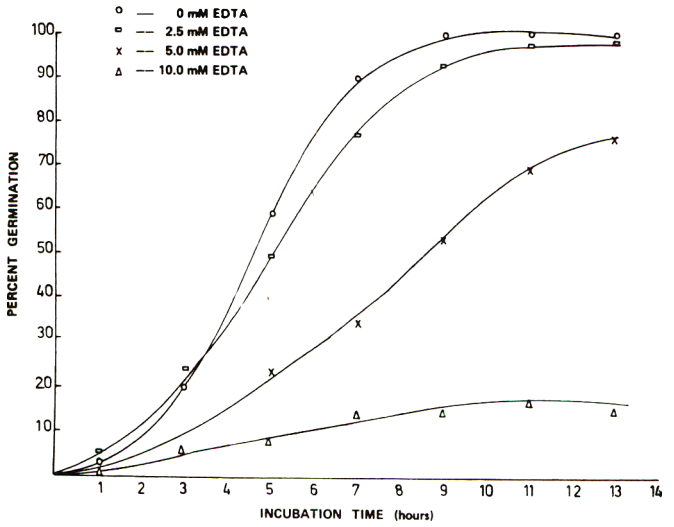

Fig. 3—The effect of different concentrations of EDTA on the germination of C. botulinum Type A.

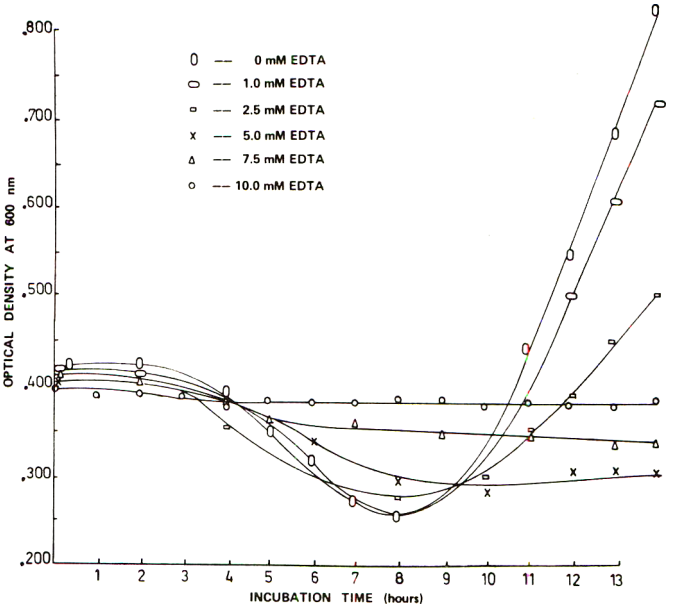

Fig. 4—The effect of EDTA at various concentrations on the germination of and outgrowth from spores of C. botulinum Type A.

inhibit, though growth and toxin production were delayed by EDTA concentrations of 0.5, 1.0 and 3.0 mM.

In a second experiment, in which different spore populations were employed, it was found that 5 mM inhibited growth in homogenate inoculated with 5 or 15 spores per ml, but was inadequate to inhibit growth in homogenate inoculated with 50, 250 or 500 spores per ml. With these higher spore levels, 7.5 mM was required to inhibit growth.

For comparison purposes, the influence of EDTA in pork infusion and polypeptone agars was investigated. Here it was found that 5 mM of EDTA was sufficient to inhibit growth regardless of initial spore concentration up to 2500 spores per ml. 4 mM failed to inhibit completely when the initial spore concentrations were 250 or 2500 per ml.

There appeared to be two possible reasons why somewhat higher concentrations of EDTA were required to inhibit growth in the fish homogenate than in the agars, namely, pH and cation concentration differences. The pH of the fish homogenate was about 6.6, while the pH of the agars was about 7.1. Since the fish were not deboned prior to homogenation, the cation (especially Ca^{++}) concentration would naturally be higher in the homogenate than in the agars. EDTA being a strong chelating agent, it appeared reasonable that its growth inhibitory capacity might be substantially reduced by its combination with cations of the medium.

In pH studies, polypeptone broth was used as the test medium and germination and outgrowth were followed by observing optical density changes during incubation. When no EDTA was added, it was found that pH in the range of 6.5–8.1 had no significant effect on germination. Germination was both slowed and reduced at pH 6.0 and virtually blocked at pH 9.0. (Reduction in optical density was considered indicative of germination.) Growth was best at pH 7.1, appreciably reduced at pH 6.0, 6.5, 7.5 and 8.1, and completely inhibited at pH 9.0.

In another study, in which 3.0 mM of EDTA was added to the polypeptone broth, it was found that germination was influenced only slightly, if at all, at pH 6.5, 7.1 and 7.5 but completely inhibited at pH 8.1. In this case, growth was best at pH 6.5. At pH 7.1 growth was reduced, at pH 7.5 further reduced, and at pH 8.1 completely inhibited.

Results of this study show that the growth inhibitory efficacy of EDTA is definitely influenced by pH in the pH range of 6.5–8.1, increasing with pH over this range. This could well account, at least in part, for the differences in EDTA inhibitory efficacy in fish homogenate and in polypeptone and pork infusion agars. However, it was still believed that cation concentration might play an important role in this respect. In further experiments it was demonstrated that 10 mM of $CaCl_2$ or $MgCl_2$ added to polypeptone broth, containing 10 mM of EDTA, completely erased the growth inhibitory action of EDTA, while 5 and 7.5 mM of these compounds appreciably reduced inhibitory action. It was therefore concluded that differences in EDTA inhibitory action in fish homogenates and in pork infusion and polypeptone agars could well be due to a combination pH and cation effect.

These studies further demonstrated that EDTA affects both germination and outgrowth. Certain concentrations which failed to inhibit germination completely were found to inhibit outgrowth. With respect to germination, findings in this study were interesting compared to those of Brown (1956) and Riemann (1961) who found that low concentrations of EDTA promoted germination of spores of the Putrefactive Anaerobe (PA) 3679, an organism which is closely related, physiologically, to *C. botulinum* in many respects. In this study there was no evidence that EDTA, in any concentration studied, promoted germination. In fact, it was demonstrated that DPA and Ca release, known to be associated with spore germination, was suppressed by EDTA in concentrations of 5.0 and 10.0 mM.

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INFLUENCE OF SEX AND POSTMORTEM AGING ON INTRAMUSCULAR AND SUBCUTANEOUS BOVINE LIPIDS

SUMMARY—Fifteen wholesale beef ribs from an equal number (5) of half-sib bulls, steers and heifers were used to study the changes in subcutaneous (SQ) and longissimus dorsi intramuscular (IM) lipids at 2, 7, 14 and 21 days post-mortem (PM). The progressively increasing free fatty acid (FFA) levels observed in both depots with time PM were paralleled by fatty acid composition changes. During the aging period the content of C14:0 in the FFA fraction decreased from 11.7–5.8%; the level of C18:1 increased from 42.6–49.7%. Cholesterol and phospholipid levels did not change with PM aging. PM aging had no effect on the composition of the IM phospholipid class, suggesting that autoxidation does not readily occur during aging of fresh beef ribs. Fatty acid composition of the bull IM lipid fractions frequently differed from the fatty acid composition of these fractions in heifers or steers; the latter two sexes had similar fatty acid compositions in their IM lipid fractions. In the SQ triglyceride fraction, heifers had higher and lower levels of C18:1 and C16:0, respectively, when compared to both bulls and steers. Traces of C22:6 were present in the IM free fatty acid fraction of heifers, whereas 3.2 and 4.1% were present in the FFA of steers and bulls, respectively. Rib steaks from heifers had significantly ($P < .05$) higher sensory panel scores for aroma and lower IM and SQ levels of FFA when compared to bulls. A significant ($P < .01$) correlation of −0.49 was obtained between aroma score and IM levels of FFA.

INTRODUCTION

THE FATTY ACID composition of meat tissue lipids in relation to sex and anatomical location of porcine (Allen et al., 1967a; Allen et al., 1967b) and bovine animals (O'Keefe et al., 1968; Terrell and Bray, 1969; Hornstein et al., 1961; Hornstein and Crowe, 1967) has been widely studied. The role of water-soluble aroma and flavor precursors in meat has been well documented (McCain et al., 1968; Zaika et al., 1968 and Dryden et al., 1969) but relatively few studies (Waldman et al., 1968, and Dryden and Marchello, 1970) relating the quantity and composition of muscle lipid to taste panel parameters have been reported.

Doty and Pierce (1961), using fat obtained from broiled rib steaks reported, without explanation, an increase in fat flavor with postmortem aging. Hornstein and Crowe (1960) heated fat from beef and pork under vacuum and in nitrogen, producing non-meaty aromas; heating in air produced characteristic odors associated with beef and pork. Since a certain degree of fat oxidation appears to be important in development of flavor, the quantity and composition of the free fatty acids, which are more easily oxidized than triglycerides (Fredholm, 1961), appear to be important factors in developing an aged beef flavor. It has been implied that free fatty acids have an important function in the development of flavor in dry sausages and country-cured hams. Peters et al. (1968) used refrozen cod muscle and Pearson (1968) used ground meat; both obtained high negative correlations between free fatty acid concentration and taste panel scores for aroma.

The present study was designed to provide a comprehensive investigation of the effect of sex and postmortem aging on the quantity and composition of bovine lipid classes and to relate these data to sensory panel scores for aroma and flavor.

EXPERIMENTAL

Samples

Fifteen wholesale ribs from an equal number (5) of 14-month-old half-sib Angus bulls, heifers and steers with a similar nutritional background (Meiske et al., 1969) were used in this study. After 2, 7, 14 and 21 days of postmortem (PM) aging at 2°C, samples of longissimus dorsi muscle and subcutaneous (SQ) rib fat were removed from between the 8th and 11th ribs and frozen at −20°C for a maximum of 3 wk. To eliminate bacterial lipase activity from surface microorganisms accumulating during the postmortem aging period, the outer layer (0.25 cm) of the subcutaneous fat was removed before freezing. After 1 and 3 wk of postmortem aging, steaks from the 10th and 8th ribs, respectively, were cut to a thickness of 1 in. and the freshly cut samples used for sensory panel evaluation. The over-all experimental design is summarized as a flow sheet (Fig. 1).

Lipid extraction

25g of still partially frozen longissimus dorsi muscle was diced and homogenized in chloroform-methanol (1:2). Extraction of the total lipid was achieved using the method described by Bligh and Dyer (1959). The extraction mixture was filtered under slight suction and phase separation allowed to proceed at 2°C for 4–5 hr. The upper alcoholic layer was removed by aspiration and the lower chloroform layer transferred to an Erlenmeyer flask and dehydrated over anhydrous sodium sulfate (Na_2SO_4). After filtration to remove Na_2SO_4, the crude lipid was evaporated to dryness at 37°C with a Buchi rotary evaporator. The dried sample was weighed and aliquots taken for lipid fractionation (Fig. 1) and quantitative determination of free fatty acid, phospholipid and cholesterol.

Subcutaneous crude lipid was isolated by homogenizing 5g of fat in chloroform at full speed in a Virtis homogenizer, dehydrating the sample over anhydrous Na_2SO_4, filtering and evaporating the sample to dryness with a Buchi rotary evaporator. Aliquots were taken from the weighed sample for free fatty acid estimation. All lipid samples were stored at −20°C in chloroform and under nitrogen.

Chromatographic lipid fractionation

Separation of the intramuscular (IM) crude lipid into neutral lipid and phospholipid (PL) fractions was achieved in a glass column (1.5 by 25 cm) containing 12g of acid-washed Florisil (Carroll, 1963). The neutral lipid and phospholipid fractions were eluted with 75 ml of chloroform and 75 ml of methanol, respectively. Acid-washed Florisil was chosen rather than silicic acid due to the inability of silicic acid to completely separate free fatty acids from phospholipids. Approximately 120 mg of the neutral lipid fraction was further separated into lipid classes using 7% hydrated Florisil as described by Carroll (1961). The triglyceride (TG), free fatty acid (FFA) and combined mono- and diglyceride (MG + DG) fractions

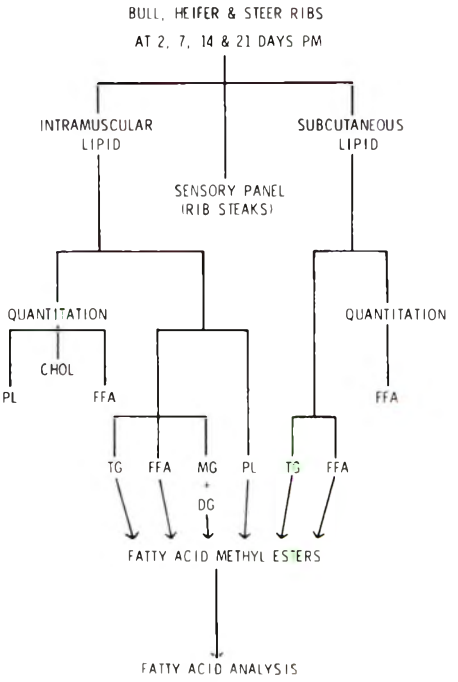

Fig. 1—Experimental flow sheet. TG = triglyceride. FFA = Free fatty acid. MG + DG = Mono- and diglyceride. PL = Phospholipid. CHOL = Cholesterol.

Table 1—Percent composition of lipid classes isolated from intramuscular and subcutaneous lipid of bulls, heifers and steers[1,2,3]

Fatty acid	Retention time	Intramuscular lipid												Subcutaneous lipid					
		Phospholipid			Free fatty acid			Triglyceride			Mono and diglyceride			Triglyceride			Free fatty acid		
		Bull	Heifer	Steer	Bull	Heifer	Steer	Bull	Heifer	Steer	Bull	Heifer	Steer	Bull	Heifer	Steer	Bull	Heifer	Steer
C14:0	0.336	0.9	1.3	1.4	8.5^{ab}	7.2^{a}	7.2^{b}	3.0	2.9	3.2	5.2	4.5	4.8	3.2^{a}	2.7^{ab}	3.3^{b}	8.6	8.8	8.0
C14:1	0.392	Tr	Tr	Tr	1.0	1.3	1.1	1.3	1.2	1.3	1.4	1.3	1.4	2.4^{a}	1.8^{ab}	2.4^{b}	2.4^{ab}	2.0^{bc}	2.6^{ac}
C15:0	0.440	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr	Tr
C16Br	0.506	1.6^{ab}	4.0^{bc}	2.7^{ac}															
C16:0	0.578	14.1^{ab}	16.7^{bc}	15.6^{ac}	19.8^{ab}	23.7^{a}	22.3^{b}	25.6^{a}	25.6^{b}	26.5^{ab}	27.3	27.4	27.0	24.5^{a}	22.5^{ab}	24.9^{b}	17.9^{a}	17.0	16.4^{a}
C16:1	0.690	1.8^{a}	2.2^{a}	2.1	3.9^{a}	4.5^{a}	4.2	4.3^{a}	4.8^{a}	4.6	4.3	4.5	4.5	6.3^{a}	5.5^{ab}	6.2^{b}	6.5^{ab}	5.9^{bc}	7.0^{ac}
C17:0	0.752	0.5	0.4	0.6	1.5^{ab}	1.9^{a}	2.0^{b}	1.6	1.8	1.8	1.8^{a}	1.7^{b}	2.1^{ab}	1.7	1.6	1.8	1.6	1.5	1.5
C18Br	0.855	1.9^{a}	2.9^{a}	2.5	0.7	0.9	0.7	0.8	1.0	0.9	0.8	0.9	1.0	1.3	1.3	1.4	1.4	1.2^{b}	1.5^{b}
C18:0	1.000	10.8^{a}	9.5^{ab}	10.2^{b}	15.8	15.6	15.2	16.0^{ab}	14.2^{a}	14.2^{b}	16.0^{ab}	13.4^{ac}	14.2^{bc}	11.0	10.3	10.2	9.3^{a}	9.4^{b}	8.2^{ab}
C18:1	1.17	17.8	17.7	17.8	25.8^{ab}	29.1^{a}	28.5^{b}	44.0	44.8	44.5	37.9^{ab}	40.5^{a}	39.8^{b}	45.1^{a}	49.7^{ab}	45.6^{b}	45.3	47.1	47.6
C18:2	1.45	30.8^{ab}	26.3^{a}	25.8^{b}	8.5^{a}	10.3^{ab}	7.5^{b}	3.1^{a}	3.3^{b}	2.6^{ab}	3.5	3.2	3.0	3.4	3.4	3.3	4.3	3.9	4.1
C20:0	1.71	Tr	Tr	Tr															
C18:3	1.87	0.6	0.7	0.6	0.4	0.9	0.4	0.5	0.5	0.4	0.8	0.7	0.7	1.1	1.2	1.0	1.5	1.5	1.4
C21:0[4]	2.26				Tr	Tr	Tr				1.0	1.9	1.5	Tr	Tr	Tr	0.7	0.8	0.6
C21:1[4]	2.65	0.6	Tr	Tr	Tr	0.4	Tr												
C22:0	2.84	2.2^{ab}	3.1^{a}	3.0^{b}	0.9^{a}	1.5^{a}	1.2							Tr	Tr	Tr	Tr	0.5	0.4
C20:4	3.29	12.5	11.6^{a}	13.6^{a}	3.2	2.9	3.1							Tr	Tr	Tr	0.5	0.6	0.6
C22:2[4]	4.29	0.9^{ab}	0.6^{a}	0.7^{b}	1.0	Tr	0.7												
C23:0[4]	4.53				5.2	Tr	3.0												
C22:3[4]	5.51	1.1^{ab}	1.5^{a}	1.6^{b}	Tr	Tr	Tr												
C22:4[4]	7.21	1.6^{a}	1.2^{ab}	1.5^{b}	Tr	Tr	Tr												
C22:6	8.60	Tr	Tr	Tr	4.1	Tr	3.2												

[1] Each figure is the average of five animals at four PM aging periods.
[2] Percent composition calculated on a weight basis.
[3] There were no significant ($P > .05$) sex X aging-time interactions.
[4] Confirmed only by semilogarithmic plot and hydrogenation.
[a,b,c] Means in the same row for each lipid class and having the same superscript are significantly different ($P < .05$).

were retained for further analyses. Methyl esters were prepared from these lipid fractions using the procedure of Metcalfe et al. (1966).

Gas-liquid chromatography of these methyl esters was conducted on a Beckman model GC4 gas chromatograph using a flame ionization detector and nitrogen as carrier gas. Samples were injected on to a 3-mm by 2-m stainless steel column packed with 15% diethylene glycol succinate (DEGS) on Chromosorb W (Beckman DEGS-lb) and maintained in an oven at 190°C. The injector and detector temperatures were 220°C. Fatty acid peak areas were measured by triangulation and all acids are expressed as relative percentages of the total fatty acids measured in each fraction. Individual fatty acids were identified by comparing retention times with those in known fatty acid mixtures (Hormel Institute). In addition, comparison of the gas chromatograms from hydrogenated and unhydrogenated lipid fractions were used for identification and confirmation of some fatty acids. The statistical analyses were conducted as described by Steel and Torrie (1960).

Quantitative determinations

Free fatty acid levels were determined according to the procedure of Duncombe (1964) with the following modification. Phospholipids were found to interfere with the color reaction, producing erroneously high results due to the formation of emulsions, which resulted in turbidity. Phospholipids were removed by chromatography on acid-washed Florisil and the FFA assay was performed on the neutral lipid fraction. Triplicate samples of neutral lipids were transferred to stoppered vials (2.5- by 9.0-cm) and dissolved in 5 ml of chloroform to which 3 ml of Duncombe's copper reagent was added. The vials were vigorously shaken along the long axis for 5 min in a mechanical shaker and the contents transferred to a 15-ml centrifuge tube and spun for 5 min. The aqueous phase was aspirated off each sample and 3 ml of the chloroform layer transferred from each tube to a colorimeter tube and mixed with 0.5 ml of Duncombe's diethyldithiocarbamate reagent. After mixing, the extinction was read with a spectrophotometer at 440 mμ against a blank carried through the complete procedure. Free fatty acid levels were determined by comparison with a standard curve prepared from palmitic acid.

Intramuscular phospholipid levels (Morrison, 1964) and cholesterol levels (Leefler, 1963) also were determined. Intramuscular lipid levels were determined by the ether extraction technique.

Sensory panel evaluation

All steaks were broiled 13 min, at which time the centers of the steaks were pink (medium). The actual internal temperature was not recorded. The rib-eye steaks were evaluated by a six-member laboratory-type panel consisting of three men and three women. Each panel member evaluated either a 1/2-(8th rib) or 1 (10th rib)-in. core (depending on rib-eye area) of the rib-eye muscle taken at a standard location for each panel member. These samples were evaluated for aroma and flavor using a 9-point hedonic scale, where a score of 9 denotes Extremely desirable.

RESULTS & DISCUSSION

Effect of sex

Fatty acid composition of the IM and

Table 2—Total lipid, cholesterol, phospholipid and free fatty acid levels in bovine longissimus dorsi muscle

PM aging time[1] (days)	Total lipid % of Muscle	Total cholesterol % of Muscle	Total cholesterol % of Fat-free muscle	Total cholesterol mg/g Lipid	Phospholipid % of Muscle	Phospholipid % of Fat-free muscle	Phospholipid mg/g Lipid	Free fatty acid mg/100g Muscle	Free fatty acid mg/g Lipid
2	5.77	0.052	0.055	9.01	0.63	0.67	109.2	6.5^{abr}	1.1^{abr}
7	5.77	0.054	0.057	9.36	0.59	0.63	102.2	8.7^{a}	1.5^{a}
14	5.77	0.049	0.052	8.49	0.57	0.61	99.1	9.4^{b}	1.6^{b}
21	5.77	0.050	0.053	8.66	0.56	0.60	97.2	10.5^{r}	1.8^{r}
Sex[2]									
Bull	4.03^{rs}	0.046^{s}	0.048^{s}	11.51^{rs}	0.56^{a}	0.58	138.4^{rs}	8.0	2.0^{rs}
Heifer	7.29^{rt}	0.057^{rs}	0.061^{rs}	7.79^{r}	0.52^{ab}	0.56	71.8^{rt}	8.7	1.2^{ar}
Steer	5.98^{st}	0.049^{r}	0.052^{r}	8.10^{s}	0.57^{b}	0.59	95.5^{st}	9.6	1.6^{as}

[1] Each figure is the average of 15 observations.
[2] Each figure is the average of 20 observations.
[a,b]Means in each column with the same superscript are significantly different ($P < .05$).
[r,s,t]Means in each column having the same superscript are significantly different ($P < .01$).

Table 3—Sensory panel scores for the aroma of rib steaks from bulls, steers and heifers

PM aging period	Heifers	Steers	Bulls
7 Days	6.6	6.9	7.1
21 Days	6.6	6.7	6.8
Average	6.6^{a}	6.8	7.0^{a}

[a]Means with the same superscript differ significantly ($P < .05$).

SQ lipid fractions from bulls, steers and heifers is shown in Table 1. The IM lipid fractions in bulls had significantly ($P < .01$) higher levels of C18:2 (PL class) and C18:0 (TG; MG + DG classes) and significantly lower levels of C16:0 and C18:1 in the FFA class than either steers or heifers. Fatty acid composition of the IM lipid fractions in heifers and steers was similar. Terrell et al. (1968) reported that differences between steers and heifers were totally associated with the fatty acids from the neutral fraction rather than the phospholipid fraction. In the SQ triglyceride fraction, which is the major lipid class in SQ adipose tissue, heifers had significantly ($P < .01$) higher C18:1 levels and significantly ($P < .01$) lower C16:0 levels than either steers or bulls. These compositional differences may be due to the influence of sex hormones on enzyme systems such as the desaturase enzyme (Marsh and James, 1962), which converts C16:0 and C18:0 to C16:1 and C18:1, respectively. The SQ depot lipid has a higher C16:1 and C18:1 content than the TG fraction from IM lipid (Table 1).

Intramuscular lipid levels (Table 2) were significantly different ($P < .01$) among bulls, heifers and steers. Phospholipid levels expressed as mg/g of lipid were significantly ($P < .01$) higher in bulls and lower in heifers when compared to steers. This suggests that marbling (visible intramuscular fat) contains little or no phospholipid, since the phospholipid content of the extracted muscle lipid decreases appreciably with increasing IM lipid levels. When expressed as percent of wet muscle, heifers had lower phospholipid levels than bulls and steers, but as reported by Link et al. (1970) with steers and heifers, this significance is lost when the data are expressed as percent of fat-free muscle. For the three sex groups, the IM cholesterol content expressed as mg/g of lipid was significantly ($P < .01$) higher in bulls and also inversely related to the IM lipid content. Heifer longissimus dorsi muscle contained higher ($P < .01$) levels of total cholesterol whether expressed as percent of wet muscle or fat-free muscle. Phospholipid and to a lesser extent cholesterol are associated almost completely with the muscle membranes (e.g., sarcoplasmic reticulum and mitochondrial membranes) rather than marbling. Stromer et al. (1966) reported that marbling contains no detectable cholesterol.

After 2 days PM there were no significant differences among the sex groups in FFA content of the SQ rib fat, but as the aging period increased, FFA levels from both steers and bulls became significantly ($P < .05$) higher than heifers (Fig. 2). In the IM fat, free fatty acid levels also increased slightly during PM aging (Fig. 3). Once again heifer IM fat contained the lowest levels of FFA while bull and steer IM fat contained higher and intermediate levels, respectively. These differences between sex groups can probably be attributed to the level or degree of activation of hormone-sensitive lipase which hydrolyzes triglycerides to release FFA and glycerol. No difference was found between the FFA levels in the outermost and innermost layers of the outer SQ fat layer, suggesting that FFA release was not due to bacterial lipase. If bacterial lipases had been active or the hydrolysis nonenzymatic, then the observed sex differences (Fig. 2 and 3) would not have occurred.

Rib steaks from the heifers had significantly ($P < .05$) higher sensory panel scores for aroma (Table 3) when compared with bulls. This difference in aroma may be attributed to fatty acid compositional differences or to the different FFA levels in the IM lipid, or both. Pearson

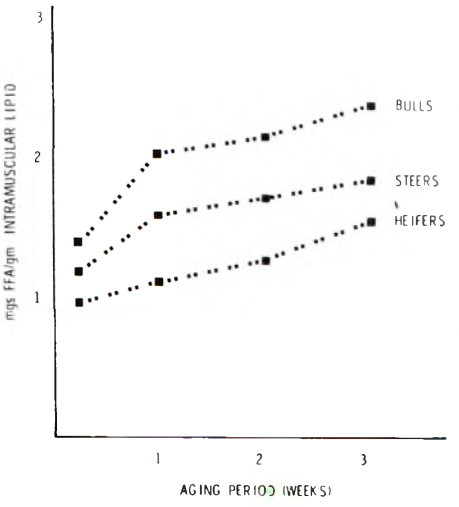

Fig. 2—Influence of sex and postmortem aging on the free fatty acid (FFA) level of subcutaneous beef fat.

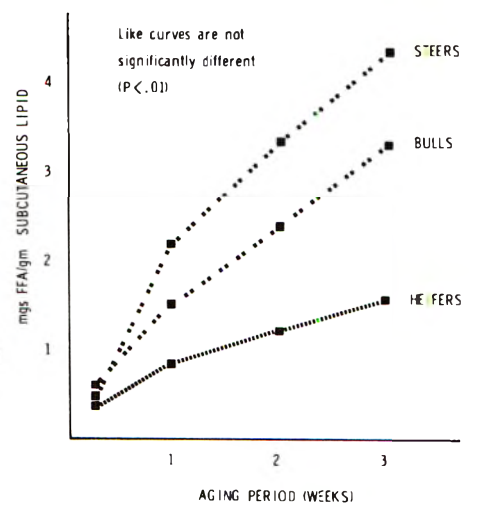

Fig. 3—Influence of sex and postmortem aging on the free fatty acid (FFA) level of intramuscular beef fat.

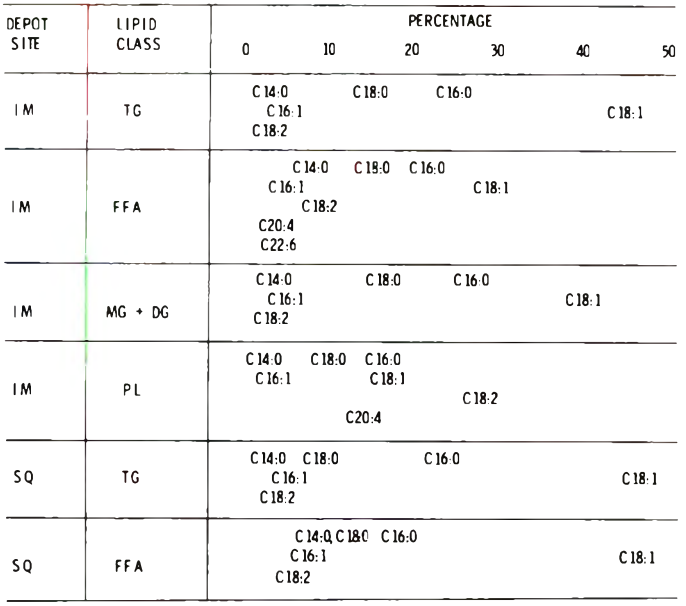

Fig. 4—Distribution of fatty acids in various lipid classes isolated from intramuscular (IM) and subcutaneous (SQ) beef fat. Each value is averaged across sex groups and aging periods. TG = Triglyceride. FFA = Free fatty acid. MG + DG = Mono- and diglyceride. PL = Phospholipid.

Table 4—Influence of postmortem (PM) aging on the fatty acid composition of various lipid classes isolated from intramuscular (IM) and subcutaneous (SQ) beef fat[1,2,3]

Depot site	Lipid class[4]	Fatty acid	PM aging period (days)			
			2	7	14	21
SQ	FFA	C14:0	11.7^{ars}	10.0^{atx}	6.3^{rx}	5.8^{st}
	FFA	C16:0	18.3^{rst}	16.8^{r}	16.8^{s}	16.6^{t}
	FFA	C16:1	6.0^{at}	6.2^{bs}	6.7^{ab}	7.0^{rs}
	FFA	C18:1	42.6^{rst}	46.1^{arx}	48.7^{abs}	49.7^{btx}
	TG	C18:1	47.1	47.1	46.6	46.3
IM	FFA	C14:0	8.9^{a}	7.9	7.2	6.7^{a}
	FFA	C18:2	7.1^{rs}	8.1^{ab}	9.9^{ar}	10.1^{bs}
	TG	C18:1	45.3^{rs}	44.5	43.9^{r}	43.9^{s}
	PL	C18:0	11.0^{abr}	9.9^{a}	9.9^{b}	9.8^{r}
	PL	C18:2	26.3	27.3	27.8	28.4
	PL	C20:4	12.2	13.0	12.1	12.7

[1] Each figure is the average of 15 animals.
[2] Percent composition calculated on a weight basis.
[3] There were no significant ($P > .05$) sex × aging-time interactions.
[4] FFA = Free fatty acid. TG = Triglyceride. PL = Phospholipid.
^{a,b}Means in the same row with the same superscript are significantly different ($P < .05$).
^{r,s,t,x}Means in the same row with the same superscript are significantly different ($P < .01$).

(1968) obtained a correlation of −0.69 between FFA level and odor score in ground meat; most meat samples were acceptable, provided the FFA level did not exceed 1.2%. A coefficient of correlation of −0.97 was obtained by Peters et al. (1968) between average FFA levels of thawed and refrozen cod, and average taste panel scores. In this study a significant ($P < .01$) correlation of −0.49 was obtained between aroma score and FFA levels for the three sex groups at 7 and 21 days of PM aging. A correlation coefficient of 0.48 ($P < .01$) was obtained between aroma score and percent C16:0 in the FFA lipid class. All other correlations between aroma score and individual fatty acids in the various lipid classes were nonsignificant ($P > .05$) and below 0.25.

Trace amounts of fatty acids C23:0 and C22:6 were found in the heifer FFA class, intermediate levels in steers and 5.2 and 4.1%, respectively, in the bull FFA lipid class. This high level of C22:6 may contribute to the less desirable aroma of meat from bulls (Table 3), since this highly unsaturated fatty acid is very susceptible to autoxidation during cooking. The role of unsaturation and autoxidation in the production of off-flavors has been demonstrated in frozen bovine muscle (Awad et al., 1968) and whale fat (Hornstein et al., 1963). Few significant and no consistently high correlations were obtained by Terrell et al. (1968), Waldman et al. (1968) or Dryden and Marchello (1970) between sensory panel measurements and individual fatty acids derived from bovine total lipid. Correlations of 0.33 and 0.36, respectively, were drawn between C18Br and C16Br fatty acids in the phospholipid class and sensory panel flavor. These were the only significant ($P < .05$) correlations found between flavor and individual fatty acids.

Effect of PM aging

Probably the most noticeable effect of PM aging is the accumulation of FFA in the SQ rib fat (Fig. 2) and IM longissimus dorsi fat (Fig. 3) with time PM. By regression analysis, the rate at which FFA progressively increases in the SQ rib fat is significantly higher ($P < .01$) in bulls and steers when compared to heifers. Although nonsignificant, bull, steer and heifer rib steaks tended to have lower sensory panel scores for flavor and aroma and higher intramuscular FFA levels (Fig. 3) at 21 days PM than 7 days PM. Since the sensory panel was conducted at two time periods only, a valid correlation coefficient could not be determined between time PM and sensory panel scores for flavor and aroma.

As documented earlier, FFA levels in SQ and IM fat increase progressively with time PM; running parallel to the increasing FFA levels are compositional changes in the lipid classes of these depot fats (Table 4). During the 3-wk aging period, the content of C14:0 and C16:0 in the FFA fraction of SQ rib fat decreased significantly ($P < .01$) from 11.7–5.8% and 18.3–16.6%, respectively, whereas the level of C18:1 and C16:1 increased significantly ($P < .01$) from 42.6–49.7% and 6.0–7.0%, respectively. No significant fatty acid composition differences due to PM aging were observed in the TG fraction from SQ rib fat. However, significant fatty acid composition differences in the IM lipid during PM aging were found in the FFA fraction (C14:0, 8.9–6.7%; C18:2, 7.1–10.1%), the TG fraction (C18:1, 45.3–43.9%) and the PL fraction (C18:0, 11.0–9.8%). The percentage of all other fatty acids in the lipid classes of the two depot fats remained unaltered with PM aging.

The composition of the FFA in the IM longissimus dorsi (Fig. 4) is intermediate in composition between the IM triglycerides and phospholipid fractions and quite different from the SQ rib fat FFA lipid class, suggesting that the IM free fatty acids are derived from both the TG and PL fractions, whereas the FFA from the SQ depot fat are derived primarily from the triglycerides. Addison et al. (1969) reported that 75% of the FFA in herring oil arose from phospholipid hydrolysis and the remaining 25% from triglyceride hydrolysis. These workers observed high levels of C22:6 in the FFA fraction of herring oil when compared to other lipid fractions, which is similar to the observation for IM lipid reported in this study. The composition of the combined MG and DG fraction (Fig. 4) is very close to that of the TG fraction, suggesting that this fraction is derived from the TG fraction and not the PL fraction by specific phospholipase action.

The only fatty acids to change appreciably in the SQ free fatty acid class were

C14:0, C16:0, C16:1 and C18:1. This can be partially explained by considering the triglyceride fatty acid distribution for beef as published by Brockerhoff (1966) and assuming that the FFA are derived primarily from the fatty acids hydrolyzed from the α positions of the nonrandomly distributed triglyceride. Fatty acids are known (Windrum et al., 1955; Berrens and Van Driel, 1962) to be associated with reticulin. Windrum et al. (1955) reported that human renal reticulin contained 10.9% bound fatty acids, 95% of which is myristic acid. This fact may explain the initial high levels of C14:0 in the FFA class, and the decreasing percentage of this acid with time PM may very well be a dilution effect.

The concentration of IM cholesterol and phospholipid found during PM aging is documented in Table 2. No significant change was observed in total cholesterol levels, suggesting cholesterol is chemically stable during PM aging. As the PM aging time increased, the level of phospholipid declined, but not significantly ($P > .05$), indicating only mild phospholipid hydrolysis. Phospholipid hydrolysis rate was greatest in bulls, intermediate in steers and lowest in heifers, which may relate to the higher aroma scores for ribs from heifers.

Several workers have studied the effect of cooking on fatty acid patterns in ground beef and pork (Campbell and Turkki, 1967), freeze-dried meat (Gian and Dugan, 1965) and bovine longissimus dorsi muscle (Terrell et al., 1968); no major compositional changes in the neutral and phospholipid fractions were observed due to cooking. If oxidative changes occur during PM aging, a decrease in the percent composition of some or all of the unsaturated fatty acids would be expected. In this study, PM aging was shown to have no effect on the composition of the major IM lipid classes. Particularly important is the PL fraction containing highly unsaturated fatty acids, which are very susceptible to autoxidation under many conditions. However, there were no significant differences in the percentage of C18:2 and C20:4 (Table 3), the two major unsaturated fatty acids in the PL fraction, during the 21-day PM aging period for the three sex groups. Since there were no significant changes in fatty acid composition, it can be concluded that autoxidation does not readily occur during the aging of fresh beef ribs. However, during cooking the application of heat to beef ribs with different quantities and composition of the FFA lipid class may result in fatty acid alterations that relate to differences in aroma. This seems particularly likely in view of the relationship between FFA levels and aroma score reported in this study.

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RELATION OF ATPase ACTIVITY AND CALCIUM UPTAKE TO POSTMORTEM GLYCOLYSIS

SUMMARY—Calcium uptake and Ca^{2+}-activated ATPase activity have been investigated in relation to pH_1 in pork muscle Longissimus dorsi, sampled 35 min postmortem. Both the rate of calcium uptake and the enzyme activity were negatively, and significantly, correlated with pH_1, except when the latter value dropped below 5.9. At $pH_1 < 5.9$, impairment of reticular function and of enzyme activity became apparent. As pH_1 is a guide to the rate of postmortem glycolysis, the significance of the findings is discussed with respect to accelerated glycolysis and the development of the PSE condition in pork.

INTRODUCTION

IN PORK, the "PSE" condition—a quality defect characterized by the pale, soft and exudative nature of the lean meat—is known to develop when a rapid pH fall occurs in the muscle postmortem, while the carcass temperature remains high. Under such conditions of high temperature, protein constituents of the muscle may be extensively denatured, with consequent changes in texture and color of the tissue and loss of water binding capacity (Briskey and Wismer-Pedersen, 1961; Briskey, 1964; Bendall and Lawrie, 1964).

In general the rate of glycolysis of a muscle is determined by the rate at which ATP is dephosphorylated to supply the energy expended by that muscle (Hallund and Bendall, 1965; Bendall, 1960). But factors responsible for the accelerated glycolysis which leads to the rapid pH fall in PSE muscle have not been fully elucidated. Work reported by Krzywicki and Ratcliff (1967) provided evidence that the rate of glycolysis or pH fall in the L. dorsi of pigs, postmortem, is related to the amount of phospholipid constituents in the so-called myofibrillar fraction. The latter included reticulum (one of the major phospholipid-containing organelles of the muscle fiber) and this was implicated as the source of this relationship.

The present work was aimed at securing further information on the relationship of reticular function and ATPase activity to rates of glycolysis postmortem, in the L. dorsi of pigs. Calcium uptake was determined in homogenates of the muscle and also in the myofibrillar fraction which contained reticulum bound to the myofibrils. ATPase activity of the myofibrillar fraction of muscle was estimated in the presence or absence of magnesium ions. The relationship between both of these parameters and pH_1 was investigated.

EXPERIMENTAL

Sampling

Pigs of the Polish Large White breed were taken from the line 35 min after slaughter at the abattoir. The pH of the L. dorsi was immediately determined with a Radiometer 24 meter, using probe electrodes inserted in the lumbar region. This value, recorded 35 min postmortem (pH_1), may be regarded as a guide to the rate of postmortem glycolysis in the muscle.

Samples (~ 10g) were excised from the same region of the L. dorsi immediately after the determination of pH_1, and stored at 4°C until required for analysis 1–2 hr later.

Analytical

All analytical operations were conducted at room temperature, 20°C. Reagent and buffer solutions were prepared with double-distilled water.

Deproteinizing reagent consisted of 0.6M $NaClO_4$, 0.04M glycine and 0.1M HCl (Mozersky et al., 1966).

Inorganic P_i was estimated by the method of Chen et al. (1956), the tubes being held 1 hr at 20°C rather than 1½–2 hours at 37°C, to minimize nonenzymatic hydrolysis of ATP.

Calcium was estimated with EDTA, using murexide as indicator. To ensure a definite and sharp end point in the titration, phosphates were previously removed by passing the solution through a column of anion-exchange resin (Amberlite IRA-410). The titration was performed with the aid of a Microdose Pump (Unipan) which facilitated the delivery of accurately measured 0.01 ml volumes of the EDTA solution.

Protein N was determined by formol titration of the diluted and neutralized protein digest (Bradstreet, 1965).

Preparation of the myofibrillar fraction

0.5g of sample was homogenized (1–2 min) with 10 ml of 0.16M KCl at 4°C, using an all-glass Potter-type of homogenizer. The homogenate was centrifuged at 3,000 × G for 10 min, and the myofibrils thus obtained were washed twice with 0.16M KCl.

ATPase activity

Ca^{2+}-activated ATPase. Washed myofibrils were suspended in 15 ml of 0.16M KCl and 5mM $CaCl_2$. To 0.5 ml of the suspension of myofibrils was added 0.5 ml of 10mM ATP in glycine buffer at pH 9.1. After 6 min incubation, with frequent shaking, the solution was deproteinized by adding deproteinizing reagent (5 ml). After 10 min the precipitate was centrifuged down at 6,000 × G for 20 min. Aliquots of the supernatant were taken for estimation of inorganic P_i.

Residual ATPase activity in presence of added Mg^{2+}. The ATPase activity measurements were repeated as above but with the addition of Mg^{2+} ions at two levels, giving Mg/Ca molarity ratios in the final solution of either 1.6/1 or 4.0/1.

Residual ATPase activity of the myofibrils used in calcium uptake estimations was also determined using buffer solution and additives appropriate to the latter (see below), except that added calcium was at the level of 0.4mM.

Calcium uptake by myofibrils and whole muscle

Myofibrils. Washed myofibrils giving a final concentration of 3–5 mg protein were suspended in 15 ml of buffer solution at pH 7.3, consisting of 0.1M KCl, 10mM histidine, 5mM $MgCl_2$ and 5mM oxalate. To 1 ml of the solution was added 1 ml of the same buffer containing ATP (10mM) and $CaCl_2$ (0.8mM). After incubation, (with frequent shaking) for 10 min, the solution was centrifuged 1 min at 3,000 × G. Calcium was determined in the supernatant. Residual ATPase activity was also estimated by determination of inorganic P_i in the supernatant, (final Ca^{2+} concentration was 0.4mM). Appropriate blanks without added ATP or myofibrils were run.

Whole muscle. A sample of muscle (0.5g) was homogenized in 5 ml of solution containing 0.16M KCl and 32mM $CaCl_2$. To 0.5 ml of homogenate was added an equal volume of buffer consisting of 40mM histidine, 10mM oxalate and containing 10mM $MgCl_2$ and 10mM ATP. Calcium was determined in the supernatant after centrifuging (1 min at 3,000 × G) at time t_0, immediately after addition of the ATP/buffer solution, and after incubation for 8 min, t_8. Calcium uptake was calculated as the difference $t_8 - t_0$ and expressed as μMoles calcium per gram tissue per minute.

RESULTS

IN FIGURE 1, the Ca^{2+}-activated ATPase activity of the myofibrillar fraction of muscle, obtained from the L. dorsi of 12 pigs, has been plotted against pH_1 (which ranges between 5.3 and 7.0). The curve shows that if two samples showing the lowest pH_1 values (5.32 and 5.50) are excepted, there appears to be a negative correlation between pH_1 and enzyme activity. The two anomalous points on the curve (obtained for muscle with pH_1 of 5.32 and 5.50) probably result from a loss of ATPase activity due to denaturation of the enzyme at these low pH_1 values (Penny, 1969). With the omission of these two samples, the correlation between pH_1 and the measured ATPase activity is highly significant, ($r = -0.93$). The regression coefficient of ATPase activity on pH_1 ($b = -0.198$) is

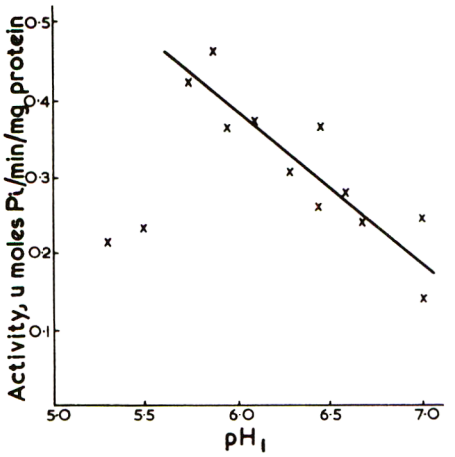

Fig. 1—ATPase activity of myofibrillar fraction of L. dorsi, without added magnesium. (Abscissa = pH_I of muscle; Ordinate = activity, μmoles P_i/min/mg protein.)

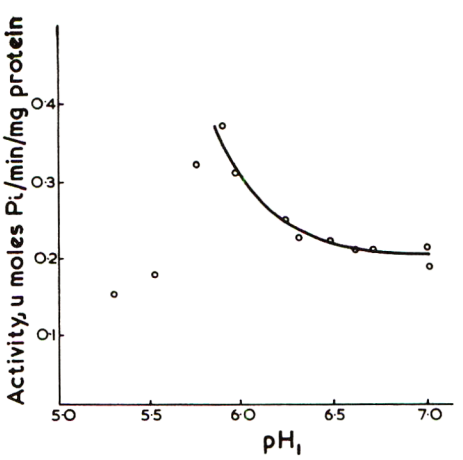

Fig. 2—ATPase activity during final 4 min incubation. (Abscissa = pH_I of muscle; Ordinate = activity, μmoles P_i/min/mg protein.)

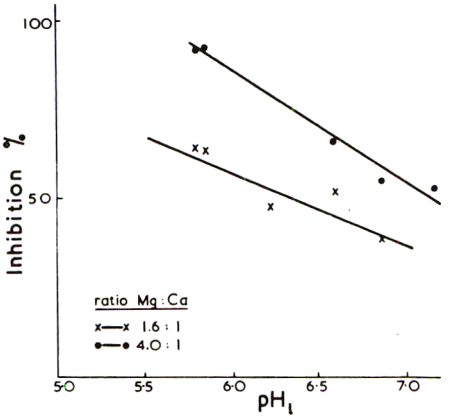

Fig. 3—Inhibition of ATPase activity of myofibrillar fraction of L. dorsi by magnesium added at two levels. (Abscissa = pH_I of muscle; Ordinate = percentage inhibition.)

significant ($P < 0.001$) by the "t" test.

This relationship was independent of the degree of contraction of the homogenized myofibrils which, it was thought, might influence the initial values obtained in the activity determinations. Thus, a similar relationship to pH_I was shown by ATPase activity measured during the final 4 min of the 6 min incubation period, as shown by the curve in Figure 2.

The determination of ATPase activity was repeated, using the myofibrillar fractions of the same samples of muscle in the presence of added magnesium ions. Since magnesium activates the calcium-pump function of the sarcoplasmic reticulum, a marked inhibition of enzyme activity was effected at both of the Mg/Ca ratios used. Figure 3 shows that the observed percentage inhibition depended not only upon the Mg/Ca ratio but also upon pH_I of the samples. Inhibition of enzyme activity was negatively related to pH_I, indicating a similar negative relationship between pH_I, and the capacity of the sarcoplasmic reticulum to sequester calcium ions.

Uptake of Ca^{2+} by the myofibrillar fraction of muscle samples, taken from another seven animals and covering a range of pH_I of 5.60–6.80, was also found to be negatively related to pH_I. Results obtained are given in Table 1 which also includes the values obtained for ATPase activity. The latter values, under the conditions of this experiment, might be expected to be reduced by the calcium-sequestering activity of the calcium pump, and indeed a positive correlation of ATPase activity with pH_I is apparent.

The results, calculated for both 1 mg of myofibrillar protein, and for 1g of muscle tissue (wet weight), show that the correlation coefficient relating pH_I and calcium uptake is higher when the latter parameter is calculated for 1g of tissue ($r = -0.89$). But with ATPase activity, which is more directly associated with the myofibrils, the correlation with pH_I is higher when calculated for 1 mg of protein ($r = +0.90$). The corresponding regression coefficients are respectively, 0.986 and 0.055, which are significant ($P < 0.05$).

The results obtained for calcium uptake in homogenates of whole muscle tissue (Table 2) showed that pH_I (in the range 5.95–6.93) was highly correlated with calcium uptake measured after 8 min incubation (t_8), with $r = -0.98$. The regression coefficient, $b = 1.005$, is significant ($P < 0.01$). The two samples with low pH_I values (5.70–5.77) were anomalous in that they showed unexpectedly low rates of calcium uptake at t_8 (Fig. 4), presumably again owing to the damaging effects of high lactic acid levels upon the functional mechanism of the reticulum. It is interesting to note (Table 2) that the initial uptake measured after 1 min incubation (t_0), is nevertheless highest in these two samples (with pH_I values of 5.70 and 5.77) and in this respect they conform with the negative relationship observed between pH_I and rate of calcium uptake. If the reticulum can be visualized as a system that both removes calcium ions from the sarcoplasm or surrounding medium, and stores them, then it may be conjectured that lactic acid, at low pH_I, damages only the storage mechanism of the reticulum. Under these conditions of low pH_I the system functions like a pump filling into a porous reservoir; despite the large calcium pump capacity of the reticulum, the amount of calcium taken up and stored over an extended period of 8 min will appear as a low rate of uptake.

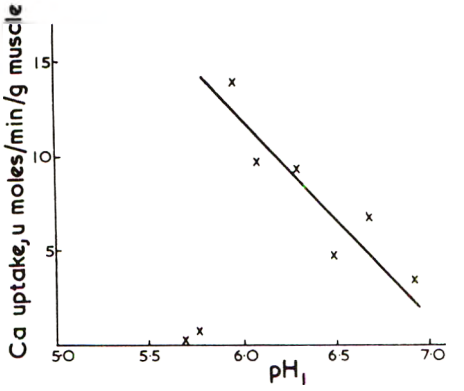

Fig. 4—Rate of calcium uptake by L. dorsi muscle. (Abscissa = pH_I of muscle, Ordinate = calcium uptake, μmoles Ca/min/g muscle tissue.)

DISCUSSION

THE DATA obtained in this work indicate that the rate of fall of pH in the L. dorsi, postmortem, is positively related to (1) the activity of myosin ATPase and (2) the calcium-sequestering capability of the tissue. The first of these findings agrees with the observations of Quass and Briskey (1971), who reported that myosin ATPase activity of the L. dorsi was higher in stress-susceptible pigs (in which pH fall at death is comparatively rapid) than in stress-resistant animals. With regard to the second finding: it is known that the specific calcium-sequestering activity of the reticulum, immediately postmortem, is significantly lower in FSE than in normal muscle (Greaser et al., 1969a). The observed negative relationship of this reticular activity to pH_I must therefore be attributed to the larger amounts of reticulum associated with muscles which

Table 1 Calcium uptake and ATPase activity of myofibrillar fraction of L. dorsi muscle in the presence of magnesium[a]

Sample no.	pH_1 of muscle	ATPase activity μmoles P_i/min per mg protein	ATPase activity μmoles P_i/min per g tissue	Calcium uptake μmoles Ca/min per mg protein	Calcium uptake μmoles Ca/min per g tissue
1	5.60[b]	0.143[b]	15.21[b]	0.042[b]	4.5[b]
2	6.00	0.057	9.70	0.060	10.2
3	6.10	0.073	6.25	0.119	10.2
4	6.20	0.070	10.24	0.035	5.1
5	6.50	0.104	8.54	0.081	6.6
6	6.65	0.089	10.52	0.033	3.9
7	6.80	0.106	10.17	0.013	1.2
Correlation coefficient "r"		+0.90**	+0.47	−0.59	−0.89**

[a]The assay medium contained 2.5mM Mg^{2+} and 0.4mM Ca^{2+}.
[b]Results not included in calculation, for reasons given in the text.
**Significant at 0.01 level.

Table 2–Calcium uptake by L. dorsi muscles of various pH_1 values

Sample no.	pH_1 of muscle	Ca uptake at t = 0 min (μmoles Ca/min/g of tissue)	Average Ca uptake $t_8 - t_0$ (μmoles Ca/min/g of tissue)
1	5.70	93.9	0.4[a]
2	5.77	77.0	0.9[a]
3	5.95	–	13.9
4	6.10	88.4	9.7
5	6.30	77.0	9.2
6	6.50	62.7	4.6
7	6.70	54.1	5.5
8	6.93	65.3	3.3
Coefficient correlation "r"		−0.81**	−0.98**

[a]Results not included in calculation, for reasons given in the text.
**Significant at 0.01 level.

develop a low pH_1 (Krzywicki and Ratcliff, 1967). This enhanced reticular function was present also in the myofibrillar fractions prepared from the muscles of low pH_1, thus confirming that reticulum remained substantially bound to the myofibrils after muscle fractionation (Krzywicki and Ratcliff, 1967; Muscatello, 1961; Hanson and Olley, 1965; Hulsmans, 1961; Wheeldon et al., 1965).

High Ca^{2+}-activated myosin ATPase activity and high calcium pump activity, together with a greater development of reticulum (Krzywicki and Ratcliff, 1967), appear to be characteristic features of a muscle that undergoes rapid postmortem glycolysis. These are among the well-recognized features of white muscle fibers (Gergely et al., 1965; Seifter and Gallop, 1966; Porter and Franzini-Armstrong, 1965) which may therefore be expected to contribute to rapid glycolysis and the PSE condition in a muscle. It is surprising, therefore, that Sair et al. (1970) reported that the ratio of white/red fibers in the L. dorsi was actually lower in pigs susceptible to the PSE condition than in stress-resistant pigs which are not prone to this condition. However, as these authors point out, their work made no distinction between white fibers and intermediate types of fiber, which were together classified as "white." More recently it has been demonstrated by Dildey et al. (1970) that in Hampshire-Yorkshire pigs, the ratio of white to red fibers is highly significantly greater ($P < 0.01$) in L. dorsi which develops the PSE condition, than in normal muscle.

It is of interest that the activity of myosin ATPase was substantially reduced at pH_1 values below 5.8 (Fig. 1), when the temperature of the muscle would be ~ 40°C. This may be compared with the results for rabbit L. dorsi reported by Penny (1967) who calculated that the activity of Ca^{2+}-activated ATPase was reduced (through denaturation) by as much as 30% when the pH had reached the critical level of 5.55 at 37°C. The same author (Penny, 1969) has shown that in pork L. dorsi, denaturation of myofibrillar protein and loss of ATPase activity (both the Ca^{2+}- and Mg^{2+}-activated enzymes) occurred when the pH at 90 min postslaughter fell below 5.9, the temperature of the muscle being at that time 33–35°C.

The calcium-retaining properties of the reticulum appeared to be severely impaired at pH_1 values below 5.9 (Tables 1 and 2 and Fig. 4). Greaser et al. (1969b) similarly reported that loss of calcium-accumulating ability in postmortem muscle was accelerated, when the pH fell below 6.0. Greaser et al. (1969c) also observed that this loss occurred more rapidly in muscle which developed the PSE condition than in normal muscle.

Attention has already been directed to the high capacity of the calcium pump at low pH_1 values, < 5.9, (as indicated by the high "t_0" values for calcium uptake, given in Table 2) and to the loss of the calcium storage ability of the reticulum under these conditions ("t_8" values in Table 2). Possibly the release of calcium by the reticulum, when damage results from pH_1 falling below 5.9, may account for the enhanced activity of residual, undenatured ATPase, which was recorded for one sample of muscle with pH_1 of 5.60 (as shown in Table 1).

When the pH_1 of postmortem muscle falls below 5.9, the reticulum apparently sustains damage, losing its capacity to store calcium ions withdrawn from the sarcoplasm. At this point calcium concentration in the sarcoplasm will increase, thereby increasing the activity of myosin ATPase, accelerating the pH fall of the muscle and leading to further denaturation and therefore to the development of the PSE condition. Hence the fall in pH in the postmortem muscle may proceed in two stages. Thus it has been found by the present author (Krzywicki, 1963, unpublished observation) that when the frequency distribution of pH_1 is plotted for a sufficiently large sample of pork carcasses, the curve obtained represents two subpopulations with average pH_1 values of 6.5 and 5.7. Whether the same discontinuity in pH_1 curves arising from similar causes, can be demonstrated in other muscles, and in other animals besides the pig, requires to be investigated.

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LIPIDS OF CURED CENTENNIAL SWEET POTATOES

SUMMARY—Lipids isolated from cured Centennial sweet potatoes were identified and quantitated by a combination of column and thin layer chromatography. These lipids were shown to consist of 42.1% neutral lipids, 30.8% glycolipids and 27.1% phospholipids. Triglycerides and steryl esters were the major lipids of the neutral fraction. Among the phospholipids, phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl inositol were the most abundant. Galactolipids and the steryl glucosides were also present. The predominant fatty acids were stearic, palmitic, oleic, linoleic and linolenic.

INTRODUCTION

OXIDATIVE deterioration of the lipids of dehydrated sweet potato flakes is a major obstacle to consumer acceptance of this food product. However, very little is known about the nature of lipids present in sweet potatoes. Boggess et al. (1967; 1970) studied lipid changes in a number of varieties of sweet potatoes including Centennials during curing and storage by separating lipids into "nonphospholipids," "cephalin" and "lecithin." These workers found the lipid content to be 0.44% (fresh weight) for cured Centennials stored for 180 days at 15.5°C.

On the other hand, the lipid make-up of white potato tubers has been studied exhaustively (Lepage, 1968; Galliard, 1968). These workers found that white potatoes contained approximately 0.10–0.13% lipids on a fresh weight basis. Phospholipids were present in the greatest amounts followed by glycolipids and neutral lipids.

It is the purpose of this paper to identify and quantitate the lipids of cured Centennial sweet potatoes.

EXPERIMENTAL

Sweet potatoes

Centennial sweet potatoes used in this study were harvested at Benson, N.C. (1969) and cured at 85°F and 80% relative humidity (r.h.) for 2 wk. The cured roots were then stored at 61°F and 60% r.h. for 9 months.

Reagents and chemicals

Only reagent grade solvents, with the exception of 95% ethanol, were used. All solvents were carefully distilled and stored in the dark in brown-glass bottles. Silicic acid (−325 mesh) (Sigma Chemical Co., St. Louis, Mo.) and silica gel G and H (Brinkmann Instrument, Westbury, N.Y.) were used for chromatography. Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N.J.) was used for lipid purification. Lipid standards were obtained from Sigma Chemical Co. and Supelco (Bellefonte, Pa.).

Lipid extraction and purification

Cured Centennial sweet potatoes from the 1969 crop were peeled, diced and a 50g sample ground with boiling 95% ethanol (3.5:1 v/wt) for 2 min in a Waring Blendor. The slurry was allowed to settle and filtered. The filter pad was then exhaustively extracted with chloroform-methanol (2:1 v/v) until extracts were colorless. The extracts were combined and an aqueous sodium chloride solution (10%) added to give two layers. The bottom layer containing the lipids was removed and the upper layer was extracted with 3 equal portions of chloroform. The chloroform extracts were combined with the original lower layer and evaporated. The extracted aqueous layer was shown to be lipid free by TLC examination of a concentrated aliquot. The lipid slurry was freeze-dried, taken up in chloroform-methanol (2:1 v/v) and filtered.

The crude lipid extract was freed from nonlipid contaminants by passage through a Sephadex G-25 column (Wuthier, 1963) followed by freeze drying to remove water. The purified lipids were dissolved in chloroform-methanol (2:1 v/v), transferred to a tared vial and the solvent evaporated under nitrogen. The vial was then placed in a desiccator and evacuated to remove the last traces of solvent. The lipid was then weighed. So that the lipid could be expressed on a dry weight basis, a 5g sample of sweet potato was dried under vacuum at 80°C to constant weight and the moisture loss determined.

Separation of lipids into classes

The silicic acid column procedure of Lepage (1968) was used to separate the purified lipid extract into neutral lipid, glycolipid and phospholipid fractions. A 1 × 15 cm silicic acid column was washed with 3 column volumes each of methanol, chloroform and hexane. Samples (ca. 100 mg) were transferred onto the column in hexane, the columns were eluted with 30 ml each of 2%, 5% and 50% ether in hexane, then with 30 ml each of 5%, 10%, 30% and 50% methanol in chloroform and finally with 50 ml of methanol. After testing each fraction by TLC, similar fractions were combined to give neutral lipid, glycolipid and phospholipid fractions. After evaporation of the solvent, the percent distribution of the three lipid classes was determined from the weights of the major fractions.

Analysis of neutral lipids

Neutral lipids were identified by comparison on TLC with authentic standards. The TLC system used was that of Nagy and Nordby (1970). Lipids in hexane were spotted onto activated silica gel H-coated plates and developed in ethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2 v/v) to a distance 10 cm above the point of sample application. After this development the plate was dried for 30 min in vacuo. The plate was then developed in hexane-benzene-acetic acid (80:25:1 v/v). The solvent was allowed to travel 16 cm above the application point. The plate was removed, dried, sprayed with 50% sulfuric acid and charred in a muffle furnace at 190°C for 35 min.

The neutral lipids were quantitated after charring by densitometry on a Densicord 542 Densitometer (Photovolt Corp., N.Y.). The light source was fitted with a 1 × 6 mm substage slit and a 0.3 × 6 mm collimating slit was placed over the phototube. Silica gel H plates (3 × 20 cm) were developed overnight in chloroform, dried and activated at 110°C for 1 hr. The silica gel layer was then scribed to give a lane 8 mm wide (Downing, 1968). The sample was applied within the lane and the plate developed and charred as described above. The plate was scanned on the densitometer and the area of each component obtained by triangulation. Three plates were run and the areas of each component averaged. Standards were run along with the unknown lipid samples. The percentage composition of each component was obtained from the areas after correction of sterol area for abnormally high carbon yields (Downing, 1968).

Analysis of glycolipids

Monogalactosyl diglyceride (MGDG), digalactosyl diglyceride (DGDG) and cerebrosides (CER) were identified by comparison on TLC with known standards. The systems used were silica gel H layers containing magnesium acetate with solvent systems (A) chloroform-methanol-water (65:25:4 v/v/v) and (B) $CHCl_3$-methanol-acetic acid-water (170:25:25:5 v/v/v/v). Visualization was by spraying with 50% H_2SO_4 and heating at 110°C. Steryl glucoside (SG) and esterified steryl glucoside (ESG) were identified by a positive Liebermann-Burchard spot on TLC. In addition these materials were isolated by TLC and acid hydrolysed. Glucose and free sterol were obtained from SG while glucose, free sterol and free fatty acid were obtained from ESG (Lepage, 1964).

Glycolipids were quantitated by isolation of the individual lipids by TLC followed by hexose measurements in duplicate on acid-hydrolysed lipids (Svennerholm, 1956). ESG, SG and MGDG were separated on plates prepared from silica gel H (20g) containing magnesium acetate (0.5g) developed in solvent system (A). After development the area corresponding to each lipid was removed, extracted with chloroform-methanol-water (30:30:1) and assayed for hexose according to Svennerholm (1956). DGDG and CER were treated in the same manner except solvent system (B) was used for TLC separation. Standard solutions of glucose and ga-

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lactose were carried through all steps with the exception of TLC. The percent distribution of each component was obtained from the hexose values.

Analysis of phospholipids

Phospholipids were identified by separation with two dimensional TLC on silica gel H impregnated with magnesium acetate according to Rouser et al. (1970). Individual lipids were identified by spraying with one of the following reagents: ninhydrin reagent (0.4% in butanol containing 2% acetic acid) and heating at 110°C for phospholipids containing free amino groups; modified Dragendorff reagent for choline-containing phospholipids (Bregoff et al., 1953); the specific phospholipid spray of Dittmer and Lester (1964); or the sulfuric acid formaldehyde spray of Rouser et al. (1970) followed by charring at 180°C. Additionally identification was accomplished by comparing R_f values with those of reference compounds.

Phospholipids were quantitated by phosphorous analysis of the individual components in duplicate after two dimensional TLC as described above. The results were converted to percent of total lipid by multiplying phosphorous values by the appropriate factor (Rouser et al., 1966).

Fatty acid analysis

The major fatty acid content of the total lipid extract and that of each of the three fractions was determined by converting an aliquot of each fraction into methyl esters (FAME). FAME were prepared by transesterification. About 5 mg of lipid was mixed with 1 ml anhydrous methanolic hydrogen chloride (5%) and sealed under nitrogen in an ampoule. The mixture was then heated at 80–85°C for 3 hr. The FAME were extracted with hexane, washed with 1N sodium carbonate solution, dried and purified by TLC.

Purified FAME were separated on an F and M model 810 gas chromatograph (Hewlett-Packard, Palo Alto, Calif.) equipped with a flame ionization detector. Dual 6 ft × 1/8 in. aluminum columns maintained at 180°C packed with EGSSX' (10%) on chromosorb P (100/120 mesh, Applied Science, State College, Pa.) were employed. Carrier gas (nitrogen) flow was 38 ml/min. Assignment of identity was based on comparison of retention times with those of authentic reference samples.

The relative quantities of fatty acids of Centennial sweet potatoes were obtained by comparing the peak areas of FAME with those of a known mixture. The percentage composition of the known mixture (Applied Science) was confirmed with better than ± 1% precision and accuracy. Gas chromatographic detector response was linear over the range studied.

RESULTS & DISCUSSION

THE LIPID content of cured Centennial sweet potatoes from this study was found to be 2.7% (dry weight). Boggess et al. (1967; 1970) found 1.6% lipids for Centennials. This discrepancy could easily be due to differences in curing and storage or methodology. Further comparison of the make-up of Centennial lipids as found by Boggess et al. (1967; 1970) and this study is not possible because of the differences in methodology.

In contrast, white potatoes were found (Galliard, 1968; Lepage, 1968) to contain about 0.5% lipid (dry weight). Although white potatoes and sweet potatoes are not closely related botanically, they are similar in that both are high in carbohydrates and low in lipids. Moreover, both are processed and sold as dehydrated food products. Possibly the relatively high lipid content of Centennials is one of the factors responsible for the greater storage stability of white potato flakes as compared to that of sweet potato flakes. Lepage (1968) found 16.5% neutral lipids, 45.5% phospholipids and 38.0% glycolipids for Netted Germ white potato tubers. We have found 42.1% neutral lipids, 27.1% phospholipids and 30.8% glycolipids for Centennial sweet potatoes.

For the purpose of this study neutral lipids are defined as those which contain neither phosphorous nor sugar in their molecules while glycolipids contain sugar and phospholipids contain phosphorous. The identity and amount of each lipid class and each component is given in Table 1.

Table 1—Lipid composition of cured Centennial sweet potatoes

Lipid	% Weight total lipid
Neutral Lipids[a]	42.1
Triglycerides	26.9
Steryl esters	6.1
Diglycerides	3.8
Hydrocarbons	2.8
Sterol (free)	2.5
Glycolipids[b]	30.8
Monogalactosyl diglyceride[c]	13.6
Digalactosyl diglyceride	6.3
Cerebroside	4.7
Esterified steryl glucoside	3.5
Unknown	2.1
Steryl glucoside	0.6
Phospholipids[d]	27.1
Phosphatidyl ethanolamine	7.8
Phosphatidyl choline	7.0
Phosphatidyl inositol	5.1
Unknown[e]	3.0
Cardiolipid	1.6
Phosphatidyl glyceride	1.2
Phosphatidyl serine	1.1
Phosphatidic acid	0.4

[a]Individual components determined by duplicate densitometric analyses.
[b]Individual components determined by duplicate hexose analyses.
[c]Possibly contains a contaminating glycolipid.
[d]Individual components determined by duplicate phosphorous analyses.
[e]Positive Dragendorff Test for choline; negative ninhydrin test for free amine; R_f on TLC similar to lyso-phosphatidyl choline.

Neutral lipids

Neutral lipids make up 42.1% of the total lipid of Centennial sweet potatoes. Of these lipids triglycerides are the most abundant followed by steryl esters, diglycerides, hydrocarbons and free sterols. No attempt was made to further study the components of the neutral fraction with the exception of the pigments. An investigation of the pigments of Centennials has been conducted previously (Purcell and Walter, 1968). Carotenoids, responsible for the orange color of sweet potatoes, were shown to consist of mostly β-carotene (86.4%) and small amounts of a large number of other carotenoids. In this study, most of the pigment was isolated in the neutral lipids and was found to amount to 2.3% of the total lipids.

Glycolipid composition

Glycolipids comprise 30.8% of the lipid fraction. This fraction contains MGDG, DGDG, cerebrosides, ESG, SG and an unidentified lipid. The lipid designated as MGDG contained a contaminating lipid. This was noted when the developed TLC plate was sprayed with 50% sulfuric acid and heated at 110°C. Under these conditions a yellow area was noted in the brown galactosyl diglyceride. If the plate is charred, the contaminating substance is not distinguishable from the galactolipid. Repeated attempts with numerous solvent systems did not resolve the two spots.

Phospholipid composition

As is usual, for nonphotosynthetic plant tissue, phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) and phosphatidyl inositol (PI) were found to

Table 2—Fatty acid composition of cured Centennial sweet potatoes[a] (Relative weight percent)

Lipid Fraction	% of Individual fatty acids in each fraction										
	14:0	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	19:0	20:0
Total Lipids	1.7	1.3	29.3	2.0	1.5	6.8	2.0	44.7	8.8		2.4
Neutral Lipids	2.3	1.7	32.2	1.8	1.7	7.8	2.0	39.0	9.6		2.2
Phospholipids	1.7	1.4	24.7	1.7	1.6	4.6	2.1	49.0	8.0		*d
Glycolipids #1[b]	4.4	1.8	21.9	3.8	2.0	7.1	3.9	43.9	7.5		1.7
Glycolipids #2[c]	2.9	2.8	17.5	2.2	3.4	5.9	2.1	49.4	9.7	3.5	*d

[a]Results based on the average of two gas chromatographic analyses.
[b]Diagalactosyl diglyceride, cerebrosides, and unknown glycolipids.
[c]Monogalactosyl diglycerides, steryl glucoside, and esterified steryl glucoside.
[d]Asterisk (*) indicates trace amounts.

be the major components of the phospholipid fraction. An unknown lipid which gave a positive Dragendorff test and a negative ninhydrin test was tentatively identified as lyso-phosphatidyl choline on the basis of similar R_f values on two dimensional TLC. Phosphatidyl glyceride (PG) and cardiolipid (DPG) were tentatively identified by their chromatographic behavior on TLC.

Fatty acid composition

The major fatty acid composition of each of the lipid fractions as well as that of the total lipid is given in Table 2. Palmitic and linoleic acids are the most abundant fatty acids in all fractions. The neutral lipids contain 52.4% unsaturated acids while phospholipid and glycolipid fractions contain greater than 60% unsaturation. Our results for the total lipid fraction are in qualitative agreement with those of Boggess et al. (1970) who found palmitic and linoleic to be the most abundant fatty acids present. These workers also found stearic and linolenic acid but in significantly smaller amounts.

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ANTIOXIDANT POTENTIAL OF TEMPEH AS COMPARED TO TOCOPHEROL

SUMMARY—The antioxidant potential of tempeh to preserve tocopherol-stripped corn oil was studied. Different levels of tempeh were mixed into corn oil, and incubated at 37°C for a maximum of 6 wk. Peroxide values were determined biweekly. Results showed that tempeh can prevent lipid oxidation. Corn oil containing 50% tempeh showed higher antioxidant potential than those containing 25% tempeh, 0.01% α-tocopherol, or 0.03% α-tocopherol. This study substantiates the antioxidant potential of tempeh and suggests its use with other foodstuffs to help preserve the lipids contained therein.

INTRODUCTION

TEMPEH, a popular food in Indonesia, is produced by fermenting soybeans with a species of *Rhizopus* mold. The antioxidants, jenistin, diadzein and 6,7,4′-trihydroxyisoflavone, which are assumed to be in a bound inactive form in soybean, are present in tempeh (György et al., 1964). The antioxidant activity is useful for the preservation of lipids in tempeh and possibly lipids in other food products when mixed with tempeh.

The nutritive value of tempeh was reported to be higher than that of unfermented soybeans (György, 1962). The nutritive advantages of tempeh over soybeans are: increase in digestibility and content of riboflavin, pantothenic acid, pyridoxine and nicotinic acid (Murata et al., 1967). Favorable flavor changes and improved stability of the dried product make tempeh a more attractive food product than soybeans. Wang and Hesseltine (1969) discovered that antibacterial compounds extracted from tempeh inhibited bacteria including species found in the human intestinal tract.

The antioxidant potential of tempeh in preserving food oils high in unsaturated fatty acids was investigated. The hypothesis suggested by the authors is that tempeh, owing to its antioxidant property, may be mixed into unstable foods containing highly unsaturated fatty acids to stabilize food lipids. This property would be useful in food preservation in developing countries and could result in nutritious, high-protein products.

EXPERIMENTAL

AIR-DRIED tempeh was produced in Indonesia by culturing the boiled soybean with local mold *Rizopus oligosporus* or *orzoe*. It was ground to a fine powder in a Wiley mill before being mixed with corn oil. Tocopherol-stripped corn oil and dl-α-tocopherol were obtained from General Biochemicals, Chagrin Falls, Ohio.

The antioxidant potential of tempeh on tocopherol-stripped corn oil was determined in Experiment 1. Three samples contained the following: (a) Tocopherol-stripped corn oil; (b) Tocopherol-stripped corn oil, 75%; tempeh, 25%; and (c) Tocopherol-stripped corn oil, 50%; tempeh, 50%. Samples were incubated at 37°C for a 5-wk period. Peroxide values were determined biweekly.

The relative antioxidation potential of tempeh and of dl-α-tocopherol on tocopherol-stripped corn oil was compared in Experiment 2. The five samples contained the following: (a) Tocopherol-stripped corn oil; (b) Tocopherol-stripped corn oil, 75%; tempeh, 25%; (c) Tocopherol-stripped corn oil, 50%; tempeh, 50%; (d) Tocopherol-stripped corn oil, 99.97%; dl-α-tocopherol, 0.03%; and (e) Tocopherol-stripped corn oil, 99.99%; dl-α-tocopherol, 0.01%. Samples were incubated at 37°C over a 6-wk period and peroxide values were determined biweekly.

Total lipid was extracted by the method of Bleigh and Dyer (1959) using a chloroform and absolute methanol mixture (2:1).

Peroxide values of the samples were determined by a modified method of Privett and Nichell (1953). The modifications were a 10–15-min equilibration before adding saturated potassium iodide solution and a 15-min incubation after adding the solution. The peroxide value of the tempeh was 4.6 meq/1000g tempeh.

RESULTS & DISCUSSION

EXPERIMENT 1 proved that tempeh contained a very potent antioxidant. Sample A, 100% tocopherol-stripped corn oil, had peroxide values of 12.6, 69.2, 242.3 and 1508.0 meq/1000g corn oil after 1, 3, 4 and 5 wk incubation times, respectively. Sample B, 75% tocopherol-stripped corn oil with 25% tempeh added, had significantly lower peroxide values of 10.2, 24.1, 63.5 and 886.8 meq/1000g corn oil at 1, 3, 4 and 5 wk incubation times, respectively. The addition of 50% tempeh, by weight, to tocopherol-stripped corn oil (Sample C) resulted in much lower peroxide levels of 4.3, 10.2, 28.6 and 38.4 meq/1000g corn oil at 1, 3, 4 and 5 wk, respectively. An indication of the potency of the antioxidant in tempeh is evident in the approximately 40-fold reduction in peroxide formation after 5 wk incubation in the corn oil sample containing 50% tempeh as compared to the corn oil sample alone. A second experiment was conducted replicating the tempeh addition and also including two levels of a natural antioxidant, tocopherol, to permit a quantitative estimation of the antioxidant potency of tempeh.

Tempeh was highly effective again in preventing peroxide formation in tocopherol-stripped corn oil in Experiment 2 (Table 1). When added at the 25% level, it was more effective than the 0.03% α-tocopherol at 2 wk incubation and remained more effective than the 0.01% α-tocopherol at 6 wk incubation. Peroxide formation increased sharply in all samples after 4 wk except in the 50% tempeh. It is clear that at the higher level (Sample C) tempeh has considerably more antioxidant potential than 0.03% α-tocopherol.

When comparing the effect of tempeh in Experiments 1 and 2, it is clear that the antioxidant is very stable and highly effective in preserving tocopherol-stripped corn oil from autoxidation. Per-

Table 1—Antioxidant potential of tempeh as compared to α-tocopherol (Experiment 2). Peroxide values of corn oil (meq/1000g)[a]

Incubation time (wk)	Sample A corn oil, 100%	Sample B corn oil, 75%; tempeh, 25%	Sample C corn oil, 50%; tempeh, 50%	Sample D corn oil, 99.97%; α-tocopherol 0.03%	Sample E corn oil, 99.99% α-tocopherol 0.01%
0	12.1	7.6	1.9	6.8	5.6
2	33.8	21.5	6.9	21.6	21.9
4	286.6		26.6	80.8	223.4
6	1649.7	1013.2	111.4	468.8	1619.8

[a]Mean value from triplicate analyses.

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oxide levels of Samples B and C remained much lower than Sample A during the entire storage periods. However peroxide values increased sharply after 4 wk storage in both experiments. It seems that the antioxidants of tempeh at 50% level had reached the maximum potential between the 5th and 6th wk of incubation. The same phenomenon is also observed with the 25% tempeh-75% corn oil mixture. This probably is due to the exhaustion of antioxidants by the high level of peroxide produced.

The quantitative effect of 0.01% and 0.03% α-tocopherol added in tocopherol-stripped corn oil could be observed at the 2nd wk (Table 1). The same quantitative effect is noticeable with 25% and 50% tempeh. In a comparison of peroxide values at the 6th wk, a 0.03% of α-tocopherol added showed a greater antioxidant activity than that of 0.01% α-tocopherol or of 25% tempeh. However, even at the 0.03% level, α-tocopherol had less than ¼ the antioxidant activity of tempeh at the 50% level, based on the calculated peroxide value for 1000g stripped corn oil in the third sample.

The results would indicate that the stability of the antioxidant in tempeh is considerably greater than that of α-tocopherol. The tempeh used in this study was more than 2 yr old (stored at room temperature) at the time of the experiment. Regulations of the U.S. Food and Drug Administration and the Food and Agriculture Organization of the United Nations do not permit the use of α-tocopherol in levels greater than 0.03%. It would appear that tempeh can be mixed with oil or other food high in oil content, such as whole fish, and dehydrated to produce food products that are stable and have a high nutritive value. If equal weights of tempeh can preserve a polyunsaturated food such as corn oil, it is likely that it would be effective in the storage of food products subject to lipid autoxidation.

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HYDRATION OF SODIUM CHLORIDE BOUND BY CASEIN AT MEDIUM WATER ACTIVITIES

SUMMARY—In dried mixtures of sodium chloride and casein, one part of the salt is bound by the protein and the other part is crystalline or amorphous, depending on drying conditions. Bound and free forms of salt are in equilibrium, which is strongly dependent on the actual water activity. If changes in water activity take place slowly, this equilibrium may be maintained; whereas, during rapid changes nonequilibrium conditions are likely to be attained. The bound, crystalline and amorphous sodium chlorides produce characteristic changes in the water vapor sorption properties of salt-casein-mixtures. Sorption isopsychric curves describing the equilibrium water content as a function of the composition at constant water activity were determined between the water activities of 0.50 and 0.75 at 25°C, using the isopiestic technique. These curves were analyzed in terms of the hydration of bound sodium chloride ion pairs. The hydration increases progressively with increasing water activity; also, with the amount of sodium chloride added up to the point where the protein becomes completely saturated with ion pairs. From the isopsychric curves, the sorption isotherms of salt-casein-complexes of definite salt contents were constructed in the water activity range investigated.

INTRODUCTION

MANY DRIED FOODSTUFFS contain proteins, neutral salts and small amounts of water. In particular, sodium chloride is present in large quantities in most food mixtures as a flavor component and preservative. However, the state of the salt in dried food products has not yet been studied in great detail. Nemitz (1962) found that the indigeneous salt contained in egg white does not form a eutectic upon freezing and must, therefore, be bound to the protein in some way. Bull and Breese (1968) came to a similar conclusion in studying the hydration of egg albumen in the presence of added salt. Gál et al. (1962) and Gál and Signer (1963) have been able to demonstrate by water vapor sorption measurements and x-ray diffraction that casein is capable of binding a certain amount of sodium chloride. Later, the dependence of the quantity of bound salt on water activity also was demonstrated, Gál et al. (1968).

In a recent paper on the kinetics of the binding and crystallization of salt in sodium chloride-casein-systems, the authors made the following observations (Gál, 1970): When such systems, originally containing a large amount of water, are dried, one part of the salt remains bound to the protein. Whether the protein becomes completely or only partially saturated with ion pairs, depends upon the water content of the mixture prior to drying and also on the conditions of dehydration, provided there is an excess of salt present. In dried mixtures, the unbound salt is normally present in its crystalline form. However, a part of this free salt may become amorphous during dehydration and crystallizes only if the water activity is raised again. On the other hand, the protein tends to bind the maximum possible number of ion pairs, being favored by the increased mobility of the ions at higher water activities. Finally, the following equilibrium exists between the two forms of the salt:

$$\text{bound salt on the protein} \rightleftharpoons \text{free salt in the crystal lattice.}$$

The position of this equilibrium and the rate of phase changes indicated by the arrows are functions of the water activity and, to some extent, the distribution of the salt, which itself is again mainly determined by conditions of dehydration.

In all practical cases, nonequilibrium conditions exist in dried systems because of the extreme slowness of some of the processes mentioned.

This paper presents results of experiments with sodium chloride-casein-systems in states of equilibrium with regard to the hydration and binding of the sodium chloride ion pairs to casein in the range of $a_w = 0.50-0.75$ at a temperature of 25°C.

EXPERIMENTAL

THE EXPERIMENTAL TECHNIQUES were described in previous papers, Signer and Gál (1961) and Gál et al. (1962), and are briefly summarized here. Substances used to prepare the mixtures were analytical-grade sodium chloride, double-distilled water and acid precipitated casein by Hammarsten from Merck. The samples of different salt content in amounts of about 150 mg were obtained by weighing together calculated amounts of freeze-dried casein and of a casein-salt-mixture treated in the same way, containing 130 moles NaCl per 10^5g casein. Mixtures between 0 and 130 moles NaCl per 10^5g casein were prepared for each 10 moles of salt content. The samples were ground in an agate micromill with a frequency of 50 Hz during 15 min to achieve complete homogeneity.

The equilibrium water contents were determined by the standard atmospheric isopiestic technique, Robinson and Sinclair (1934) using weighing bottles of stainless steel. No corrosion was detected on the inside of the bottles after using them in several sorption cycles. The weighing bottles could be covered in the vacuum desiccator used for water content determinations and also in the isopiestic apparatus by means of a simple mechanism before opening the desiccators, Gál (1967). As the lids were not absolutely airtight, several weighings were made with each bottle after taking out of the desiccator, and the right weights were obtained by extrapolating the individual weight-time curves to zero time. No correction for buoyancy effects was found to be necessary. Throughout the work a Mettler ultramicro balance was used for weighing the samples, with a maximal deviation of ± 0.01 mg.

Water activities were obtained by aqueous sulfuric acid solutions. Concentration of the solutions was determined after each experiment by titrating with 0.1N sodium hydroxide solution. Activities could be determined with maximal deviations of ± 0.002 unit.

Equilibria of the ion binding and of the water vapor sorption were achieved as follows: After weighing and grinding the samples, the water activity was raised to 0.7528 during a period of at least 8 days, using a saturated sodium chloride solution instead of sulfuric acid in the desiccators. Thereafter, the samples were rapidly dried at room temperature in a vacuum desiccator over phosphorous pentoxide at 0.02 Torr for 48 hr. The dry samples were ground again for 15 min to initiate crystallization of the sodium chloride. The samples were then put back into the isopiestic apparatus conditioned to the lowest water activity selected. After equilibration the samples were weighed and dried as just described, to obtain the equilibrium water content. The dry samples were used for x-ray investigations, Gál (1970). The samples were then equilibrated at the next higher water activity. The equilibration in the course of which the final state of salt binding and water sorption were attained was continued for 30 days at the water activity of 0.510 and 7 days at $a_w = 0.738$. The other intervals were between these two extremes.

RESULTS & DISCUSSION

Binding of NaCl-ion pairs by casein

The water vapor sorption capacity of

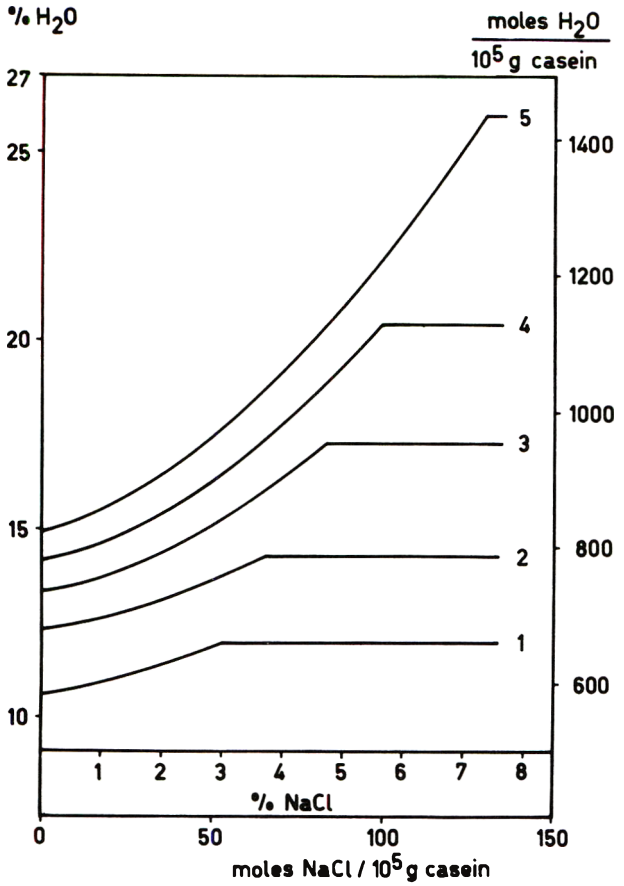

Fig. 1—Water vapor adsorption isopsychric curves of sodium chloride-casein-mixtures at 25° C.

Curve 1, a_W = 0.510. *Curve 4, a_W = 0.706.*
Curve 2, a_W = 0.603. *Curve 5, a_W = 0.738.*
Curve 3, a_W = 0.664.

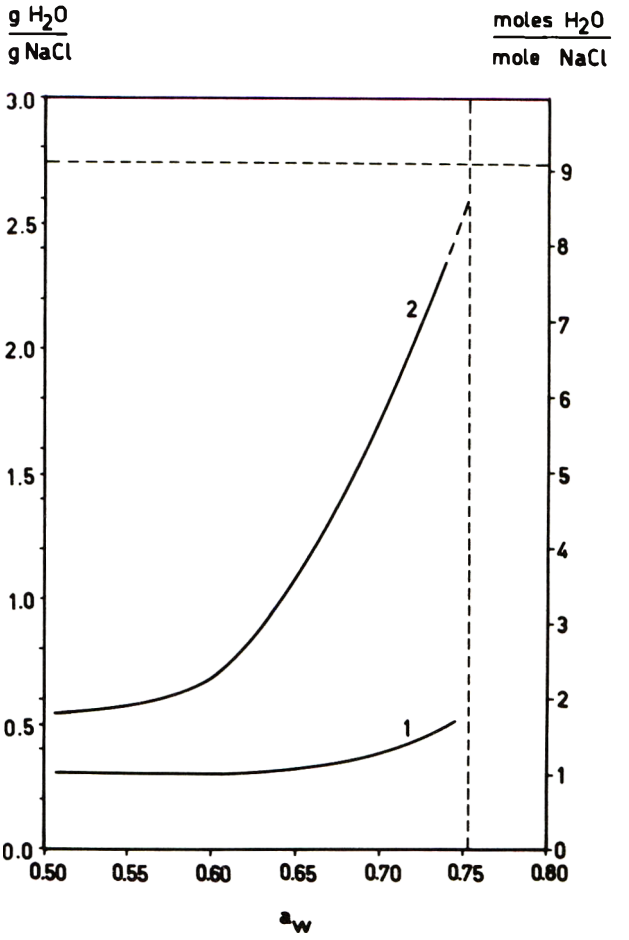

Fig. 2—Hydration of sodium chloride-casein-complexes.

Curve 1, first moles of bound NaCl.

Curve 2, last moles of bound NaCl.

casein is increased by the bound sodium chloride ion pairs. The relationship between the equilibrium water content and the amount of salt bound at constant water activities (isopsychric curves) is shown in Figure 1. In this Figure, as well as in Figures 2 and 3, the water content and quantity of salt are expressed as a percentage or in units of moles per 10^5 g of the water- and salt-free casein.

Range of the water activity was determined by experimental conditions. At water activities below 0.50, migration of salt in the systems investigated becomes so slow that equilibrium cannot be attained in a reasonable time. Above the water activity of 0.7528, the excess salt goes into solution, a condition not covered by the investigation from which the diagram originates.

From Figure 1 it can be seen that the equilibrium water content of casein increases steadily with increasing salt content until there is a break in the curve at the point where the protein becomes saturated with ion pairs. From here the line is straight and horizontal, indicating that the excess salt is crystalline and does not adsorb water vapor at the water activities concerned. It is also worthy of note that the curves are turned upwards, showing less interaction between the ion pairs and the protein. This results in the components becoming increasingly hygroscopic. It is evident also that the capacity of casein to bind NaCl ion pairs is strongly dependent on the water activity.

The curved part of the isopsychric lines of Figure 1 can be approximated very accurately by the method of least squares with a polynomial of second order

$$y = a + bx + cx^2$$

where y = equilibrium water content as a percentage of casein
x = amount of sodium chloride as moles per 10^5g casein.

The constants of the equation and the coefficients of determination at different water activities are shown in Table 1. With the ordinates of the horizontal part of the curves, the figures of the last column of Table 1 were calculated. They agree well with the results of x-ray diffraction measurements on the same systems, Gál et al. (1968).

Hydration of sodium chloride-casein-complexes

The slopes of the curves in Figure 1 at any point correspond to the change in the hydration of the sodium chloride-casein-complex with the quantity of salt in terms of g H_2O/g NaCl or in any other unit. The functions were differentiated analytically at two points: at the beginning (at zero percent bound salt) and at the point of the break (maximum possible amount of bound salt). Values of the slopes are shown graphically in Figure 2. Curve 1 represents the hydration of the complex per g or mole for the first bound ion pairs and curve 2 the same for the last ones. The first ion pairs exhibit an hydration of 1.0–1.6 moles of water per mole of sodium chloride, whereas the last ones

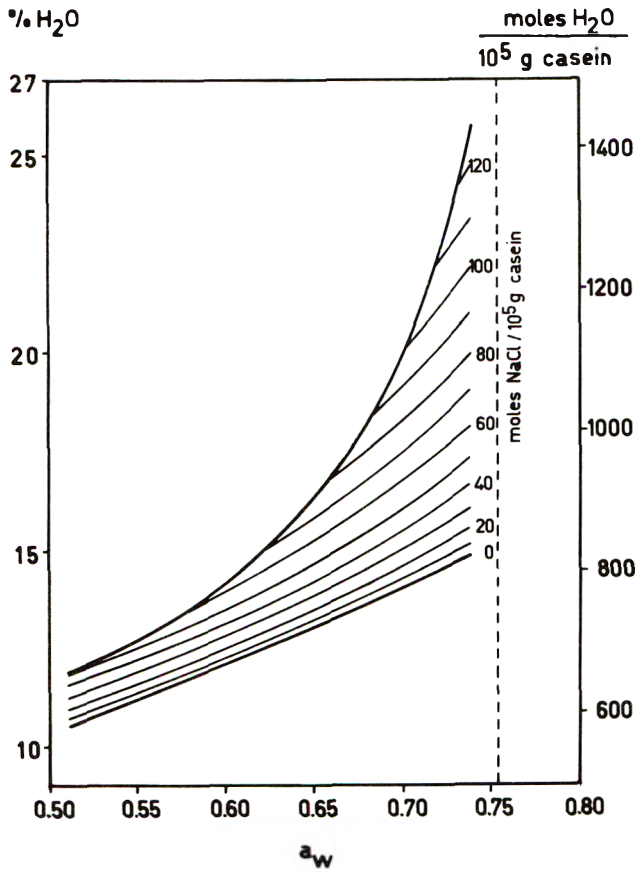

Fig. 3—Adsorption isotherms of sodium chloride-casein-complexes.

Table 1—Constants of the equations of isopsychric curves

a_w	a	b .10^2	c .10^4	Coefficient of determination	NaCl-binding (%)
0.510	10.51	2.057	1.106	0.9991	3.1
0.603	12.22	1.702	2.094	0.9999	3.8
0.664	13.28	1.963	3.257	0.9999	4.8
0.706	14.07	2.326	4.031	0.9996	5.7
0.738	14.81	2.909	4.446	0.9998	7.5

Table 2—Hydration of NaCl ion pairs at a_w = 0.7528

Method		g H_2O / g NaCl	moles H_2O / mole NaCl
Extrapolation of present results		2.67	8.7
Obtained experimentally by water vapor adsorption measurements	– adsorption	2.92	9.5
	– desorption	2.58	8.7
Calculated from concentration of the saturated solution		2.79	9.1

adsorb 1.8–7.8 moles water per mole sodium chloride in the range of water activity investigated. The horizontal dashed line in Figure 2 corresponds to the hydration of sodium chloride in the saturated solution and the vertical one indicates the water activity of the saturated salt solution, both at 25°C.

By extrapolating curve 2, the hydration of the complex per mole of sodium chloride for the last bound ion pairs at a_w = 0.7528 could be determined. Results in Table 2 show this value, along with the results of direct sorption measurements at the same water activity, Gål and Signer (1965), and also with the hydration of the ions in the saturated salt solution.

This comparison indicates that the last molecules of sodium chloride bound at the water activity of the saturated solution are so loosely attached to the protein that they are hydrated practically to the same extent as the dissolved free molecules. The transition of the ion pairs from the bound to the free state at this water activity seems to be quite smooth.

Sorption isotherms of the sodium chloride-casein-complexes

From results shown in Figure 1 the water vapor sorption isotherms of the casein with different amounts of bound sodium chloride can also be determined. These are shown in Figure 3. The lowest curve of Figure 3 represents the sorption isotherm of pure casein. The uppermost curve corresponds to the isotherm of casein saturated with sodium chloride. Because saturation is a function of the water activity, this curve involves two kinds of equilibria: 1) equilibrium of the binding of sodium chloride by casein; 2) equilibrium of the sorption of water vapor by the sodium chloride-casein-complex.

The isotherms of casein loaded with different quantities of sodium chloride lie between these two extremes. It should be mentioned that all curves shown in Figure 3 are adsorption isotherms.

Some conclusions can be drawn from the data presented in Figure 3 regarding the sorption processes in dried sodium chloride-casein-mixtures. In the presence of an excess of salt the adsorption follows the highest curve. If only a small amount of salt is present, say less than 8%, the adsorption follows the uppermost curve only up to the point where all the salt is in the bound state. From here the curve follows a flatter isotherm corresponding to this particular salt concentration and shows a break at the transition between the two parts of the curve. It should be emphasized that the binding process of salt to protein is generally a very slow one, so that in the times normally encountered in water vapor adsorption measurements no true salt-binding equilibria can be expected.

In slow dehydration, the processes take place in the opposite direction. During fast dehydration, on the other hand, nonequilibrium conditions can again be attained and the water vapor desorption isotherms run without a break into the area above the uppermost curve of Figure 3, where the salt set free remains amorphous and is markedly hygroscopic.

It should be mentioned in conclusion that the results presented in this paper were obtained on a very special model system. They cannot be transferred to other neutral salt-protein-mixtures, even though there may be a qualitative resemblance between different pairs of substances. The work will be continued with the aim of producing a complete phase diagram for the sodium chloride-casein-system.

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CAROTENOID TRANSFORMATIONS IN RIPENING APRICOTS AND PEACHES

SUMMARY—β-Carotene was the dominant pigment of apricot tissue and accumulated rapidly during ripening; however, in peach tissue, β-carotene along with lutein and violaxanthin were synthesized in almost equal amounts during the ripening process. In the latter tissue small amounts of zeaxanthin were detected mid-point in the ripening sequence. Peach and apricot tissue incorporated [2-^{14}C] mevalonic acid into β-carotene, β-cryptoxanthin, lutein and also into violaxanthin in peach tissue. Incorporation of [2-^{14}C] mevalonic acid into the above carotenoids was greatest during the initial stages of ripening in the peach and then declined with senescence especially in the xanthophylls lutein and violaxanthin.

INTRODUCTION

ACCORDING to Mackinney's classification of fruits (Mackinney, 1966) the mature or ripe apricot and peach are representatives of the two sub-categories of yellow-to-orange fruits in that β-carotene accounts for some 60% of the total carotenoid content of the apricot, while peaches contain large quantities of xanthophylls, of which violaxanthin and β-cryptoxanthin are dominant. Like most mature fruits, the carotenoid makeup of apricots and peaches is complex with many hydrocarbon and xanthophyll pigments present in smaller quantities. Curl (1959, 1960) has reviewed previous work and gives a detailed list of the carotenoids present in these two fruits as separated by counter-current distribution.

The carotenoids of ripe fruit have been extensively studied, but the changes in the individual pigments and their transformations during the developing, ripening and senescent stages of fruit ontogeny are not well understood. Several workers (Goodwin and Jamikorn, 1952; Zscheile, 1966; Meredith and Purcell, 1966; Edwards and Reuter, 1967; Raymundo et al., 1967, 1970) have followed the changes in the individual pigments of the ripening tomato fruit but generally the pigment content of ripening fruits has been measured at a wavelength of about 450 nm (Miller et al., 1941, 1944; Gortner, 1965; Reid et al., 1970).

It has been shown that lycopene and β-carotene are labeled when [^{14}C] acetate and [^{14}C] mevalonic acid (MVA) are injected into ripe fruit (Shneour and Zabin, 1957; Purcell et al., 1959; Anderson et al., 1960). The pathway of lycopene synthesis has been outlined by genetic and radiotracer methods (Porter and Anderson, 1967) but the point where cyclization occurs to form β-carotene remains undecided. Recent evidence strongly suggests the existence of two separate pathways for β-carotene formation (Raymundo et al., 1970).

While the route of carotene synthesis in ripening fruit has been generally established (Porter and Anderson, 1967), the pathway of xanthophyll biosynthesis is unknown. It is a reasonable assumption that the xanthophylls are formed from the parent carotene (Chichester and Nakayama, 1967); the changes in and the incorporation of [2-^{14}C] MVA into β-carotene and the major xanthophylls of ripening apricots and peaches support this thesis.

EXPERIMENTAL

Materials

Apricots (*Prunus armeniaca* cv. 'Royal') and peaches (*Amygdalas persica* cv. 'Halford') were picked in the University of California, Davis orchard at weekly intervals during ripening. Fruit samples were collected from the same trees during the experimental period. Fruit maturity was judged visually and samples were matched as closely as possible. Fruit at the ripe stage were fully colored (orange) and edible. Three samples of fruit were analyzed at each maturity level and the results averaged. All solvents and chemicals were of analytical reagent grade and used as received except ethyl ether which was purified on a column of basic alumina to remove peroxides.

Extraction of carotenoids

The flesh (100g per sample) was blended with acetone in a Waring Blendor after removal of the pits and skin. The homogenate was exhaustively extracted with acetone after which the pigments were transferred to petroleum ether, washed with water and saponified (Goodwin, 1955). The unsaponifiable material was transferred to petroleum ether, washed with large quantities of water and dried over anhydrous Na_2SO_4.

Separation of carotenoids

The unsaponifiable material was partitioned between petroleum ether and 90% (v/v) MeOH saturated with petroleum ether. The hypophasic or xanthophyll fraction was chromatographed on Kieselguhr paper (Schleicher and Schull No. 967) (Jensen and Liaaen-Jensen, 1959; Jensen, 1959) developed with 10% (v/v) acetone in petroleum ether (b.p. 60–70°C). Each band was cut from the developed chromatogram and extracted with ethyl ether. The identity of the major components was determined by cochromatography with authentic pigments prepared from spinach (lutein, zeaxanthin, violaxanthin and neoxanthin) and lemons (β-cryptoxanthin), by visible absorption spectra and by the epoxide test (Curl and Bailey, 1961).

The epiphasic or carotene fraction was chromatographed on MgO-impregnated paper and developed with 3% (v/v) acetone in petroleum ether (b.p. 60–70°C). The separated bands were cut from the paper and eluted with ethyl ether. The identity of the major carotenes was established by cochromatography with authen-

Table 1—Incorporation of [2-^{14}C] MVA into the carotenoids of apricots picked at the green-yellow maturity stage

Pigment	Radioactivity cpm/mg pigment
β-carotene	19,900
β-cryptoxanthin	1,923
lutein	1,242

Table 2—Incorporation of [2-^{14}C] MVA into the carotenoids of ripening peaches

	Maturity Stage[a]				
	3	4	5	6	7
Pigment	Radioactivity (cpm/mg pigment)				
β-carotene	9,980	58,100	57,600	48,000	33,400
β-cryptoxanthin	55,900	65,500	35,700	35,500	43,600
lutein	5,400	3,420	2,260	1,620	–
violaxanthin	703	482	495	–	–

[a]See Figure 2 for explanation of maturity stages.

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tic pigments prepared from tomato fruit (lycopene), carrot (γ-carotene) and spinach (β-carotene). The papers were prepared by dipping Whatman filter paper (No. 4) two to three times into a slurry of 20g MgO suspended in 200 ml 50% aqueous MeOH. After preliminary drying, the papers were placed in an air oven at 100°C for 6 hr and then stored in a desiccator until required.

Administration of [2-^{14}C]MVA and radioassay

An aqueous solution of [2-^{14}C]MVA was prepared from the N,N'-dibenzylethylenediamine salt and contained approximately 1×10^6 cpm/ml. The solution (0.25 ml for each apricot and 0.15 ml for each peach) was injected into the fruit. 24 hr later, the carotenoids were extracted and separated as previously described. Radioautographs were prepared by placing X-ray film in contact with the circular chromatograms. The film was exposed for 2 days, whereupon it was developed and examined for radioactivity. The labeled pigment bands were cut from a duplicate chromatogram, eluted and counted (Yokoyama et al., 1962).

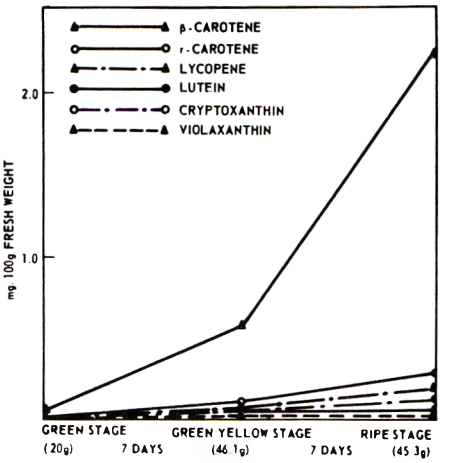

Fig. 1—Carotenoids of ripening apricots. Fruit weight (avg of 5 fruits) at each maturity stage is given on the X-ordinate.

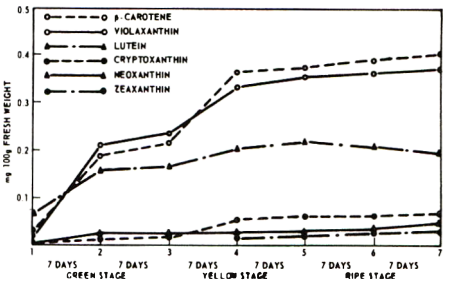

Fig. 2—Carotenoids of ripening peaches.

RESULTS & DISCUSSION

THE PATTERNS of the major carotenoids during the ripening of apricots and peaches are shown in Figures 1 and 2. In the ripening apricot, β-carotene increased rapidly to become the predominant pigment; moderate increases were found for γ-carotene, lycopene, lutein, β-cryptoxanthin and violaxanthin. However, these pigments together constituted a small percentage of the total pigment content. On the other hand, violaxanthin and lutein along with β-carotene increased substantially during the ripening of the peach, while β-cryptoxanthin and neoxanthin increased slightly. The three major pigments showed marginal increases after the fourth maturity stage or 3 wk after the commencement of the experiment; in fact the lutein content decreased slightly. At this stage, zeaxanthin was first detected in peach tissue and furthermore by maturity stage 6, most of the xanthophylls were esterified confirming previous work (Curl, 1959). Thus the ripening peach, in many respects, is similar to the autumn leaf where the xanthophylls are synthesized and gradually esterified (Goodwin, 1958; Chichester and Nakayama, 1967).

It is generally agreed that the xanthophylls are formed from a parent carotene and do not arise independently of the related compounds (Chichester and Nakayama, 1967). When apricots at the green-yellow stage were supplied with [2-^{14}C]MVA, the β-carotene, β-cryptoxanthin and lutein fractions were strongly labeled (Table 1), with β-carotene having ten-fold the radioactivity of the latter two fractions. The normal carotene intermediates such as phytoene and phytofluene contained practically no radioactivity after 24-hr metabolism which suggested that these compounds were rapidly turned over. During this period of active carotenoid synthesis β-carotene may be regarded as the major end product. It is probably then more slowly transformed into the monol β-cryptoxanthin and the diol lutein by oxidation and isomerization steps.

This work was extended by supplying [2-^{14}C]MVA to peaches picked at several maturity stages (Table 2). During the period of rapid carotenoid synthesis (stages 3 and 4) β-carotene and the xanthophylls β-cryptoxanthin, lutein and violaxanthin were labeled; however, as the fruit approached maturity, the tendency to become labeled became less and at complete maturity there was little or no labeling of the xanthophylls other than β-cryptoxanthin. β-Carotene and β-cryptoxanthin were strongly labeled at all maturity stages studied although there was some decrease at full maturity of the fruit. Zeaxanthin was found in small amounts from maturity stage 4 onwards but no radioactivity was detectable in this fraction. One would expect zeaxanthin as an intermediate between β-cryptoxanthin and violaxanthin on structural considerations (Davies et al., 1970) and this conversion has been demonstrated in higher plants (Yamamoto and Chichester, 1965); however, it seems likely that the small quantities present were rapidly turned over. The results suggest that both peach and apricot tissue rapidly synthesized β-carotene which was then transformed quite rapidly to β-cryptoxanthin and then more slowly to the more oxygenated compounds. Recent work of Walton et al. (1969) indicated that the hydroxyl functions in β-cryptoxanthin and lutein were introduced by sterospecific replacement of one hydrogen atom at C-3 or C-3′ thus eliminating the possible formation of a keto intermediate.

Recent work in our laboratory has shown the transformation of β-carotene to the oxygenated derivatives echinenone and canthaxanthin by the brine shrimp fed with [^{14}C]β-carotene (Hsu et al., 1970; Davies et al., 1970). In contrast to marine organisms and other animals which transform the ingested carotene molecule, ripening fruits can synthesize the complete carotene molecule before transforming it. It might be pointed out that the present approach along with the analysis of mutants provides only limited information concerning the mechanisms of xanthophyll biosynthesis; enzyme studies are required to complete the picture.

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Contribution No. 1388 of the Rhode Island Agricultural Experiment Station.

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GAMMA GLUTAMYL TRANSPEPTIDASE OF SPROUTED ONION

SUMMARY—An enzyme capable of liberating p-nitroaniline from γ-L-glutamyl p-nitroanilide has been purified 800-fold from sprouted onions. The enzyme acts optimally at pH 9.0, is activated by amino acids and inhibited by borate and competitively by γ-glutamyl derivatives. With the p-nitroanilide as substrate it exhibits a K_m of 14.3 mM whereas the K_i values with glutathione and γ-glutamyl-S-methyl-L-cysteine as inhibitors are 0.20 and 2.14 mM respectively. From chromatography of the reaction products and from kinetic considerations it is concluded that the purified enzyme is a transpeptidase (E.C.2.3.2.1) whereas crude onion extract may, in addition, possess a true γ-glutamyl hydrolase. It is suggested that such enzymes may play a role in the disappearance of peptides in post-dormant onions and may be useful evoking the full flavor potential of onion.

INTRODUCTION

STUDIES in our laboratory strongly indicate that most of the sensory attributes developed as a result of cellular disruption of onion can be traced to the action of L-cysteine sulfoxide lyase on the endogenous S-propenyl derivative of L-cysteine sulfoxide and, to a less extent, its methyl and propyl derivatives (Schwimmer, 1968; 1969; 1970; 1971). However, there is substantial evidence that a significant portion of the propenyl derivative is not available for flavor production in some foods because it is bound to glutamic acid as a γ-glutamyl peptide (Matikkala and Virtanen, 1967). In a previous communication we showed that although there is no enzyme in the fleshy layers of mature dormant onion bulbs capable of releasing this flavor precursor, sprouted onions are capable of releasing p-nitroaniline (PNA) from γ-L-glutamyl p-nitroanilide (GPNA) (Austin and Schwimmer, 1971). No evidence was obtained as to whether this enzyme activity is due to a nontransferring γ-glutamyl hydrolyase as found in chive seedlings (Matikkala and Virtanen, 1965) or to one of the more ubiquitous transpeptidases (E.C.2.3.2.1). The latter class of enzymes has been studied in some detail in animals (Orlowski and Meister, 1970) and plants, including beans (Goore and Thompson, 1967) asparagus (Fujii and Izawa, 1967) and germinated soy beans (Kasai and Obata, 1967). We report here that transpeptidase action can be demonstrated in purified onion enzyme preparations. There is some evidence for a hydrolase in crude onion extracts. In addition the purification and other properties of this enzyme are herein set forth.

EXPERIMENTAL

Materials

The principal substrate was γ-L-glutamyl nitroanilide (GPNA) synthesized as previously described (Austin and Schwimmer, 1971). γ-L-glutamyl-S-methyl-L-cysteine (GSMC) was synthesized by Dr. J. F. Carson of this laboratory using a new procedure (manuscript in preparation). All other amino acids and cysteinyl glycine (Aldrich) were obtained from biochemical suppliers. Suitable sprouted onions were either Southport White Globe or Nevada Globe variety obtained from onion dehydrators.

Enzyme assay

For routine standard assay of the enzyme 0.7 ml of the stock substrate solution (Austin and Schwimmer, 1971) was incubated with 0.3 ml of enzyme at 37°C for varying times depending on the activity of the enzyme, along with two blanks, each containing either substrate alone or enzyme alone. The reaction was terminated by the addition of 2 ml of 1.5N acetic acid. The absorbance at 410 mμ (molar extinction coefficient for p-nitroaniline was found to be 8,750, from a standard curve) was read in a 1 cm cuvette after, if necessary, centrifuging the acid-treated mixture for 10 min at 20,000 × G.

One unit of γ-glutamyl peptidase activity is defined as that amount of enzyme which will liberate one μmole of p-nitroaniline in 1 min under standard assay conditions. Results herein are reported as milliunits (mU). Specific activity (Sp.Ac.) is mU per mg of protein as determined previously (Austin and Schwimmer, 1971). Toluene was added to all reaction mixtures incubated longer than 1 hr.

Chromatography of enzyme reaction products

For paper chromatography, 12 mU of either kidney (Sp.Ac. = 94) or onion enzyme (Sp.Ac. = 61) were incubated at 37°C in separate reaction mixtures containing, in μmoles per ml: GPNA, 12; Tris-HCl buffer, 200. After various times, 25 μl were added to 3 μl of 1.5N acetic acid. Samples were spotted on Whatman #1 filter paper along with appropriate standards. Ascending chromatography overnight was conducted in n-butanol/pyridine/water, 1:1:1 (Orlowski and Meister, 1965). After drying the chromatogram was sprayed with the ninhydrin reagent of Moffat and Lytle (1959). The characteristic hues of the developed spots aid in product identification.

In another experiment designed to identify reaction products, onion enzyme (Sp.Ac. = 48) was incubated with reaction mixture containing, in μmoles per ml: glutathione (GSH) 5; S-methyl-L-cysteine (SMC), 50; and Tris buffer pH 8.0, 100. After 3 hr at 37°C multiple 2 μl aliquots of the reaction mixture were spotted, along with appropriate standards, on TLC plates (microcrystalline cellulose, 250 μl no binder, Mann Research Lab.). The chromatograms were developed in phenol/H_2O (8:3), dried and sprayed with the Moffat-Lytle reagent.

Gel exclusion chromatography

50 ml of an enzyme preparation purified by procedure A (Sp.Ac. = 73) in 1 ml of 0.04M Tris-HCl buffer at pH 7.0 was placed on a 19 × 0.9 cm column of Biogel P-100 (50–150 mesh) and equilibrated with the same buffer (void volume with blue dextran, ca 4 ml). The protein was eluted with the same buffer at the rate 1.1 ml/min at 4°C.

Variation in pH

Crude onion extract, 0.3 mU was incubated for 15 hr under standard conditions except for variation of pH which was accomplished by adding HCl to Tris above pH 7 or acetic acid to sodium acetate below pH 7. Similarly, 1.5 mU of enzyme preparation purified by procedure B (Sp.Ac. = 57) was incubated for 1 hr under standard conditions.

RESULTS

Purification

Purification of the PNA-releasing activity was achieved by two procedures, A and B. In essence, procedure B differs from A in the use of a relatively low $CaCl_2$ concentration instead of NaCl for

Table 1—Purification of onion peptidase

Fraction	Treatment	Vol ml	Total activity mU	Specific activity mU/mg protein
1	Acme extract	890	648	0.095
2	Centrifuged precipitate	179	1028	0.84
3	Addition of NaCl	90	440	10.4
4	Centrifuged supernatant	90	454	15.3
5	Pressure dialysis conc.	11.5	314	52
6	Addition of $(NH_4)_2SO_4$, 0.5–1.0 saturation	3.7	284	77

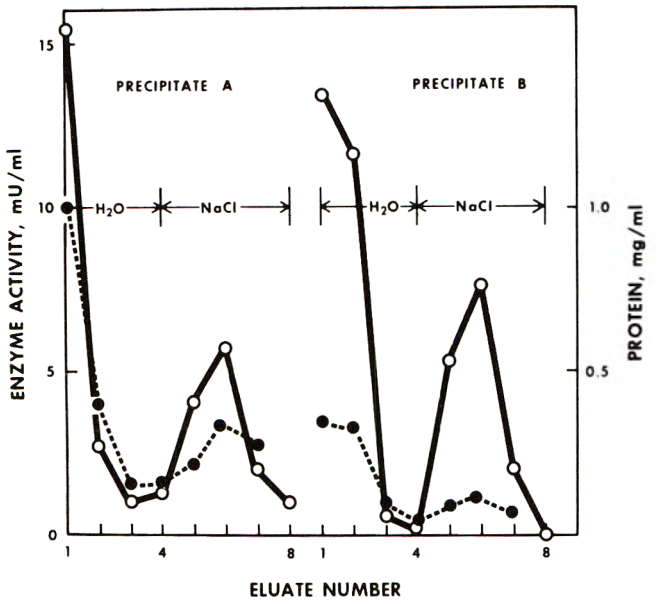

Fig. 1—Elution of PNA-releasing activity from Celite-salt precipitates. Ammonium sulfate was added to a preparation (20 ml, 8.5 mU/ml, Sp.Ac. = 16, corresponding to Fraction 3, Table 1) to 0.4 saturation and filtered through Whatman #1 paper to obtain precipitate A. Ammonium sulfate to 0.7 saturation and 0.4g Celite were added to the filtrate to obtain precipitate B. Each precipitate was then eluted with 2 ml aliquots of H_2O followed by 0.5N NaCl as indicated; (○), activity; (●), protein.

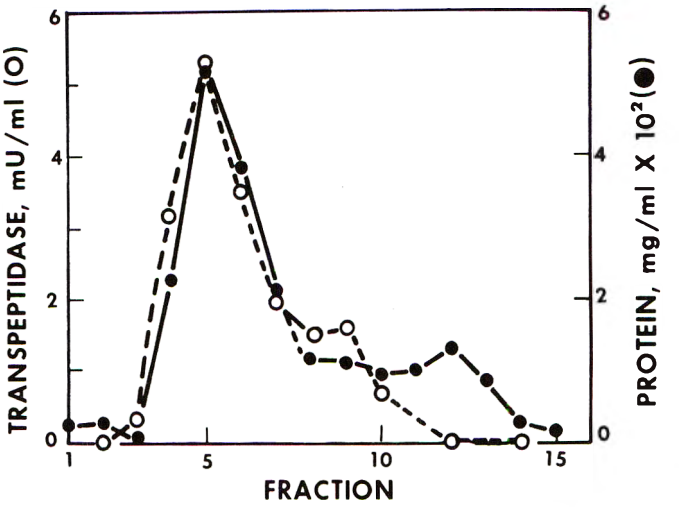

Fig. 2—Gel exclusion chromatography of onion transpeptidase. Details described under Experimental.

solubilization of the activity present in crude pellet. A typical purification of onion peptidase by method A follows:

Step 1. 1100g of chilled sprouted onion bulbs, divested of skin, roots, most of the stems, lateral buds and bladeless leaves were sliced into wedges small enough to pass through the hopper (3 x 5 cm) of an Acme fruit and vegetable juicer fitted with a strip of Whatman No. 1 filter paper to cover the perforations in the side of the cylindrical rotatable basket. The combined grinding, centrifugation and filtration accomplished by this device resulted, after washing the pulp with 100 ml H_2O, in 890 ml of Fraction 1 (see Table 1).

Step 2. The slightly turbid extract was centrifuged at 27,000 x G for 30 min at 0°C. The supernatant liquid was discarded, the precipitate washed with 179 ml H_2O and recentrifuged to yield Fraction 2.

Step 3. To Fraction 2 was added 90 ml of 0.5M NaCl. 1 hr after thorough mixing (Omni-Mixer) to insure complete dispersal, the resulting suspension was centrifuged, and the supernatant was discarded. The precipitate was dispersed in 90 ml of 2M NaCl. After 1 hr the suspension was again centrifuged and the precipitate discarded to yield Fraction 3.

Step 4. After 10 days at 4°C, the precipitate which developed in Fraction 3 was centrifuged off to yield Fraction 4.

Step 5. Fraction 4, which was quite dilute, was concentrated at 0°C by means of pressure dialysis (Richardson and Kornberg, 1964) for 16 hr against 4 liter of 0.005M Tris-HCl buffer pH 8.0 and centrifuged to remove precipitate to yield 11.5 ml of supernatant Fraction 5. (The volume in the dialysis bag initially increased but started to decrease after 2 hr.)

Step 6. To Fraction 5 was added, at 4°C, 3.4g ammonium sulfate to 0.5 saturation. After centrifugation at 34,000 x G for 10 min, 4.6g of ammonium sulfate (0.99 saturation) was added to the supernatant. After again centrifuging at 34,000 x G for 20 min, the resulting precipitate was dissolved in 3.7 ml 0.002M Tris-HCl pH 8.0 (Fraction 6), to give a final yield of 44% of the activity measured in the original extract (or 27% based on Fraction 2) an 800-fold increase in specific activity. A summary is shown in Table 1.

In one preparation considerable purification could be obtained by elution from Celite (Fig. 1). In this experiment in NaCl extract was fractionated with ammonium sulfate to yield a 0—0.4 and a 0.4—0.7 saturated fraction. In attempting to dissolve these fractions, Celite and small amounts of H_2O were added to the precipitates. The first filtrates contained considerable activity, but after removal of the ammonium sulfate no further activity could be washed from the precipitates. However, upon further washing with 0.5M NaCl, the remaining activities were apparently eluted from the Celite, with considerable increase in specific activity in the 0.4—0.7 saturated fraction.

In method B, onions were blended with 25% of their weight of Celite. After removal of solubles by squeezing through cheese cloth and centrifuging the enzyme was extracted from the residue with two volumes of 0.25M $CaCl_2$ in 0.01M Tris-HCl buffer, pH 9.0. The resulting filtrate was treated with ammonium sulfate. The resulting precipitate was extracted 4—6 times with 10% by volume of water. Fractions (usually the second to the fourth) with high activities were combined to yield preparations with about the same specific activity as those of B but with about 2/3 recovery of original activity.

Figure 2 shows gel exclusion chromatography of a transpeptidase preparation purified by Procedure A. Although complete recovery of neither protein nor activity was achieved, most of the surviving protein co-eluted with the activity as one principal peak. A small amount of activity trailed between the main peak. The specific activity of the enzyme activity in the peak tube was about 100. From the void volume and data of the gel supplier one can estimate a molecular weight in the order of 10,000. Attempts to apply chromatography on DEAE were unsuccessful due to unrecoverable enzyme activity.

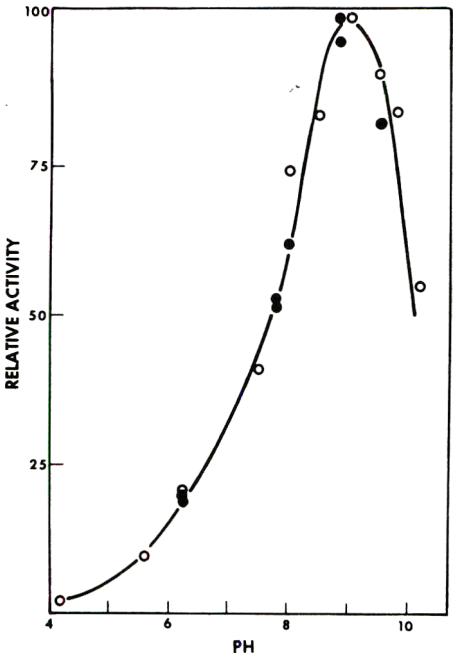

Fig. 3–pH-rate profile of PNA-release from GPNA by crude (•) and purified (○) enzyme preparations. Details described under Experimental.

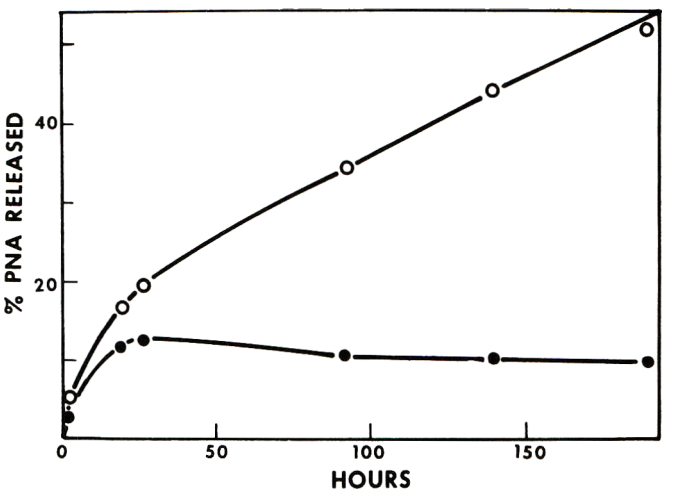

Fig. 4–Course of PNA release by 1.5 mU each of purified (•, Sp.Ac. = 70) and crude (○, Sp.Ac. = 0.5) onion enzyme fractions under standard conditions over an extended time period.

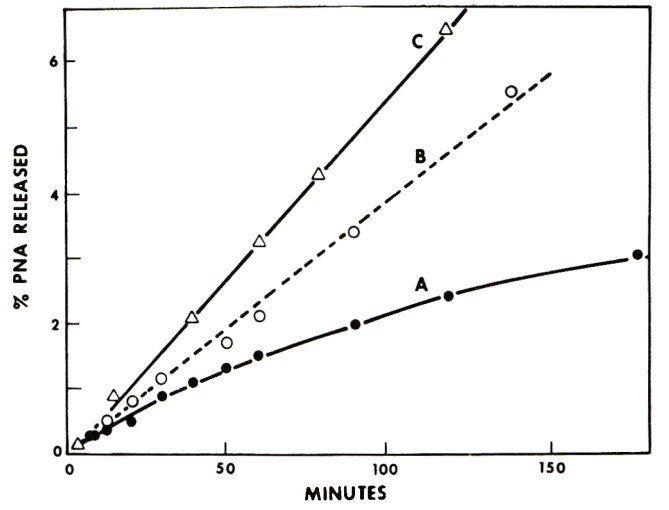

Fig. 5–A, B, liberation PNA by same fractions as in Fig. 4; C same as A except that 10 μmoles of S-methyl-L-cysteine were added to 1 ml of enzyme reaction mixture.

Effect of pH

The pH-activity profile of purified enzyme preparation made by Method A (Fig. 3) exhibits a pH optimum of pH 9.0. Also included are data with a crude extract as enzyme source. From the data shown, it is apparent that the pH profile was not influenced by purification.

Course of PNA release from GPNA

The course of release of PNA by crude enzyme is bimodal (Fig. 4, curve B) consisting of an initial fast phase up to about 15–20% PNA release, followed by a slow and an apparently linear phase to at least 50% PNA release over an extended period. By contrast, the purified preparation (Fig. 4, curve A) although having the same initial rate, reached a maximum of 15% hydrolysis after which the amount of PNA appeared to either remain constant or to slowly taper off. Figure 5 shows data for early stages of hydrolysis and also includes data on the effect of adding S-methyl-L-cysteine to the reaction mixture (curve C) containing purified enzyme. This diminishing reaction rate of enzyme action appears to be characteristic of crude kidney transpeptidase action in the absence of acceptor (Austin and Schwimmer, 1971).

Effect of various additives

Table 2 shows the effect of various substances at 0.01M on the activity of partially purified onion enzyme. In contrast to the kidney transpeptidase, Mg^{++} did not appear to affect the activity. As for the Lima bean enzyme (Goore and Thompson, 1967), borate also inhibited the onion enzyme 61% at 0.1M. However neither citrate nor carbonate, reported to influence Lima bean transpeptidase, affect onion enzyme activity. Added free amino acids did stimulate activity, presumably due to their function as glutamyl acceptors. Glutamic acid, (0.1M, 60% inhibition) and its derivatives all inhibited the release of PNA from GPNA. The inhibition by the γ-glutamyl peptides, expected since they are probably better substrates than is GPNA, was investigated in greater detail.

Effect concentration of substrate and of γ-glutamyl derivatives

The rate of PNA release by 0.9 mU of enzyme (Method A, Sp.Ac. = 65) at varying PNA concentrations is shown in Figure 6. The smooth curves were obtained (assuming the Michaelis equation, $v = VS/(K_m + S)$, where v is the initial rate and S the substrate concentration)

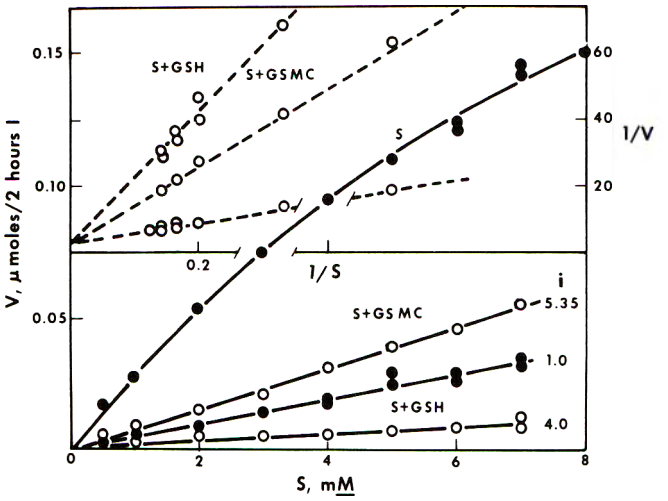

Fig. 6—Effect of concentration of substrate alone (S = GPNA) and of glutathione (S+GSH) or of γ-glutamyl S-methyl cysteine (S+GSMC) on the rate of PNA release by onion transpeptidase. The millimolar concentrations of GSH and GSMC are indicated by "i." Broken lines show Lineweaver-Burk double-reciprocal plots of same data.

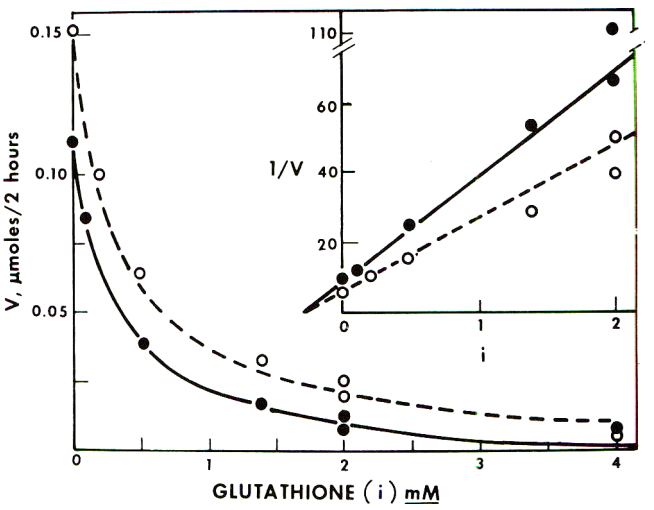

Fig. 7—Effect of concentration glutathione (i, mM) on the rate of release of PNA at 5 (•) and at 8 (○) mM, substrate GPNA. Other conditions are the same as for Figure 6. The inset shows a Dixon plot of 1/v vs. i. The smooth curves for both main figure and inset are those calculated using the constants in Figure 6.

by an approximation method similar to that of Cohen (1970). The calculated values for V (maximum velocity) and for K_m (Michaelis constant) were 0.43 μmoles of PNA released in 2 hr and 14.3 mM, respectively. Because of the limited solubility of GPNA (S), the data were confined to values decidedly lower than one should use to obtain reliable K_m value. With this proviso, the action of the purified enzyme obeys simple Michaelis kinetics with respect to substrate concentration. The Lineweaver-Burk reciprocal plots are shown by broken lines at the top of Figure 6.

Figure 6 also demonstrates the inhibition of GPNA release by glutathione (S+GSH) and by γ-glutamyl-S-methyl cysteine (S+GSMC). The data in Figure 6, in which the substrate was varied at constant inhibitor concentration, suggest that the inhibition is fully competitive. This is corroborated by the Dixon plot of 1/v vs. inhibitor concentration (i) at constant substrate in Figure 7. The estimated values of the inhibitor-enzyme dissociation constants K_i were calculated from the standard competitive inhibition equation,

$$v = VS/[K_m (1 + i/K_i) + S].$$

The K_i values were found to be 0.204 mM for GSH and 2.14 mM for GSMC.

Identification of reaction products

The enzyme action products were subjected to both paper and TLC chromatography (Table 3). Using paper chromatography the results with crude kidney enzyme acting on GPNA are in agreement with the observations of Orlowski and Meister (1965). At early stages of the reaction, the main ninhydrin-positive component had the same R_f as di-γ-glutamyl nitroanilide with smaller amounts of substrate and glutamic acid. Spots corresponding to γ-glutamyl glutamic acid and triglutamyl nitroanilide were also present. After 16 hr the main component appeared to glutamic acid and the substrate had completely disappeared. With onion enzyme acting on GPNA, the main component after 2 hr was the substrate with smaller amount of γ-diglutamyl nitroanilide. Glutamic acid was also detectable. After 16 hr, GPNA was still detectable, but the main products appear to be the γ-diglutamyl nitroanilide and glutamic acid. Spots corresponding to γ-glutamyl glutamic acid and γ-triglutamyl nitroanilide were absent.

Table 2—Effect of various substances (0.01 M) on PNA release from GPNA

Substance	Activity
Magnesium chloride	0.99
Calcium chloride	1.02
Calcium acetate	0.91
Sodium carbonate	1.03
Sodium acetate	0.96
Sodium citrate	1.02
L-Methionine	1.21
Sodium borate	0.89
S-methyl-L-cysteine	1.16
S-propyl-L-cysteine	1.27
L-glutamate	0.78
L-glutamine	0.85
γ-L-glutamyl S-methyl-L-cysteine	0.27
Glutathione	0.02

Table 3—Demonstration of transpeptidase activity[a]

Substrate-GPNA						Substrates-GSH + SMC		
		Spot Intensity						Spot
Standards[b]		Kidney		Onion		Standards[c]		Onion
	R_{GPNA}	2h	16h	2h	16h		R_{SMC}	3h
GPNA	1.00	+	–	+++	++	SMC	1.00	++
G_2PNA	0.78	+++	+	+	+++	GSH	0.76	–
G_3PNA	0.51	+	–	–	–	GSMC	0.85	+++
G	0.30	+	++++	+	++	CG	0.53	+
G_2	0.20	++	+	–	–	G	0.62	++

[a]Experimental details are described under Experimental.
[b]Abbreviations in descending order: γ-glutamyl, di-γ-glutamyl and tri-γ-glutamyl nitroanilides, glutamic acid and γ-glutamyl-glutamate. R_{GPNA} is migration rate relative to GPNA. Values for G_2PNA, G_3PNA, and G_2 were calculated from the data of Orlowski and Meister (1965).
[c]Abbreviations in descending order: S-methyl-L-cysteine, glutathione, γ-L- glutamyl-S-methyl-L-cysteine and L-cysteinyl glycine. R_{SMC} is migration rate relative to SMC.

After incubation of the onion enzyme with glutathione and S-methyl-L-cysteine, we consistently found a spot corresponding to γ-glutamyl-S-methyl-L-cysteine as well as glutamic acid and cysteine glycine. Some of the chromatograms showed another elongated spot in area of glutamic acid. Oxidized glutathione acted in this manner in the TLC system.

DISCUSSION

THE MOST cogent evidence pointing to the transpeptidase nature of the enzyme herein purified is the data summarized in Table 3. With both GPNA in absence of an amino acid as acceptor, and with glutathione as substrate in the presence of S-methyl-cysteine as acceptor, the onion enzyme produced predicted transpeptidase products. Further evidence for the transpeptidase nature of our enzyme is afforded by the stimulation of PNA release by various amino acids (Table 2, Fig. 5) and by the observation, as shown in Figures 4 and 5, that the reaction slows up at rather early stages of the reaction in absence of acceptor, a phenomenon also shown by kidney transpeptidase. The enzyme differs from that of the kidney in that it is not activated by Mg^{++} (Orlowski and Meister, 1965) or by citrate or carbonate as is kidney bean transpeptidase (Goore and Thompson, 1967) but, like the latter, is inhibited by borate.

While the purified enzyme is apparently a transpeptidase, the crude onion extract may contain a second enzyme activity, perhaps true γ-glutamyl hydrolase, as was found by Matikkala and Virtanen (1965) in chive seedlings. The suggestive evidence for this is the biphasic mode of PNA liberation by such extracts as shown in Figure 4. Also the PNA-releasing activity of such crude extracts is not consistently stimulated by the addition of potential acceptor amino acids.

The observed competitive inhibition of PNA liberation by glutathione and by γ-glutamyl-S-methyl cysteine (Fig. 6 and 7) probably reflect their efficacy compared to GPNA as substrate for the enzyme. Thus, the low K_i value for glutathione suggests that the enzyme has 70-fold greater "affinity" for the enzyme than does GPNA. It is of interest to note that removal of glycine from and S-methylation of glutathione renders the latter a much more less efficacious inhibitor of PNA release by onion transpeptidase. These data suggest that the nine endogenous γ-glutamyl peptides which have been detected in onion, especially γ-glutamyl-S-propenyl-L-cysteine sulfoxide, probably disappear from sprouting onion via the route of transpeptidation. Onion transpeptidase preparations may thus be useful in evoking the full flavor potential of onions.

Orlowski and Meister (1970) have recently presented evidence that transpeptidase functions in kidney as integral part of an amino acid transport system. If such a system is functional in onion, it is sluggish indeed, since γ-glutamyl peptides of amino acids do accumulate. Perhaps in the case of onion the operation of such a γ-glutamyl cycle may be part of a mechanism for protection of the endogenous L-cysteine sulfoxide derivatives from degradation by the associated lyase (Schwimmer, 1971).

Although the data show that γ-glutamyl transfer to the substrate does occur, the rate of this transpeptidase action in absence of a nonsubstrate acceptor must be so small as to be experimentally indiscernible from PNA-release kinetics alone. A simplified formulation of the mechanism action in absence of acceptor other than substrate follows:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P_1$$

$$ES + S \xrightarrow{k_4} E_2 + P_1 + P_2$$

where S is GPNA; E is the enzyme, P_1 is PNA and P_2 is G_2PNA (see Table 3). The steady state rate equation is:

$$V = \frac{k_1 (k_3 + k_4 S) S}{k_m + S}$$

where v = rate of PNA release; $K_1 = e\, k_1/(k_1 + k_4)$ where e is the total enzyme concentration; and $K_m = (k_2 + k_3)/(k_1 + k_4)$. This equation shows that the usual hyperbolic Michaelis relation between v and S holds only if k_4 is small compared to k_3 at relatively low concentration of substrate S. At high S values, the rate v would be proportional to S with no tendency to reach a limiting maximum value. As previously mentioned, the limited solubility of the substrate precludes testing of this hypothesis at high S values. The data do fit the traditional Michaelis equation for enzyme action. The chromatographic observations adumbrated in Table 3 suggest that transpeptidation to substrate by kidney transpeptidase appears more intense per unit of activity than that by the onion enzyme due, perhaps, to the presence of a nontransferring peptidase in the latter preparation.

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POST-IRRADIATION INACTIVATION OF HORSERADISH PEROXIDASE

SUMMARY—Aqueous solutions of purified horseradish peroxidase lost enzymic activity not only during irradiation with gamma rays but also after irradiation. The higher the dose of irradiation, the greater the rate of post-irradiation inactivation. Enzyme activity loss after irradiation increased at higher temperatures of post-irradiation storage. Adjusting the pH of the irradiated enzyme solution to 9 resulted in greater inactivation than adjusting to pH 5 or 7. When the enzyme solution was diluted before irradiation the inactivation proceeded more rapidly after irradiation than in the more concentrated solution. When, however, the already irradiated enzyme was diluted, it was the concentrated enzyme solution that lost more activity. Glycerol added to irradiated enzyme diminished rate of inactivation. Irradiating a frozen enzyme solution resulted in very little or no loss of activity during post-irradiation storage. Neither irradiated water nor irradiated enzyme solution affected the activity of non-irradiated enzyme mixed with them. The following mechanism for the post-irradiation inactivation of HRPO is proposed: A portion of the enzyme which is not inactivated during irradiation is modified so as to result in subsequent inactivation by reacting with itself.

INTRODUCTION

INTERACTION between ionizing radiation and enzymes is of theoretical as well as practical interest, in view of efforts being made to preserve food and sterilize commercial enzyme preparations by radiation. Inactivation of enzymes by irradiation has been reviewed by Bacq and Alexander (1966) among others; the enzymological aspects of food irradiation, along with the preparation of sterile enzymes, has been discussed recently by an international panel, International Atomic Energy Agency (1969). Baker and Goldblith (1961) reported the complementary effects of thermal and ionizing energies on the peroxidase activity of green beans, and Al-Jasim (1966) studied the effect of gamma rays and accelerated electrons on the peroxidase activity of horseradish and turnips.

The original objective of this study was to investigate the possibility of reactivation of horseradish peroxidase after it had been inactivated by gamma radiation. This enzyme is a classic example of reactivation after thermal inactivation. In preliminary experiments, however, not only was reactivation not observed but the enzyme continued losing activity at a rather rapid rate after irradiation. It was then deemed desirable to study this post-irradiation inactivation in some depth; results of this work are reported here.

MATERIALS & METHODS

HORSERADISH peroxidase (HRPO) type VII-L, "electrophoretically purified, lyophilized powder, RZ approximately 3.0" was used (Sigma Chemical Co.). Although this preparation was reportedly free from other enzymes, upon starch gel electrophoresis 11 different entities of the enzyme were detected (Scandalios, 1970, personal communication). The composite enzyme was used in this research. All other reagents (guaiacol, glycerol, H_2O_2, KH_2PO_4, NaOH) were of analytical grade. Distilled water, redistilled in an all-glass apparatus, was used to prepare the enzyme and reagent solutions. The irradiations were performed in a 35,000 Curie Cobalt-60 gamma facility, located at the Food Science Building of Michigan State University. The Fricke dosimeter determined the radiation dose received by the samples.

The peroxidase activity was measured in terms of increasing absorbance resulting from oxidation of guaiacol by hydrogen peroxide in the presence of enzyme, Chance and Maehly (1955). The absorbance measurements were made with a Beckman DU spectrophotometer connected to a Ledlang log-converter and a Sargent recorder. The reaction mixture consisted of the following: 2.90 ml of 10 mM phosphate buffer, pH 7.0; 0.05 ml of 20 mM guaiacol solution; 0.01 ml of 40 mM H_2O_2 solution; 0.02 to 0.05 ml of enzyme solution; water to make a total volume of 3.1 ml.

The H_2O_2 solution was added last to the reaction mixture which had been assembled in a 1-cm lightpath Beckman cuvette. For the transfer and mixing of the H_2O_2 solution a square, perforated Teflon plunger, connected to a stainless steel rod, was used. The reaction was followed by automatically recording the change in absorbance at 470 nm. Absorbance change with time of reaction was linear in all measurements and the slope of the line was used as a measure of the reaction rate. In dilution experiments, the enzyme concentration was proportional to the slope of the reaction rate line.

Triplicate sets of 3-ml aliquots of solution were transferred to Pyrex glass test tubes, 7.5 by 0.8 cm, closed with rubber stoppers and irradiated in the center well of the radiation source. The dose rate in that location was 1450 Krad per hour. In the range used (0–24 Krad) the irradiation was completed in times up to 1 min. After irradiation all samples were kept at room temperature (22°C), except in the experiment in which the effect of the post-irradiation temperature was studied.

RESULTS & DISCUSSION

NINE EXPERIMENTS corresponding to nine factors tested for their effect on the post-irradiation activity of HRPO were conducted. All results are expressed as percentage enzyme activity, 100% being the activity of the enzyme immediately after irradiation. Loss of activity occurring during irradiation is also reported.

Radiation dose

An enzyme solution containing 7.5×10^{-7} M protein (assuming M.W. = 40,000) was exposed to 0, 8, 16 and 24 Krad of radiation. Losses of enzyme activity during irradiation were 0, 52, 66 and 84%, respectively. The enzyme activity was also measured 3, 6, 9, 12 and 24 hr after irradiation. Results are shown in Figure 1, and indicate that a) the post-irradiation inactivation of HRPO was greater when the enzyme was exposed to higher levels of irradiation, and b) loss of activity was more rapid soon after irradiation than later on. The storage time-activity loss data did not indicate a first-order reaction, but they fit a second-order reaction equation. In such a reaction all enzymatically active molecules should react and inactivate each other. In view of subsequent experiments, however, an explanation based on the reaction of excited molecules only was found preferable and is proposed at the end of this discussion.

Post-irradiation storage temperature

The enzyme solution containing 4.1×10^{-7} M protein was irradiated with 24 Krad and subsequently stored at 0, 10, 20 and 30°C. The HRPO activity of the solution was determined at 0, 3, 6, 9, 12

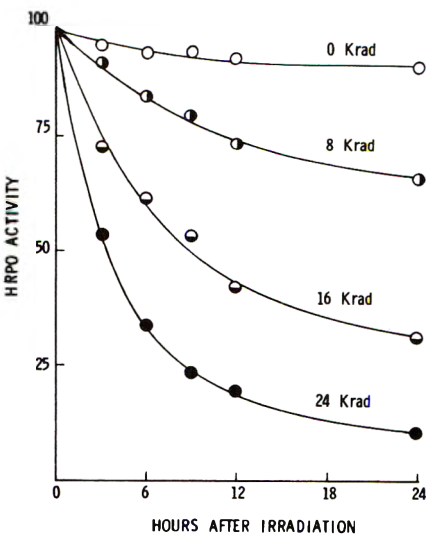

Fig. 1—Effect of irradiation dose on the post-irradiation inactivation of HRPO. (Activity is expressed as percent of the value assessed immediately after irradiation.)

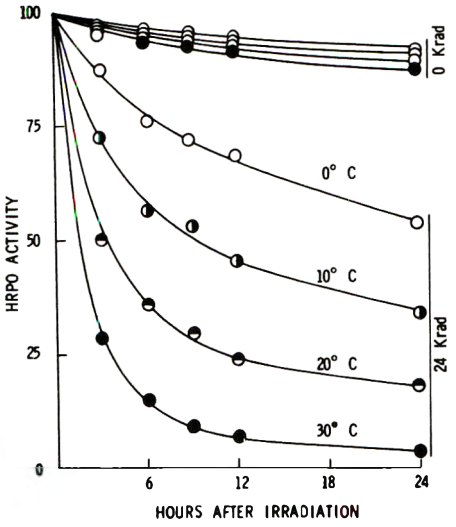

Fig. 2—Effect of post-irradiation storage temperature on HRPO activity. (Activity is expressed as percent of the value assessed immediately after irradiation.)

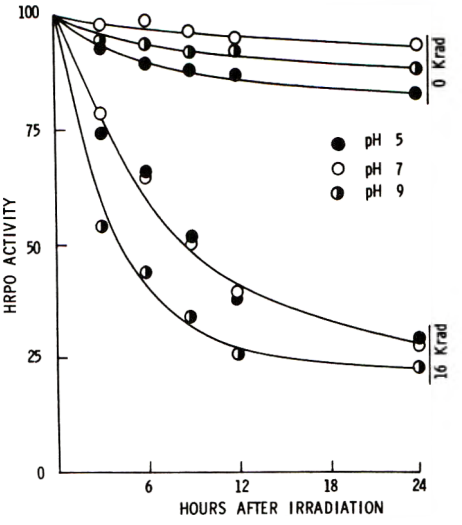

Fig. 3—Effect of post-irradiation pH on HRPO activity. (Activity is expressed as percent of the value assessed immediately after irradiation.)

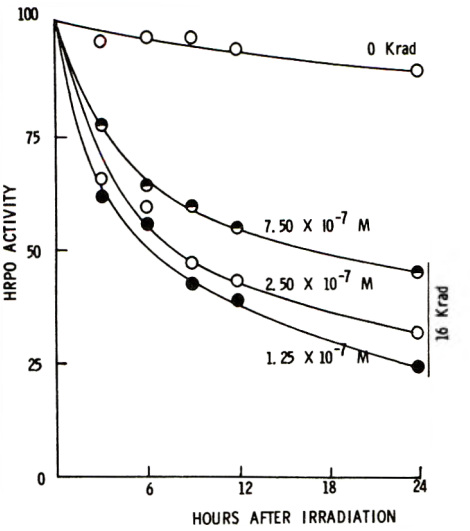

Fig. 4—Effect of enzyme concentration on the post-irradiation inactivation of HRPO. (Activity is expressed as percent of the value assessed immediately after irradiation.)

and 24 hr after irradiation. There was an 80% loss of activity during irradiation. The effect of post-irradiation storage temperature on the remaining enzyme activity is shown in Figure 2. The higher the post-irradiation temperature, the greater the decrease in enzyme activity.

pH

An enzyme solution containing 3.7×10^{-7} M protein was irradiated with 16 Krad. Immediately after irradiation, equal volumes of irradiated solution and 20 mM phosphate buffer, pH 5, 7 or 9, were mixed and the enzyme activity of each mixture determined. Loss of activity due to the irradiation alone was 68%. Similar activity measurements were made 3, 6, 9, 12 and 24 hr later. Results are summarized in Figure 3 and show that at pH 5 and 7 the rate of decrease of the post-irradiation enzyme activity appeared to be the same, while pH 9 resulted in greater rate of activity loss.

Enzyme concentration

Three solutions of HRPO containing 1.25×10^{-7}, 2.5×10^{-7} and 7.5×10^{-7} M protein were exposed to 16 Krad of radiation. Immediately after irradiation activity losses were 75, 64 and 49%, respectively. The increase in activity loss with dilution has been observed previously with other enzymes, Dale (1940), and is indicative of the indirect action mechanism operating in enzyme inactivation during irradiation in solution, Bacq and Alexander (1966). The fate of the enzyme during the 24 hr after irradiation is summarized in Figure 4. Again, the more dilute enzyme solutions lost activity at a greater rate during storage. If some enzyme molecules are slightly modified during irradiation and these molecules later lose activity, the faster post-irradiation inactivation of the more dilute solutions indicates that indirect action is also the probable mechanism of the radiation-induced modification.

Glycerol addition

An enzyme solution containing 7.5×10^{-7} M protein was divided into three parts immediately after irradiation with 16 Krad. The first part was mixed with water, 2 vol solution to 3 vol water, the second part with 50% aqueous glycerol, again 2:3 v/v, and the third part with 100% glycerol, also 2:3 v/v. The mixtures, 0, 30 and 60% in glycerol, were analyzed for HRPO activity immediately after mixing and at 3, 6, 9, 12 and 24 hr later. Results are summarized in Figure 5, clearly showing the protective effect which glycerol exerted on the HRPO activity in post-irradiation storage.

Irradiation in the frozen state

Part of an enzyme solution containing 4.0×10^{-7} M protein was frozen at −38°C and exposed to 24 Krad of gamma radiation; another part was irradiated at ambient temperature. Non-irradiated frozen and unfrozen controls also were used. No provision was made to maintain the temperature of the samples (three tubes containing 3 ml solution each, wrapped together with paper) at −38°C during irradiation, but at the end of the 1-min irradiation period the solutions were still frozen. Immediately after irradiation the samples were thawed under running water, analyzed for HRPO activity and stored at room temperature for subsequent analysis. The frozen solution lost 19% of its activity during irradiation; the unfrozen one, 80%. Results of the post-irradiation inactivation are illustrated in Figure 6 and show that very little enzyme activity was lost in storage when the enzyme solution was irradiated in the frozen state, in contrast to the large loss in the sample irradiated unfrozen. It appears that the theory which states that free radicals, of water origin, are immobi-

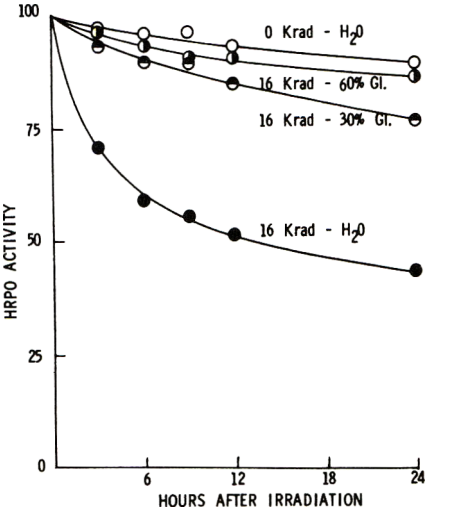

Fig. 5—Effect of glycerol addition to irradiated HRPO on the post-irradiation inactivation of the enzyme. (Activity is expressed as percent of the value assessed immediately after irradiation.)

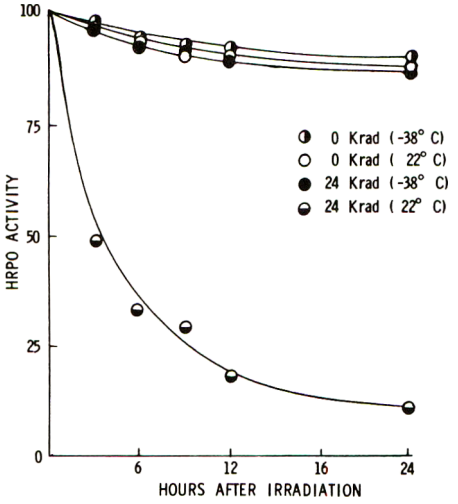

Fig. 6—Effect of freezing during irradiation on the post-irradiation activity of HRPO. (Activity is expressed as percent of the value assessed immediately after irradiation.)

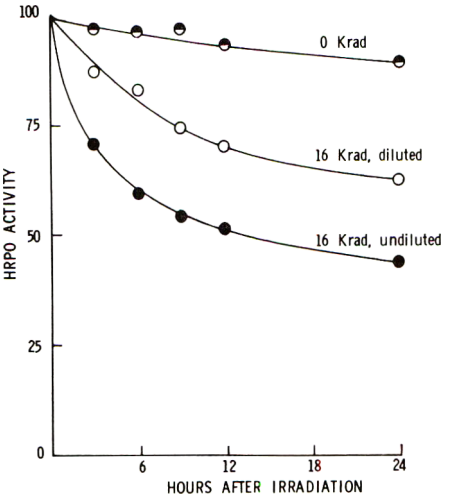

Fig. 7—Effect of dilution of irradiated HRPO on its post-irradiation inactivation. (Activity is expressed as percent of the value assessed immediately after irradiation.)

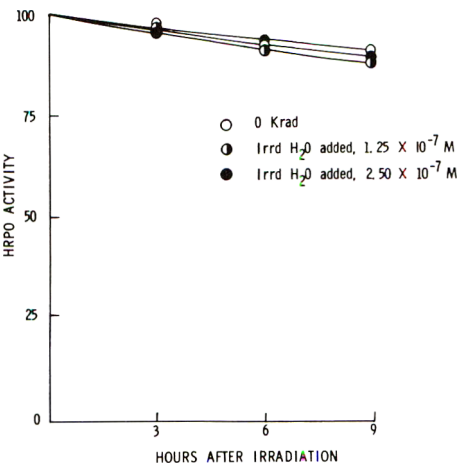

Fig. 8—Effect of addition of irradiated water on the activity of HRPO. (Activity is expressed as percent of the value assessed immediately after irradiation.)

lized in the frozen solutions and "die" before they have the chance to meet and react with enzyme molecules explains not only the low enzyme inactivation during irradiation, but also the modification which could result in inactivation subsequent to irradiation.

Dilution of irradiated enzyme

HRPO solution containing 7.5×10^{-7} M protein was irradiated with 16 Krad and immediately after irradiation the solution was diluted with glass-redistilled water to a final concentration of 3.0×10^{-7} M protein. The enzyme activity was measured at 0 (dilution time), 3, 6, 9, 12 and 24 hr later. Results are shown in Figure 7 and indicate that dilution of the irradiated enzyme considerably decreased the post-irradiation rate of enzyme inactivation. This is in contrast to the observed increase in HRPO inactivation during and after irradiation when the enzyme was diluted before irradiation.

Addition of irradiated water

To test the possibility that the post-irradiation enzyme inactivation may be caused by radiolysis products of the water, glass-redistilled water was exposed to 24 Krad of gamma radiation and mixed with a non-irradiated enzyme solution containing 1.0×10^{-6} M protein. Two mixtures were prepared, one containing 2.5×10^{-7} M protein and the other 1.25×10^{-7} M protein. The enzyme activity of the mixtures was measured at 0, 3, 6, 9, 12 and 24 hr after mixing. Results are shown in Figure 8 and indicate that radiolysis products of water did not act on the enzyme after radiation was terminated.

Addition of intact enzyme to irradiated enzyme solution

The purpose of this experiment was to determine whether radiolysis products originating from the irradiated enzyme solution would inactivate added intact enzyme. An irradiated (24 Krad) enzyme solution was prepared containing 3.0×10^{-7} M protein. Immediately after irradiation, 0.9 ml of the irradiated solution was mixed with 0.1 ml intact enzyme solution containing 3.0×10^{-6} M protein. The mixture, as well as a non-irradiated control and the unmixed irradiated solution, was analyzed for HRPO activity at 0, 3, 6 and 9 hr after irradiation. The enzymatic activity, A, of the intact enzyme in the mixture was calculated from the following equation:

$$A \text{ intact} = A \text{ total} - 0.9\ A \text{ irr.}$$

The 10-fold enzyme concentration in the intact enzyme solution cancels the 1:10 dilution in the mixture.

Results are summarized in Figure 9 and indicate that no radiolysis products originating from the irradiated enzyme solution were capable of reacting with non-irradiated enzyme and causing its inactivation.

Based on these experiments, the following mechanisms of HRPO inactivation during and after irradiation may be deduced: Indirect action must be the prevalent mechanism during irradiation, since a) dilution of the enzyme prior to irradiation resulted in greater inactivation, and b) irradiation of the enzyme solution in the frozen state greatly reduced inactivation in comparison to the irradiation at room temperature.

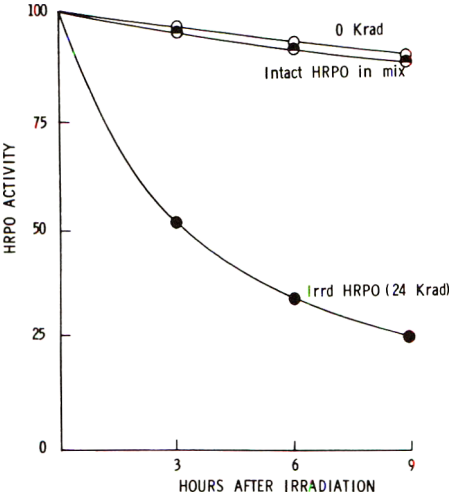

Fig. 9—Effect of addition of irradiated HRPO on the activity of non-irradiated HRPO. (Activity is expressed as percent of the value assessed immediately after irradiation.)

During irradiation some enzyme molecules are subjected to sufficient change to become inactive, other molecules remain intact, and there must be a third category of enzyme molecules which, although they have not lost their activity, have become sensitized to further changes leading to inactivation during storage (metastable molecules).

The following two observations agree with the presence of the metastable molecules: Irradiated water added to non-irradiated enzyme did not cause inactivation. Therefore, the existence of stable radiolytic products of water capable of damag-

ing the enzyme after irradiation is excluded. Irradiated enzyme solution added to non-irradiated enzyme did not result in inactivation of the latter. This indicates that no radiolysis products originating from the enzyme or the water are capable of reacting with intact enzyme present in the irradiated enzyme solution.

One is led to the thought that the partially modified enzyme molecules present in the irradiated solution must react among themselves, for the enzyme inactivation to continue after irradiation. Such a possibility is supported by the fact that dilution after irradiation resulted in lower rates of post-irradiation inactivation. Incorporation of other solutes, such as glycerol, which might act as dampers in the collision between metastable molecules, would slow down the post-irradiation inactivation; this was observed. The increase in the rate of post-irradiation inactivation as a result of increased temperature of post-irradiation storage is also compatible with this hypothesis, since higher temperatures should increase the effective collisions among metastable molecules.

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ODOR THRESHOLD LEVELS OF PYRAZINE COMPOUNDS AND ASSESSMENT OF THEIR ROLE IN THE FLAVOR OF ROASTED FOODS

SUMMARY—The concentration of alkylpyrazine compounds in roasted peanuts, coffee and potato chips was determined quantitatively. Ten pyrazines, purified by gas-liquid chromatography, were subjected to sensory evaluation studies in both aqueous and lipid media. Both the odor-detection threshold levels and a subjective evaluation of the odor of the compounds were obtained. This information, coupled with the quantitative knowledge of the pyrazine content of roasted foods, allows an assessment of the probable significance of the pyrazines in the aroma of these food products.

INTRODUCTION

DETERMINATION OF the flavor constituents of food products is complicated by the large number of components present in natural products and the minute quantities in which many occur. Sensory evaluation studies are often necessary to determine which of the many components present are actively involved in the flavor of the product.

A number of alkylated pyrazine compounds have been observed in the volatile aroma constituents of peanuts, coffee, cocoa and potato chips (Mason et al., 1966; Marion et al., 1967; Goldman et al., 1967; Rizzi, 1967; Bondarovich et al., 1967; Deck and Chang, 1965). In view of their presence in the volatile aroma fraction of peanuts and other roasted foods, it was deemed desirable to subject a series of pyrazine compounds to sensory evaluation, to determine their role, if any, in the flavor of these foods. Since only a few of the alkylpyrazine compounds are available commercially, synthesis of several of the commercially unavailable alkylpyrazines was undertaken.

The simplest technique and still the most popular for determining the olfactory threshold is sniffing (Amerine et al., 1965). Jones (1955a; 1955b) compared several methods and concluded that sniffing was an adequate technique for threshold studies.

EXPERIMENTAL

Synthesis of alkylpyrazine compounds

The synthesis of alkylpyrazine compounds for use in the sensory evaluation studies was based on the procedure described by Klein and Spoerri (1950; 1951). Because of the extreme reactivity of alkyllithium reagents with water vapor and carbon dioxide, it was necessary to utilize a closed reaction system with a continuous nitrogen flush.

In the synthesis of trimethylpyrazine, commercial 2,5-dimethylpyrazine was used as the starting material and was alkylated with methyllithium. Using a large syringe, 60 ml of a 2.3 M solution of methyllithium in diethyl ether was cautiously transferred to a cooled (0°C), nitrogen-flushed reaction flask. A solution of 11 ml of 2,5-dimethylpyrazine in 20 ml of diethyl ether was then added dropwise through a dropping funnel while stirring. After about two-thirds of the 2,5-dimethylpyrazine had been added, a dark reddish-orange precipitate caused the reaction mixture to become too viscous to stir; therefore, 50 ml of diethyl ether was added to facilitate stirring. The 2,5-dimethylpyrazine was added dropwise over a period of 40 min and the reaction permitted to continue at 0°C for 1 hr after the last addition. The reaction flask was warmed to 20°C and allowed to stand an additional hour. After cooling again to 0°C any excess methyllithium was cautiously decomposed with water. The dark red precipitate dissolved completely in about 50 ml of water and formed a yellow solution.

The aqueous and ether layers were separated and the aqueous layer extracted five times with 10 ml of diethyl ether. The ether extracts were combined with the original ether layer and reduced in volume on a rotary evaporator prior to gas-liquid chromatography.

The same equipment and similar procedures were used for the synthesis of other alkylpyrazine compounds. The synthesis of 2,5-dimethyl-3-ethylpyrazine involved alkylation with ethyllithium. Alkylation of 2,6-dimethylpyrazine and 2,3-dimethylpyrazine with ethyllithium yielded 2,6-dimethyl-3-ethylpyrazine and 2,3-dimethyl-5-ethylpyrazine. The ethyllithium alkylations were all conducted in benzene rather than diethyl ether. The monoethylpyrazines, 2-methyl-3-ethyl, 2-methyl-5-ethyl and 2-methyl-6-ethylpyrazine, were synthesized simultaneously by alkylating 2-methylpyrazine with ethyllithium.

Purification of pyrazines

Preparative gas-liquid chromatographic purification of the pyrazines was effected with an

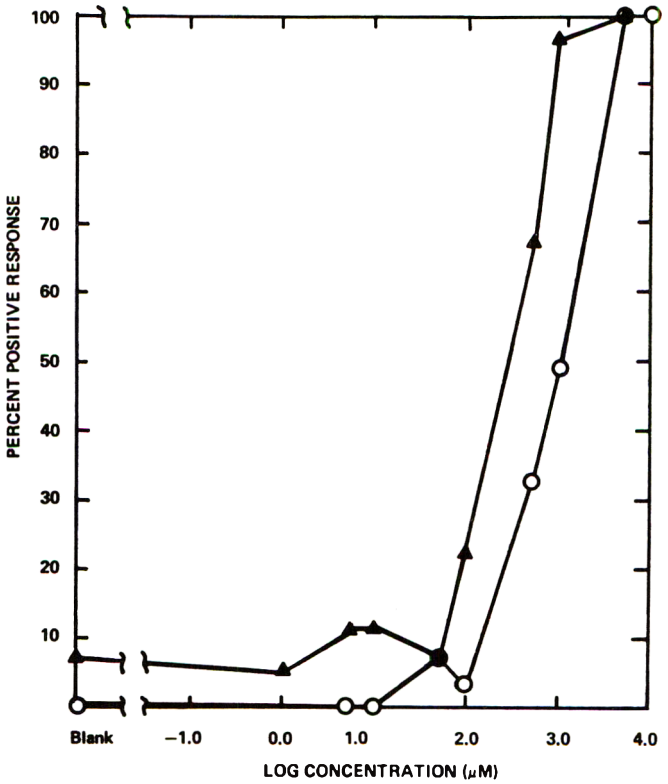

Fig. 1—Odor detection threshold profile for 2-methylpyrazine in water ○ and Oil ▲.

Water ○ Oil ▲

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F&M Model 500 gas chromatograph equipped with a four-filament thermal detector. The 3/8-in. by 12-ft aluminum column packed with 15% (w/w) Carbowax 20 M on Gas Chrom Q (100/120-mesh) was operated isothermally at 160°C with a helium flow rate of 200 ml per minute. The appropriate alkylpyrazine peak was collected in a U-shaped trap loosely filled with stainless steel shavings.

The identity and purity of the compounds were confirmed by mass spectral analysis obtained with a LKB 9000 combination gas chromatograph-mass spectrometer (Waller, 1968), infrared spectra obtained on a Perkin-Elmer Model 457 grating infrared spectrophotometer and nuclear magnetic resonance spectra from a Varian 60 megacycle instrument.

Sensory evaluation of pyrazines

The sensory evaluation tests were conducted in a special 10- by 18-ft room with a separate exhaust system. The samples to be tested were prepared in another laboratory to avoid a build-up of background odors in the room.

The sensory evaluation panel was composed of 20 members. Ten were also members of a trained taste panel which scored peanut butter and roasted peanut samples. The other 10 members were graduate students and technicians. The panel was 75% female, with most of the members between the ages of 18 and 25.

Each panel member was presented two separate groups of 10 samples. Each group contained the sample under study at eight different, randomly ordered levels of concentration, along with both an identified and a hidden blank containing only the dilution solvent. Heavy, white paraffin oil (Saybolt viscosity 335/350) and deionized, distilled water were used as dilution solvents. The samples were presented in ground-glass-stoppered, 16-oz wide-mouthed reagent bottles maintained at 25°C in a temperature-controlled water bath.

All of the bottles were well shaken before the panel members initiated a test. Each member was instructed to shake each bottle, remove the stopper, sniff the contents and mark on a score sheet whether or not any odor was detected. When odor was detected, the panel member described it on the score sheet if possible. The bottle was shaken again before being returned to the water bath. At least 15 min were allowed for the samples to re-equilibrate before they were introduced to the next panel member.

Quantitative survey of pyrazine compounds in foods

Ground, roasted coffee (350g Cain's Hotel and Restaurant coffee-fine grind) was placed in a large (90- by 200-mm) Soxhlet extraction thimble and extracted 12 hr with 1,500 ml of redistilled dichloromethane. The coffee was then removed from the thimble, ground twice in a Wiley mill using a fine (0.5-mm) screen, returned to the Soxhlet and extracted for an additional 18 hr using the same dichloromethane. After the dichloromethane extract was reduced to 150 ml on a vacuum rotary evaporator, the reduced extract was passed slowly three times down a steam-jacketed falling-film evaporator (Hertz and Chang, 1966). The volatile materials were collected on a liquid nitrogen (−196°C) cold-finger trap. The collected volatiles were thawed and extracted seven times with 7-ml portions of dichloromethane. This extract was reduced to a small volume on a rotary evaporator, transferred quantitatively to a 5-ml volumetric and diluted to exactly 5 ml with redistilled dichloromethane. Koehler and Odell (1970) observed that a single pass through the falling-film evaporator removed 98% of the pyrazines.

Quantitative gas-liquid chromatographic analyses were performed on a Perkin-Elmer 801 equipped with a dual hydrogen flame ionization detector. A 20-ft by 0.25-in.-o.d. glass column containing 15% (w/w) Carbowax 20 M Gas Chrom Q (100/120 mesh) was used at 150°C with a nitrogen flow rate of 60 ml per minute. Gas chromatographic peaks were quantitated by comparison of peak areas of samples with those of known concentrations of standard chromatographed the same day under the same conditions. Three injections of each of the samples and standard were made and the results averaged.

Extraction of alkylpyrazines from 550g of roasted peanuts and 560g of potato chips (Kitty Clover) was conducted by a similar procedure. The peanuts were roasted whole and passed through a food grinder before extraction. The potato chips were crushed and ground. Both were extracted 18 hr with dichloromethane, dried, reground twice in a Wiley Mill as described previously, then extracted for 24 additional hours. The remainder of the extraction and quantitation was effected as described for the coffee sample.

Table 1—Odor detection threshold levels in water and mineral oil

Compound	Concentration in water		Concentration in oil	
	Micromolar	ppm	Micromolar	ppm
2-Methylpyrazine	1,122	105	282	27
2,5-Dimethylpyrazine	320	35	159	17
2,6-Dimethylpyrazine	501	54	71	8
2-Ethylpyrazine	200	22	159	17
2,3,5-Trimethylpyrazine	71	9	224	27
Monomethylmonoethylpyrazine	4	0.5	7	0.9
2,3,5,6-Tetramethylpyrazine	71	10	282	38
2,5-Dimethyl-3-ethylpyrazine	316	43	180	24
2,6-Dimethyl-3-ethylpyrazine	112	15	178	24
2-n-Pentylpyrazine	7	1	57	9

Table 2—Pyrazine content of some roasted foods

	Pyrazine		2-Methylpyrazine		Dimethylpyrazine	
	μmoles/kg	mg/kg	μmoles/kg	mg/kg	μmoles/kg	mg/kg
Coffee	57	5	695	65	178	19
Coffee (2nd extraction)	ND		ND		ND	
Roasted peanuts	ND		66	6	97	11
Roasted peanuts (2nd extraction)	ND		ND		ND	
Potato chips	ND		ND		1.6	0.2
Potato chips (2nd extraction)	ND		ND		ND	

ND = Not Detected.

RESULTS & DISCUSSION

Sensory evaluation of pyrazines

The odor threshold of detection for the compounds tested was defined as that concentration which produced a positive (odor) response by the panel members 50% of the time. Each compound was presented to the 20 panel members on three different and isolated days. Thus, in every case, each point on the threshold profile curves reflected at least 60 responses. Threshold levels for each pyrazine were determined in both water and mineral oil so that the results could be related to many types of foods and food products.

A typical threshold profile curve (2-methylpyrazine) is shown in Figure 1. Ten pyrazine compounds were evaluated. The results are summarized in Table 1. The subjective descriptions of the odor of the various alkylpyrazines encompassed a wide range of responses from the various panel members. Responses for a given compound were often much different in water than in mineral oil. In some cases, the nature of the odor of a compound also changed dramatically with the concentration.

A sample of oil from roasted peanuts, freshly prepared, elicited 100% "good peanut" response from the panel. When the volatiles removed from the oil of 1 lb of peanuts were made up to a 1% solution in paraffin oil, the response was again 100% "peanut odor." However, a 1% solution of the volatiles in water elicited

responses ranging from "disagreeable" to "burned" or "over-roasted peanut." This again points out the very critical nature of sensory evaluation work and the necessity of testing compounds in media similar to those in which they occur naturally.

The great diversity of responses prevented statistical or even tabular presentation of the subjective odor descriptions. It is probably sufficient to note that none of the compounds evaluated gave a true peanut or coffee odor at any concentration tested. There were several "roasted," "burned" or "nutty" responses for a few of the alkylpyrazines, especially 2-ethylpyrazine, 2-methylpyrazine and the methylethylpyrazines.

Quantitative estimation of pyrazine compounds in food products

Although it has been definitely established that pyrazine compounds occur in a number of roasted foods, no quantitative data on the amount of these compounds actually present have been published. The common technique of isolating aroma constituents of foods by pumping off the volatile compounds under high vacuum and isolating them on cold-finger traps (Hertz and Chang, 1966; Mason et al., 1966) is satisfactory for isolation of compounds of the volatile aroma fraction, but it fails to quantitatively remove all of the aroma compounds from foods. Experience in this laboratory has shown that even prolonged vacuum degassing of homogenized roasted peanut slurries over a period of four or five days does not completely remove the pyrazine compounds and other flavor constituents from the system.

An exhaustive procedure of extraction and grinding was employed to obtain the quantitative data presented in Table 2. After the foods had been exhaustively extracted as described previously, they were again subjected to the same extraction procedure. No pyrazines were detected in the second extracts, indicating that the procedure was sufficient to remove pyrazine compounds from the foods completely.

The levels of some pyrazine compounds found in three common foods (Table 2) are significant in view of the thresholds of detection levels presented in Table 1. The two pyrazines occurring in the largest amounts in roasted food products are 2-methylpyrazine and the dimethylpyrazines. Methylpyrazine was found in coffee at levels of 65 mg per kg. The positional isomers of dimethylpyrazine were not separated. Many higher molecular weight alkylpyrazines are known to be present in coffee and roasted peanut volatiles. Quantitative work indicates that these other pyrazines are minor components.

The level of pyrazine compounds in these three roasted food products appears proportional to the extent of browning the food undergoes. Coffee, clearly the most darkly roasted product, has the highest concentration of pyrazine compounds. Potato chips, which are only lightly browned, contain very small amounts of alkylpyrazines.

Not only do the pyrazine levels as a whole vary from food to food, but also the ratio of the various alkylpyrazines to each other varies. In coffee, for instance, the ratio of dimethylpyrazine to 2-methylpyrazine is 0.3. However, in roasted peanuts the ratio is 1.7. Some possible explanations for these variations in the amounts of the various pyrazines have been discussed in a previous paper (Koehler and Odell, 1970).

The concentration of pyrazines in coffee is considerably above the odor threshold levels in oil for both 2-methylpyrazine and dimethylpyrazine. The probability that pyrazine compounds play a role in the aroma of roasted coffee is thus rather high. The 2-methylpyrazine concentration in roasted peanuts is somewhat below the odor threshold level, whereas the dimethylpyrazine concentration is near the threshold level. The very low concentration of dimethylpyrazine in potato chips makes doubtful its contribution to the aroma of the product. It should be kept in mind, however, that synergistic effects between two or more compounds in solution can cause the threshold of detection of the mixture to be considerably lower than that of either component individually (Amerine et al., 1965). Antagonistic effects can also produce the opposite effect. Also, the odor of a mixture of two nonreactive compounds may bear no similarity to either of the pure compounds (Amerine et al., 1965). These effects make the evaluation of flavor compounds extremely difficult.

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MINOR CONSTITUENTS OF WHISKY FUSEL OILS
1. Basic, Phenolic and Lactonic Compounds

SUMMARY—Basic, phenolic and lactonic fractions of the fusel oils obtained from Japanese and Scotch whiskies were analyzed mainly by means of gas-liquid chromatography coupled with mass spectrometry. Two picolines, three lutidines, two ethyl pyridines, α-isopropyl and 2-methyl-5-ethyl pyridines, quinoline, 2- and 6-methyl quinolines, eight pyrazines, guaiacol and its three alkyl derivatives, phenol, two ethyl phenols, o-isopropyl phenol, two cresols, 2,6-xylenol, eugenol, o-hydroxy acetophenone, 2-hydroxy-5-methyl acetophenone, γ-nonalactone, cis- and trans-3-methyl-4-hydroxy caprylic acid γ-lactone and five furan compounds were found in Japanese or Scotch whiskies. Origins of these compounds are discussed.

INTRODUCTION

IT HAS been well known by those analyzing the volatile constituents of alcoholic beverages that the aroma fractions of them produced from quite different raw materials principally consist of the same compounds. Kahn (1969) published a table of the compounds found hitherto in wine, beer, whisky and other yeast fermentation beverages, as well as compounds recently identified in his laboratory. Most of these compounds seemed to be produced by yeast during fermentation and very few compounds were known to be derived from other sources. Basic, phenolic and lactonic compounds, contained in very minute quantities in alcoholic beverages, are among those originated from other than yeast fermentation.

For the past 10 yr, analyses on the components of the fusel oils produced from fermentation of different kinds of raw materials have been carried out in our laboratory for discovering the compounds characteristic of the sources, Hirose et al. (1961; 1962a; 1962b) and Ogawa and Hirose (1962). This paper deals with the analyses of basic, phenolic and lactonic compounds of fusel oils obtained from Japanese and Scotch whiskies using gas-liquid chromatography and mass spectrometry connected with gas-liquid chromatography.

EXPERIMENTAL

Materials

Japanese whisky. A sample of Japanese whisky (850 liters) was manufactured from barley malt, distilled through a pot still and aged 5 yr in an oak barrel at our own factory.

Scotch whisky. 6-months-aged malt whisky (872 liters) was purchased from Scotland for these analyses.

Isolation of basic, phenolic and lactonic compounds

3-liter portions of both whiskies were fractionally distilled under an atmospheric pressure to obtain fusel oils with a distillation column of 2.5-cm i.d. by 2-m length, packed with stainless steel helices. 1,600 and 1,500 ml of fusel oils were obtained from Japanese and Scotch whiskies, respectively. These oils were distilled using a 1.5-m by 25-mm i.d. Heli-Grid column to remove the bulk of amyl alcohols. The remaining fractions were divided into basic, acidic and phenolic fractions by extracting with 5 N-hydrochloric acid, aqueous sodium bicarbonate and 2 N-sodium hydroxide successively.

As phenolic fractions appeared to contain fatty acids and lactones, chromatography on DEAE-Sephadex A-25 column was adopted to separate those compounds. The column was prepared according to the method of Rowe and Zinkel (1964). Lactones were first eluted by passing a mixture of ether, methanol and water (89:10:1), then phenols with a mixture of ether and methanol (90:10) saturated with CO_2. Fatty acids were eluted finally with a mixture of ether, methanol and formic acid (86:10:4).

Analyses on the acidic fractions were excluded from this study because most of them were common to other alcoholic beverages.

Methods of analyses

Basic and phenolic compounds were so limited in amounts that analyses were carried out by means of the GC-MS method without isolating compounds. Lactonic compounds were isolated in the pure state by means of preparative GLC. Identification of the compounds was attained by comparing their MS spectra and retention times of GLC with those of authentic samples obtained through the commercial route or synthetic preparation. IR spectrum served as an additional method of identification for lactones.

Estimation of the concentration of phenols was based on the amounts of isolated fractions and peak areas measured by triangulation of gas chromatograms.

Apparatus

Gas-liquid chromatography (GLC). Analyses of phenolic and lactonic compounds were performed on an Hitachi K-53 gas chromatograph with a flame ionization detecter containing a 45-m by 0.25-mm i.d. stainless steel capillary column coated with Ucon oil HB-2000 at a nitrogen inlet pressure of 1.5 kg/cm². The column temperature was isothermal at 150°C. For basic compounds we used a 25-mm i.d. by 4-m stainless steel column packed with 10% of polyethylene glycol 20M on Celite at a nitrogen flow rate of 70 ml/min. The column temperatures were programmed in two different ways: linear programmed from 80–200°C at 3°C/min or isothermal at 80°C for 26 min, then programmed to 200°C at approximately 3.3°C/min, then isothermal at 200°C for 30 min. Preparative separations for lactones were performed using a Varian model 90-P gas chromatograph fitted with a 20-ft by ¼-in. i.d. aluminum column packed with 10% polyethylene glycol 20M on Celite material. The helium flow rate was 90 ml/min and the column was operated at 150°C isothermal.

GLC and fast-scan mass spectrometry method (GS-MS). Analyses via direct connection of the GLC with a fast-scan mass spectrometer were performed using an Hitachi mass spectrometer RMU-6 and Hitachi K-53 gas chromatograph containing a 0.5-mm by 45-m capillary column coated with Ucon oil HB-2000 for phenols and a 25-mm i.d. by 4-m stainless steel tube packed with 10% of polyethylene glycol 20M on Celite for basic compounds. Helium was adopted as the carrier gas. The effluent from the column was introduced into an ionization chamber after passing through a carrier gas separator designed by Bieman. The mass spectrum was scanned at 20–300 mass units in 4 sec. Part of the total ion current was multiplied and recorded as a monitor of the gas chromatography.

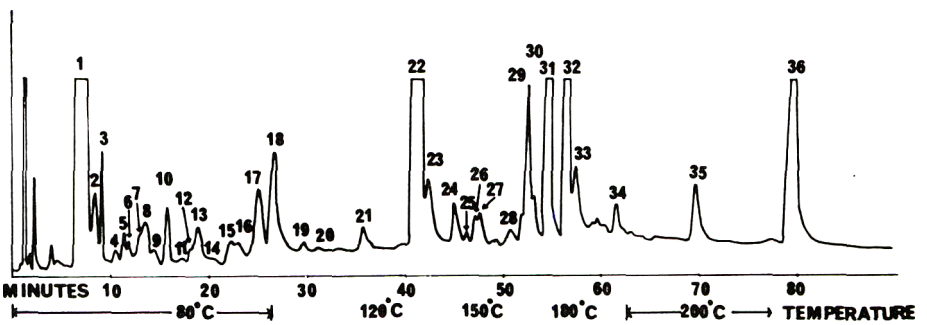

Fig. 1—Gas chromatogram of the basic fraction of Japanese whisky fusel oil.

Table 1—Compounds identified in the basic fraction of Japanese whisky

Peak No.	Compounds	Methods of identification MS.	GLC
1	Isoamyl alcohol	+	+
2	α-Picoline	+	+
3	2,6-Lutidine	+	+
4	α-Ethyl pyridine	+	+
5	γ-Picoline	+	+
6	α-Isopropyl pyridine	+	+
7	2,5-Dimethyl pyrazine	+	+
8	2,5-Lutidine	+	+
9	Unknown		
10	Unidentified acetal	+	
11	Unknown		
12	2-Methyl-5-ethyl pyrazine	+	+
13	Trimethyl pyrazine	+	+
14	2-Methyl-5-ethyl pyridine	+	+
14	3,5-Lutidine	+	+
15	2,5-Diethyl pyrazine	+	+
16	2,5-Dimethyl-3-ethyl pyrazine	+	+
17	Furfural	+	+
18	Tetramethyl pyrazine	+	+
19	2-Acetyl furan	+	+
20	Unidentified pyrazine	+	
21	5-Methyl furfural	+	+
22	Furfuryl alcohol	+	+
23	Unidentified compound of M.W. 172	+	
24	Unidentified compound of M.W. 119	+	
25	Unknown		
26	Unknown		
27	Unknown		
28	Unknown		
29	Unknown		
30	Unidentified compound of M.W. 155	+	
31	β-Phenethyl alcohol	+	+
32	Quinoline	+	+
33	2-Methyl quinoline	+	+
34	6-Methyl quinoline	+	+
35	Unidentified compound of M.W. 169	+	
36	Unidentified acetal	+	

Table 2—Compounds identified in the basic fraction of Scotch whisky

Peak No.	Compounds	Methods of identification MS.	GLC
1	Isoamyl alcohol	+	+
2	n-Amyl alcohol	+	+
3	α-Picoline	+	+
4	α-Ethyl pyridine	+	+
5	α-Isopropyl pyridine	+	+
6	γ-Picoline	+	+
7	2,5-Dimethyl pyrazine	+	+
8	2-Ethyl pyrazine	+	+
9	2,3-Dimethyl pyrazine	+	+
10	Unidentified acetal	+	
11	γ-Ethyl pyridine	+	+
12	2-Methyl-5-ethyl pyrazine	+	+
13	Trimethyl pyrazine	+	+
14	2,5-Dimethyl-3-ethyl pyrazine	+	+
15	Furfural	+	+
16	Tetramethyl pyrazine	+	+
17	2-Acetyl furan	+	+
18	Unknown		
19	3-Acetyl furan	+	+
20	5-Methyl furfural	+	+
21	5-Methyl-2-acetyl furan	+	+
22	Furfuryl alcohol	+	+
23	Unknown		
24	Unknown		
25	Unidentified compound of M.W. 163	+	
26	Unknown		
27	β-Phenethyl alcohol	+	+
28	Quinoline	+	+
29	2-Methyl quinoline	+	+
30	Unidentified compound of M.W. 151	+	

RESULTS & DISCUSSION

Basic compounds

Gas chromatograms of the basic fractions of Japanese and Scotch whiskies are presented in Figures 1 and 2. Analytical results are shown in Tables 1 and 2.

It is not surprising that the water-soluble neutral compounds such as i-amyl alcohol, β-phenethyl alcohol, furfuryl alcohol and furfural are predominant in these fractions.

Eighteen nitrogen-containing compounds were found in Japanese or Scotch whiskies but the contents of them were very minute and even those of the most predominant compound, quinoline, were not more than 0.1 ppm. The difference between these two whiskies as to basic components is quantitative rather than qualitative. In Japanese whisky, quinoline and its methyl derivatives are predominant; in Scotch whisky, pyrazine compounds are major components.

There have been a few literature citations on the volatile bases in alcoholic fermentation products. Pyridine and 2,5-dimethyl pyrazine were found by Taira (1936) and trimethyl, tetramethyl and diethyl pyrazines by Chapman and Hatch (1929) in beet molasses fusel oil, and methyl triethyl pyrazine in potato fusel oil by Shoruigin et al. (1933). Liebich et al. (1970) found seven pyrazines in the volatile components of rum. Recently, many pyrazine compounds

Table 3—Phenolic compounds found in Japanese and Scotch whisky

Compounds	Japanese ppm	Japanese Peak No.	Scotch ppm	Scotch Peak No.	MS.	GLC
Guaiacol	0.006	1	0.012	1	+	+
o-Hydroxy acetophenone	–		0.001	2	+	+
2,6-Xylenol	–		0.001	3	+	+
6-Methyl guaiacol	+	2	–		+	+
Phenol	0.012	3	0.003	4	+	+
o-Cresol	0.013	4	0.015	5	+	+
4-Methyl guaiacol	+	4	0.005	6	+	+
2-Hydroxy-5-methyl acetophenone	–		+	6	+	+
o-Ethyl phenol	0.002	5	0.002	7	+	+
p-Cresol	0.010	6a	0.009	8	+	+
p-Ethyl guaiacol	0.002	6b	0.035	9	+	+
o-Isopropyl phenol	+	7	–		+	+
p-Ethyl phenol	0.002	7	0.009	10	+	+
Eugenol	0.011	8	0.032	11	+	+

have been isolated from some roasted foods including potato chips, Deck and Chang (1965); peanut, Mason et al. (1966); cacao, Flament et al. (1967) and Rizzi (1967); coffee, Bondarovich et al. (1967) and Goldman et al. (1967) and barley, Wang et al. (1969), and their significance on cooking flavor noted.

Their formation has been correlated to the reaction of amino acids and sugars by Mason et al. (1966) and Newell et al. (1967). Dawes and Edwards (1966) obtained 2,5-dimethyl pyrazine by heating the mixture of fructose and glycine or β-phenylalanine, and suggested the formation mechanism starting with the reaction of α-diketo compounds and amino acids, followed by condensation and dehydrogenation of the products to pyrazines. Wang et al. (1969) proved that pyruvaldehyde produced 2,5-dimethyl pyrazine when heated with leucine or isoleucine.

Nevertheless, it was believed these pyrazines in whisky might arise from cooked grain during the mashing process. This was supported by pyrazines such as 2-methyl, 2,5-dimethyl, 2-methyl-5-ethyl and 2,3,6-trimethyl derivatives being found in the volatiles of barley malt, Nishimura and Sugibayashi (1970).

Higman et al. (1970) have shown that nitrogen-containing compounds such as proteins and amino acids produced pyridine and quinoline derivatives in rather high yields when degradated at 840°C under nitrogen. However, this is not true for the origin of pyridine and quinoline compounds in whisky, because the whisky manufacturing process is too mild to produce these compounds from proteins or amino acids in grains. Larsson (1942) found pyridine, picoline and lutidine in peat tar. In our recent study, it was found that the volatile bases in the barley malt kilned with peat fire contained pyridine and quinoline derivatives together with pyrazines, whereas malt kilned without peat contained only pyrazines. This fact implies that pyridine and quinoline derivatives in whisky can be attributed to peat smoke.

Phenolic compounds

Gas chromatograms of the phenolic compounds of both whiskies are shown in Figures 3 and 4. Compounds found in both whiskies are listed in Table 3. Guaiacol, phenol, o-cresol, 4-methyl guaiacol, o-ethyl phenol, p-cresol and 4-ethyl guaiacol have already been listed in Kahn's table. o-Hydroxy acetophenone, 6-methyl guaiacol, o-isopropyl phenol, p-ethyl phenol, eugenol, 2-hydroxy-5-methyl acetophenone and 2,6-xylenol are compounds recently found. Steinke and Paulson (1964) have proved that 4-ethyl guaiacol, 4-methyl guaiacol, 4-vinyl guaiacol, p-ethyl phenol and p-vinyl phenol were produced from the coumaric and ferulic acids in grains at the mashing process by heating or by action of fungi. Guaiacol, phenol and p-cresol may be formed by further degradation of those compounds.

Among o-substituted phenols found in this study, o-cresol and 2,6-xylenol are probably derived from lignin-like material. The former compound has been found in grain spirits fusel oil by Braus and Miller (1958). The origin of other o-alkylated phenols, such as o-isopropyl phenol, o-hydroxy acetophenone and 2-hydroxy-5-methyl acetophenone, remains to be studied. Eugenol was found

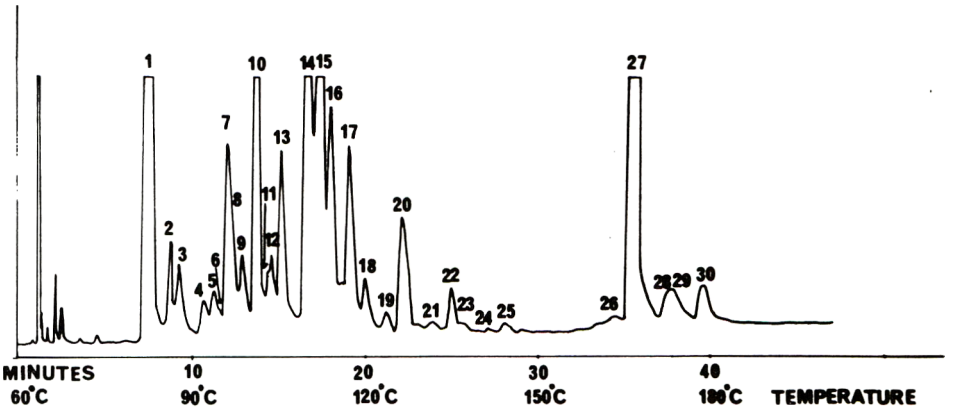

Fig. 2—Gas chromatogram of the basic fraction of Scotch whisky fusel oil.

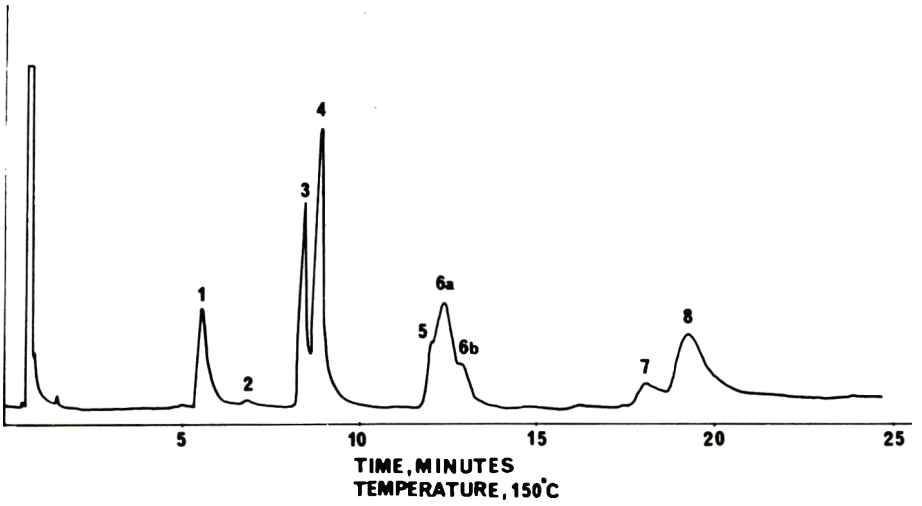

Fig. 3—Gas chromatogram of the phenolic fraction of Japanese whisky fusel oil.

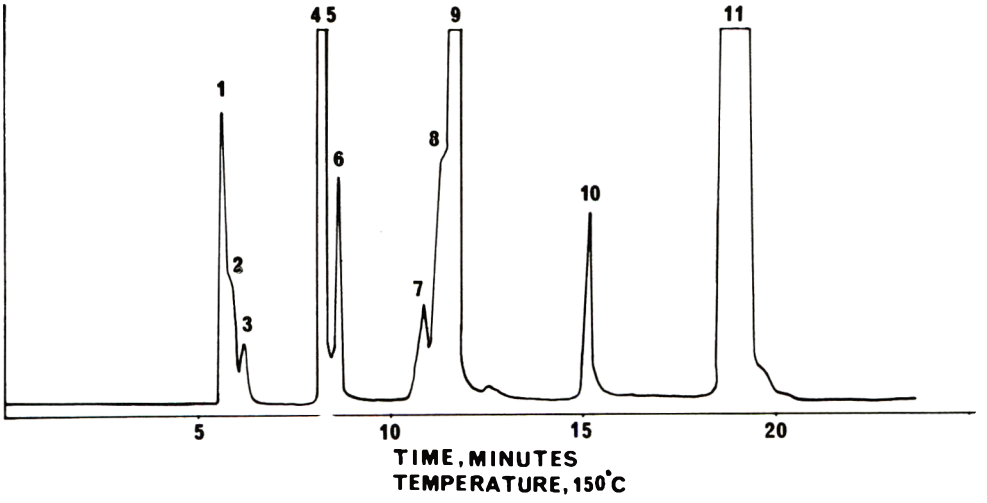

Fig. 4—Gas chromatogram of the phenolic fraction of Scotch whisky fusel oil.

both in the volatile fraction of barley malt and in the methanol extracts of oak woods. Perhaps it arose from ferulic acid of barley and from the lignin material of oak wood.

Lactones

Gas chromatograms of lactonic compounds of both whiskies were nearly the same, exhibiting three peaks: γ-nonalactone, cis-3-methyl-4-hydroxy caprylic acid γ-lactone and trans-3-methyl-4-hydroxy caprylic acid γ-lactone, in increasing order of retention times of GLC. Contents in whiskies could not be calculated in this study because major parts of them seemed to remain in neutral fractions.

γ-Nonalactone has been found in whiskey by Kahn et al. (1969) and one of the diastereomers of 3-methyl-4-hydroxy caprylic acid γ-lactone by Suomalainen and Nykänen (1969). All of these lactones were proved by us, Masuda and Nishimura (1971), to be the ingredients of the woods of white oak and some other *Quercus* species such as *Q. mongolica* Fisch. var. *grosseserrata* Rehd. et Wils. and *Q. serrata* Thunb. and their odor seemed to represent the raw, woody aroma of whisky.

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ISOLATION AND CHEMICAL PROPERTIES OF CAPSANTHIN AND DERIVATIVES

SUMMARY—A solvent system for thin layer chromatography on silica gel was developed to separate the six classes of carotenoid pigments in paprika. The isolation, R_f values, visible, infra-red, and NMR spectra of capsanthin and its derivatives (capsorubin, capsanthol, 3-ketokryptocapsone, 3-keto-beta-apo-8'-carotenal, capsanthin dilaurate, capsanthin (trimethyl silyl) diether) are presented. NMR and mass spectra of capsanthin are also given as well as beta-carotene and canthaxanthin for comparison.

INTRODUCTION

A NUMBER of carotenoids have been isolated and characterized from red peppers (*Capsicum annum*) or the dehydrated product paprika (Curl, 1962; Cooper et al., 1962; Cholnoky et al., 1963; de la Mar and Francis, 1969). The major red pigment is capsanthin which was isolated in crystalline form as early as 1927 (Karrer and Jucker, 1950) but the current structure was assigned only recently (Barber et al., 1960; 1961). Faigle and Karrer (1961) determined the asymmetry of the C5-position in the five-membered ring of capsanthin and Cooper et al. (1962) established that the hydroxyl in the cyclopentane ring is trans to the polyene chain. Capsanthin occurs as the dilaurate in paprika (Philip et al., 1971). The oxidation degradation sequence for capsanthin in oxygen was worked out by Philip and Francis (1971).

Column chromatography has been the traditional method for separation of carotenoids (Davies, 1965). For example, with paprika carotenoids, Sea Sorb 43 is effective, but it does cause some isomerization and oxidation of pigments, hence a more rapid method is desirable. Paper chromatography based on impregnated papers (Booth, 1962) and papers with suitable fillers (Jensen and Jensen, 1959; Jensen, 1960) has been suggested. Separations are described based on thin layer systems on alumina (benzene), silica gel G (methylene chloride-ether, petroleum ether-benzene, undecane-methylene chloride and methylene chloride-ethyl acetate), calcium hydroxide (hydrocarbon mixture-methylene chloride), secondary magnesium phosphate (carbon tetrachloride, benzene and petroleum ether-ether), silica gel G mixed with rice starch (m-hexane-ether), calcium hydroxide (benzene, benzene-methanol and petroleum ether-benzene) (Stahl et al., 1963; Demole, 1958, 1959; Bunt, 1964; Bollinger, 1965). Stahl and coworkers (1963) reported that all carotenoid mixtures could not be separated with a single solvent and a single absorbent in a thin layer system.

The present investigation reports the development of a solvent system for the thin layer separation of paprika carotenoids as well as the physico-chemical properties of capsanthin and derivatives. Capsanthone, 3-keto-kryptocapsone, and 3-keto-beta-apo-8'-carotenal were included because they were isolated as degradation products of capsanthin (Philip and Francis, 1971). Capsorubin was included because it usually occurs with capsanthin in paprika. Canthaxanthin and beta-carotene were included for reference purposes.

MATERIALS & METHODS

THE CAROTENOIDS of whole ground paprika (Columbia Gem obtained from Cal-Compack Foods, Inc., Santa Ana, Calif.) were extracted and saponified according to the method of Curl (1962). The saponified and purified carotenoids were then counter current fractionated in a 200 tube run at 25°C with 2 min shaking and 1½ min resting time using a solvent system (Curl, 1960) consisting of hexane, benzene, methanol and water in the ratio 1:1:1:0.15 (v/v). The fractions were collected into six fractions based on the optical density at 450 nm and the solvent evaporated. These fractions were used to develop a solvent system to separate the pigments in each fraction. Thin layer chromatographic plates were prepared from a silica gel G:water slurry (1:2 w/v), at 0.250 mm thickness, dried at room temperature for 30 min, and activated at 105°C for 30 min.

Isolation of capsanthin

For the isolation of capsanthin in larger quantities, ground paprika (one Kg) was extracted as described by Philip and Francis (1971).

Silylation

Capsanthin (20 mg) was dissolved in N,N-dimethyl formamide (1 ml) and an excess of bis-(trimethyl silyl) trifluoroacetamide (2 ml) added. The reaction was carried out in a 5 ml screw cap bottle. After flushing the bottle with nitrogen, the reaction mixture was warmed to 50°C for 5 min. The solvent was removed under a stream of nitrogen and capsanthin (trimethyl silyl) diether was purified by thin layer chromatography on silica gel G using 10% acetone in petroleum ether as the developing solvent.

Reduction

Capsanthin (25 mg) was dissolved in 20 ml ethanol in a screw cap bottle (30 ml capacity). An excess of sodium borohydride (3g) was added and the bottle left in the refrigerator overnight with the cap loose. The whole mixture was transferred to a separatory funnel containing 50 ml ethyl ether. The mixture, after shaking, was washed with distilled water, dried over anhydrous sodium sulfate and the solvent removed. The capsanthols were purified by thin layer chromatography on silica gel G using a solvent system consisting of 3.5% ethanol, 10% benzene and 20% acetone in petroleum ether.

Oppenauer oxidation

A mixture of capsanthin (20 mg) and aluminum t-butoxide (0.8g) in acetone (20 ml) and benzene (20 ml) was refluxed for 36 hr, then cooled and shaken with N sulfuric acid. The organic layer was washed with distilled water, followed by saturated sodium bicarbonate solution and distilled water, dried over anhydrous sodium sulfate and the solvent removed. Capsanthone was purified by thin layer chromatography on silica gel G using a solvent system consisting of 3.0% ethanol, 10% benzene and 20% acetone in petroleum ether.

3-keto-kryptocapsone was obtained by refluxing a solution of capsanthone in acetone under a stream of oxygen and purifying by thin layer chromatography on silica gel G using the same solvent system as in the case of capsanthone.

Alkali fission

A solution of capsanthin (20 mg) in benzene (15 ml) and 10% methanolic KOH (60 ml) was refluxed for 2 hr. The mixture was cooled, diluted with benzene (300 ml), washed consecu-

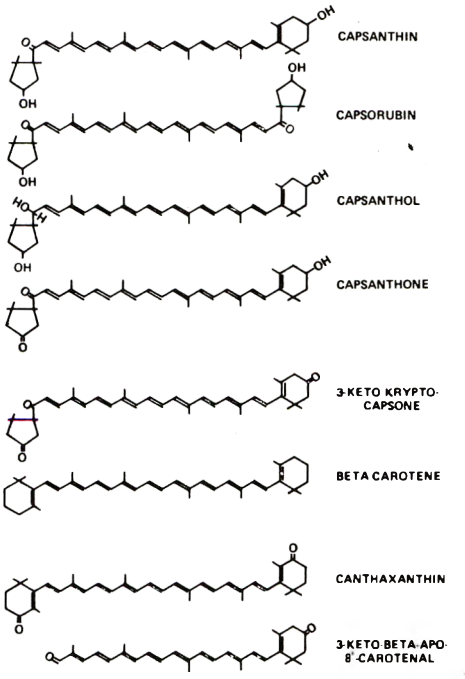

Fig. 1—Structures of capsanthin and related compounds.

Table 1—Counter current distribution of carotenoids of paprika (Columbia Gem)[a]

Fraction No.	Tube No.	Percentage[b]	Class of carotenoid	Major components
1	1 – 30	5	Polyols	Neoxanthin, hydroxy capsanthin, hydroxy capsolutein 5,6-epoxide
2	31 – 60	8	Diketo diols	Capsorubin, capsochrome, capsanthin 5,6-epoxide, hydroxy capsolutein
3	61 – 110	47	Diepoxide diols, mono keto diols, monoepoxide diols	Capsanthin, violaxanthin, antheraxanthin mutatoxanthins, luteoxanthin, capsolutein 5,6-epoxide, capsolutein 5,8-epoxide
4	111 – 150	17	Diols	Zeaxanthin, capsolutein
5	151 – 170	2	Monols	Cryptocapsin, cryptoxanthin
6	171 – 200	21	Hydrocarbons	Beta-carotene, phytoene, phytofluene, alpha-carotene, delta-carotene

[a]Solvent system: hexane, benzene, methanol and water in the ratio of 1:1:1:0.15 (v/v).
[b]Calculated based on the optical density at 450 nm as beta-carotene.

tively with distilled water, 0.5N sulfuric acid, saturated solution of sodium bicarbonate and distilled water, then dried over anhydrous sodium sulfate and the solvent removed. 3-keto-beta-apo-8′-carotenal which presumably arose from the oxidation of beta-citraurin, was purified by thin layer chromatography on silica gel G using a solvent system consisting of 2.0% ethanol, 10% benzene and 20% acetone in petroleum ether.

Esterification

The esterification of capsanthin to the dilaurate form was described previously (Philip et al., 1971).

Spectra

The visible and UV spectra were recorded in a Perkin-Elmer Model 450 UV-Vis-NIR spectrophotometer. The infra-red data were obtained with a Perkin-Elmer 337 IR spectrophotometer using KBr discs. The NMR data were obtained with a Varian A-6 NMR spectrophotometer in $CDCl_3$. The mass spectra were obtained with a Hitachi Model 6E medium resolution Mass Spectrometer (courtesy of Perkin-Elmer Corp., Norwalk, Conn.).

Nomenclature

Karrer's nomenclature is followed in this report. The carbon atoms are numbered 1 to 15 and 1′ to 15′, beginning at the carbon atoms that carry gem-dimethyl groups and the beta-ionine ring carries the unprimed numbers. Trivial names are used for the sake of convenience.

RESULTS & DISCUSSION

Separation of pigments

The six classes of carotenoids fractionated by counter current distribution of paprika carotenoids are listed in Table 1. Each class contains a number of carotenoids (de la Mar and Francis, 1969) and the major ones are listed in Table 1. The individual pigments can be easily separated on silica gel G by different proportions of the same solvent as listed in Table 2. The separations are better if the chambers are presaturated prior to development.

Structure

The structures of capsanthin and related compounds are shown in Figure 1.

R_f values

Carotenoids can be classified into different classes based on their adsorption affinity such as carboxyls, polyols, keto diols, ketones, epoxides, diols, monols and hydrocarbons. The adsorptive power depends on the functional groups, their steric disposition, the adsorbent and the solvent.

Table 3 shows the R_f values of capsanthin and related compounds. Capsanthin being a monoketo diol, has an absorption affinity between that of polyols and monols and the conversion of hydroxyl groups to carbonyls (capsanthone and 3-keto-kryptocapsone) reduces the adsorption affinity markedly. On reduction, capsanthin gives two capsanthols having widely different R_f values. Model studies revealed the possibility of steric hindrance between the newly formed hydroxyl group and the dimethyl groups in the cyclopentane ring of capsanthol. Consequently, the hindered isomer has higher R_f value and is produced in greater quantity (2:1) during reduction of capsanthin by sodium borohydride than the

Table 2—Solvent systems for the separation of different classes of carotenoids

Class of carotenoids	Ethanol % (v)	Benzene % (v)	Acetone % (v)	Petroleum Ether % (v)
Polyols	3.5	10	20	66.5
Diketo diols	3.5	10	20	66.5
Diepoxide diols, monoketo diols & monoepoxide diols	3.0	10	20	67.0
Diols	2.0	10	20	68.0
Monols	0.0	10	20	70.0
Hydrocarbons	0.0	0.0	5	95.0

Table 3—Rf values of capsanthin and related compounds (silica gel G; thickness 0.250 mm; temperature 25° C)

Carotenoid	R_f value	Solvent system
Capsanthin	0.53	3.5% ethanol, 10% benzene and 20% acetone in petroleum ether
Capsorubin	0.47	3.5% ethanol, 10% benzene and 20% acetone in petroleum ether
Capsanthols	0.47 0.27	3.5% ethanol, 10% benzene and 20% acetone in petroleum ether
3-keto-krypto-capsone	0.71	3.5% ethanol, 10% benzene and 20% acetone in petroleum ether
Capsanthone	0.65	3.5% ethanol, 10% benzene and 20% acetone in petroleum ether
3-keto-beta-apo-8′-carotenal	0.82	3.5% ethanol, 10% benzene and 20% acetone in petroleum ether
Capsanthin di-laurate	0.52	10% acetone in petroleum ether
Capsanthin mono-laurate	0.21	10% acetone in petroleum ether
Capsanthin (trimethyl silyl) diether	0.64	10% acetone in petroleum ether

unhindered isomer which has the expected R_f value (Lederer and Lederer, 1955). Silylation and esterification of capsanthin reduced the adsorption affinity considerably.

Electronic spectra

Carotenoids having 7–12 double bonds exhibit a three-banded spectrum in the visible region. The position and the shape of the spectra are dependent on the structural features as well as the solvent used. Nonpolar solvents allow considerable fine structure to be preserved in the structure of carotenoids, and broad bands (e.g., canthaxanthin) are typical of ketocarotenoids.

Table 4 shows the visible spectral maxima of capsanthin and related compounds. The main band is attributed to the $\pi \rightarrow \pi^*$ transition of the electrons of the conjugated system and the longest wavelength band is attributed to the $\eta \rightarrow \pi^*$ transition of the nonbonding electrons of the carbonyl group. Capsorubin has two conjugated carbonyl groups and as a result, the longest wavelength band is well separated and occurs with greater intensity than that of capsanthin. The $\pi \rightarrow \pi^*$ transition band is shifted to longer

Table 4–Visible spectral maxima of capsanthin and related compounds

Carotenoid	Hexane nm			Benzene nm			Ethanol (95%) nm			Carbon tetrachloride nm			Carbon disulfide nm		
Beta-carotene	476	448	427	485	460	435	–	–	–	–	–	–	–	–	–
Canthaxanthin	–	466	–	–	476	–	–	–	–	–	–	–	–	–	–
Capsorubin	499	465	440	(502)	476	–	–	–	–	–	–	–	–	–	–
Capsanthin	492	464	(442)	502	478	–	–	468	–	502	478	(458)	(525)	496	–
Capsanthone	(495)	472	–	–	483	–	–	–	–	–	–	–	–	–	–
3-keto-kryptocapsone	(495)	467	(442)	–	482	–	–	–	–	–	–	–	–	–	–
3-keto-beta-apo-8′-carotenal	472	449	(429)	–	457	–	–	–	–	–	–	–	–	–	–
Capsanthols	468	440	(422)	482	453	(433)	470	443	(424)	–	–	–	496	467	(447)
	464	438	(418)	481	453	(431)	469	442	(422)	–	–	–	496	467	(444)
Capsanthin dilaurate	495	467	(447)	(505)	482	–	–	469	–	505	478	–	–	–	–
Capsanthin (trimethyl silyl) diether	489	465	(445)	(502)	480	–	–	468	–	502	478	(457)	520	495	(472)

Table 5–Infra-red spectral absorptions of capsanthin and related compounds

Carotenoid	C–H Stretch[a] cm^{-1}	C=O Stretch[a] cm^{-1}	CH Out-of-plane deformation[a] cm^{-1}	Other vibrations[a] cm^{-1}
Capsanthin	3020(w), 2950(s), 2920(s), 2855(s)	1660(s)	960(s), 947(vs)	3600–3200 (b, O–H stretch), 1580(s), 1550 (vs), 1520(s), 1420(b), 1385(s), 1370(s), 1225(s), 1155(s), 1030(vs, sec. H), 988(s), 745(s)
Capsanthin dilaurate	3030(w), 2960(s), 2930(vs), 2860(vs)	1730 (vs, ester C=O, 1670(s)	960(s)	1583(s), 1550(s), 1510(w), 1460(s), 1380(s), 1245(s), 1165 (vs), 1040(s), 1000(s), 980(w), 720(s)
Capsanthin (trimethyl silyl) diether	3030(w), 2965(vs), 2925(vs), 2860(s)	1660(s)	960(vs)	1580(s), 1545(vs), 1505(s), 1445(b), 1375(s), 1355(s), 1240(vs, Si–$(CH_3)_3$ rock), 1070(s), 1045(s), 1000(s), 890(s), 840(vs, Si–C stretch), 750(s)
Canthaxanthin	3020(s), 2950(s), 2910(s), 2850(s)	1650(vs)	958(vs), 946(s)	1575(s), 1550(s), 1450(b), 1385(w), 1360(w), 1350(s), 1340(w), 1320(w), 1300 (w), 1250(w), 1178(w), 1072(w)
Capsanthone[b]		1740 (cyclopentanone), 1660		
3-keto-kryptocapsone[b]		1740 (cyclopentanone), 1710 (cyclohexenone), 1660		
3-keto-beta-apo-8′-carotenal[b]		1710 (cyclohexenone), 1660		

[a]b–broad; vs–very strong; s–strong w–weak.
[b]Spectra recorded in spectral grade chloroform (0.1 mm cell).

wavelength and the $\eta \rightarrow \pi^*$ transition band is shifted to shorter wavelength as the polarity of the solvent is increased and as a result, polar solvents give broad and featureless bands. The capsanthols have identical spectra with considerable detail as is usually the case with hydroxy-carotenoids. The maxima are shifted to shorter wavelengths (24–26 nm) corresponding to the loss of one double bond from the conjugated system. Esterification and silylation do not affect the shape and maxima of capsanthin significantly and oxidation of the hydroxyl groups to carbonyl groups (capsanthone and 3-keto-kryptocapsone) results in the loss of fine structure.

Interpretation of structural features of carotenoids based on the visible spectra requires considerable practice and reported maxima often differ considerably. Therefore, it is reliable to interpret changes in the spectra, only if one used spectra obtained in the same laboratory.

Infra-red spectra

Carotenoids, in general, and symmetrical carotenoids, in particular, do not give significant absorptions in the infra-red region of the spectrum. However, different types of carbonyls and hydroxyls and cis-isomers can be easily recognized by their characteristic infra-red absorptions. Table 5 shows the infra-red absorption bands of capsanthin and related carotenoids. The O-H stretching band of capsanthin appears as a broad band (3600–3200 cm^{-1}) and the strong absorption at 1030 cm^{-1} is characteristic of secondary alcohols (Weedon, 1965). The O-H stretching absorption of the hydrogen attached to the carbon-carbon double bonds appears as a weak band at about 3020 cm^{-1}. The bands at 2960–2850 cm^{-1} are due to C-H stretching (asym and sym) absorptions and are very strong in capsanthin dilaurate and capsanthin (trimethyl silyl) diether due to the methylene and methyl groups of these compounds. The very strong ester carbonyl stretching absorption of capsanthin dilaurate occurs at 1730 cm^{-1}. The carbonyl stretching band of capsanthin is of low intensity as is the case with polyene ketones of high molecular weight (Bellamy, 1954). The frequency (1660 cm^{-1}) of the carbonyl stretching band is lowered due to the conjugated carbon-carbon double bonds. The carbonyl stretching frequencies of the cyclopentanone (capsanthone and 3-keto-kryptocapsone) and the cyclohexenone (3-keto-kryptocapsone and 3-keto-beta-apo-8′-carotenal) appear at 1740 and 1710 cm^{-1} respectively. The conjugated polyene chain of carotenoids contains two types of carbon-carbon double bonds, methylated and unmethylated and these give rise to more than one band around 960 cm^{-1} and are attributed to CH out-of-plane deformations (Lunde and Zechmeister, 1955). The carbon-carbon double bond stretching vibrations occur in the region 1585–1550 cm^{-1}. Capsanthin (trimethyl silyl) diether gives strong absorptions at 1240, 840, and 750 cm^{-1}. The band at 1240 cm^{-1} is assigned to the rocking vibration of the methyl groups attached to the silicon and the lower bands are due to Si-C stretching absorptions. The C-O-C and Si-O-C stretching vibrations overlap with C-O stretching vibrations and occur in the region 1200–1000 cm^{-1} (Bellamy, 1954).

NMR spectra

The "in-chain" methyl bands (19, 20, 19′ and 20′) of carotenoids occur at 7.95–8.15 Tau in the NMR spectrum. The "end-of-chain" methyls (16, 17, 18, 16′, 17′ and 18′) are more shielded than the "in-chain" methyls and as a result, the bands occur at higher fields, the location of which is related to the molecular environment.

Table 6 shows the NMR spectral bands of capsanthin, canthaxanthin and beta-carotene. The "in-chain" methyl bands of these carotenoids occur at about 8.00 T. The dimagnetic effect of the 4-keto group in canthaxanthin lowers the "end-of-chain" methyl bands to slightly lower fields as compared to beta-carotene. The NMR spectrum of capsanthin shows seven bands at 9.14, 8.91, 8.79, 8.74, 8.62, 8.25 and 8.01 T in the ratio 1:1:1:1:1:1:4. The bands at 9.14, 8.79 and 8.62 T are attributed to the methyl groups in the cyclopentane ring and the band at 8.25 T is assigned to the C_5-methyl group of the cyclohexane ring (Barber et al., 1961). The bands at 8.91 and 8.74 are, therefore, due to the gem-dimethyl group of the cyclohexene ring.

Mass spectra

Schwieter et al. (1965) provided a mechanism for the formation of M-92 (toluene) and M-106 (xylene) ions in the mass spectra of carotenoids. Baldas et al. (1969), Francis (1969) and Philip (1970) have recently discussed the mass spectra of carotenoids and related compounds. Table 7 shows the m/e values of the peaks at the high-mass-unit end of the mass spectra of capsanthin, canthaxanthin and beta-carotene. The high intensity of the molecular ion is indicative of the stability of these compounds. The ratios of the intensity of M-92/M-106 ions are 0.1:1.1:9.2 respectively for capsanthin, canthaxanthin and beta-carotene. The mass spectra of capsanthin shows a peak at m/e 429 (M-155) which presumably

Table 6—NMR spectral signals of capsanthin, beta-carotene and canthaxanthin

Carotenoid	Value[a]	Methyl groups involved
Capsanthin	9.14(1), 8.79(1), 8.62 (1)	16′, 17′, 18′
	8.91(1), 8.74(1)	16, 17
	8.25(1)	18
	8.01(4)	19, 20, 19′, 20′
Beta-carotene	8.95(2)	16, 17, 16′, 17′
	8.27(1)	18, 18′
	8.01(2)	19, 20, 19′, 20′
Canthaxanthin	8.82(2)	16, 17, 16′, 17′
	8.13(1)	18, 18′
	8.00(2)	19, 20, 19′, 20′

[a]The value in parentheses indicates relative intensity.

Table 7—The m/e values of the peaks at the high-mass-unit end of the mass spectra of capsanthin, beta-carotene and canthaxanthin

	Capsanthin[a] m/e	Beta-carotene[a] m/e	Canthaxanthin[a] m/e
Molecular ion	584 (61.5)	536 (100)	564 (100)
Loss of methyl (M-15)	–	521 (4.8)	549 (20.6)
Loss of water (M-18)	566 (16.7)	–	–
Loss of C_4H_8 (M-56)	–	–	508 (8.4)
Loss of C_6H_8 (M-80)	–	456 (5.8)	484 (9.4)
Loss of toluene (M-92)	492 (11.5)	444 (73.1)	472 (44.9)
Loss of xylene (M-106)	478 (100)	430 (7.0)	458 (12.2)
Loss of water and toluene (M-114)	460 (32.3)	–	–
Loss of $C_9H_{15}O_2$ (M-155)	429 (41.7)	–	–

[a]The value in parentheses denotes the relative percentage intensity.

arose by the scission of the chain at the carbon-carbon bond alpha to the carbonyl group.

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CHANGES IN CARBOHYDRATES OF ALFALFA DURING ARTIFICIAL DRYING

SUMMARY—The present study was undertaken to examine the effect of different temperatures and duration of artificial drying treatment of herbage tissue on changes of some carbohydrates, lignin and methoxyls. Significant changes were established with seven of ten constituents examined. The contents of monosaccharides, disaccharides, starch and hemicellulose were lower in the samples of dehydrated alfalfa than in the samples of fresh herbage tissue under all experimental conditions. Protopectin content was lower in all samples of dried alfalfa, except when samples of fresh herbage tissue were dried at extremely high temperatures to a final moisture level below 4%. On the contrary, the content of free galacturonic acid was higher in all samples, except under the latter mentioned conditions. Cellulose content decreased in the herbage tissue during artificial drying, except when drying was conducted at higher temperatures and longer periods; that is, when the herbage tissue was dried to a moisture level below 7%. The contents of pectin, lignin and methoxyls did not show regularity of quantitative change during the artificial drying of herbage tissue. Duration of temperature was one of the main factors, other than temperature, affecting the change in constituents and was directly related to the final moisture level of dried herbage tissue.

INTRODUCTION

ARTIFICIAL drying of plant material causes changes in the composition and quantity of nutrients (Livingston et al., 1966; 1968). During artificial drying the biological value of feeds is reduced due to changes in the retention of energy, determined from retention of nitrogen and carbon (Ekern et al., 1965). Underwood and Deatherage (1952) found that the content of monosaccharides and disaccharides in plant tissue was decreased by dehydration. Lower molecular constituents of the saccharose and glucose type were formed by degradation of higher carbohydrate polymers. These data are in agreement with the findings of Raguse and Smith (1965), who reported that the heat treatment of feeds caused a progressive increase in soluble sugars. It is clear that simple sugars undergo changes during the time the plant tissue is exposed to the drying temperature and that they react with the other constituents (Goering and Van Soest, 1967; Van Soest, 1962) or disappear by decarboxylation or dehydration.

Uronides, holocellulose and hemicellulose of the plant tissue undergo slight changes during artificial drying, but they are significant particularly from the standpoint of nutritive value of the feed. Hudson et al. (1941) and Thompson and Wolfrom (1958) found that complex carbohydrates degraded under the influence of temperature, by cleavage of the basic matrix structure with a probable shift of the bonds resulting in formation of water soluble sugars. Waite and Boyd (1953) reported that during artificial drying of plant tissue at a temperature above 100°C hemicellulose degraded some into xylan, xylose and smaller amounts of arabinose. Determination of xylan and xylose in the extracts of such samples is rather complicated as there is no direct method available. The furfural method does not give the exact results of the amounts of pentosans and pentoses (Kertesz, 1951).

Reynolds et al. (1962) found that the content of pectin substances decreased when the drying temperature increased.

Enzymes play a significant role in the change of carbohydrates and remain active for a certain period of time after harvesting. The temperature treatment hinders further enzyme activity and the temperature itself is a significant factor in the breakdown of some constituents in alfalfa. Short-term drying caused certain losses in sugars as reported by Salo (1965) while longer periods of drying gave greater losses and marked changes (Laidlaw and Wylam, 1952). Salo (1965) reported that pure sugars at the 100°C temperature did not undergo major changes; however, fructose melted, became brown and the volume of sugar was reduced. If fructose was heated at 70°C in vacuo for 20 hr no changes in the amount and in reducing ability of fructose were observed. From the above it can be concluded that specific temperature conditions and interaction with other substances may be responsible for decomposition of carbohydrates.

EXPERIMENTAL

Material and methods

Alfalfa (*Medicago sativa* cultivar Panonska) from the second cutting, at bud stage, was used as the experimental material. After cutting, one sample of the whole plant was taken for analysis. Leaves were separated from stems and their weight ratio determined. The second sample (200g) was treated with boiling 2-propanol until the final concentration of 2-propanol was 70%. This included the water from the herbage tissue. After boiling for 5 min, the tissue was macerated and extracted by the Soxhlet procedure for 5 hr.

The remaining plant material was mechanically cut (about 5 cm length) was dried in a Van der Broeck industrial dryer with the inlet air temperature from 400–835°C, or in a conveyer-type dryer with an inlet air temperature of about 135°C. The duration of drying of the given material varied from 4–10 min in the Van der Broeck dryer and from 22–35 min for the conveyer dryer. Time of drying changed as temperatures used were varied.

Dried alfalfa was ground in industrial mills. For laboratory examinations dehydrated alfalfa meal was ground a second time so it would pass a 60 mesh screen. About 20g of fresh herbage and about 5g of the meal in two replicates were used for chemical analyses. Analysis of carbohydrates included a multiphase procedure of extraction, purification of the extract, separation of carbohydrates and final analysis of carbohydrate content.

Extraction

After inactivation of enzymes present in the sample with boiling 2-propanol, the samples were macerated. Mono- and disaccharides were extracted with 70% 2-propanol for 5 hr in a Soxhlet apparatus.

Purification of the extract

Alfalfa extracts in 70% 2-propanol were evaporated to dryness in a rotary vacuum evaporator at 35°C. The residue was dissolved in about 70 ml of deionized water, 2.5g of the solid adsorbent Polyclar AT (crosslinked polyvinylpyrrolidone from Antara Chemical Div. of General Aniline and Film Co., New York) and 2.5g anion and cation exchange resins, Lewatit MIH and Lewatit PN (Bayer AG., Leverkusen, Germany) were added to the solution to eliminate reducing substances and substances of the polyphenol type which interfered with the analysis. The suspension was gently stirred on a magnetic stirrer for 1 hr.

Mono- and disaccharides

The purified extracts were brought to 100 ml volume with deionized water. Two 25-ml aliquots were used for determination of the reducing sugars. One was used directly and the other was hydrolyzed with 0.02N H_2SO_4, and then neutralized with 0.05N NaOH, as described by Salo (1961).

Starch

The residue of the sample, after the extraction with 70% 2-propanol, was incubated with 100 ml 2% solution of α-amylase at 38°C for 24 hr. The resulting digested sample was filtered

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and the content of reducing sugars in 25 ml of clear solution determined using the method of Salo (1961).

Hemicellulose and cellulose

The washed wet residue, after digestion with α-amylase, was hydrolized with 100 ml of 0.7N HCl under reflux for 5 hr in a boiling water bath. After hydrolysis, the mixture was filtered through a sintered-glass filter, packed with 2 cm layer of Celite 545, into a 500 ml volumetric flask. After filtering, it was brought to volume with deionized water. A 25-ml aliquot was neutralizated and purified with anion and cation exchange resins and with an insoluble cross linkage polyvinylpyrrolidone adsorbent in the previously described manner. The solution was filtered through dry filter paper and a 5-ml aliquot tested for reducing sugars by the standard procedure. A portion of the purified filtrate was evaporated to a small volume and used for chromatographic evaluation. Amounts of individual sugars were determined from the chromatogram. These results were used to calculate the percentage of hemicellulose.

The residue on the glass filter, after hydrolysis with 0.7N HCl, was dried at 45°C and transferred to a 500 ml round bottom flask, to which 25 ml 72% H_2SO_4 was added, and thoroughly mixed with a glass rod. The reaction mixture was kept at room temperature for 5 hr with occasional stirring. The contents of the flask were transferred with 800 ml of deionized water and the mixture was refluxed for 4 hr. The mixture was filtered through an asbestos-covered sintered-glass filter into a 1000 ml volumetric flask. The pooled filtrate was neutralized with NaOH and brought to volume with deionized water. A 25 ml-aliquot was deionized and purified in the previously described manner. The percentage of reducing sugars was determined using the standard procedure and the percent of cellulose in the sample was calculated.

Uronides

Free galacturonic acid, pectin and protopectin were determined in a separate sample after the inactivation of enzymes with boiling 80% ethanol and after elimination of the extracted substances dissolved in 80% ethanol. The macerated sample was washed with 80% ethanol, dried at room temperature until the ethanolic odor disappeared. The sample was then extracted in three steps with 60% ethanol, water and 0.75% ammonoxalate solution. Extraction at 70°C for 12 hr with each of the three solvents removed free galacturonic acids, pectin and protopectin, respectively. The content of uronic acids was determined in the 1.5 ml aliquots of the three different extracts, using the carbasol method of Rouse and Atkins (1955). The absorption values at 550 mμ were compared to those for the standard curve of pure galacturonic acid, and the content of uronic acids in all three fractions was calculated. These results corresponded to free galacturonic acid, pectin and protopectin in the sample.

Lignin and methoxyls

About 10g of the fresh herbage and about 2.5g of the meal, in duplicate, were dried in vacuo, over P_2O_5 at 45°C, to constant weight. In these prepared samples, lignin was determined using the method of Thacker (1954). The content of methoxyls were determined in the lignin fraction using the method of Mathers and Pro (1958).

RESULTS & DISCUSSION

ARTIFICIAL drying of alfalfa herbage tissue caused significant changes in six of eight carbohydrate constituents examined. The contents of lignin and methoxyl in the lignin fraction of fresh herbage tissue were also changed by dehydration.

Results regarding the changes in alfalfa carbohydrates during artificial drying are presented in Tables 1 and 2. Results in Table 1 show that the quantity of total carbohydrates decreased about 10% in alfalfa herbage tissue when dehydration was conducted with hot air. This decline is especially evident in the amounts of monosaccharides, hemicellulose, cellulose and uronides.

A 20% decrease in the amount of monosaccharides, using both types of drying, is significant and it is apparent that a part of the monosaccharides have been degraded during artificial drying by decarboxylation or dehydration as reported by Underwood and Deatherage (1952). Also, it is possible that some of the remaining monosaccharides have interacted with other compounds, possibly those having a free amino group, forming complex compounds of the melanoidin type (Goering and Van Soest, 1967; Van Soest, 1962; Anet, 1960, 1961; Burton et al., 1963; McWeeny and Burton, 1963). A larger decrease in the content of monosaccharides in the herbage tissue under the influence of temperature was expected. However, it seems that monosaccharides are formed as degradation products of di- and polysaccharides (Reynolds et al., 1962).

Starch present in concentrations of less than 1% in alfalfa herbage tissue undergoes partial pyrolysis resulting in

Table 1—Changes of some carbohydrate constituents, lignin and methoxyl in alfalfa during artificial drying (% d.w.)

	Fresh alfalfa	Conveyer dryer (135°C) Time of drying, min			Rotopneumatic dryer Inlet air temperatures			
		22	35	35[a]	400°C	600°C	700°C	800°C
Moisture content,%	81.36	7.37	7.83	17.56	7.43	7.59	7.28	7.61
Total carbohydrates	54.66	52.35	52.60	50.17	49.35	49.82	50.07	50.72
Monosaccharides	5.37	4.13	4.23	4.07	4.87	5.12	4.93	5.00
Disaccharides	2.64	2.06	2.12	2.16	2.10	2.11	2.09	2.23
Starch	0.70	0.51	0.12	0.22	0.25	0.09	–	–
Hemicellulose	12.98	12.54	12.00	12.12	11.93	11.40	11.05	11.02
Cellulose	20.66	21.97	22.98	20.41	19.77	20.56	21.52	22.20
Total uronides	12.13	11.14	11.15	11.19	10.43	10.54	10.48	10.27
– FGA[b]	2.53	2.30	2.76	3.23	2.24	2.64	2.88	3.02
– Pectin	2.13	2.20	2.02	1.98	2.18	1.80	1.80	2.29
– Protopectin	7.47	6.64	6.31	5.98	6.10	6.10	5.80	4.96
Lignin	7.63	7.80	7.47	7.45	7.88	8.01	7.96	8.12
– Methoxyls ($-OCH_3$)[c]	2.50	2.53	2.43	2.41	2.48	2.61	2.57	2.60

[a]The sample had a higher moisture level than the similar sample dried at 135°C for 35 min.
[b]The methoxyl content was calculated on the sample of alfalfa.
[c]Free galacturonic acid.

Table 2—Changes in some carbohydrate constituents, lignin and methoxyl in alfalfa as related to temperatures and final moisture levels of samples during artificial drying (% d.w.)

	Fresh alfalfa	Temperature of the inlet air								
		—500°C—		600°C		——735°C——		——835°% ——		
Moisture content, %	80.02	20.05	9.63	7.17	3.67	7.47	11.29	3.87	6.94	12.43
Total carbohydrates	60.91	51.11	57.23	55.59	59.40	55.73	52.87	59.20	57.14	55.02
Monosaccharides	5.87	5.72	5.63	5.83	5.66	5.42	5.03	5.60	5.27	5.00
Disaccharides	3.05	2.88	2.73	2.60	2.40	2.20	2.05	2.47	2.30	2.10
Starch	1.57	1.05	0.45	0.38	0.12	0.42	0.55	–	0.10	0.44
Hemicellulose	12.80	11.35	11.07	10.83	10.10	11.43	11.84	10.15	11.90	12.45
Cellulose	24.95	19.98	24.23	23.08	26.79	23.88	22.31	26.82	25.39	24.56
Total uronides	12.40	10.15	12.37	12.12	12.45	11.87	11.05	12.20	11.53	10.8
– FGA[a]	2.30	2.25	2.40	2.44	2.25	2.47	2.63	2.10	2.45	2.5
– Pectin	2.08	2.01	2.00	1.86	1.82	2.01	2.18	1.75	1.98	?
– Protopectin	8.02	5.99	7.97	8.00	8.38	7.39	6.24	8.45	7.10	
Lignin	8.54	8.78	8.89	8.90	8.25	8.60	9.07	8.37	8.89	
– Methoxyls ($-OCH_3$)[b]	2.89	2.93	2.97	3.05	2.70	2.92	3.10	2.87	3.07	

[a]Free galacturonic acid.
[b]The methoxyl content was calculated on the sample of alfalfa.

the formation of dextrin and 1,6-anhydro-beta-D-glucopyranose in the samples of alfalfa meal. The content of starch decreased from 0.70–0.12% when using the conveyer technique of drying. Artificial drying of alfalfa in a rotopneumatic dehydrator caused almost a complete degradation of starch.

The quantity of total uronides decreased from 12.13–11.14% when alfalfa was dried with the conveyer system and to 10.27% using the rotopneumatic equipment. The greatest changes were found in protopectin. Similar results have been reported by Wiering (1960).

The concentration of hemicellulose was about 12.98% in fresh alfalfa herbage tissue. This decreased to 12.00% with conveyer dryer during 35 min drying time, and to 11.02% during rotopneumatic dehydration. Degradation of hemicellulose amounted to only 10–15% of the total amount in fresh alfalfa tissue.

Results reported in Table 1 are interesting as they indicate a relative increase in the cellulose content from 20.66% in the fresh tissue to 22.98% (conveyer dryer, 35 min), and to 22.20% (rotopneumatic dehydrator, 800° C) in the samples of artificially-dried alfalfa meal. The studies repeated in the following year (Table 2) showed a similar change in the quantity of this compound in some cases. An increase in the amount of cellulose in the process of drying can be explained as the result of a relative decrease of the other constituents. Another possibility is that there is some variation between samples when using the analytical procedure for determination of cellulose content both in fresh herbage tissue and alfalfa meal. Probable changes in the spatial structure of this polysaccharide, and the formation of the strong-bonded complex prevent elimination of the substances occluded with the cellulose membrane so that these substances may interfere in the carbohydrate determination on the cellulose digests after hydrolysis with H_2SO_4. A higher content of cellulose was found in the samples of herbage tissue dried at higher temperatures (700–835°C) when the moisture levels in alfalfa meals were below 7%.

Lignin content increased slightly, in some cases, during artificial drying. This was probably due to changes in the structure of lignin, that is, the basic unit of phenylpropane polymer. A higher content of this substance in the meals of artificially-dried alfalfa, relative to fresh herbage tissue, could be explained by linking of N-alkylamide to form a phenylpropane unit. A greater share of N-alkylamide was found in the lignin fraction in some samples of nontreated alfalfa.

Discussing the experimental results shown in Table 2, it could be stated that there are less total carbohydrates, monosaccharides and uronides in the samples of artificially-dried alfalfa than in samples of fresh herbage tissue. This is in agreement with previously shown results in Table 1. One of the very important factors influencing the changes of carbohydrate constituents is the final moisture level of the dehydrated herbage tissue. The samples of alfalfa dried at 735° C or 835° C to a final moisture level of about 12% had less total carbohydrates, monosaccharides and uronides than samples dried to moisture levels of 3–7%.

The conclusion could be drawn, that the nutritive quality of dehydrated alfalfa meal is higher if it contains a larger quantity of total carbohydrates. However, on the basis of the relationship between the quantities of galacturonic acid, pectin, protopectin, hemicellulose and other constituents, as amino acids and vitamins, it is clear that the meal samples with a higher moisture content, produced by drying at the same inlet air temperature, have the greater biological value in comparison with the samples with a lower moisture content. They contain larger amounts of available amino acids and vitamins and more readily available carbohydrates.

Some of these statements are in agreement with the previous results obtained in our laboratory (Milić et al., 1965). Results from other experimental trials with rats and mice which support these statements will be published in the near future.

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JFS RESEARCH NOTES

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A Research Note

SPOILAGE UNITS OF RADURISED BOMBAY DUCK (Harpodon nehereus)

INTRODUCTION

THE CORRELATION between spoilage of fishery products and temperature has been well established, the rate of deterioration increasing with rise in temperature. In this connection, the mathematical relationship adopted by Spencer and Baines (1964) for deriving spoilage units serves as a useful parameter. It has been conclusively established (Coleby and Shewan, 1965; Kumta and Sreenivasan, 1966) that dose levels in the range of 0.1–1.0 Mrad gamma radiation successfully extend the storage life of sea foods at melting-ice temperatures. However, although the terminal spoilage of fresh fishery products closely compares with that of fishery products irradiated in the radurisation range (i.e., up to 0.3 Mrad), the pattern of terminal spoilage of sea foods irradiated at higher doses is quite different.

Earlier observations in this laboratory showed that quality indices such as total bacterial count (TBC), trimethylamine (TMA) and total volatile basic nitrogen (TVBN) show marked irregularities depending on the radiation dose administered, the storage temperature and the species of sea foods irradiated (Alur et al., 1971a). These findings are attributed to differential radio-sensitivity of fish microflora (Lewis et al., 1971; Kumta and Mavinkurve, 1971) and variations in the spoilage potential of these microorganisms (Alur et al., 1971b). It was therefore of interest to examine the usefulness of the equation of Spencer and Baines (1964) as a parameter for defining the spoilage units of radurised fishery products. This communication reports evidence on the suppression of spoilage units in Bombay duck (*Harpodon nehereus*) exposed to a radurisation dose of 0.1 Mrad.

EXPERIMENTAL

FRESH BOMBAY duck obtained from the local market were washed, eviscerated and filleted. Representative samples were homogenized with saline (1:10). Aliquots of fish homogenate were exposed to gamma radiation at a dose level of 0.1 Mrad; the unirradiated samples served as controls.

Organoleptic evaluation

Sensory scoring was done according to the Miyauchi et al. (1964) reference scale which is based upon changes in odors.

Chemical analysis

TMA and TVBN were determined in TCA extracts according to the methods adopted previously for Bombay duck (Sawant et al., 1967).

Bacteriological examination

Total aerobic bacterial count (TBC) was assessed by the pour-plate method.

Spoilage units

Spoilage rates of the samples in terms of TBC, TVBN and organoleptic ratings were determined by the least-square method. TMA content in fish muscle was converted into a TMA-index, defined as $10 \times \log (1 + \text{TMA content})$ (Shewan and Ehrenberg, 1957) for determining the spoilage units.

RESULTS & DISCUSSION

SINCE BACTERIAL spoilage or deterioration in quality during storage is a progressive reaction, the rate of spoilage expressed in terms of units/day serves as an important parameter of storage changes. The magnitude of suppression in the spoilage rate can thus be used for defining the process treatment.

Figure 1 depicts the spoilage rates for unirradiated and irradiated (0.1 Mrad) Bombay duck stored at 0–2°C and 10–12°C; these have been computed from spoilage units derived for OS, TMA-index, TBC and TVBN using the least-square method. The rate of increase in these spoilage units was found to be a function of temperature. These findings are in agreement with those of Spencer and Baines (1964). As can be seen from Figure 1, the spoilage rates are higher for unirradiated Bombay duck than for irradiated samples. The inverse relationship between fall in OS and rise in TBC, TMA

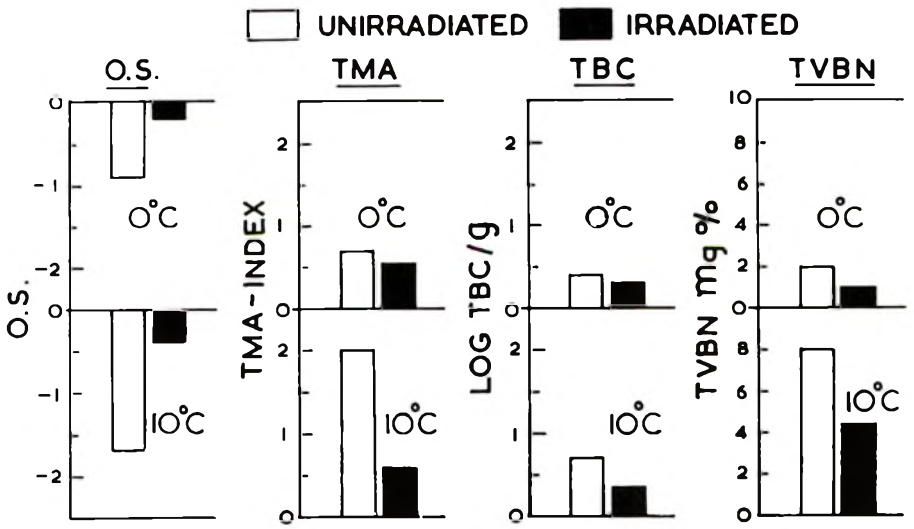

Fig. 1—Spoilage rates of unirradiated and irradiated (0.1 Mrad) Bombay duck fillets stored at 0–2° C and 10–12° C as determined by the least-square method.

and TVBN is seen from the negative slope values for OS and positive slope values for the other indices.

The spoilage rate of 0.86 calculated for OS of unirradiated Bombay duck stored at 0°C is found to be much higher than the rates reported by Spencer and Baines (1964) for cod varieties from Hull and Aberdeen. These were found to be in the range of 0.23–0.3 at 0°C. These differences in the spoilage rates may be ascribed to variations in spoilage organisms and compositional differences of fish species. *Pseudomonas* which is considered to be one of the predominant spoilage groups in fishery products (Shaw and Shewan, 1968) could not be detected in unirradiated spoiling Bombay duck (Mavinkurve et al., 1967). The observed retardation in spoilage rates of irradiated Bombay duck may be attributed to survival of relatively weak spoilers (Kumta and Mavinkurve, 1971; Lewis et al., 1971).

Since spoilage units are temperature dependent, a rise in spoilage units of radurised fishery products during commercial transport would serve as a useful parameter for assessment of storage properties and also for establishing microbial safety in terms of *Clostridium botulinum*. It is now generally accepted (IAEA, 1970) that radurisation of fishery products is a feasible process if the irradiated products are stored at temperatures below 3°C which control outgrowth of *C. botulinum* spores. Thus, if the spoilage units of radurised products held under these conditions are predetermined, any higher value for such samples that are likely to be abused under practical conditions of refrigeration storage and distribution systems, would serve as a guide-line for rejection of the samples. Also, the shelf life of fishery products can be computed with sufficient accuracy using spoilage rates as determined by Spencer and Baines' equation (1964) and taking a sensory score of 5 as a critical level of spoilage. On the basis of these considerations, the storage life of radurised Bombay duck was 26 days as against 6 days for unirradiated samples stored at 0°C. The validity of spoilage units as a parameter is being tested for a large number of fishery products dispatched in ice containers to remote centers within the country. Detailed reports of these investigations will appear elsewhere.

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A Research Note

IMPACT OF LOW DOSES OF GAMMA RADIATION AND STORAGE ON THE MICROFLORA OF GROUND RED MEAT

INTRODUCTION

THE COMMON USE of ground red meats provides a major potential vector for microorganisms of public health significance. The magnitude of the problem can be surmised from literature reporting contamination of commercial products, Kirsch et al. (1952) and Rogers and McCleskey (1957). Concern for the pathogenic contaminants has been directed primarily to the salmonellae, Cherry et al. (1943), Felsenfeld et al. (1950) and Weissman and Carpenter (1969).

Irradiation processing has potential for destroying microorganisms of public health significance and extending the shelf life while maintaining the fresh meat characteristics, Wolin et al. (1957), Niven (1958) and Lea et al. (1960). No data have been reported on the potential benefits from a combination of high-quality meat products plus low-dose irradiation.

MATERIALS & METHODS

FRESH GROUND BEEF and fresh pork sausage were obtained from six supermarkets to determine the common level of microbial contamination. Ground beef at a quality level attainable in central processing was obtained from a commissary and used for the study of the impact of low-dose irradiation on the microflora and its subsequent fate during storage at 2 and 5°C. Irradiation was with a ^{60}Co source providing 17,000 rad/min in a physical facility similar to that reported by Teeny and Miyauchi (1970). Organoleptic quality evaluation of meat samples was done by the laboratory personnel serving as a panel. General sample preparation, plating and counting procedures were those described in Recommended Methods for the Microbiological Examination of Foods (APHA, 1966). Colonies were picked by random design from countable pour plates for further study according to the Manual of Microbiological Methods (Society of American Microbiologists, 1957) and were grouped according to major identifying characteristics, Breed et al. (1957). The enterococcal counts were made using Citrate Azide Agar of Saraswat et al. (1963). Staphylococcus Medium #110 (Difco) was used for staphylococcal counts. Pigmented colonies from Staphylococcus Medium were transferred into Brain-Heart Infusion (Difco) broth and tested for coagulase production.

RESULTS

IN A SURVEY of samples from six local supermarkets, the ground beef had an average total count of 3.5×10^7/g and a coliform count of 1.3×10^5/g. After storage for 6 days at 5°C, the average counts were 4.0×10^9/g and 7.8×10^7/g, respectively. Samples after storage were judged organoleptically unacceptable.

Fresh pork sausage from these stores had an average total count of 3.1×10^7/g and an average coliform count of 9.5×10^4/g. The increase in microflora on storage was similar to that in ground beef, indicating the same magnitude of public health problem.

Six samples of fresh ground beef from the commissary had an average total count of 1.1×10^5/g and a coliform count of 1.1×10^2/g (Table 1), which is under 1% of comparable counts on the retail products. The total count on the commissary product was reduced to 1.8×10^4/g and 2.8×10^3/g by 34 and 68 Krad of gamma radiation, respectively, and the coliform organisms were destroyed by this treatment. Total count in the control samples as well as in the irradiated samples increased during storage. However, the samples irradiated with 68 Krad of gamma radiation had only 0.2% as many microorganisms as in unirradiated control samples stored for 6 days at 5°C. Both the enterococcal count and the coagulase-positive staphylococci were reduced markedly by irradiation and neither recovered to the preirradiation level during storage.

In fresh unirradiated samples, gram-positive nonsporeforming rods, micrococci and pseudomonads constituted the major portion of the microflora. On storage, the major change was an increase in proportion of gram-negative rods and a decreased proportion of micrococci.

Irradiation eliminated most of the gram-negative rods, leaving predominantly micrococci, gram-negative diplococci (*Acinetobacter-Moraxella* group) and a few gram-positive rods. On storage, however, gram-positive nonsporeforming rods became predominant.

The above-reported studies involved storage at 5°C to simulate conditions of household refrigerators and in supermarkets. To obtain comparative data on behavior of microflora at a lower storage temperature, microbial counts in unirradiated and irradiated ground beef were obtained after various time intervals at 2 and 5°C. Previous observations had indicated an objectionable off-odor was commonly apparent when the microbial population reached 50 to 100 million per gram, in general agreement with observations on other foods, Punch et al. (1965) and Adamcic et al. (1970). A count of 5.0×10^7 to 1.0×10^8/g, therefore, was taken as an indication of deterioration of a sample to the threshold of unacceptability, and observations were continued until the counts reached this range.

Figure 1 shows average data of three trials for the comparative microbial growth occurring in unirradiated ground beef at 2 and 5°C through 6 days of storage. The total count did not increase appreciably until 48 hr, after which multiplication was much faster at 5°C than at

Table 1—Microbial counts per gram of unirradiated and irradiated freshly ground beef before and after storage at 5° C

	Total count			Coliform			Enterococci			Staphylococci		
	Storage time (days)											
Radiation dose	0	2	6	0	2	6	0	2	6	0	2	6
0 rads	1.1×10^5	3.7×10^6	1.3×10^9	1.1×10^2	4.5×10^2	2.2×10^4	44	67	51	20	30	<10
34 Krads	1.8×10^4	3.0×10^4	5.7×10^7	<10	<10	13	<10	<10	<10	<10	<10	<10
68 Krads	2.8×10^3	5.5×10^3	2.6×10^6	<10	<10	<10	<10	32	14	<10	<10	<10

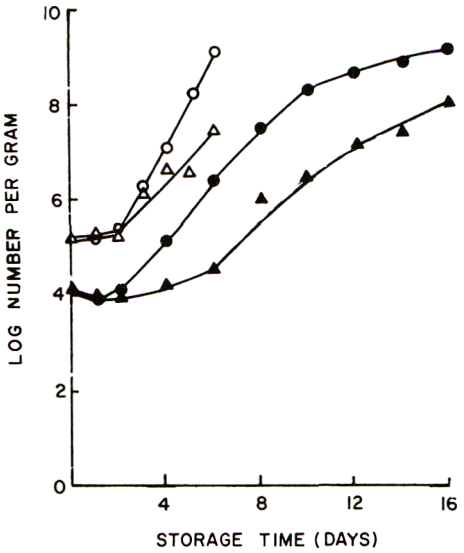

Fig. 1—Total microbial count of unirradiated and irradiated (68 Krad) ground beef stored at 2 and 5° C. Symbols: ○—○, unirradiated sample stored at 5° C; △—△, unirradiated sample stored at 2° C; ●—●, irradiated sample stored at 5° C; ▲—▲, irradiated sample stored at 2° C.

2°C, with the result that at 6 days the count was 1.1×10^9/g at 5°C but was only 5.0×10^7/g at 2°C.

Average data for three trials with irradiated ground beef showing the effect of storage are also plotted in Figure 1. At 2°C it took 14 days for the samples to reach a count of 5.0×10^7/g; at 5°C the samples reached this level in 8 days. The combination of irradiation and storage at 2°C up to 9 days limited the count to less than that obtained in the best of the fresh samples from commercial retail stores.

DISCUSSION

THE MICROBIAL LOAD in retail ground beef indicates it is on the threshold of organoleptic spoilage. The diverse sources of contamination in ground beef and its potential as a carrier into the home warrant new methods for public health protection. Our data indicate central processing, irradiation and storage at 2°C provided a product with far superior microbial quality as exemplified by total, coliform, staphylococcal and enterococcal counts. The microbial quality could no doubt be improved further by increased irradiation dose. Dose levels of 34 and 68 Krad chosen here were arbitrary to explore the potential of low-dose irradiation for public health protection. Final dose-level selection will have to be made considering the desired extent of microbial destruction, safety level to the consumer and organoleptic quality acceptance, Goldblith (1970).

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A Research Note

RAPID DETERMINATION OF WATER IN MEAT BY THE SAPONIFICATION REACTION

INTRODUCTION

THE ACCURATE monitoring of the fat, moisture and protein content is a critical factor in the quality control of meat products. Governmental regulations specify fat levels in sausages and decree that the moisture content be a function of protein levels. Furthermore, accurate determination of fat and moisture, combined with careful control of additives, permits the determination of protein by difference. These determinations are a requirement for effective least-cost formulation of sausages. The availability of reasonably rapid and accurate fat determinations (Whitehead, 1966) has increased the potential value of a rapid moisture test.

Glass (1970) reported a new approach to rapid moisture analysis in neutral organic liquids which is based on the saponification reaction. This study was conducted to explore the use of the saponification method for rapid moisture analysis in a variety of meat products.

EXPERIMENTAL

OUTLINE of the procedure is as follows: (1) Water is extracted from the meat by homogenization with anhydrous methanol; and (2) An aliquot of the extract supernatant is reacted with the reagent (methanolic sodium methoxide plus ethyl acetate) at 50°C. The sodium hydroxide formed by hydrolysis of the methoxide is irreversibly consumed by saponification of the ethyl acetate. The decrease in titer with standard ethanolic HCl is equivalent to the water present.

Preparation of reagents

Approximately 23g metallic sodium is dissolved in 1 liter ice-cold anhydrous methanol. To 650 ml of reagent-grade anhydrous ethyl acetate is added 350 ml of the methoxide solution and 0.15g phenolphthalein. The resulting reagent, which may be used immediately, is stable for at least several months. It is conveniently stored at room temperature in the reservoir of a self-leveling 10 ml pipette protected from atmospheric moisture by a tube of indicating Drierite attached to the air-inlet. Prepared as described, the reagent has a capacity of 6.3 mg water per ml. This will be decreased somewhat by water present in the reagents and by atmospheric moisture. Ethanolic HCl is prepared by passing the anhydrous gas into ice-cold absolute ethanol. A stock solution, approximately 5M, is prepared and stored at −10°C. As needed, 20 liters of approximately 0.15M acid is prepared by diluting the stock acid solution with absolute ethanol. The dilute acid, standardized as described, is stored in an appropriately-sized bottle attached to a self-leveling 25 ml burette and with a tube of Drierite attached to the air-inlet. It is quite stable losing its strength only slowly over a 2-month period at room temperature. A standard water solution (1.0M) is prepared by diluting 9 ml water to 500 ml with anhydrous methanol.

Standardization of methanolic-HCl

To a 50 ml glass-stoppered Erlenmeyer flask is added 1.0 ml of the standard water solution and 10.0 ml of the water reagent. To another flask is added 1.0 ml of methanol and 10.0 ml of the reagent. The flasks are stoppered and placed in a water bath at 50°C for 15 min. They are then removed and the contents titrated with the acid. The normality (N) of the acid is given by $N = 1/(T_b - T_a)$ where T_b is the titer of the methanol solution and T_a is the titer of the standard water solution.

Determination of water in meat

A quantity of meat containing < 2.5g of water is accurately weighed into a 90 ml stainless steel extraction chamber (Omni-mixer from Sorvall Inc.). To the sample is added 50 ml of anhydrous methanol and the mixture is homogenized 1 min. The mixture is transferred to a 50 ml tube with a screw-top cap and centrifuged at 250 × G for 5 min. The process is then carried out as previously described for the standardization of the acid but with a 1.0 ml aliquot of the extract replacing the standard water solution. A methanol blank is included.

Calculations

The amount of water present in the 1.0 ml aliquot of the extract is given by

$$\text{g water} = (T_b - T_a)\,(N)\,(0.018)$$

where T_a is the titer of the sample extract. Because the methanol has been diluted with water, this must be taken into consideration. It is convenient and essentially correct to consider the total volume as 50 ml plus a volume equal to the g water found per ml × 50. Thus, if 0.024g water/ml were found in the assay of a 2.5g sample, the percent water would be given by

$$\%\ \text{water} = \frac{(\text{g water/ml})\,[50 + (50 \times 0.024)]}{2.5} \times 100 = 49.2$$

Failure to make the correction for the volume of water results in a calculated water content of 48.0.

The saponification method was compared to the oven drying (108°C, 18 hr) and to the toluene distillation method. Sample size for the saponification method was ca 2.5g except for jowl fat (ca 5g). The time required to carry out an analysis by the new procedure is about 30 min.

RESULTS & DISCUSSION

COMPARISON of the results obtained from the saponification method with those of conventional methods of moisture analysis reveals very good correspondence (Table 1). This was true over a wide range of composition and chemical characteristics. The standard deviations calculated appeared to be sufficiently small to make the saponification method usable in both research and quality control work. The magnitude of the standard deviations was more a reflection of sampling error than error in the methods of analysis. Replicate (20) analyses by saponification of a single extract gave a standard deviation of ±0.054% (jowl fat, reduced from ±0.6). These findings indicate that most of the error was due to inaccurate sampling—probably reflecting

Table 1—Water content of various meats and sausage formulation materials as measured by the saponification and conventional methods

Sample	Moisture %	
	Saponification[a,c]	Conventional[a,b]
Turkey		
Light, uncooked	74.8 ± 0.5	74.4 ± 0.2
Light, cooked	67.6 ± 0.2	67.5 ± 0.1
Dark, uncooked	76.1 ± 0.4	76.1 ± 0.3
Dark, cooked	65.6 ± 0.4	65.1 ± 0.3
Ham	69.7 ± 0.4	69.3 ± 0.7
Fat, jowl	13.7 ± 0.6	12.9 ± 0.6
Emulsion, uncooked	56.8 ± 0.8	54.8 ± 1.8
Frankfurters	52.5 ± 0.4	52.1 ± 0.2

[a]The mean of 10 individual determinations ± S.D.
[b]Oven drying at 108°C for 18 hr except for uncooked emulsion and jowl fat which were determined by the toluene distillation procedure.
[c]Sample size 2.5g except for jowl fat (5g).

the small sample sizes used and inferior equipment for mixing meat in the laboratory.

The only other method known to the authors that compares with the saponification method for speed and accuracy is the Karl Fischer potentiometric titration. Glass (1970) concluded that the saponification method is superior to the Karl Fischer titration.

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A Research Note
PHOSPHOLIPID CONCENTRATION ESTIMATED FROM TOTAL MUSCLE LIPID

PHOSPHOLIPID concentration in muscle lipid decreases as the concentration of total lipid in beef muscle increases, Callow (1962) and Turkki and Campbell (1967). Turkki and Campbell (1967) also reported that the relationship appeared to be curvilinear for eight psoas major and eight extensor carpi radialis muscles. When phospholipid and total lipid values obtained in several later studies in this laboratory were plotted, all of the points were on or near the curve for the 1967 study. The 67 samples were diverse, including several different muscles of pork and of beef, light and dark portions of a single pork muscle, light and dark muscles of young and mature poultry and muscles of grain- and grass-finished beef. A log-log plot of all of the data results in a straight line (Fig. 1), for which the regression equation is: $Y = 1.7676 - 0.9256X$, in which Y = log percent phospholipid in muscle lipid and X = log percent total lipid in muscle, wet weight basis. The regression coefficient is significant ($P < 0.01$).

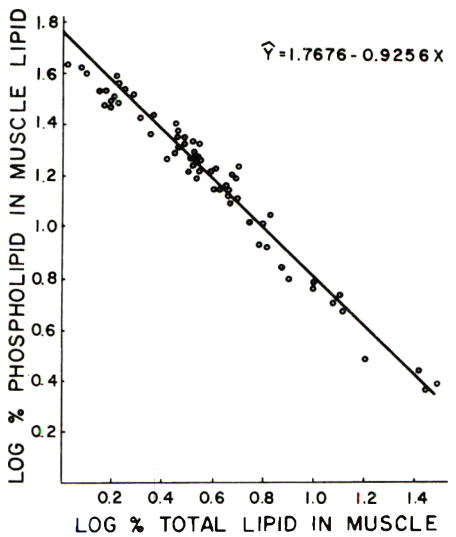

Fig. 1—Relation of phospholipid concentration in lipid to muscle total lipid concentration, wet weight basis.

Table 1—Calculated and analytical values for phospholipid concentration in lipid of four beef muscles

Muscle	% Total lipid in muscle[a]	% Phospholipid in muscle lipid	
		Calculated from regression equation	Based on P analysis
Extensor carpi radialis	2.35	26.6	27.7
Gastrocnemius	3.02	21.0	21.7
Semitendinosus	4.70	14.0	12.4
Psoas major	7.81	8.7	7.5

[a]Wet weight basis.

All of the total lipid data on which the equation is based were obtained through chloroform-methanol extraction essentially by the method of Bligh and Dyer (1959), with re-extraction of the residue. Phospholipid values were obtained by column chromatography or phosphorus analysis, or both; the two methods gave similar results. The lipid extraction procedure affects applicability of the equation for estimation of phospholipid concentration from total lipid values. A method that does not include a polar solvent results in incomplete extraction of phospholipid, Giam and Dugan (1965), which would contribute to error in predicting phospholipid concentration from the equation.

Phospholipid analyses require considerable time beyond that involved in lipid extraction. In addition, these and further analyses, for example thin-layer chromatography of phospholipids, require proper dilution of lipid extracts. If actual analysis of phospholipid concentration is necessary, the regression equation presented herein at least should eliminate trial and error in dilution. In Table 1, reasonably close agreement between calculated and analytical values for phospholipid concentration is seen for four samples of beef muscle lipid extracted after the regression analysis had been completed.

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A Research Note

LYSOSOMAL ENZYME ACTIVATION AND PROTEOLYSIS OF BOVINE MUSCLE

INTRODUCTION

THE SIGNIFICANCE of catheptic activity in postmortem proteolysis is questionable. The questionable status may be attributed, in part, to the method of analysis: previous studies used amounts of proteolytic product as indices of catheptic activity and there are suggestions that catheptic activity is accompanied by transpeptidation and peptide elongation reactions (Fruton et al., 1951; Durell and Fruton, 1954; Bodwell and Pearson, 1964; Maggi et al., 1966; Maggi and Fosella, 1966). Cathepsin is associated with other hydrolases within the lysosomes and, generally, simultaneous activation of these hydrolases is associated with rupture of the lysosomal membrane. I suggest that a more substrate-specific and more active lysosomal enzyme than cathepsin might be used to follow the course of activation and to indicate the course of proteolysis due to cathepsin.

EXPERIMENTAL

Fractionation

Portions of semitendinosus muscles from seven 5-yr old Angus steers were trimmed of fat and ground twice, about 3 hr after slaughter. Remaining muscles were stored at 3°C and processed at 1, 2, 4, 6 and 10 days, thereafter. For each specimen, 100g of ground meat was mixed with 200 ml of 0.175M KCl in 0.25M sucrose and blended for 20 sec. All blendings were performed with an Omni-mixer running at maximum speed. The suspension was homogenized with one pass of a motor-driven teflon pestle revolving at an air speed of 1000 RPM and centrifuged at 650 × G for 10 min at 2°C. The supernatant was filtered through glass wool to remove lipid debris. The residue was resuspended in 200 ml of the solvent, blended for 10 sec and centrifuged at 650 × G for 10 min. The supernatant fractions were combined and centrifuged at 22,000 × G for 20 min. The brown lysosome-rich pellet (I) was separated from the combined supernatants (II), which contained the soluble proteins and most of the solubilized lysosomal enzymes, by decantation. Two 3-ml portions of (II) were treated with an equal volume of 10% TCA for amino acid analyses. Meanwhile the residue from the 650 × G centrifugation was suspended in 200 ml of the solvent containing 2g of deoxycholate, blended for 10 sec and allowed to stand at 3°C. After 2 hr, the suspension was centrifuged at 22,000 × G for 20 min and the supernatant (III), which contained the deoxycholate-solubilized lysosomal enzymes, was collected by filtration.

Fractions (I), (II) and (III) were lyophilized and the residues were dissolved in 50, 100 and 100 ml, respectively, of 0.1M acetate buffer, pH 5.0.

Enzymatic assay

Ribonuclease activity was determined by the method of de Duve and coworkers (1955) except that a 2-hr incubation period was used.

Essentially, the incubation mixtures of Gianetto and de Duve (1955) and that of Sellinger et al. (1960) were used to assay for β-glucuronidase and β-galactosidase, respectively. The reconstituted fractions (I), (II) and (III) were freeze-thawed eight times to obtain complete lysis of the lysosomes. After incubating for 2 hr at 37°C, 3 ml of the incubate was treated with 3 ml of cold 10% TCA, allowed to stand for 30 min, and spun at 12,000 × G for 10 min. A 2-ml portion of the supernatant was neutralized with 0.5 ml of 1N NaOH and made alkaline with 2.0 ml of 1M glycine buffer, pH 10.5. The mixture was spun at 18,500 × G for 20 min. Absorption of phenolphthalein was read at 550 mμ and that of o-nitrophenol at 410 mμ.

β-galactosidase recovery

Commercial β-galactosidase was added, just before the initial blending, to 100g ground semitendinosus muscle suspended in 0.176M KCl:0.25M sucrose and the usual extraction procedure was followed until just prior to the addition of the deoxycholate. The blended suspension was divided into two equal parts and one was treated with 1g of deoxycholate. Both were kept at 3°C for 2½ hr and then centrifuged. After lyophilization and reconstituting in the buffer, the mixture was allowed to react for 20 min in a reaction mixture similar to that used for the other assays.

Amino acid analysis

A modified ninhydrin method (Rosen, 1957) was used.

RESULTS & DISCUSSION

OF THE THREE lysosomal enzymes tested, β-galactosidase was most suited for following the course of activation. The ribonuclease assay had high blanks and low activities. Since part of the β-glucuronidase may be distributed in the microsomal fraction (de Duve et al., 1955; Gianetto, 1964), this enzyme was less desirable to study than β-galactosidase.

The mean ± standard error of the mean of two replications indicates that 78.1 ± 4.3% and 66.4 ± 3.6% of added β-galactosidase activity were recovered from deoxycholate-treated and nontreated mixtures, respectively. Therefore, the total activated enzymes include the enzymes in Fraction II plus those in the deoxycholate-treated Fraction III.

Figure 1 shows the distributions of β-galactosidase, β-glucuronidase and acid ribonuclease activities in the lysosome-rich fraction (I). Analysis of variance by one-way classification indicates significant changes ($P < .05$) occur between 0–4 days but the changes between 4 and 10 days are nonsignificant ($P > .05$), suggesting that activation is essentially complete in 4 days.

Figure 2 compares the rate of amino acid liberation and total β-galactosidase activity present in the soluble fraction (II + III). Analysis of variance indicates that there are no significant differences ($P > .05$) among the means of o-nitrophenol liberated at 0, 4 and 10 days, al-

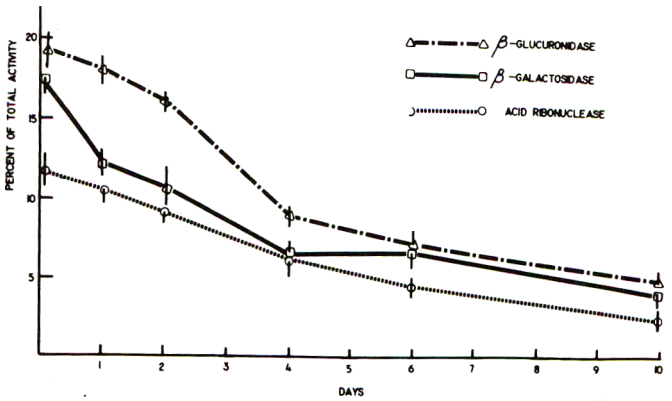

Fig. 1—Changes, with aging, in percent distributions of β-glucuronidase, β-galactosidase and acid ribonuclease activities in the lysosomal fraction (I) of beef semitendinosus muscle. Vertical lines represent standard error of the mean (n = 7).

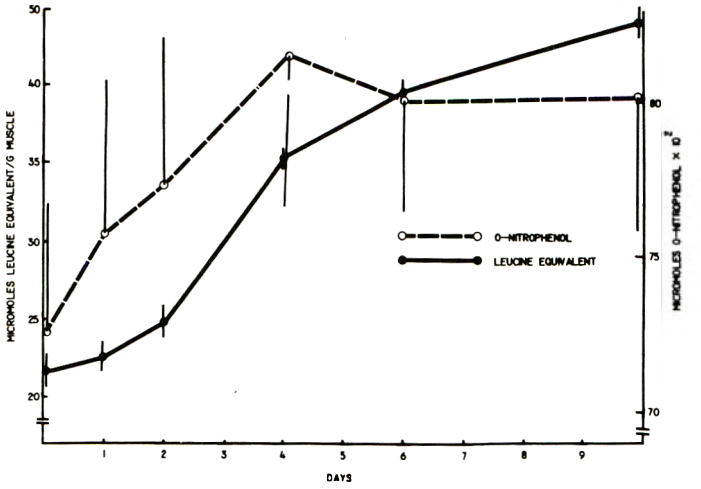

Fig. 2—Effect of postmortem aging of bovine semitendinosus muscle on rates of amino acid formation and solubilization of β-galactosidase into the soluble fraction (II + III). Vertical lines represent standard error of the mean (n = 7).

though Figure 2 shows a rising trend up to 4 days. When this trend is coupled with the significant decrease, within 4 days, in the β-galactosidase activity in Fraction I (Fig. 1), there is a strong suggestion that lysosomal enzyme activations are complete, essentially in 4 days. Sampling was not adequate to overcome the within-sample effect when Fractions II and III were analyzed for β-galactosidase activities. However, there are significant differences among the amounts of amino acid liberated at 0, 4 and 10 days. The large differences in the slopes of the amino acid curves during the first 4 days (Fig. 2) may reflect changing concentrations of cathepsin while the relatively constant slope after 4 days may indicate that cathepsin concentration is constant.

Canonico and Bird (1970) reported that, in the rat, there is a distinct species of lysosome in the muscle and another species which originates from macrophages and connective tissue cells. If such distinct species also are present in bovine muscle, perhaps the relationship between muscle cathepsin activation, proteolysis and tenderization can be elucidated further by using β-galactosidase activation as a model.

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