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Volume 35 : Number 6
Pages 703–888

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ABSTRACTS:

IN THIS ISSUE

MOLECULAR PROPERTIES OF POSTMORTEM MUSCLE. 8. Effect of Postmortem Storage on α-Actinin and the Tropomyosin-Troponin Complex. N. ARAKAWA, D. E. GOLL & J. TEMPLE. *J. Food Sci.* **35,** 703–711 (1970)–Effects of postmortem storage of rabbit muscle at 25° on the properties of α-actinin and tropomyosin-troponin-containing fractions prepared from this muscle were studied. Myosin B (natural actomyosin) prepared from postmortem muscle undergoes turbidity development much faster than myosin B prepared from muscle immediately after death. The activities of α-actinin and the tropomyosin-troponin complex in the ATPase and turbidity assays decreased slightly during postmortem storage, but both of these fractions still possessed considerable activity, even after 14 days of postmortem storage at 25°. Some evidence suggested that the F-actin-tropomyosin-troponin interaction was gradually weakened during postmortem storage without any evident degradation of F-actin and the tropomyosin-troponin complex themselves. This weakening probably accounts for loss of Ca^{++}-sensitivity in myosin B prepared from postmortem rabbit muscle. The results indicate that postmortem changes in α-actinin and the tropomyosin-troponin complex per se are not the primary cause of the postmortem modification in the actin-myosin interaction.

MOLECULAR PROPERTIES OF POST-MORTEM MUSCLE. 9. Effect of Temperature and pH on Tropomyosin-Troponin and Purified α-Actinin from Rabbit Muscle. N. ARAKAWA, D. E. GOLL & J. TEMPLE. *J. Food Sci.* 35, 712–716 (1970)–A study was done on the effects of in vitro storage of purified α-actinin, troponin, tropomyosin, and the tropomyosin-troponin complex on the activity of these protein fractions in the ATPase and superprecipitation assays. Storage was done at various combinations of temperatures between 0 and 40°C and pH values between 5.7 and 7.0. Even after 40 hr of storage, activities of purified tropomyosin and the tropomyosin-troponin complex were not affected by any combination of temperature and pH included in this study, but activities of purified α-actinin and troponin were almost completely lost after 16 hr at 40°C and pH 5.7. Storage for 40 hr at low pH (5.7) and 0°C temperature did not affect the activity of either α-actinin or troponin, but 40 hr of storage at high temperatures (40°C) and neutral pH caused some loss in activity for both these proteins. This loss of activity caused by 40°C, pH 7.0 storage was much more noticeable in the case of troponin than in the case of α-actinin. Storage periods of 40 hr or longer were required before any loss of α-actinin activity could be detected at pH 7.0 and 40°C. Since most meat animal carcasses are chilled soon after exsanguination and attain muscle temperatures of 25°C or lower before the pH falls below 6.2, it is probable that α-actinin and tropomyosin-troponin activity remain almost unchanged in meat handled through normal market channels. However, myofibrillar tissue in those porcine animals whose musculature undergoes a very rapid post-mortem decline in pH so that values of 5.7 or less are reached while muscle temperatures are still 37°C or higher may lose much of its α-actinin and tropomyosin-troponin activity during the first 24 hr post-mortem.

MUSCLE CONTRACTION AND POSTMORTEM pH CHANGES IN PIG SKELETAL MUSCLE. J. V. McLOUGHLIN. *J. Food Sci.* 35, 717–719 (1970)–The initial (5 min) pH of longissimus dorsi muscle taken from live pigs under anesthesia was 7.0; in muscle taken immediately after slaughter it was 6.3. When the neuromuscular blocking agent, curare, was given intramuscularly before slaughter the initial pH was raised to 6.8. The gastrocnemius muscle was stimulated to contract via the sciatic nerve in vivo and the pattern of pH change compared with that of the unstimulated muscle in the other side of the animal. Stimulation caused a fall in initial pH and an acceleration in the subsequent rate of pH fall in the excised muscle under nitrogen at 37°. It was concluded that neural stimuli entering the muscle at the time of death were the major factors involved in the rapid postmortem glycolysis observed in the pigs studied.

EFFECT OF MICROBIAL GROWTH UPON SARCOPLASMIC AND UREA-SOLUBLE PROTEINS FROM MUSCLE. T. HASEGAWA, A. M. PEARSON, J. F. PRICE, J. H. RAMPTON & R. V. LECHOWICH. *J. Food Sci.* 35, 720–724 (1970)–Aseptically removed rabbit and pig muscles were inoculated with *Pseudomonas fragi, Micrococcus luteus, Leuconostoc mesenteroides* and *Pediococcus cerevisiae*. Uninoculated control and inoculated samples were stored at 0, 8 and 20 days at 10°C. Comparisons between control and inoculated samples were made at all storage periods for the sarcoplasmic and urea-soluble proteins using starch gel and disc gel electrophoresis. *P. fragi* caused the most extensive breakdown of the sarcoplasmic proteins from both rabbit and pig muscle. *L. mesenteroides* caused extensive alteration in the sarcoplasmic proteins of rabbit muscle and had only slightly less effect upon porcine muscle. *P. cerevisiae* acted upon the same sarcoplasmic fractions in rabbit muscle as *L. mesenteroides*, but had no effect upon the sarcoplasmic proteins of porcine muscle. *M. luteus* caused only minor breakdown of rabbit muscle sarcoplasmic proteins and had no effect upon the sarcoplasmic proteins in porcine muscle. Both *P. fragi* and *P. cerevisiae* caused considerable breakdown of the 8 M urea-soluble rabbit muscle proteins. Neither *M. luteus* nor *L. mesenteroides* exerted any measurable effect upon the urea-soluble proteins.

DENATURATION THERMOPROFILES OF SOME PROTEINS. E. KARMAS & G. R. DiMARCO. *J. Food Sci.* 35, 725–727 (1970)–Denaturation thermoprofiles of raw beef muscle tissue and egg albumin were determined. Irreversible changes occurred in two stages: the first peak was observed at 65° and 73°C and the second peak at 82° and 83°C, respectively for beef muscle tissue and egg albumin. Pertinent calculations suggest that the second peak is due to changes in the water structures. It may be hypothesized that protective, semicrystalline water structures surrounding the nonpolar amino acid radicals of proteins were collapsed by heat, followed by the formation of hydrophobic bonding yielding aggregated denatured state. Thermodynamic information was obtained from the thermoprofiles.

FLAVOR QUALITY IN EXPLOSION PUFFED DEHYDRATED POTATO. 1. A Gas Chromatographic Method for the Determination of Aldehydes Associated with Flavor Quality. G. M. SAPERS, J. F. SULLIVAN & F. B. TALLEY. *J. Food Sci.* 35, 728–730 (1970)–An objective method based on the analysis of headspace vapor by GLC was developed to measure volatile components associated with an off-flavor produced in explosion puffed dehydrated potatoes. The vapor above a hot potato slurry containing an internal standard was analyzed by GLC for peaks corresponding to 2-methylpropanal plus acetone and 2-methylbutanal plus 3-methylbutanal. The intensity of the off-flavor was associated with the height of these peaks. The method was found to be precise, accurate, and rapid.

FLAVOR QUALITY IN EXPLOSION PUFFED DEHYDRATED POTATO. 2. Flavor Contribution of 2-Methylpropanal, 2-Methylbutanal and 3-Methylbutanal. G. M. SAPERS. *J. Food Sci.* 35, 731–733 (1970)–Flavor notes resembling some but not all elements of an off-flavor in explosion puffed dehydrated potatoes could be produced by the addition of 2-methylpropanal, 2-methylbutanal and 3-methylbutanal to potatoes. These compounds and acetone are major headspace vapor components of explosion puffed potatoes. Acetone is a major headspace vapor component of fresh boiled potatoes and is produced along with smaller amounts of 2- and 3-methylbutanal by prolonged cooking.

FLAVOR VOLATILES OF SOME COOKED VEGETABLES. A. J. MacLEOD & G. MacLEOD. *J. Food Sci.* 35, 734–738 (1970)–Using previously described sampling techniques, the flavor volatiles of a number of cooked vegetables were collected and analyzed by gas chromatography. The vegetables investigated were Brussels sprouts, cauliflower and runner beans and, in addition to the fresh vegetables, some preserved samples were examined. Quantitative measurements were made of the individual flavor components and the results were compared with those for fresh cabbage.

THE FLAVOR VOLATILES OF DEHYDRATED CABBAGE. A. J. MacLEOD & G. MacLEOD. *J. Food Sci.* 35, 739–743 (1970)–Using previously described sampling techniques the flavor volatiles of a number of dehydrated cabbage samples were collected and then analyzed by gas chromatography. Quantitative measurements were made of the individual components and results compared with those for fresh cabbage. Many differences were observed, in general indicating the poor nature of the preserved product as a substitute for the fresh vegetable. In particular allyl cyanide (3-butene nitrile) was much increased in the dehydrated samples, whereas the important allyl isothiocyanate was virtually lost altogether. Keeping qualities of the dehydrated samples were also investigated.

EFFECTS OF VARIATIONS IN COOKING METHODS ON THE FLAVOR VOLATILES OF CABBAGE. A. J. MacLEOD & G. MacLEOD. *J. Food Sci.* 35, 744–750 (1970)–Cabbage was cooked by a number of different methods and the flavor volatiles liberated were collected and analyzed by gas chromatography. The relative percentage abundance of each volatile was calculated and the values compared for the different cooking variations. The fates of certain individual volatiles were particularly interesting and are described. The cooking variations considered include cooking for different lengths of time, using microwave radiation instead of conventional heating and using different parts of the plant.

VARIANT AND INVARIANT PROPERTIES OF THE MITOCHONDRIAL FRACTION ISOLATED FROM RIPENING BANANA FRUIT. N. F. HAARD & H. O. HULTIN. *J. Food Sci.* 35, 751–756 (1970)–The mitochondrial characteristics of oxidation of Krebs cycle intermediates, respiratory control by ADP, succinate-linked oxidative phosphorylation, and overall morphology, and the amount of protein in the fraction did not vary as the ripening of banana fruit progressed. However, stimulation of succinoxidase activity by calcium and succinate-driven calcium accumulation underwent striking changes as terminal senescence ensued. The results are consistent with observations of the controlled nature of terminal-senescence and to the possible involvement of ancillary work performances, such as active transport, with climacteric respiration.

ALCOHOL-INSOLUBLE CONSTITUENTS OF JUICE VESICLES OF CITRUS FRUIT. S. V. TING. *J. Food Sci.* 35, 757–761 (1970)–Proteins, ash and polysaccharides of the alcohol-insoluble solids (AIS) of juice vesicles of several varieties of citrus fruit were determined. The polysaccharides were differentially extracted, hydrolyzed and analyzed by paper chromatography and gas chromatography of the trimethylsilyl ether derivatives. No apparent differences were found in the polysaccharide fractions, either due to maturity or variety of the fruit studied. Arabinose, galactose and a trace of glucose and rhamnose were identified in fractions containing pectin. Xylan was the main polysaccharide in the hemicellulose, and glucose was accompanied by arabinose in the cellulose fraction.

POLYPHENOLS IN MONTMORENCY CHERRIES. D. R. SCHALLER & J. H. VON ELBE. *J. Food Sci.* 35, 762–765 (1970)–An examination of the polyphenols of Montmorency cherries resulted in the separation and identification of six isomers of caffeoyl-quinic acid, four isomers of p-coumaryl-quinic acid, caffeic acid, and p-coumaric acid. In addition two flavonols, kampferol-3-rhamnoglucoside and kampferol-3-monoglucoside were also identified. Four additional polyphenols were separated but could not be identified.

RELATIVE RECOVERY AND IDENTIFICATION OF CARBONYL COMPOUNDS FROM CELERY ESSENTIAL OIL. C. W. WILSON, III. *J. Food Sci.* 35, 766–768 (1970)–The identity and quantitative estimation of carbonyl compounds in celery essential oil recovered from celery leaves and stalks by two different recovery methods was determined by chromatographic and spectroscopic methods. Two epoxides, five ketones, five esters, three acids and three phthalides were reported. Two of the three phthalides had a strong flavor and aroma of celery. Sixteen of the 18 compounds have not been reported as constituents of the essential oil from fresh celery.

IDENTIFICATION OF CARBONYL AND SULFUR COMPOUNDS FROM NONENZYMATIC BROWNING REACTIONS OF GLUCOSE AND SULFUR-CONTAINING AMINO ACIDS. P. T. ARROYO & D. A. LILLARD. *J. Food Sci.* 35, 769–770 (1970)–Odor evaluations, chemical analyses and the effect of pH on the nonenzymatic browning reaction products between equimolar concentrations of glucose and each of the sulfur-containing amino acids, methionine, cysteine and cystine, were conducted. None of the 3 mixtures emitted an odor associated with meat. The cystine-glucose mixture produced no significant aroma, the cysteine-glucose mixture an odor resembling over-boiled egg and the methionine-glucose mixture an objectionable odor associated with boiled potato, the most interesting of the 3 mixtures. The characteristic odor of the last-named mixture was attributed to mercaptans. Only the methionine-glucose mixture was affected by an increase in pH.

COLORIMETRY OF FOODS. 3. Carrot Puree. I-LO HUANG, F.J. FRANCIS & F. M. CLYDESDALE. *J. Food Sci.* 35, 771–773 (1970)–A series of carrot purees was prepared with FD&C Green No. 1 in added increments of 0.015 ppm. They were ranked visually under a 7400°K light and instrumentally with a G.E. Spectrophotometer and a Hunterlab D_{25} colorimeter, using cells varying from 2–8 mm in thickness against a white and black background. The panelists could rank samples varying by 0.10–0.15 delta units most efficiently, using sample thickness of 5 mm or more against a black background. Adequate instrument rankings required all 3 color parameters. Excellent separation of samples was obtained with a Hunterlab D_{25} colorimeter using a 2-in.-diameter opening, cells 4 mm or more in thickness and a white background. This method was superior to a trained panel. Calculation of Hunter data in K/S ratios was slightly better but more laborious. Individual K and S values may be useful in characterizing purees for colorimetry.

DISC GEL ELECTROPHORESIS OF PROTEINS IN NATIVE AND HEAT-TREATED ALBUMEN, YOLK, AND CENTRIFUGED WHOLE EGG. P. CHANG, W. D. POWRIE & O. FENNEMA. *J. Food Sci.* 35, 774–778 (1970)–Polyacrylamide gel electrophoresis was an effective technique for separating the proteins of unheated and heat-treated albumen, yolk, and centrifuged whole yolk. Bands of ovalbumins, conalbumins, and globulins were formed in the gels, but ovomucoid could not be detected and lysozyme did not migrate. Livetins and lipovitellins of yolk separated into distinct bands. The addition of EDTA to diluted yolk brought about mobility of phosvitin which otherwise did not move into the gel. With centrifuged whole egg, the electrophoretic pattern consisted of livetin and albumen protein bands but no evidence was obtained for the formation of new complexes. Alteration of gel patterns occurred when albumen, yolk, and centrifuged whole egg samples were heated to pasteurization temperatures of 61.7°C and above.

ABSTRACTS:

IN THIS ISSUE

EFFECTS OF FOUR SPECIES OF BACTERIA ON PORCINE MUSCLE. 1. Protein Solubility and Emulsifying Capacity. R. J. BORTON, L. J. BRATZLER & J. F. PRICE. *J. Food Sci.* **35**, 779–782 (1970)–Aseptic porcine muscle samples were inoculated with cultures of *Pediococcus cerevisiae, Micrococcus luteus, Leuconostoc mesenteroides* and *Pseudomonas fragi*. The inoculated samples were compared with an aseptic control for protein solubility and emulsifying capacity throughout a 20-day storage period at temperatures of 2 and 10°C. All organisms grew at 10°C, but only *L. mesenteroides* and *P. fragi* grew at 2°C. Protein solubility studies showed a loss in water-soluble fraction during storage for all samples except those inoculated with *P. fragi*. The solubility of meat proteins in a salt solution increased during the first 8 days of storage, then decreased or remained relatively constant for the controls and all treatments. Insoluble protein in the *P. fragi*-inoculated samples decreased, but increased in all other samples. Nonprotein nitrogen increased slightly during storage of all samples. The pH increased during growth of *M. luteus* and *P. fragi* and decreased during growth of *P. cerevisiae* and *L. mesenteroides*. Inoculation with *L. mesenteroides* resulted in a decreased emulsifying capacity, whereas *M. luteus* or *P. cerevisiae* had no effect, and *P. fragi* resulted in an increased emulsifying capacity.

EFFECTS OF FOUR SPECIES OF BACTERIA ON PORCINE MUSCLE. 2. Electrophoretic Patterns of Extracts of Salt-Soluble Protein. R. J. BORTON, L. J. BRATZLER & J. F. PRICE. *J. Food Sci.* **35**, 783–786 (1970)–Porcine muscle samples were inoculated with *Micrococcus luteus, Leuconostoc mesenteroides, Pediococcus cerevisiae* and *Pseudomonas fragi* organisms and compared with aseptic control samples after storage at 2 or 10°C for 0, 8 and 20 days. Electrophoresis of 0.6 M KCl sample extracts indicated that the *P. fragi* organism caused a loss in the number of protein bands present. Extracts of the 3 other inoculated samples did not differ from control sample extracts when examined by starch-gel and disc-gel electrophoresis.

THE INFLUENCE OF LACTIC CULTURES ON GROUND BEEF QUALITY. S. G. REDDY, R. L. HENRICKSON & H. C. OLSON. *J. Food Sci.* **35**, 787–791 (1970)–The effect of adding different lactic cultures (*S. lactis* and *L. citrovorum*) concentrations, combinations, and forms to ground beef stored at refrigeration temperature (7°C) were studied. CVT (crystal violet tetrazolium) count for gram-negative bacteria, pH, VNC (volatile nitrogen content) and organoleptic observations were evaluated. The addition of 10% lactic culture grown in 20% milk solids was very effective in preventing the growth of inherent gram-negative bacteria in ground beef stored at 7°C. CVT count in the uncultured meat significantly increased ($P \leqslant .01$) as the storage time progressed. Cultured meat did not exhibit a significant increase in CVT count until 7 days of storage. The pH of cultured meat significantly declined ($P < .01$) whereas the uncultured meat indicated a significant increase in pH ($P < .01$) during storage. VNC was significantly higher ($P < .01$) in the uncultured meat. Cultured meat with 450 ppm of added ascorbic acid was consistently preferred for flavor, aroma, and color when compared with uncultured meat and the meat with culture alone.

SUSCEPTIBILITY TO AMYLOLYSIS OF GAMMA-IRRADIATED WHEAT. H. N. ANANTHASWAMY, U. K. VAKIL & A. SREENIVASAN. *J. Food Sci.* **35**, 792–794 (1970)–Initial reducing sugars and diastatic activity, expressed as "maltose value," are increased in irradiated wheat and are functions of dose levels in the range 20–200 Krad. Both alpha- and beta-amylases retain their activities in irradiated wheat, but the sensitivities of starch to amylolysis are increased with radiation dose levels. Latent beta-amylase, present in resting seeds, is also radioresistant. Susceptibility of starch to radiation treatment is more pronounced with moist than dry grain.

EFFECT OF GAMMA RADIATION ON WHEAT STARCH AND ITS COMPONENTS. H. N. ANANTHASWAMY, U. K. VAKIL & A. SREENIVASAN. *J. Food Sci.* **35**, 795–798 (1970)–Studies on the susceptibility of irradiated wheat starch, amylose and amylopectin to alpha- and beta-amylolysis reveal that they are more susceptible to enzyme actions, compared to their unirradiated controls. However, irradiated amylose seems to be comparatively more vulnerable. From irradiated starch, series of oligosaccharides of the maltose series are discernible, while glucose appears only above the 200 Krad dose level. Quantitative analysis of the radiolytic breakdown products of starch reveal that at high dose levels (1 Mrad) maltose, maltotriose and maltotetrose are the main products. Results on the separation of radiolytic breakdown products suggest they resemble those produced by alpha-amylolysis of starch.

COMPUTER-AIDED PREDICTIONS OF EXTENT OF BROWNING IN DEHYDRATED CABBAGE. S. MIZRAHI, T. P. LABUZA & M. KAREL. *J. Food Sci.* **35**, 799–803 (1970)–Kinetic data on the browning reaction were obtained for samples maintained at various constant-moisture contents, and rate of browning was correlated with moisture content. The kinetic data were combined with mass transfer characteristics of the packages. The validity of the assumptions was tested and the magnitudes of needed corrections evaluated. The standard deviation ranged from 2–10% for the low and high extent of browning, respectively.

FEASIBILITY OF ACCELERATED TESTS FOR BROWNING IN DEHYDRATED CABBAGE. S. MIZRAHI, T. P. LABUZA & M. KAREL. *J. Food Sci.* **35**, 804–807 (1970)–Feasibility of using accelerated tests for deterioration of dehydrated vegetables through nonenzymatic browning was evaluated. Studies were made on unsulfited freeze-dehydrated cabbage. Two procedures for shortening time required to predict shelf life were used: (1) Extrapolation from high to low moisture contents; and (2) Extrapolation from high to low temperatures. A combination of the two procedures was also evaluated. It was determined that activation energy for browning was dependent on moisture content, and an equation expressing this dependence was developed. Equations relating rates of browning to moisture content at a given temperature were also obtained. Extrapolations based on either procedure, or on a combination of both, gave satisfactory results. The variance of the extrapolation-based estimates of browning rates was dependent on the number of points used for the extrapolation. The procedures used reduced the time required to obtain browning-rate data at low moisture contents from over a year to only 10 days.

THERMAL BROWNING OF TOMATO SOLIDS AS AFFECTED BY CONCENTRATION AND INHIBITORS. M. T. DANZIGER, M. P. STEINBERG & A. I. NELSON. *J. Food Sci.* **35**, 808–810 (1970)–Tomato solids at concentrations of 5.6 to 98% were heated for 15 min at 95°C. The optical density increased slowly with increasing concentration to about 50% solids. Thereafter, the O.D. increased rapidly to a peak at 95% and decreased to half this value at 98%. The effect of heating time at 55, 75 and 95°C and at 5.6 and 45% solids was studied. The browning rate was slight at the lower 2 temperatures relative to that at 95°C, and was much more rapid at the higher concentration than at single strength. Under each temperature and solids condition, the rate of browning was highest at the earlier times.

EXPLORING AIRBORNE SOUND IN A NONVACUUM FREEZE-DRYING PROCESS. JAMES H MOY & G. ROBERT DiMARCO. *J. Food Sci.* **35**, 811–817 (1970)–In studying the effects of sound energy on freeze-drying rates of foods, it was found that sample temperature, inlet air pressure, the freeze-dryer tube size and the sound source, all had significant effects. Freeze-drying rates were 11–100% higher in the stem-jet whistle runs than in the dummy whistle runs. Seven percent of this improvement was attributed to the thermal effect on the air resulting from friction and adiabatic compression. The remaining increase was due to the sound pressure energy, the increase in heat and mass transfer coefficients, and the "reduced-pressure" effect in the sweeping air stream during the rarefaction cycle of the sonic vibration.

THE NITRATE DETINNING REACTION IN MODEL SYSTEMS. R. P. FARROW, N. T. LAO & E. S. KIM. *J. Food Sci.* **35**, 818–822 (1970)–As part of a coordinated research program on internal can corrosion, the action of nitrates on tinplate was studied in model systems simulating foods of varying acidities. Detinning rates were measured in citrate buffered nitrate solutions ranging in pH from 3.0–7.0. At pH values less than about 5.2–5.5 the detinning rate is extremely rapid, with slower rates at higher pH values. Nitrate concentrations of 125 ppm in pH 4 buffers completely detinned cans in about 2 months at ambient temperatures. Ammonia is the principal nitrate reduction product in the pH range of acid products. Nitrous oxide is also produced and acts as an effective detinner at pH 4 and 5. Nitrogen may also be produced at pH 5.

FABRICATION AND CORROSION PERFORMANCE OF TIN-MILL PRODUCTS MADE FROM CONTINUOUSLY CAST STEEL. A. N. LAUBSCHER & G. N. WEYANDT. *J. Food Sci.* **35**, 823–827 (1970)–RIBAND 1, a continuously-cast steel developed by U.S. Steel, contains up to 0.08% silicon. It has excellent fabrication performance for food container parts such as beer can bodies, beaded can bodies, can ends, tapered 5 gal can bodies and domed tops for aerosol cans. In plain can test packs, corrosion resistance was equal to that of ingot-cast steels. In fully lacquered cans, continuously-cast steel was better in one red tart cherry pack, but had somewhat shorter pack life in a 2nd cherry pack and a blueberry pack. With orange soda, similar corrosion performance was attained for the two steels. It is concluded that RIBAND 1 meets the requirements of the container industry.

THE HELICAL PUMP: USE AS A MULTIZONE PROCESSING SYSTEM. W. C. ROCKWELL, D. F. FARKAS & M. E. LAZAR. *J Food Sci.* **35**, 828–830 (1970)–A hydrostatic processing system consisting of a compression zone, a holding zone, and a decompression zone has been built from a single coil of pipe. Theoretical maximum operating pressure is the product of the number of turns in the compression zone and the diameter of the coil. Problems of air compression and disruptive air expansion, noted in a previous model, have been overcome by an innovative air feedback system. Crosstubes, another innovation, are used to maintain a constant pressure in the holding zone of the coil. Experimental results on a model operating at 9 psig show that hydrostatic pressures in the holding zone under continuous conditions are about 90% of theoretical. Packages moving through the unit were found to have a pressure history similar to conventional hydrostatic processing systems that use single tall vertical legs for compression and decompression.

DEVELOPMENT OF A NEW PRODUCT FOR THE CIVIL DEFENSE PROGRAM. J. N. TATE JR., R. D. MATHEWS & H. STONE. *J. Food. Sci.* **35**, 831–833 (1970)–A new cereal-based product was developed to replace existing civil defense rations, which are nutritionally inadequate. Materials were selected to provide a proper amount and balance of protein, vitamins, and minerals. An animal feeding study indicates the new ration meets most nutritional requirements and costs about 20 ¢/lb.

INTEGRAL TECHNIQUES APPLIED TO SUBLIMATION DRYING WITH RADIATION BOUNDARY CONDITION. J. W. McCULLOCH & J. E. SUNDERLAND. *J. Food Sci.* **35**, 834–838 (1970)–An analytical solution is presented for the temperature distribution in a semi-infinite slab with a radiation boundary condition and sublimating phase front. 2 cases are considered. First, a solution is presented for the transient surface temperature holding the heater temperature constant. Secondly, a solution is presented for the heater temperature variation required to hold the surface at the same prescribed value reached in the first case. The application of this technique to sublimation drying is considered, along with calculations to demonstrate the effect of various parameters on the drying time. For example, a steak ½-in. thick with a porosity of 0.8 can be dried 35% faster if the initial heater temperature is 200 rather than 150°F. In contrast, for the same sample, the drying time is increased by only 15.5% if the initial heater temperature is 250 instead of 200°F. Graphs are included to show how optimum drying rates may be obtained.

AN EXPERIMENTAL DRY CAUSTIC PEELER FOR CLING PEACHES AND OTHER FRUITS. M. R. HART, R. P. GRAHAM, C. C. HUXSOLL & G. S. WILLIAMS. *J. Food Sci.* **35**, 839–841 (1970)–An experimental cling peach peeler was developed for continuous removal of alkaline peel as a solid rather than the dilute slurry of common industrial practice. By processing waste peel as a semi-solid, waste disposal and pollution problems associated with conventional lye peeling can be lessened. The peeler consists of rows of soft rubber disks rotated in such a manner that peel material is wiped clean from peach halves and flung into collectors. Peeling losses were as low as 4% after suitable lye treatment. Limited tests were successful on whole Bartlett pears with a slight modification of the peeler. In single tests, whole apricots and freestone peaches were also successfully peeled.

BACTERIOLOGICAL ANALYSIS OF FROZEN SHRIMPS. 1. Total Plate Count–Coliforms and Enterococci in Precooked Frozen Chilean Shrimp. R. VIRGILIO, C. GONZÁLEZ, S. MENDOZA, S. AVENDAÑO & N. MUÑOZ. *J. Food Sci.* **35**, 842–844 (1970)–392 samples of precooked frozen shrimps from 2 Chilean industries (A and B) were bacteriologically analyzed throughout a period of 8 months. Total bacterial counts ranged from 10^4 to 10^5 organisms per g. 89.6% of the samples from A and 50.1 % from B contained not more than 50 coliforms per g. When some sanitary measures were adopted, coliform counts improved. 98% of the samples belonging to A contained enterococci, as did 66% of the samples from B. Coliform and enterococci counts are in contradiction in plant A and better correlated in plant B, probably due to differences in precooking systems employed.

BACTERIOLOGICAL ANALYSES OF FROZEN SHRIMP. 2. Staphylococci in Precooked Frozen Chilean Shrimp. R. VIRGILIO, C. GONZÁLEZ, S. MENDOZA, S. AVENDAÑO & N. MUÑOZ. *J. Food Sci.* **35**, 845–848 (1970)–392 samples of precooked frozen shrimp were quantitatively examined for the presence of *Staphylococcus aureus* by direct plating on Difco mannitol salt agar (MSA, 10% NaCl). 140 samples (35.7%) contained Staphylococci but only half of these had counts of over 100, 82.4% remaining within the acceptable limit. Frozen shrimps are prone to contamination by Staphylococcus during processing, especially if hand-processed, but these results show that it is possible to obtain a good-quality product when stringent sanitary measures are observed.

PREMORTEM STRESS AND POSTMORTEM BIOCHEMICAL CHANGES IN SKIPJACK TUNA AND THEIR RELATION TO QUALITY OF THE CANNED PRODUCT. L. CRAWFORD, E. J. IRWIN, J. SPINELLI & W. D. BROWN. *J. Food Sci.* **35**, 849–851 (1970)–Live skipjack were caught, brought to shoreside and held in tanks 24–48 hr. At this time some were exercised to simulate stress during commercial capture, sacrificed, held at 74 or 32°F for 6 hr, sampled for chemical analyses, then canned. Unstressed (rested) skipjack were treated similarly. Some autolytic degradation products post-mortem were measured and the differences noted. Organoleptic evaluation was made on canned fish from the various treatment groups. While there were differences in various organoleptic parameters among the groups subjected to different treatments, there was no overwhelming evidence connecting stress or temperature of holding to quality in the canned product.

STABILITY OF OIL-IN-WATER EMULSIONS. 1. Effects of Surface Tension, Level of Oil, Viscosity and Type of Meat Protein. J. C. ACTON & R. L. SAFFLE. *J. Food Sci.* **35**, 852–855 (1970)–Surface tension responses for solutions of salt-soluble protein from five different meat sources followed the Type III curve typical of surface-active agents. Stability of the emulsions increased when either the concentration of the protein or oil were increased. Highly significant correlation was found between protein surface activity and emulsion stability. Little change in emulsion viscosity was found except at the upper protein and oil levels tested.

ABSTRACTS:
IN THIS ISSUE

CALCIUM POLYPHOSPHATE INTERACTION IN CURING PICKLES AND THE EFFECT OF CALCIUM ON CURED HAM YIELDS. G. R. HEGARTY, R. B. RENDEK & P. ZALOUDEK. *J. Food Sci.* 35, 856–859 (1970)–The formation of a calcium polyphosphate precipitate in curing pickles is dependent upon brine strength, brine temperature, tripolyphosphate concentration and calcium concentration. Graphs are presented to indicate what combination of the above factors will result in a precipitate. The addition of $CaSO_4$ to cured ground ham caused a significant increase in water holding capacity. The yield of fully cooked cured hams was not affected by curing with phosphate pickles containing abnormally high levels of $CaSO_4$.

MICROFLORA OF FRESH PORK SAUSAGE CASINGS. 2. Natural Casings. W.E. RIHA & M. SOLBERG. *J. Food Sci.* 35, 860–863 (1970)–Aerobic total plate counts on TGE Agar at 28°C varied from a level greater than 30,000 to 59,000,000 microorganisms per gram in salt-packed natural casings and from 180,000 to 23,000,000 organisms per gram in wet-packed natural casings. Both salt-packed and wet-packed casings supported similar growth patterns in selective media. 38 isolates were identified from the salt-packed casings and 53 from the wet-packed. Of the salt-packed isolates, 60.5% were of the genus *Bacillus*, 7.9% *Pseudomonas*, 15.8% *Clostridium*, 1.6% *Micrococcus* and 5.3% *Gaffkya*. Those isolates obtained from the wet-packed casings included 62.3% *Bacillus*, 7.5% *Pseudomonas*, 7.5% *Clostridium*, 7.5% *Micrococcus*, 5.6% *Proteus*, 1.9% *Lactobacillus* and 5.7% unidentified.

EFFECT OF HEAT TREATMENT ON VISCOSITY OF YOLK. P. K. CHANG, W. D. POWRIE & O. FENNEMA. *J. Food Sci.* 35, 864–867 (1970)–The viscosity-shear rate curves for the yolk samples were typical for a pseudoplastic, non-Newtonian fluid. The apparent viscosity of native yolk with a solids content of 52.5% dropped gradually from 23 to 18 poises with an increase in shear rate from 1.9 to 76.8 sec^{-1}. The apparent viscosity of pasteurized yolk (65.6°C for 3 min) dropped drastically from 200 to 100 poises with an increase in shear rate from 1.9 to 19.2 sec^{-1}. Addition of thin albumen at levels of 5, 10 and 20% to native yolk brought about a considerable decline in the viscosity.

TEMPERATURES IN EGG PRODUCT FOAMS DURING VAT PASTEURIZATION. R.W. DICKERSON JR., R.W. PARKER & R.B. READ JR. *J. Food Sci.* 35, 868–871 (1970)–Using a 100-gal vat, we generated foams on egg products and measured foam temperatures during vat pasteurization. The minimum temperature of a 6.5-in. liquid whole egg foam was below pasteurization temperature for 11 min after start of pasteurization. The delayed heating effect of a 1-in. foam on sugared yolk (10% sucrose by weight) was 13 min. Yolk and salted yolk (10% salt by weight) foams were below liquid temperature during heating; however, in both cases liquid and foam reached pasteurization temperature simultaneously. Minimum temperature of plain egg white (pH 9.0) foam did not reach pasteurization temperature during the holding period. A holding time of 30 min was recommended for vat pasteurization with the following temperatures for specific products: liquid whole egg, 133°F; yolk and yolk blends, 135°F; sugared and salted yolk, 139°F.

RHEOLOGY OF FRESH, AGED AND GAMMA-IRRADIATED EGG WHITE. M. A. TUNG, J. F. RICHARDS, B. C. MORRISON & E. L. WATSON. *J. Food Sci.* 35, 872–874 (1970)–Rheology of unmixed egg white at 2°C was studied with a narrow-gapped rotational viscometer over a 20-fold range of shear rates. For a constant shear rate, egg white consistency decreases with time and approaches an equilibrium value in a few minutes. Flow behavior is pseudoplastic at 2°C between shear rates of 8.1–147 sec^{-1}, and the shear stress-shear rate relation is accurately described by the power law and Casson models. The effects of gamma irradiation dose on Haugh unit score and equilibrium shear stress are discussed.

CHEESE COLORS FROM PLANT SOURCES. 1. Preparation and Properties of Color from Pepper and Safflower. S. EL-SHIBINY & M. H. ABD EL-SALAM. *J. Food Sci.* 35, 875–876 (1970)–A cheese color was prepared from the extract of pepper (*Capsicum frutescens* var. California wonder) and safflower (*Carthamus tinctorius*) pigments. The prepared color had a good keeping quality, slightly affected by temperature and sunlight. The prepared color proved successful in cheese coloration and resisted the biochemical changes in cheese during ripening. However, a slight loss in the color of cheese was observed during storage.

JFS BASIC SCIENCE

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MOLECULAR PROPERTIES OF POSTMORTEM MUSCLE. 8. Effect of Postmortem Storage on α-Actinin and the Tropomyosin-Troponin Complex

SUMMARY—Effects of postmortem storage of rabbit muscle at 25° on the properties of α-actinin and tropomyosin-troponin-containing fractions prepared from this muscle were studied. Postmortem storage did not affect the amount of α-actinin or tropomyosin-troponin that could be extracted from rabbit myofibrils by water washes or by a 3-day, pH 8.5, low-ionic strength extraction. Myosin B prepared from postmortem muscle underwent turbidity development much faster than myosin B prepared from muscle immediately after death. This increased rate of turbidity development was probably not due to postmortem changes in α-actinin, since the ability of α-actinin to accelerate the ATPase (for abbreviations used in this paper, see list under References) activity or the turbidity response of reconstituted actomyosin suspensions gradually decreased, rather than increased, during postmortem storage. However, even after 14 days postmortem at 25° α-actinin retained some activity in both the ATPase and turbidity tests. Moreover, the increased rate of turbidity development of postmortem myosin B was probably not due to degradation of the tropomyosin-troponin complex, since postmortem storage affected the activity of this complex only slightly and, even after 14 days post-mortem, the tropomyosin-troponin complex still conferred some Ca^{++}-sensitivity on reconstituted actomyosin suspensions. Myosin B prepared from postmortem muscle did not contain more active α-actinin than myosin B prepared from muscle immediately after death, but the F-actin-tropomyosin-troponin interaction was gradually weakened during postmortem storage without any evident degradation of F-actin and the tropomyosin-troponin complex themselves. The weakened F-actin-tropomyosin-troponin interaction probably caused loss of Ca^{++}-sensitivity in myosin B prepared from postmortem muscle. Results of this study indicate that postmortem changes in α-actinin and the tropomyosin-troponin complex per se are not the primary cause of postmortem modification in the actin-myosin interaction.

INTRODUCTION

IN PREVIOUS papers in this series, we have shown that the Mg^{++}- and Ca^{++}-modified ATPase activities of myosin B or myofibrils prepared from muscle after 24 hr of postmortem storage are 20–80% higher than the corresponding activities of myosin B or myofibrils prepared from muscle immediately after death (Goll and Robson, 1967; Robson et al., 1967). However, the cause of this increased ATPase activity was not clear from these earlier studies, although it presumably indicated that postmortem storage induced or was accompanied by some alteration in the actin-myosin interaction. Since both troponin and α-actinin, two recently discovered regulatory protein components of the myofibril (Ebashi and Ebashi, 1964; 1965; Ebashi and Kodama, 1965; 1966a; Maruyama and Ebashi, 1965), are ostensibly able to modify the actin-myosin interaction in vivo, it occurred to us that the increased ATPase activity in myosin B or myofibrils from postmortem muscle might originate from postmortem changes in α-actinin or troponin, or both. This possibility is strengthened by the recent discoveries that α-actinin is located in or near the Z-line (Goll et al., 1967; 1969; Masaki et al., 1967) and that the Z-line characteristically undergoes gradual disintegration during postmortem storage (Davey and Gilbert, 1967; 1969; Fukazawa and Yasui, 1967; Goll, 1968; Henderson, 1968; Stromer and Goll, 1967). Furthermore, troponin is very labile to tryptic proteolysis (Ebashi and Ebashi, 1964; Ebashi and Kodama, 1966b), and if any catheptic proteolysis does, in fact, occur in postmortem muscle (Parrish et al., 1969), it may be expected to result in considerable hydrolysis of troponin. Proteolytic hydrolysis of troponin would cause "derepression" of the inhibitory effect that this protein exerts on the actin-myosin interaction. Z-line disintegration, on the other hand, may result in release of α-actinin, permitting it to interact in a more favorable manner with actin and thereby cause the characteristic α-actinin-induced increase in the Mg^{++}-modified ATPase activity of actomyosin (Arakawa et al., 1970b; Goll et al., 1969). Consequently, changes in either α-actinin or troponin could cause the increased ATPase activity of myosin B or myofibrils prepared from postmortem muscle. In this paper, we describe the preparation and properties of crude α-actinin and tropomyosin-troponin-containing fractions from post-mortem muscle and compare these fractions with similar fractions prepared from muscle immediately after death.

MATERIALS & METHODS

EXCEPT when specifically noted otherwise, preparation of all samples was performed at 0–3° using precooled solutions. All solutions were prepared by using double-deionized, distilled water that had been redistilled in glass and stored in polyethylene containers. Reagents were the finest grade available, and only biological grade potassium chloride (Baker and Adamson Products, Allied Chemical Corp.) was used.

Rabbit muscle was used for all experiments described in this paper. Rabbits were given sodium pentobarbitol (90 mg) and d-tubocurarine (1.5 mg) prior to exsanguination. The back and leg muscles were excised immediately, immersed in ice and then trimmed free of fat and connective tissue. The trimmed muscles from a single rabbit (about 400–500 g) were pooled and a small sample of this pooled muscle ground in a meat grinder for preparation of at-death protein fractions. Other samples of the pooled muscle were stored at 2, 16 or 25° and sampled after varying periods of postmortem time. The time and temperature of postmortem storage will be specified in the individual experiments. To prevent bacterial growth, all stored muscle samples were wrapped in paper towels soaked in 10 mM sodium azide. Drying of the

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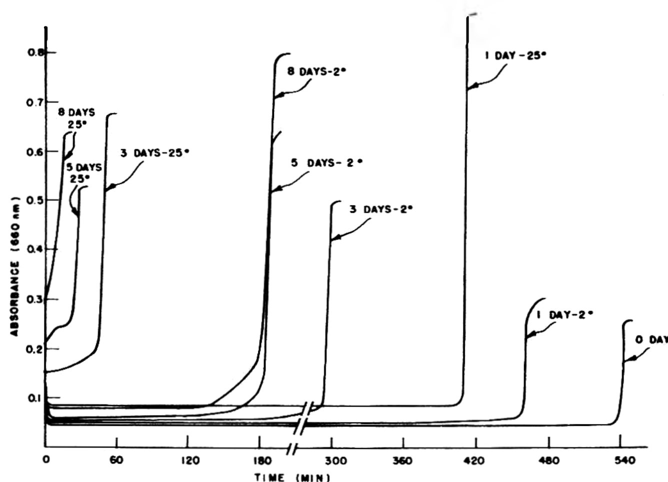

Fig. 1–Effect of postmortem storage on superprecipitation of rabbit myosin B at low Ca^{++} concentration. Conditions of superprecipitation assay: 100 mM KCl, 10 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM ATP, 0.4 mg myosin B/ml, 27°. Time and temperature of postmortem storage indicated.

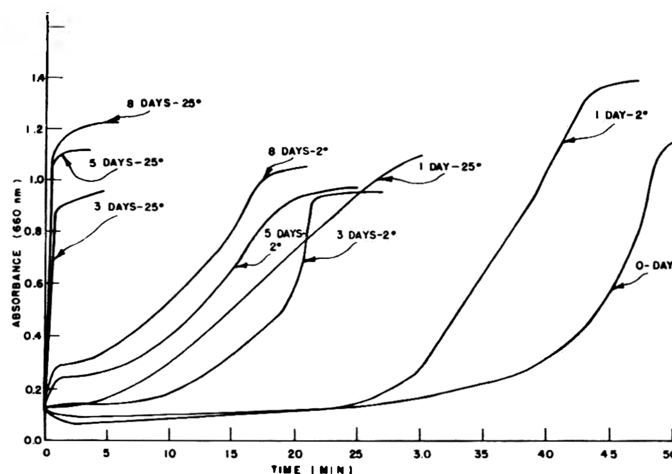

Fig. 2–Effect of postmortem storage on superprecipitation of rabbit myosin B in the presence of added Ca^{++}. Conditions of superprecipitation assay: 100 mM KCl, 10 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM ATP, 0.4 mg myosin B/ml, 27°. Time and temperature of postmortem storage indicated.

paper towels was prevented by an outer layer of Saran wrap. Under these conditions, no bacterial growth could be detected, even in muscle samples stored for 14 days at 25°.

Myosin and α-actinin-free actin were prepared according to the methods described by Arakawa et al. (1970b) and Seraydarian et al. (1967). Reconstituted actomyosin was made by mixing 1 part of actin with 2 parts of myosin by weight in 400 mM KCl and then washing as decribed by Arakawa et al. (1970b). Actomyosin was made within 1 hr after fresh actin and myosin became available and all experiments reported here were done within 5 days after actomyosin preparation.

Myosin B ("natural actomyosin") was made by suspending 10 g of ground muscle in 60 ml of Weber-Edsall solution (0.6 M KCl, 0.03 $KHCO_3$, 0.01 K_2CO_3) by using a Waring Blendor for 10 sec. This suspension was allowed to extract for 16–24 hr at 2° and then centrifuged at 15,000 × g for 20 min. The supernatant, containing the extracted myosin B together with sarcoplasmic proteins, was diluted to 150 mM KCl to precipitate the myosin B. The precipitate was collected by centrifugation at 15,000 × g for 10 min and dissolved in 1 M KCl. The volume was adjusted to a final KCl concentration of 0.5 M and the precipitation-dissolution cycle repeated twice. After the final precipitation, the myosin B was adjusted to 0.5 M KCl, dissolved by gentle magnetic stirring overnight, and then clarified by centrifugation at 15,000 × g for 20 min.

Crude α-actinin and tropomyosin-troponin-containing solutions were prepared by low ionic strength extraction of myofibrils as described previously (Arakawa et al., 1970b; Goll et al., 1970). Briefly, this procedure involves preparation of myofibrils, followed by washing the myofibrils with water to lower the ionic strength, and then extraction of the swollen myofibrils with 1–2 mM Tris · HCl at pH 8.5 and 2° for 64–72 hr. These crude extracts were fractionated between 0 and 30% (or in some cases between 15 and 25%) and between 30 and 75% (or in some cases between 40 and 75%) ammonium sulfate saturation to produce a crude α-actinin extract (the 0–30% or 15–25% fractions) and a crude tropomyosin-troponin extract (the 30–75% or 40–75% fractions). Hereafter, these crude extracts will be referred to as crude α-actinin (P_{0-30}), crude α-actinin (P_{15-25}), crude tropomyosin-troponin (P_{30-75}) or crude tropomyosin-troponin (P_{40-75}) fractions. Since it seemed likely that postmortem storage would result in some disruption of the myofibrillar structure and that this disruption might increase the extractability of α-actinin and the tropomyosin-troponin complex, the water washes of the myofibrils, beginning with the 1 mM EDTA wash (Step II in Arakawa et al., 1970b; or Step II in Goll et al., 1970), were saved and also fractionated with ammonium sulfate to produce crude α-actinin (P_{0-30}) wash and crude tropomyosin-troponin (P_{30-75}) wash fractions.

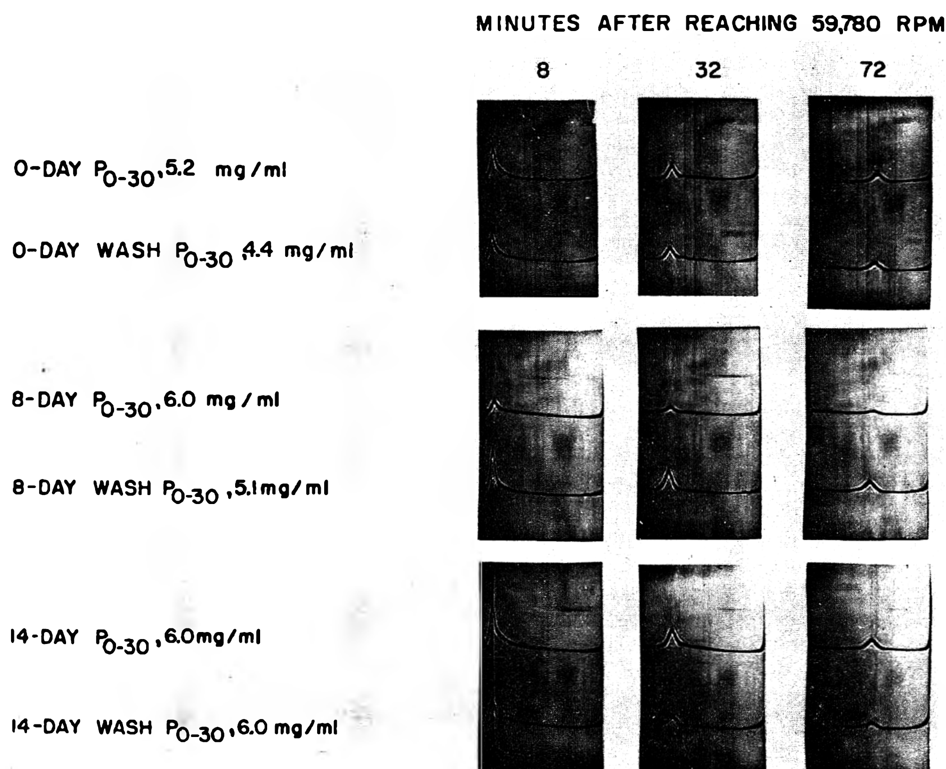

Fig. 3–Analytical ultracentrifuge diagrams of crude α-actinin (P_{0-30}) fractions prepared from postmortem rabbit muscle. All samples were dissolved in 100 mM KCl, 20 mM Tris-acetate, pH 7.5, at the concentrations indicated. Temperature of run = 20.0°, phase plate angle = 65°. Muscle stored at 25° for the times indicated.

Table 1–Effect of postmortem storage at 25° on the amount of protein extracted from rabbit myofibrils by water washing or by a 3-day, pH 8.5 extraction.[a]

Time of postmortem storage (hr)	mg Protein fraction/g of whole muscle			
	P_{0-30} (wash)	P_{0-30} (3-day)	P_{30-75} (wash)	P_{30-75} (3-day)
0	0.91	1.10	3.68	1.46
72	0.79	0.88	3.69	2.94
96	0.87	0.83	4.08	1.92
120	0.74	1.23	3.56	2.09
168	0.52	0.62	2.02	1.31
192	0.96	1.76	2.96	2.67
336	–	1.17	–	2.24

[a]See Materials & Methods section for a complete description of extraction procedure.

ATPase activities were measured according to the procedure of Goll and Robson (1967). The α-actinin and tropomyosin-troponin-containing fractions were premixed with the actomyosin preparations (either reconstituted actomyosin or myosin B) as described by Arakawa et al. (1970b). All assays were done at 25° and the inorganic phosphate released was measured by the method of Taussky and Shorr (1953). The electrolyte medium will be specified in the individual experiments.

The turbidity test as originally described by Ebashi (1961) was used as an in vitro assay of contraction. The conditions were those described by Arakawa et al. (1970b). The time required for initiation of turbidity was used as the measure of contractile activity rather than the amount of the absorbance increase. The reasons for this choice and some of the difficulties encountered in the turbidity assay of α-actinin and tropomyosin-troponin activity have been discussed by Arakawa et al. (1970b).

DEAE-cellulose chromatography of α-actinin-containing fractions was done according to the procedures described by Robson et al. (1970). "Cellex D," exchange capacity of 0.9 meq/g, was obtained from Bio-Rad Laboratories, Richmond, California, and washed according to the procedure described by Robson et al. (1970).

Analytical ultracentrifugation studies were conducted on a Spinco Model E ultracentrifuge using Kel-F centerpieces. Sedimentation coefficients were measured by using a Nikon Profile Projector. Peak size in schlieren diagrams of the α-actinin-containing fractions was estimated by projecting the peak on to the screen of the Nikon Profile Projector, tracing the projected peak on to a sheet of acetate paper, and then measuring the area under the traced peak with a compensating polar planimeter. The areas measured by this procedure were compared to areas under the schlieren diagrams of a sample of purified serum albumin, sedimented and measured under identical conditions. Since α-actinin and serum albumin have similar refractive indexes, and since the serum albumin sample is of known purity, comparison of areas under schlieren diagrams of the α-actinin fractions with those of serum albumin affords an estimate of the composition of the α-actinin fractions. Most of the α-actinin-containing samples sedimented primarily as one major boundary; therefore, Johnson-Ogston corrections were not necessary. The schlieren diagrams of the tropomyosin-troponin-containing fractions were usually hypersharp; consequently, measurement of peak areas was not done routinely on these fractions.

Protein concentrations were measured by the biuret test (Gornall et al., 1949) as modified by Robson et al. (1968).

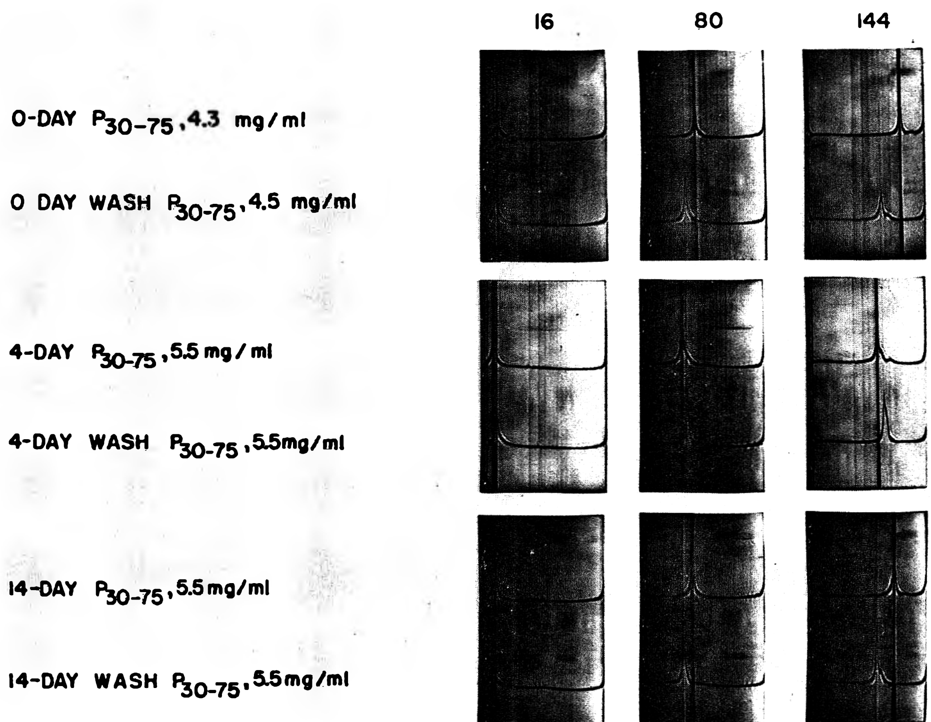

Fig. 4–Analytical ultracentrifuge diagrams of crude tropomyosin-troponin (P_{30-75}) fractions prepared from postmortem rabbit muscle. All samples were dissolved in 98 mM KCl, 20 mM Tris-acetate, pH 7.5, at the concentrations indicated. Temperature of the run = 20.0°, phase plate angle = 65°. Muscle was stored at 25° for the times indicated.

RESULTS

Postmortem changes in myosin B superprecipitation

The idea that postmortem changes in the actin-myosin interaction might originate from postmortem changes in α-actinin or troponin first clearly emerged when we observed that postmortem storage had large and dramatic effects on the turbidity response of myosin B suspensions. These effects are summarized in Figures 1 and 2. It is clear that the time required for onset of turbidity development in myosin B suspensions (which presumably corresponds to the time required for onset of contraction) is shortened drastically by postmortem storage of the muscle from which the myosin B is prepared. This increased rate of turbidity development is larger and occurs more rapidly post-mortem as a result of storage at 25° than it does as a result of storage at 2°. The decrease in time required for onset of turbidity occurs at very low Ca^{++} concentrations (Fig. 1), indicating that postmortem storage causes loss of the ability of the tropomyosin-troponin fraction to repress the actin-myosin interaction in the absence of Ca^{++}. However, the data in Figure 2 show that the increased rate of turbidity development in myosin B prepared from postmortem muscle occurs also in the presence of added Ca^{++}(0.05 mM).Thus, even after 8 days of storage at 25°, the rate of turbidity development is faster in the presence of added Ca^{++} than in the presence of 0.1 mM EGTA (about 10^{-8} M Ca^{++}) (cf. Fig. 1 and 2). This latter observation indicates that, besides its effect on the tropomyosin-troponin complex, postmortem storage has some positive, accelerating effect, independent of Ca^{++} concentration, on the rate of turbidity development. This positive, accel-

erating effect may originate from a postmortem-induced increase in activity of α-actinin, or from the presence of larger amounts of α-actinin in myosin B prepared from postmortem muscle than in myosin B prepared from at-death muscle. Consequently, our subsequent efforts were directed toward isolation of α-actinin and tropomyosin-troponin-containing fractions from postmortem muscle and assay of the activity of these fractions on reconstituted actomyosin or myosin B suspensions.

Yields of crude α-actinin (P_{0-30}) and crude tropomyosin-troponin (P_{30-75}) fractions

The effect of postmortem storage on yields of α-actinin and tropomyosin-troponin-containing fractions is shown in Table 1. There is no clear trend toward either more or less extraction of α-actinin or the tropomyosin-troponin complex with increasing time of postmortem storage. Neither is there any increase in the amount of α-actinin or tropomyosin-troponin complex extracted by the water washes in the preparation procedure. This latter result is unexpected, since it was supposed that postmortem disintegration of the myofibril would increase the extractability of α-actinin and the tropomyosin-troponin complex. Indeed, Fukazawa et al. (1970) have found that the amount of protein extractable by water at pH 7 from a washed muscle residue increased with increasing time of postmortem storage. They did not estimate the proportion of 6S α-actinin species in their water-soluble protein extracts, but careful measurements of peak areas in schlieren diagrams of our crude α-actinin (P_{0-30}) and crude α-actinin (P_{0-30}) wash fractions showed that the proportion of 6S α-actinin species in these fractions exhibited no obvious increasing or decreasing trend during postmortem storage (Table 2). Therefore, when the preparation procedure described by Arakawa et al. (1970b) is used, the extractability of α-actinin and the tropomyosin-troponin complex does not change during postmortem storage.

Sedimentation patterns of crude α-actinin (P_{0-30}) and crude tropomyosin-troponin (P_{30-75}) fractions

Schlieren diagrams from the analytical ultracentrifuge (Fig. 3 and 4) show that postmortem storage has no obvious effect on the sedimentation rate of α-actinin or the tropomyosin-troponin complex. Even after 14 days of postmortem storage at 25°, the crude α-actinin (P_{0-30}) and crude α-actinin (P_{0-30}) wash fractions sedimented primarily as 1 major boundary with an observed sedimentation coefficient of 6.0–6.1S (in 100 mM KCl, 20 mM Tris-acetate, pH 7.5) and were ultracentrifugally similar to corresponding fractions prepared from at-death muscle. In some crude α-actinin (P_{0-30}) samples, it was possible to detect traces of an 8.5S component in addition to the principal 6.1S component (cf. 0-day wash, Fig. 3). This 8.5S species probably is the dimeric form of phosphorylase, often found in crude α-actinin preparations (Arakawa et al., 1970b). The presence of the 8.5S component was not related to time of postmortem storage. In 2 or 3 instances, the crude α-actinin (P_{0-30}) or crude α-actinin (P_{0-30}) wash fraction was opalescent and cloudy, even after clarification at 27,000 rpm for 45 min. These particular fractions always contained a large proportion of rapidly sedimenting aggregates and a correspondingly lower proportion of the 6S α-actinin species (cf. the 8-day sample in Fig. 3). Evidently, in these samples a large amount of denatured actin had been solubilized by the extraction procedure. However, there was no obvious correlation between this abnormal solubilization of actin and time of postmortem storage.

Table 2–Percent of the 6S α-actinin species in P_{0-30} fractions prepared from at-death and postmortem muscle as measured by peak area in schlieren diagrams.[a]

Time post-mortem (days)	Fraction	
	P_{0-30}	P_{0-30} (wash)
0	30.2	29.8
3	37.2	35.5
4	30.2	41.5
5	31.9	63.3
8	33.7	40.5
14	30.5	18.7

[a]See Materials & Methods section for a description of extraction of P_{0-30} fractions.

Similarly, the sedimentation patterns of the crude tropomyosin-troponin (P_{30-75}) and crude tropomyosin-troponin (P_{30-75}) wash fractions prepared after 14 days of postmortem storage are nearly identical to those of the corresponding fractions prepared from at-death muscle. Most crude tropomyosin-troponin (P_{30-75}) fractions consisted primarily of a 4.0–4.6S component with traces of a 4.5–5.1S component also present. These sedimentation coefficients are characteristic of the tropomyosin-troponin complex (Hartshorne and Mueller, 1967; 1969). Sedimentation rate of the tropomyosin-troponin complex is very

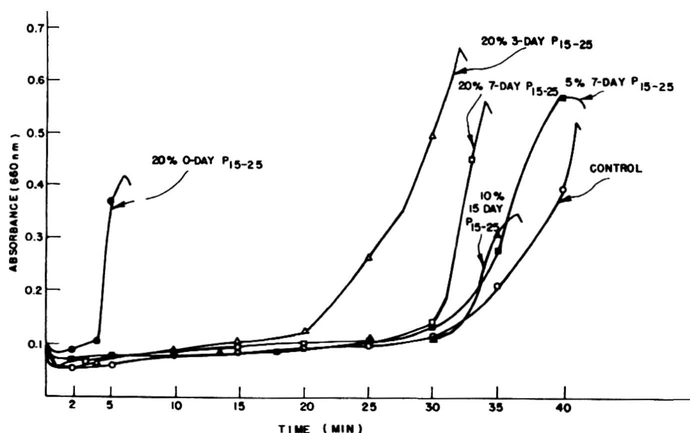

Fig. 5–Effect of crude α-actinin (P_{15-25}) fraction prepared from postmortem muscle on superprecipitation of reconstituted actomyosin. Conditions of superprecipitation assay: 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM ATP, 0.4 mg actomyosin/ml, the percent figures indicate amount of P_{15-25} added as percent of actomyosin, 27°. Muscle was stored at 25° for the times indicated.

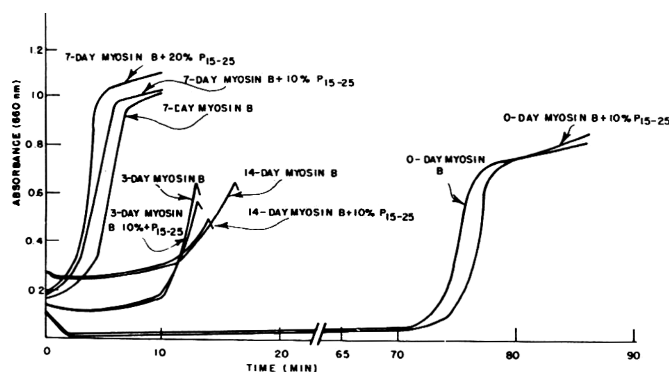

Fig. 6–Effect of a crude α-actinin (P_{15-25}) fraction on superprecipitation of myosin B prepared from postmortem rabbit muscle. Conditions of superprecipitation assay: 125 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 1 mM ATP, 0.4 mg myosin B/ml, the percent figures indicate amount of P_{15-25} added as percent of myosin B, 26°. Muscle for myosin B preparation was stored at 16° for the times indicated; P_{15-25} fraction was prepared from at-death muscle.

Table 3—Effect of α-actinin and tropomyosin-troponin fractions prepared from postmortem muscle on the ATPase activity of reconstituted actomyosin.

Fraction[a]	Quantity of fraction added as percent of actomyosin 0	5	10	20
P_{15-25}, 0 day	0.114[b]	0.140	0.140	0.147
P_{15-25}, 7 day	0.114	0.140	0.158	0.160
P_{40-75}, 0 day	0.266	–	0.220	0.166
P_{40-75}, 3 day	0.266	–	0.208	0.176
P_{40-75}, 7 day	0.266	–	0.195	0.164

[a] P_{15-25} is an α-actinin-containing fraction, P_{40-75} is a tropomyosin-troponin-containing fraction; "days" refers to number of days of postmortem storage at 25° before extraction of the P_{15-25} and P_{40-75} fractions.

[b] Figures are μmoles Pi/mg actomyosin/min. Conditions of assay for P_{15-25} fraction: 1 mM $MgCl_2$, 1 mM ATP, 0.05 mM $CaCl_2$, 100 mM KCl, 0.4 mg actomyosin/ml, 25.0°; for P_{40-75} fraction: 1 mM $MgCl_2$, 1 mM ATP, 0.05 mM EGTA, 67 mM KCl, 0.4 mg actomyosin/ml, 25.0°.

Table 4—Effects of α-actinin and tropomyosin-troponin fractions prepared from postmortem rabbit muscle on the ATPase activity of myosin B also prepared from postmortem rabbit muscle.[a]

Modifiers	Time of postmortem storage at 16° before myosin B preparation (days) 0	3	4	7
1 mM Mg^{++}, 125 mM KCl[b]	0.038[e]	0.105	–	0.152
1 mM Mg^{++}, 125 mM KCl, 10% P_{0-30} from 0-day muscle[b]	0.040	0.110	–	0.166
1 mM Mg^{++}, 125 mM KCl, 20% P_{0-30} from 0-day muscle[b]	0.040	0.106	–	0.180
1 mM Mg^{++}, 0.05 mM Ca^{++}, 75 mM KCl[c]	0.217	–	0.322	–
1 mM Mg^{++}, 0.05 mM EGTA, 75 mM KCl[d]	0.078	–	0.164	–
1 mM Mg^{++}, 0.05 mM EGTA, 75 mM KCl, 40% P_{30-75} from 0-day muscle[d]	0.055	–	0.070	–
1 mM Mg^{++}, 0.05 mM EGTA, 75 mM KCl, 40% P_{30-75} from 8-day muscle[d]	–	–	0.091	–

[a] Immediately after death, the excised muscle was wrapped in towels soaked in 10 mM sodium azide. Muscle for myosin B preparation was stored at 16° for the designated time. Muscle for α-actinin (P_{0-30}) or tropomyosin-troponin (P_{30-75}) preparation was stored at 25° for the designated time.

[b] Conditions for ATPase assay: 125 mM KCl, 1 mM Mg^{++}, 0.05 mM Ca^{++}, 0.2 mg myosin B/ml, 1 mM ATP, 25.0°, P_{0-30} indicated as percent of myosin B present.

[c] Conditions for ATPase assay: 75 mM KCl, 1 mM Mg^{++}, 0.05 mM Ca^{++}, 0.2 mg myosin B/ml, 1 mM ATP, 25.0°.

[d] Conditions for ATPase assay: 75 mM KCl, 1 mM Mg^{++}, 0.05 mM EGTA, 0.2 mg myosin B/ml, 1 mM ATP, 25.0°, P_{30-75} indicated as percent of myosin B present.

[e] Figures are μmoles Pi/mg protein/min.

concentration dependent; this accounts for the wide range of observed sedimentation coefficients found in our experiments. Occasionally, the crude tropomyosin-troponin (P_{30-75}) and crude tropomyosin-troponin (P_{30-75}) wash fractions would contain large amounts of a 3.2–3.8S component (cf. the 0-day wash and 14-day wash samples in Fig. 4). Evidently, such preparations consisted of a tropomyosin-troponin complex containing an excess of either tropomyosin or troponin (probably the former). The occurrence of such preparations was not related in any way to length of postmorten storage, but did appear more frequently in the wash preparations than in preparations from the 3-day extracts.

Effect of crude α-actinin fractions on reconstituted actomyosin or myosin B suspensions

The effect of post-mortem storage on the ability of a crude α-actinin (P_{15-25}) fraction to accelerate the turbidity response of a reconstituted actomyosin is shown in Figure 5. α-Actinin activity of this fraction decreases with increasing time of postmortem storage, but it is obvious that even after 15 days at 25°, the crude α-actinin (P_{15-25}) fraction still accelerates the turbidity response of reconstituted actomyosin suspensions. In the ATPase test, the crude α-actinin (P_{15-25}) fraction retains almost all its ability to increase the ATPase activity of reconstituted actomyosin suspensions, even after 7 days post-mortem at 25° (Table 3). These results are clearly contrary to the hypothesis that postmortem storage may increase the activity of α-actinin in the ATPase and turbidity tests.

Although it is a relatively straightforward procedure to show that α-actinin activity decreases during postmortem storage, it is considerably more difficult to ascertain whether myosin B prepared from postmortem muscle contains more α-actinin than myosin B prepared from at-death muscle. This difficulty is due to several reasons: 1) It is technically difficult to extract and quantitatively measure with any degree of precision how much α-actinin is in myosin B. 2) It is difficult to prove that a given procedure is extracting all the α-actinin in a myosin B preparation. 3) It is presently impossible to tell whether all the α-actinin in a myosin B preparation is combined with actin in a manner that allows it to accelerate the turbidity response or the ATPase activity of the myosin B. We term such a combination an "effective combination." Because of these difficulties, the best method of measuring how much α-actinin is "effectively" present in a myosin B preparation is to test the response of myosin B preparations to addition of at-death α-actinin. If 1 myosin B preparation has less effective (or "active") α-actinin than another, then addition of α-actinin to the first myosin B should cause a larger response than addition of α-actinin to the second. Results of such experiments are shown in Figure 6 and Table 4. These results clearly show that addition of α-actinin to myosin B has no or very little effect on either rate of turbidity development or ATPase activity of the myosin B, regardless of whether the myosin B was prepared from muscle immediately after death or from muscle after 14 days of postmortem storage at 25°. In fact, if there is any response at all to added α-actinin, it appears that myosin B prepared from muscle after 7 days of postmortem storage has less α-actinin than myosin B prepared from muscle immediately after death. Thus, within the inherent limitations of this experimental test, it appears that myosin B prepared from postmortem muscle does not contain any more effective α-actinin than myosin B prepared from at-death muscle.

DEAE-cellulose chromatography of crude α-actinin (P_{0-30}) fractions

Since the preceding experiments indicated that α-actinin undergoes some loss in activity during postmortem storage, a few preliminary attempts were made to ascertain the nature of the changes in α-actinin that might be responsible for this loss in activity. Robson et al. (1970) have shown that crude α-actinin (P_{0-30}) elutes with a characteristic profile from

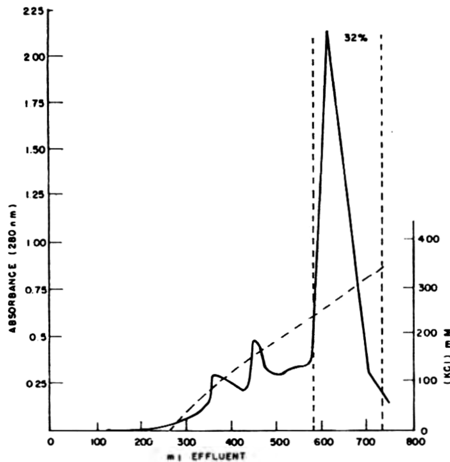

Fig. 7–Elution profile of crude α-actinin (P_{0-30}) prepared from at-death rabbit muscle from a 2.5- by 25-cm DEAE-cellulose column. Elution buffer: 25 mM Tris-acetate, pH 7.5, with a KCl gradient. The 6S α-actinin species eluted in the peak between the vertical dotted lines. 400 mg P_{0-30} was applied to the column and 32% of this protein eluted between the vertical dotted lines.

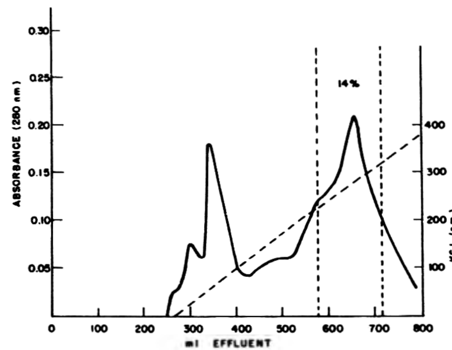

Fig. 8–Elution profile of crude α-actinin (P_{0-30}) prepared from 3-day, 25° rabbit muscle from a 2.5- by 25-cm DEAE-cellulose column. Elution buffer: 25 mM Tris-acetate, pH 7.5, with a KCl gradient. The 6S α-actinin species eluted in the peak between the vertical dotted lines. 108 mg of 3-day P_{0-30} was applied to the column and 14% of this protein eluted between the vertical dotted lines.

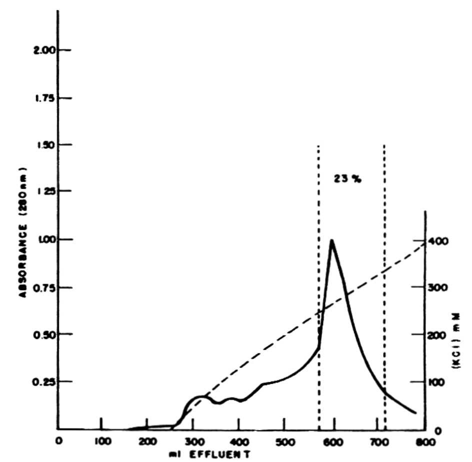

Fig. 9–Elution profile of crude α-actinin (P_{0-30}) prepared from 7-day, 25° rabbit muscle from a 2.5- by 25-cm DEAE-cellulose column. Elution buffer: 25 mM Tris-acetate, pH 7.5, with a KCl gradient. The 6S α-actinin species eluted in the peak between the vertical dotted lines. 313 mg of 7-day P_{0-30} was applied to the column and 23% of this protein eluted between the vertical dotted lines.

DEAE-cellulose columns, so the effect of postmortem storage on the elution profiles of crude α-actinin (P_{0-30}) fractions from DEAE-cellulose columns was examined (Fig. 7, 8 and 9). Postmortem storage causes little change in elution patterns of crude α-actinin (P_{0-30}) from DEAE-cellulose columns, although in some instances a larger proportion of protein in the crude α-actinin (P_{0-30}) fraction prepared from postmortem muscle eluted at low KCl concentrations, preceding the 6S, α-actinin-containing peak (cf. Fig. 7 and 8). However, even after 7 days of postmortem storage at 25°, the 6S α-actinin species eluted between 250 and 310 mM KCl, the same KCl concentration required to elute the 6S α-actinin species prepared from at-death muscle (Fig. 9).

Since Robson et al. (1970) have also shown that DEAE-cellulose chromatography is a very effective means for purifying the 6S α-actinin species from crude α-actinin (P_{0-30}) fractions, DEAE-purified α-actinin prepared from muscle after 7 days of postmortem storage at 25° was tested in the turbidity assay to determine whether purified α-actinin from postmortem muscle would be as active as purified α-actinin prepared from at-death muscle. Results shown in Figure 10 indicate that even after DEAE-purification, α-actinin prepared from muscle after 7 days of postmortem storage is not as active in the turbidity assay as α-actinin

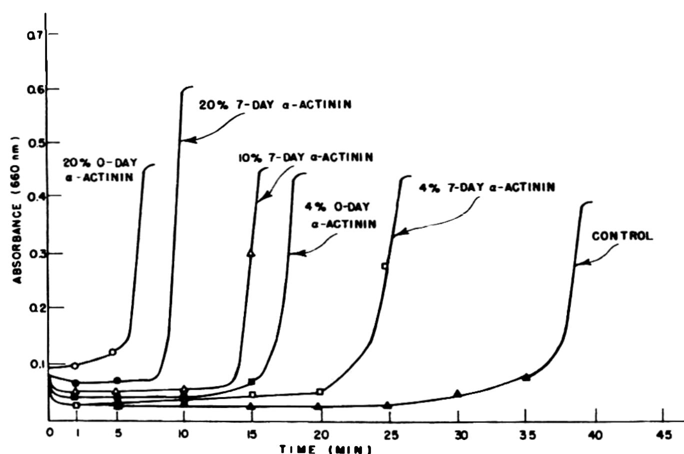

Fig. 10–Effect of α-actinin prepared from 7-day, 25° rabbit muscle and purified by DEAE-cellulose chromatography on superprecipitation of reconstituted actomyosin. Conditions of superprecipitation assay: 100 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.05 mM $CaCl_2$, 0.4 mg actomyosin/ml, α-actinin indicated as percent of actomyosin, 27°.

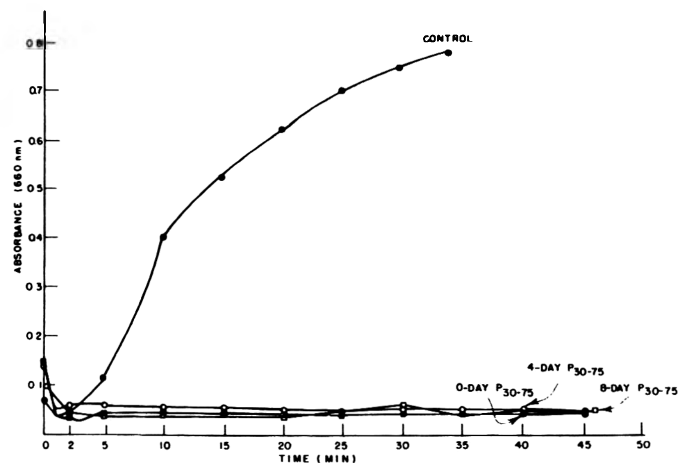

Fig. 11–Effect of a crude tropomyosin-troponin (P_{30-75}) fraction prepared from postmortem muscle on superprecipitation of reconstituted actomyosin. Conditions of superprecipitation assay: 75 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM ATP, 0.4 mg actomyosin/ml, 0.16 mg P_{30-75} when added, 25.0°. Muscle was stored at 25° for the times indicated.

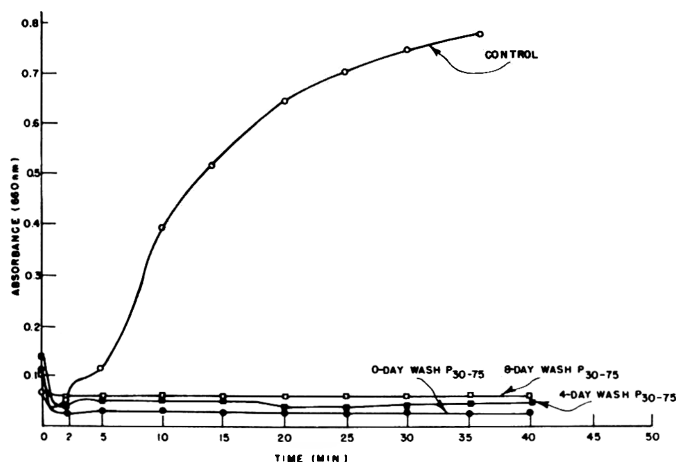

Fig. 12–Effect of a crude tropomyosin-troponin (P_{30-75}) fraction prepared from washes of myofibrils from postmortem muscle on superprecipitation of reconstituted actomyosin. Conditions of superprecipitation assay: 75 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.1 mM EGTA, 1 mM ATP, 0.4 mg actomyosin/ml, 0.16 mg P_{30-75} wash when added, 25.0°. Muscle was stored at 25° for the times indicated.

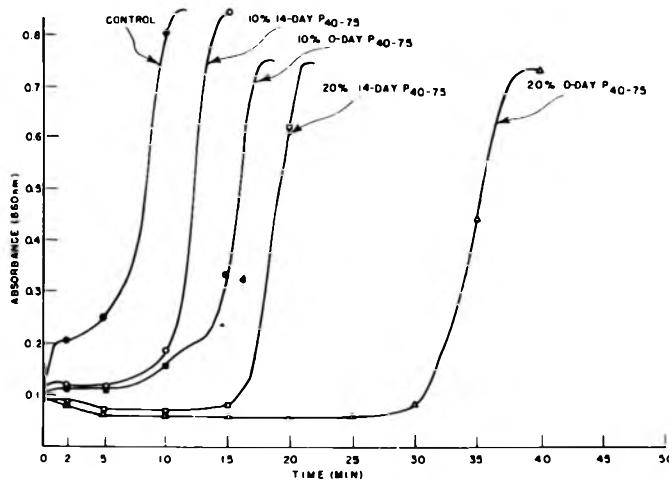

Fig. 13–Effect of crude tropomyosin-troponin (P_{40-75}) fraction prepared from 14-day, 25° muscle on superprecipitation of reconstituted actomyosin. Conditions of superprecipitation assay: 67 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 0.05 mM EGTA, 1 mM ATP, 0.4 mg actomyosin/ml, the percent figures indicate amount of P_{40-75} added as percent of actomyosin, 27°. Muscle was stored at 25° for the times indicated.

prepared from at-death muscle, although DEAE-cellulose purification has decreased the difference between at-death α-actinin and the 7-day α-actinin slightly (cf. Fig. 3 and 10). Thus, the postmortem decrease in α-actinin activity persists, even after purification by DEAE-cellulose chromatography.

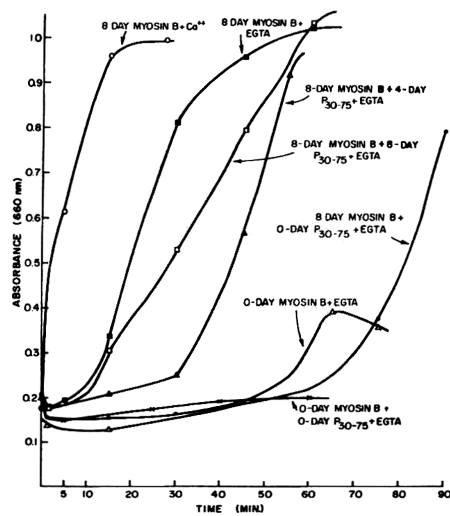

Fig. 14–Effect of a crude tropomyosin-troponin (P_{30-75}) fraction prepared from postmortem muscle on superprecipitation of myosin B prepared from postmortem muscle. Conditions of superprecipitation assay: 75 mM KCl, 20 mM Tris-acetate, pH 7.0, 1 mM $MgCl_2$, 1 mM ATP, 0.4 mg myosin B/ml, 0.16 mg P_{30-75} added when present, 25.0°. In addition to the preceding, the assay marked Ca^{++} contained 0.05 mM $CaCl_2$; assays marked EGTA contained 0.1 mM EGTA. Muscle for myosin B preparation was stored at 16° for the times indicated; muscle for P_{30-75} preparation was stored at 25°.

Effect of crude tropomyosin-troponin fractions on reconstituted actomyosin or myosin B suspensions

The effect of postmortem storage on activity of the crude tropomyosin-troponin (P_{30-75}) and crude tropomyosin-troponin (P_{30-75}) wash fractions is shown in Figures 11 and 12. Under the experimental conditions used in Figures 11 and 12, postmortem aging had no effect on ability of the tropomyosin-troponin complex to inhibit or repress the turbidity response of reconstituted actomyosin suspensions in the absence of Ca^{++}. If, on the other hand, the KCl concentration was lowered slightly from 75 to 67 mM, it was possible to demonstrate that the tropomyosin-troponin complex lost part of its repressor activity after long periods of postmortem storage (Fig. 13 and Goll et al., 1970). However, even after 14 days of postmortem storage, the tropomyosin-troponin complex still clearly possesses some ability to inhibit or repress the turbidity response of actomyosin suspensions (Fig. 13). Moreover, ability of the tropomyosin-troponin complex to inhibit or decrease the ATPase activity of reconstituted actomyosin remains unchanged after 7 days postmortem at 25°, even at 67 mM KCl (Table 3).

These results clearly show that the tropomyosin-troponin complex itself does not undergo extensive degradation during postmortem storage, even though myosin B prepared from postmortem muscle does not exhibit the Ca^{++}-sensitivity characteristically induced by the tropomyosin-troponin complex (cf. Fig. 1). Again, for the same reasons listed in connection with the α-actinin assays discussed in the preceding sections, it is very difficult to determine whether myosin B prepared from postmortem muscle contains less effective tropomyosin-troponin than myosin B prepared from muscle immediately after death. Experiments in which the tropomyosin-troponin complex, prepared from either at-death or postmortem muscle, was added to myosin B that had been prepared from postmortem muscle (Fig. 14 and Table 4) gave variable results. In almost all cases, addition of tropomyosin-troponin prepared from muscle immediately after death to myosin B prepared from postmortem muscle restored Ca^{++}-sensitivity to the postmortem myosin B (Fig. 14 and Table 4). However, addition of tropomyosin-troponin prepared from postmortem muscle to myosin B prepared from postmortem muscle often did not completely restore Ca^{++}-sensitivity to these postmortem myosin B preparations. This loss of ability to restore Ca^{++}-sensitivity was more noticeable if both the myosin B and tropomyosin-troponin were prepared from muscle stored for 4 days or longer post-mortem (cf. 8-day tropomyosin-troponin plus 8-day myosin B, Fig. 14).

These conclusions are substantiated by the ATPase results shown in Table 4. The 1 mM Mg^{++}, 0.05 mM Ca^{++}-modified ATPase activity increases by 48% between 0 and 4 days post-mortem, confirming the earlier report by Robson (Robson et al., 1967) that the ATPase activity of myosin B increases during postmortem storage. The 1 mM Mg^{++}, 0.05 mM EGTA-modified ATPase increases by 110% during the first 4 days

post-mortem, indicating that much of the Ca^{++}-sensitivity of myosin B is lost during the first 4 days post-mortem. However, addition of a crude tropomyosin-troponin (P_{30-75}) fraction prepared from 8-day muscle is not as effective in restoring Ca^{++}-sensitivity to the 4-day myosin B as is addition of crude tropomyosin-troponin (P_{30-75}) fraction prepared from at-death muscle. Thus, both the ATPase and turbidity tests suggest that postmortem storage causes subtle modifications in both actin and the tropomyosin-troponin complex; these modifications evidently cause some weakening of the interaction between actin and the tropomyosin-troponin complex. Weakening of the actin-tropomyosin-troponin interaction probably results in partial solubilization and loss of the tropomyosin-troponin complex during preparation of myosin B and may thereby account for loss of Ca^{++}-sensitivity in myosin B prepared from postmortem muscle.

DISCUSSION

RESULTS of this study add to the rapidly accumulating evidence that the actin-myosin interaction is modified during postmortem storage of muscle in situ. Although the nature of this modification remains unclear, its existence has now been detected because of at least 4 independent effects that it causes. Thus, Arakawa, Fujimaki and coworkers (Fujimaki et al., 1965b; 1965c) have reported that both the Mg^{++}- and Ca^{++}-modified ATPase activities of myosin B extracted from rabbit muscle after 2 days of postmortem storage at 4° were 15–25% higher than the corresponding activities of myosin B extracted from at-death muscle. This finding was subsequently extended to bovine muscle, to a number of other modifiers and to a second substrate by Robson (Goll and Robson, 1967; Robson et al., 1967). Soon afterward, Galloway and Goll (1967) found that the Mg^{++}- and Ca^{++}-modified ATPase and ITPase activities of porcine myofibrils also exhibited a postmortem increase in specific activity. This suggests that the postmortem increase in nucleoside triphosphatase activity is a universal feature, at least among mammalian species.

Almost simultaneously with their finding that postmortem storage caused an increase in the Mg^{++}- and Ca^{++}-modified ATPase activities of myosin B, Arakawa and Fujimaki (Fujimaki, 1965a; 1965b) reported that myosin B prepared from postmortem muscle exhibited an increased sensitivity to dissociation by ATP. Thus, myosin B prepared from at-death muscle and dissolved in 0.5 M KCl at pH 7.0 requires 0.6 mM ATP before it is fully dissociated; whereas, myosin B prepared from muscle after 7 days of postmortem storage at 2° requires only 0.1 mM ATP for complete dissociation.

A third line of evidence suggesting that the actin-myosin interaction is modified during postmortem storage was provided by independent and simultaneous investigations on bovine (Gothard et al., 1966; Stromer et al., 1967) and chicken muscle (Takahashi et al., 1967) showing that rigor-shortened sarcomeres lengthen during prolonged postmortem storage. Since this lengthening occurs in the absence of ATP, it suggests that some weakening of the actin-myosin interaction occurs during postmortem storage (Goll, 1968) and that this weakening then allows some slippage at the points where the myosin cross-bridges interact with actin.

Results of our present study now show that the postmortem increase in nucleoside triphosphatase activity is paralleled by a postmortem increase in the rate of superprecipitation. However, the postmortem increase in rate of superprecipitation is much larger than the 25–85% increase that Robson (Goll and Robson, 1967; Robson et al., 1967) observed in the nucleoside triphosphatase activity of actomyosin from postmortem muscle. This circumstance, together with the ease and convenience of performing the turbidity test, should make the turbidity method a valuable assay for study of the nature of the postmortem changes that cause modification of the actin-myosin interaction.

Although this study has provided a fourth line of evidence that the actin-myosin interaction is modified during postmortem storage, we have been unable to provide a full explanation of the changes that cause this modification. Our results indicate that during postmortem storage, α-actinin gradually loses its ability to accelerate ATPase activity and the turbidity response, a finding directly contrary to that expected if α-actinin were responsible for the increased ATPase activity and rate of superprecipitation of myosin B prepared from postmortem muscle. Moreover, the tropomyosin-troponin complex is not extensively degraded during postmortem storage, even though there does appear to be some weakening of the interaction between this complex and actin. Isolation of intact and functionally active tropomyosin-troponin from postmortem muscle adds to the already abundant evidence that there is no extensive proteolysis in postmortem muscle. This question has been discussed more completely by Goll et al. (1970).

Results of this study, therefore, indicate rather strongly that modifications in α-actinin or the tropomyosin-troponin complex are not primarily responsible for postmortem modification in the actin-myosin interaction, even though α-actinin and the tropomyosin-troponin complex themselves do undergo some changes during postmortem storage. The cause of these postmortem changes in α-actinin will be the subject of a subsequent paper (Arakawa et al., 1970a). Levy and Ryan (1965) and others (Maruyama and Ishikawa, 1964; Tonomura and Yoshimura, 1960) have shown that subtle modifications of sulfhydryl groups in actomyosin will cause a marked increase in the rate of turbidity response as well as make the turbidity response insensitive to Ca^{++}. Moreover, it has been shown by many investigators (Blum, 1962; Morales and Hotta, 1960; Sekine and Yamaguchi, 1966) that blocking of part of the sulfhydryl groups of actomyosin will cause its nucleoside triphosphatase activity to increase. Therefore, it now seems possible that postmortem modification of the actin-myosin interaction is a consequence of changes in the sulfhydryl groups of actin or myosin or both. This possibility is now under investigation in our laboratory.

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Ms. received 1/21/70; revised 6/13/70; accepted 7/14/70.

Journal Paper No. J-6460 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project 1549.

This investigation was supported in part by Public Health Service Training Grant UI-01043-03 from the National Center for Urban and Industrial Health.

We are indebted to Jean Preston and Nona Starr for technical assistance, to Richard M. Robson for helpful discussions and to Karen Schwarz for assistance with the manuscript. We thank Dr. T. Fukazawa for discussing some of his results with us prior to their publication. During this investigation, Dr. Arakawa was the recipient of a visiting professorship from Training Grant UI-01043-03, National Center for Urban and Industrial Health, U.S. Public Health Service.

Abbreviations used in this paper: ATP, adenosine 5′-triphosphate; ATPase, adenosine triphosphatase; EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane; ITP, inosine 5′-triphosphate; ITPase, inosinetriphosphatase; myosin B, natural actomyosin extracted directly from muscle by high ionic strength salt solution; Pi, inorganic phosphate; Tris, tris-(hydroxymethyl)-aminomethane.

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MOLECULAR PROPERTIES OF POST-MORTEM MUSCLE. 9. Effect of Temperature and pH on Tropomyosin-Troponin and Purified α-Actinin from Rabbit Muscle

SUMMARY—A study was done on the effects of in vitro storage of purified α-actinin, troponin, tropomyosin, and the tropomyosin-troponin complex on the activity of these protein fractions in the ATPase and superprecipitation assays. Storage was done at various combinations of temperatures between 0 and 40°C and pH values between 5.7 and 7.0. Even after 40 hr of storage, activities of purified tropomyosin and the tropomyosin-troponin complex were not affected by any combination of temperature and pH included in this study, but activities of purified α-actinin and troponin were almost completely lost after 16 hr at 40°C and pH 5.7. Storage for 40 hr at low pH (5.7) and low temperatures (0°C) did not affect the activity of either α-actinin or troponin, but 40 hr of storage at high temperatures (40°C) and neutral pH caused some loss in activity for both these proteins. This loss of activity caused by 40°C, pH 7.0 storage was much more noticeable in the case of troponin than in the case of α-actinin. Storage periods of 40 hr or longer were required before any loss of α-actinin activity could be detected at pH 7.0 and 40°C. Since most meat animal carcasses are chilled soon after exsanguination and attain muscle temperatures of 25°C or lower before the pH falls below 6.2, it is probable that α-actinin and tropomyosin-troponin activity remain almost unchanged in meat handled through normal market channels. However, myofibrillar tissue in those porcine animals whose musculature undergoes a very rapid post-mortem decline in pH so that values of 5.7 or less are reached while muscle temperatures are still 37°C or higher may lose much of its α-actinin and tropomyosin-troponin activity during the first 24 hr post-mortem.

INTRODUCTION

IN THE PREVIOUS paper in this series (Arakawa et al., 1970a), we showed that the ability of α-actinin and tropomyosin-troponin fractions to modify the ATPase activity and turbidity response of reconstituted actomyosin suspensions decreased slightly with increasing time of post-mortem storage. However, the cause of this slight decrease in activity was not ascertained. α-actinin is not particularly labile to proteolysis by trypsin (Ebashi and Ebashi, 1965; Goll et al., 1969), and more-over, the sedimentation patterns of both α-actinin and tropomyosin-troponin fractions did not change during post-mortem storage (Arakawa et al., 1970a). Therefore, it seemed unlikely that the slight post-mortem loss in activity of these two protein fractions was due to their destruction by catheptic proteolysis. Post-mortem muscle undergoes a variable decline in pH, and it seemed possible that lowered pH may contribute to the slight loss in α-actinin and tropomyosin-troponin activity in post-mortem muscle. Consequently, we designed a series of experiments in which tropomyosin-troponin-containing fractions and purified α-actinin fractions were stored at varying pH values and temperatures to ascertain whether pH and temperature had any effect on α-actinin and tropomyosin-troponin activity. The results show that a combination of low (less than 5.8) pH and high (above 30°C) temperature lowers the activity of α-actinin and troponin.

Abbreviations

Abbreviations used are: ATP, adenosine 5′-triphosphate; ATPase, adenosine triphosphatase; EGTA, 1,2-bis-(2-dicarboxymethylaminoethoxy)-ethane; Pi, inorganic phosphate; Tris, tris-(hydroxymethyl)-aminomethane.

MATERIALS & METHODS

RABBIT MUSCLE was used for all experiments described in this paper. Exsanguination of the rabbits and handling of the muscles have been described (Arakawa et al., 1970b).

The preparation of α-actinin-free actin, purified myosin, reconstituted actomyosin, and crude α-actinin and tropomyosin-troponin-containing fractions have been described in preceding papers (Arakawa et al., 1970a, 1970b). All α-actinin preparations used in this study were purified by double DEAE-chromatography according to the procedure of Robson et al. (1970), and as judged by analytical ultracentrifugation, 80–90% of their protein consisted of the 6S α-actinin species.

Partially purified preparations of troponin and tropomyosin were made by a modification of Ebashi's (Ebashi et al., 1968) procedure. The P_{30-75} tropomyosin-troponin complex, prepared according to Arakawa et al. (1970b), was dialyzed against two to four changes of 1mM $KHCO_3$ for 16–30 hr. The volume of this dialyzed solution was measured and enough 3M KCl was added to make the final solution 1M KCl. This solution was clarified at 15,000 × *g* for 20 min, and the pH of the supernatant adjusted to 4.6 by addition of 1N HCl. The solution was carefully maintained at pH 4.6 and 0°C for 20 min (more 1N HCl added if necessary), and then centrifuged at 15,000 × *g* for 10 min. The precipitate (partially purified tropomyosin) was dissolved in 1M KCl at pH 7.2–7.7 (add 1N KOH) by gentle magnetic stirring for 45 min at 2°C. The resulting solution was clarified at 15,000 × *g* for 20 min, and the supernatant again subjected to isoelectric precipitation at pH 4.6 by adding 1N HCl. The suspension was kept at pH 4.6 and 0°C for 15 min, and then centrifuged at 15,000 × *g* for 10 min. The supernatant from this second isoelectric precipitation was added to the supernatant from the first isoelectric precipitation, and the pH of the combined supernatants adjusted to

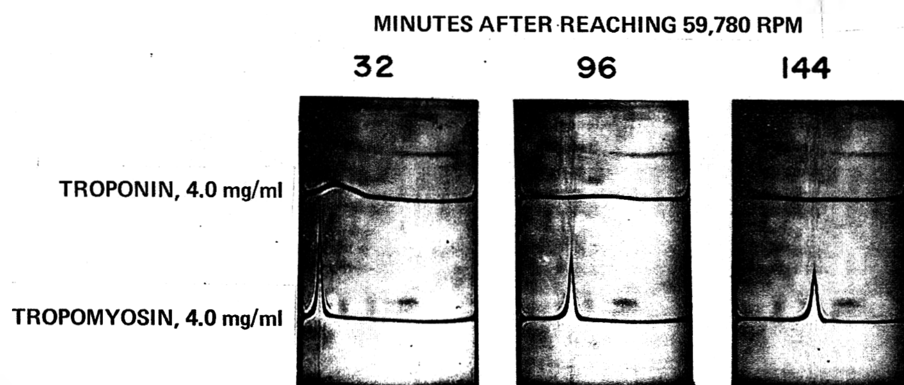

Fig. 1—Analytical ultracentrifuge diagrams of purified tropomyosin and troponin. Both samples were dissolved in 100mM KCl, 20mM Tris-acetate, pH 7.5, at the concentrations indicated. Temperature of the run = 20.0°C; phase plate angle = 65°C.

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7.5 by addition of 1N KOH. The combined supernatants were salted out at 60% ammonium sulfate saturation, the precipitate collected by centrifugation at 15,000 × *g* for 10 min, dissolved in 1mM $KHCO_3$, 0.05% 2-mercaptoethanol, and dialyzed for 48–60 hr against four to six changes of 1mM $KHCO_3$. The dialyzed solution was clarified at 27,000 rpm for 45 min (Spinco 30 rotor) to yield a partially purified troponin preparation. Although this troponin preparation appeared slightly heterogeneous in the analytical ultracentrifuge (Fig. 1), it is likely that this heterogeneity is at least partly due to variations in the subunit composition of troponin (Hartshorne et al., 1969). As judged by both ATPase and turbidity assays, troponin preparations made according to this procedure had no Ca^{++}-sensitizing activity on reconstituted actomyosin suspensions but did confer Ca^{++}-sensitivity when added to reconstituted actomyosin in the presence of purified tropomyosin; this indicates that these preparations did not contain any tropomyosin.

The precipitate from the second isoelectric precipitation was used to prepare purified tropomyosin. The precipitate was dissolved in 1mM $KHCO_3$, 0.05% 2-mercaptoethanol at pH 7.2–7.7 by gentle magnetic stirring for 45 min at 2°C, and then dialyzed for 12–24 hr against two to three changes of 1mM $KHCO_3$. The volume of the solution was measured and 32g of ammonium sulfate and 32.6 mg of potassium carbonate added for every 100 ml of supernatant. After setting at 0°C for 20 min, the tropomyosin-troponin precipitate was removed by centrifugation at 15,000 × *g* for 15 min. Four additional grams of ammonium sulfate were added for every 100 ml of the supernatant, and this suspension was allowed to set at 0°C for 60–90 min. The tropomyosin precipitate was collected by centrifugation at 15,000 × *g* for 45 min and was then dissolved in 100mM KCl, dialyzed for 48–60 hr against four to six changes of 100mM KCl, 1mM $KHCO_3$, and finally clarified at 27,000 rpm for 45 min (Spinco 30 rotor). The resulting tropomyosin solution sedimented as a single hypersharp boundary in the analytical ultracentrifuge (Fig. 1), and exhibited no or only slight Ca^{++}-sensitizing activity on reconstituted actomyosin suspensions; this indicates that these tropomyosin preparations did not contain troponin.

In these experiments described in this paper, protein solutions were incubated at the specified pH values and temperatures for a given period of time, then placed at 0°C, and the pH adjusted to 7.0 (if necessary). ATPase and turbidity assays of activity were done within 24 hr after termination of the time-temperature treatment.

The procedure used for ATPase and turbidity assays have already been described (Arakawa et al., 1970b). The electrolyte medium for the ATPase and turbidity assays will be specified in the individual experiments. Protein concentrations were measured by the biuret method (Gornall et al., 1949) as modified by Robson et al. (1968).

RESULTS

Effect of storage temperature and pH on purified α-actinin

The data in Table 1 and in Figures 2 and 3 clearly show that the ability of purified α-actinin to increase either the Mg^{++}-modified ATPase activity or the rate of turbidity response of actomyosin suspensions is lost after 16 hr at 40°C and pH 5.7. Storage for 16 hr at 40°C and pH 7.0, or at 25°C and pH 5.7, however, does not affect the activity of purified α-actinin in either the ATPase or the turbidity assay. After 40 hr, the α-actinin stored at pH 5.7 and 40°C appears to actually inhibit the turbidity response of actomyosin suspensions (Fig. 3); this inhibition is also evident in the ATPase test (Table 1). Storage for 40 hr at pH 7.0 and 40°C reduces but does not completely remove the activity of α-actinin in the turbidity test (Fig. 3). This latter effect, however, is not evident in the ATPase test for α-actinin activity. No other combination of time and temperature of storage used in this study had any effect on the activity of α-actinin in either the ATPase or the turbidity assays. Therefore, a combination of low pH (less than 6.0) and high temperature (greater than 35°C) is most effective in causing

Table 1–Effect of α-actinin stored at different temperatures and pH values on ATPase activity of reconstituted actomyosin.[a]

Sample	Time of storage (hr)	
	16	40
Control actomyosin (no α-actinin added)	0.136[b]	0.120
2°C, pH 7.0[c]	0.236	0.214
25°C, pH 7.0	0.268	0.222
40°C, pH 7.0	0.256	0.239
2°C, pH 5.7	0.228	0.227
25°C, pH 5.7	0.266	0.225
40°C, pH 5.7	0.123	0.083

[a]Conditions: α-actinin was purified by DEAE-cellulose chromatography followed by rechromatography on a second DEAE cellulose column and then was stored in H_2O solution at the designated temperatures and pH values. After the designated time, the sample was adjusted to pH 7.0, chilled to 0°C, and assayed in the ATPase test. Conditions of ATPase assay: 1mM $MgCl_2$, 1mM ATP, 0.05mM $CaCl_2$, 100mM KCl, 0.4 mg actomyosin/ml, 0.08 mg α-actinin/ml when added, 25.0°C.

[b]Figures are μmoles Pi/mg protein/min.

[c]Sample was stored at 2°C and pH 7.0 for the indicated length of time.

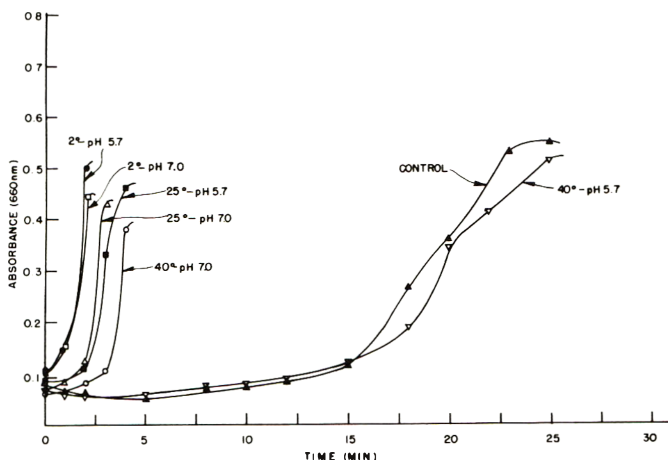

Fig. 2–Effect of 16 hr storage at different temperatures and pH values on activity of purified α-actinin in the turbidity assay. Conditions of superprecipitation assay: 100mM KCl, 10mM Tris-acetate, pH 7.0, 1mM $MgCl_2$, 0.05mM $CaCl_2$, 1mM ATP, 0.4 mg reconstituted actomyosin/ml, 0.06 mg treated α-actinin/ml when added, 29°C. α-actinin in 1mM $KHCO_3$ was adjusted to the pH indicated with dilute HCl, and stored at the specified temperature for 16 hr before readjustment to pH 7.0 and 2°C.

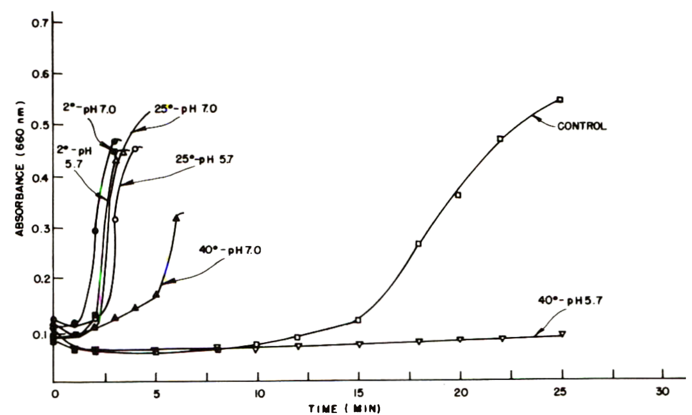

Fig. 3–Effect of 40-hr storage at different temperatures and pH values on activity of purified α-actinin in the turbidity assay. Conditions of superprecipitation assay: 100mM KCl, 10mM Tris-acetate, pH 7.0, 1mM $MgCl_2$, 0.05mM $CaCl_2$, 1mM ATP, 0.4 mg reconstituted actomyosin/ml, 0.06 mg treated α-actinin/ml when added, 29°C. α-actinin in 1mM $KHCO_3$ was adjusted to the pH indicated with dilute HCl, and stored at the specified temperatures for 40 hr before readjustment to pH 7.0 and 2°C.

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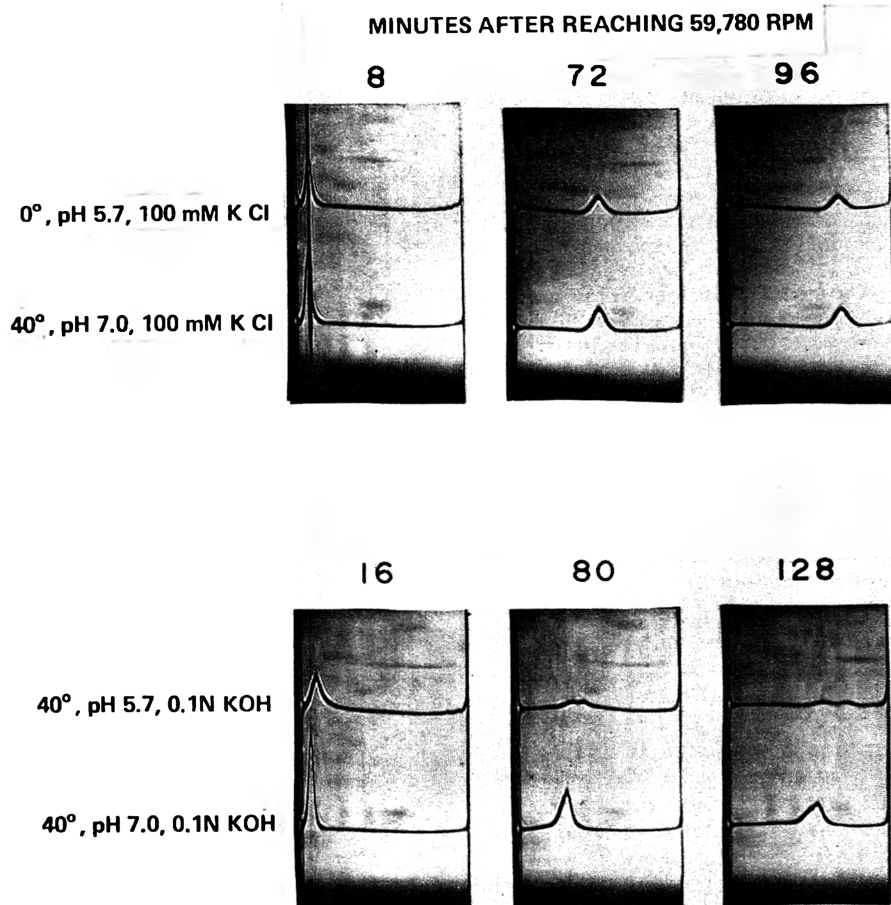

Fig. 4—Analytical ultracentrifuge diagrams of purified α-actinin after 16-hr storage. The pH 5.7, 0°C, and pH 7.0, 40°C samples (two upper diagrams) were each dissolved in 100mM KCl, 20mM Tris-acetate, pH 7.5 at a concentration of 4.0 mg/ml. The pH 5.7, 40°C and pH 7.0, 40°C samples (two lower diagrams) were each dissolved in 0.1N KOH at a concentration of 5.0 mg/ml. Temperature of both runs = 20.0°C; phase plate angle = 65°C for both runs.

loss of α-actinin activity during storage for 40 hr. Although caution must be exercised in extrapolating these in vitro results to the in situ situation in postmortem muscle, it may be noted that Sayre and Briskey (1963) have found that this same combination of high temperature and low pH is associated with a higher incidence of pale, soft, exudative porcine muscle.

Several experiments were done in an effort to ascertain the nature of the changes caused in α-actinin by storage at pH 5.7 and 40°C for 16 hr. Ultracentrifugal patterns of some of the stored α-actinins are shown in Fig. 4. α-Actinin precipitates at pH 5.7 (Ebashi and Ebashi, 1965), so all α-actinin samples stored at pH 5.7 were in the precipitated state. The α-actinin samples stored at pH 5.7 and temperatures of 25°C or less redissolved readily when the pH was adjusted to 7.0 at the end of the storage period, but α-actinin samples stored at pH 5.7 and 40°C did not redissolve when the pH was adjusted to 7.0. The 40°C, pH 5.7 samples had evidently undergone some change in conformation that resulted in extensive aggregation of the α-actinin molecules, and pH values of 12.0 or higher were necessary to dissolve these samples. This pH- and temperature-induced change in α-actinin conformation was not readily reversible because even after the 40°C, pH 5.7 samples had been dissolved at pH 12.0, lowering the pH to 8.0 resulted in precipitation of part of the protein in these samples. Because of these effects, ultracentrifugal studies on the 40°C, pH 5.7 samples had to be conducted in 0.1N KOH. The pH-induced precipitation of α-actinin at 40°C also caused part of the 40°C, pH 5.7 samples to be insoluble in the superprecipitation and ATPase assays, even though these samples were stirred gently for 40–64 hr at 2°C and pH 8.0 to minimize this effect.

When sedimented in 100mM KCl, 20mM Tris-acetate, pH 7.5, α-actinin samples that had been stored for 16 hr at 0°C and pH 5.7 or at 40°C and pH 7.0 exhibited an ultracentrifugal diagram identical to that of unstored α-actinin (cf. patterns in Fig. 4, top two rows, with those in Fig. 8, Robson et al., 1970). The 0°C, pH 5.7 and the 40°C, pH 7.0 samples both sediment with an observed sedimentation coefficient of 6.0S, characteristic of purified α-actinin (Robson et al., 1970). When the 40°C, pH 7.0 sample is sedimented in 0.1N KOH (Fig. 4, bottom row), the observed sedimentation coefficient decreases to 3.3–3.5S. Goll (unpublished results) had previously noted that the sedimentation coefficient of purified α-actinin is lowered to 3.0–3.5S when it is dissolved in 0.1N KOH. This lowered sedimentation coefficient may indicate either some unraveling of the ordered conformation of the native α-actinin molecule into a random coil, or a dissociation of the α-actinin molecule into smaller subunits by the 0.1N KOH solution. The present results do not permit a final decision between these two alternatives, but recent preliminary results in our laboratory (Suzuki et al., unpublished observations) suggest that the 6S α-actinin molecule has a subunit structure; thus, both alternatives are possible.

Whereas α-actinin samples stored for 16 hr at 40°C and pH 7.0 exhibited one principal ultracentrifugal boundary in 0.1N KOH, α-actinin samples stored for 16 hr at 40°C and pH 5.7 sedimented as two distinct species in the same solvent (Fig. 4, third row). The observed sedimentation coefficient of the slower boundary was 3.5S, similar to the main peak in the 40°C, pH 7.0 sample, but the observed sedimentation coefficient of the more rapidly moving component was 4.5S. Since these samples originally consisted only of α-actinin that was 90–95% ultracentrifugally homogeneous, the faster sedimenting boundary must represent an aggregate, possibly a dimer, of the monomeric α-actinin molecule. These results suggest that storage of α-actinin at 40°C and pH 5.7 causes aggregation of the monomeric α-actinin species. This aggregation is probably initiated by a conformational change in the α-actinin molecule that exposes hydrophobic or ionic surfaces. The ATPase and turbidity results reported earlier in this paper further indicate that this aggregated α-actinin is not active in accelerating the contractile response of reconstituted actomyosin suspensions.

Robson et al. (1970) have shown that at pH 7.5, α-actinin always elutes from DEAE-cellulose columns between 250 and 300mM KCl. Consequently, the elution profile of α-actinin stored at 40°C and pH 5.7 was examined to determine

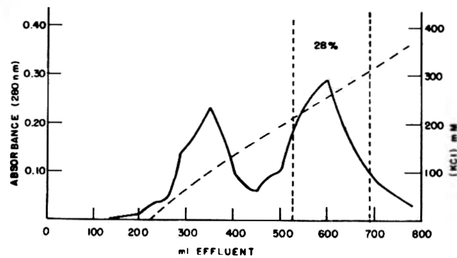

Fig. 5—Elution profile of DEAE-purified α-actinin after storage at pH 5.7 and 40°C for 150 min. Elution buffer: 25mM Tris-acetate, pH 7.5 with a KCl gradient indicated by the dotted line, 107 mg of treated α-actinin was applied to a 2.5 × 25 cm DEAE-cellulose column.

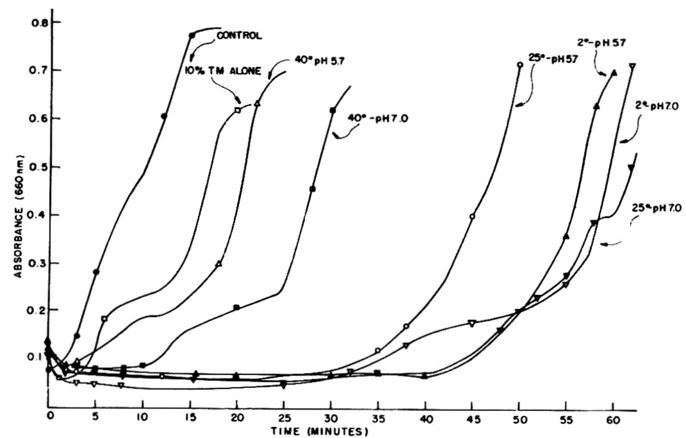

Fig. 6—Effect of 16-hr storage at different temperatures and pH values on the ability of troponin to inhibit the superprecipitation of reconstituted actomyosin. Conditions of superprecipitation assay: 75mM KCl, 10mM Tris-acetate, pH 7.0, 1mM $MgCl_2$, 0.05mM EGTA, 1mM ATP, 0.40 mg reconstituted actomyosin/ml, 0.04 mg purified tropomyosin/ml added to all samples except the control, and 0.04 mg treated troponin/ml when added, 26°C. Troponin in 1mM $KHCO_3$ was adjusted to the pH indicated with dilute HCl and stored at the specified temperature for 16 hr before readjustment to pH 7.0 and 2°C.

whether these storage conditions would alter the elution pattern of α-actinin from DEAE-cellulose columns. In order to preserve the ability of α-actinin to redissolve at pH 7.5 after being stored at 40°C and pH 5.7, storage times in these experiments were limited to 2.5 hr. Even after this abbreviated storage period, however, much of the ability of α-actinin to accelerate the ATPase activity or turbidity response of actomyosin suspensions had been lost. The results of these experiments show that storage at 40°C and pH 5.7 for 2.5 hr produces a marked change in the elution profile of α-actinin from DEAE-cellulose columns (Fig. 5). Only 28% of the 40°C, pH 5.7 sample eluted between 250 and 300mM KCl where 95–100% of control α-actinin samples eluted under these same conditions (cf. Robson et al., 1970). Approximately 20% of the protein in the 40°C, pH 5.7 samples eluted between 0 and 150mM KCl (Fig. 5), and the remaining 52% was very tightly bound to the DEAE-cellulose and could not be eluted with 2M KCl. Since alteration from an α-helical or other highly ordered structure to a random coil configuration characteristically causes proteins to be very tightly bound to ion-exchange columns, these elution patterns from DEAE-cellulose columns confirm the analytical ultracentrifugal data that storage at 40°C and pH 5.7 causes some unfolding of the tertiary structure of α-actinin and that this unfolding is then followed by aggregation among the unfolded α-actinin molecules. However, the results of DEAE-cellulose experiments do not clarify the exact nature of the changes in α-actinin that cause the unfolding.

Effect of storage temperature and pH on troponin and tropomyosin

Several experiments were done in which the tropomyosin-troponin complex (P_{30-75} fraction, Arakawa et al., 1970a) was stored at 40°C and pH 5.7 for periods up to 40 hr. Although these storage conditions caused complete loss of activity of purified α-actinin, they had no appreciable effect on ability of the tropomyosin-troponin complex to confer Ca^{++}-sensitivity on reconstituted actomyosin. Therefore, in subsequent experiments purified troponin or purified tropomyosin was stored at various combinations of temperatures between 2°C and 40°C and pH values between 5.7 and 7.0 for periods up to 40 hr. The treated protein was then combined with its untreated companion (i.e., treated tropomyosin was combined with untreated troponin and treated troponin was mixed with untreated tropomyosin) in a 1:1 ratio by weight, and the resulting tropomyosin-troponin complex was assayed for Ca^{++}-sensitizing activity in the ATPase and turbidity tests. These experiments showed that Ca^{++}-sensitizing activity of the treated tropomyosin-untreated troponin complex was identical to that of the untreated tropomyosin-untreated troponin complex even after the tropomyosin had been stored for 40 hr at pH 5.7 and 40°C. This result is not unexpected since several investigators have shown that tropomyosin is a very stable protein and is not denatured by a variety of conditions, including 100°C temperatures for 10 min at pH 6.3 (Bailey, 1948), ethanol or ether treatment at 0°C (Bailey, 1948), ethylene glycol treatment (Kay and Brahms, 1963), concentrated urea (Bailey, 1948), trichloroacetic acid precipitation (Bailey, 1948) lyophilization, or precipitation at low pH (Mueller, 1966).

On the other hand, when treated troponin was combined with untreated tropomyosin, the resulting complex exhibited a variety of Ca^{++}-sensitizing activities depending on the storage conditions to which the troponin had been subjected. These results are summarized in Figure 6. Under the conditions used in these experiments (75mM KCl and 0.05mM EGTA), the control reconstituted actomyosin undergoes turbidity development in 2–10 min. Ca^{++}-sensitivity under these conditions is reflected by a much longer time required for the turbidity response. The effect of purified tropomyosin alone on the turbidity response of reconstituted actomyosin is presented in Figure 6 to demonstrate that the tropomyosin used in this study is not contaminated by troponin; purified tropomyosin had almost no Ca^{++}-sensitizing activity. Figure 6 also shows that storage at 40°C and either pH 5.7 or 7.0 caused almost complete loss of the Ca^{++}-sensitizing ability of troponin. Storage at 25°C and pH 5.7 also appeared to cause some slight loss of the Ca^{++}-sensitizing ability of troponin, but storage at 2°C and pH 5.7 or at 25°C and pH 7.0 had no effect on the ability of troponin to sensitize reconstituted actomyosin to Ca^{++}. Results similar to these were also obtained in the ATPase assay for tropomyosin-troponin activity. These findings indicate that high temperature (above 25°C) is much more effective in denaturing troponin than low pH (below 6.0). This is not surprising since Levy and Ryan (1967) have shown that the Ca^{++}-sensitivity of myosin B (natural actomyosin) is lost by heating to 43°C for 5 min, and since

isoelectric precipitation at pH 4.6 and 2°C is routinely used to separate troponin from tropomyosin (see MATERIALS & METHODS section) without any adverse effects on troponin activity.

DISCUSSION

RESULTS OF THIS study show that in vitro storage of purified α-actinin or troponin at temperatures near those existing in the living mammalian organism causes loss of the biological activities of these proteins. This temperature-induced loss of activity is much more noticeable in the case of troponin than it is in the case of α-actinin, and storage periods of 40 hr or longer are required before any loss of α-actinin activity can be detected at pH 7.0 and 40°C. If the storage at 40°C is done at pH values of 5.7, almost complete loss of both α-actinin and troponin activity occurs in 16 hr. Tropomyosin, on the other hand, is not affected by storage at 40°C and pH 5.7 for periods up to 40 hr. Furthermore, the presence of tropomyosin appears to confer some protection against temperature-inactivation of troponin since the tropomyosin-troponin complex is not completely inactivated by storage at 40°C and pH 5.7.

Our previous study (Arakawa et al., 1970a) used intact muscle and post-mortem storage at 2°C and 25°C. In view of the findings presented in the present paper, it is not surprising that we observed only a very slight loss of tropomyosin-troponin activity during post-mortem aging in our previous study. Furthermore, although most meat animal carcasses are routinely chilled soon after exsanguination, it requires several hours before muscle temperature is reduced below 35°C (Parrish et al., 1969). If muscle pH does not fall rapidly during this period, it is probable that activity of α-actinin and the tropomyosin-troponin complex would remain unimpaired for post-mortem periods of several weeks. Indeed, Goll and Robson (1967) have found that myofibrils isolated from bovine muscle after aging for 13 days at either 2°C or 16°C are still fully sensitive to Ca^{++}. Assuming that muscle pH in most meat animal carcasses does not fall below 6.0 during the first 2–3 hr post-mortem, it would be expected that α-actinin and tropomyosin-troponin activity would remain almost unchanged in meat handled through normal market channels. On the other hand, Briskey and coworkers (Briskey, 1964; Briskey and Wismer-Pedersen, 1961; Sayre and Briskey, 1963) have recently described a class of porcine animals whose musculature undergoes a very rapid post-mortem decline in pH, so that pH values of 5.7 or less are reached while carcass temperature is still 37°C or higher. Since both α-actinin and the tropomyosin-troponin complex are adversely affected by this condition, it is possible that myofibrillar tissue from such animals would lose much of its α-actinin and Ca^{++}-sensitizing activity, and it may be interesting to compare the Ca^{++}-sensitivity and α-actinin activity of myofibrils from such animals with the corresponding activities in myofibrils from normal animals.

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Ms. received 3/2/70; revised 4/27/70; accepted 5/1/70.

Journal Paper No. J-6511 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project 1549. This investigation was supported in part by Public Health Service Training Grant UI-01043-03 from the National Center for Urban and Industrial Health. We are indebted to Jean Preston, Nona Starr, and Jean Fatka for technical assistance; to Dr. Richard M. Robson for many helpful discussions; and to Karen Schwarz for assistance with the manuscript. We thank Dr. T. Fukazawa for discussing some of his results with us prior to their publication. During this investigation, Dr. Arakawa was the recipient of a Visiting Professorship from Training Grant UI-01043-03, National Center for Urban and Industrial Health, U.S. Public Health Service.

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MUSCLE CONTRACTION AND POSTMORTEM pH CHANGES IN PIG SKELETAL MUSCLE

SUMMARY–The initial (5 min) pH of longissimus dorsi muscle taken from live pigs under anesthesia was 7.0; in muscle taken immediately after slaughter it was 6.3. When the neuromuscular blocking agent, curare, was given intramuscularly before slaughter the initial pH was raised to 6.8. The gastrocnemius muscle was stimulated to contract via the sciatic nerve in vivo and the pattern of pH change compared with that of the unstimulated muscle in the other side of the animal. Stimulation caused a fall in initial pH and an acceleration in the subsequent rate of pH fall in the excised muscle under nitrogen at 37°. It was concluded that neural stimuli entering the muscle at the time of death were the major factors involved in the rapid postmortem glycolysis observed in the pigs studied.

INTRODUCTION

THE RATE of pH fall post-mortem varies considerably in pig skeletal muscle. Low pH values (below 6.0) are frequently reached in certain muscles within 1 hr after death (Ludvigsen, 1954; Wismer-Pedersen, 1959; Briskey and Wismer-Pedersen, 1961; Wismer-Pedersen and Briskey, 1961; McLoughlin, 1963; Elliot, 1965). At this time post-mortem, the temperature of the deep-lying musculature of the carcass is usually still above 35°C. The combination of relatively high temperature and low pH brings about changes in the properties of the muscle proteins (Bendall and Wismer-Pedersen, 1962; McLoughlin, 1963; Sayre and Briskey, 1963; Goldspink and McLoughlin, 1964; Trautmann, 1966) which make muscle, normal in appearance at the time of death, become pale, soft and exudative as it goes into rigor-mortis (Sayre and Briskey, 1963); Sayre et al., 1964; 1966; Borchert and Briskey, 1964; 1965). The longissimus dorsi muscle has been most often used in studies of this type. It is a muscle which frequently becomes pale, soft and exudative post-mortem and it is a large and commercially important muscle.

McLoughlin (1964) reported that a rapid fall in the pH of the longissimus dorsi muscle frequently occurred when pigs were stunned through the forebrain, using a captive-bolt pistol, and exsanguinated. Pigs slaughtered in this way frequently struggled violently. When curare was given intramuscularly before slaughter, the rate of pH fall was considerably reduced. Curare blocks the excitation of muscle via its motor nerve and thus prevents the contraction of muscle (Bernard, 1857).

The observations of other workers have also indicated the role of muscle contraction at death in causing rapid postmortem glycolysis. Hallund and Bendall (1965) reported that Danish Landrace pigs given myanesin (a muscle relaxant which acts by depressing the activity of the anterior horn cells of the spinal cord) exhibited rates of postmortem pH fall which were much slower than those hitherto observed in this breed of pig. Bendall (1966) found that the rate of pH change and of rigor onset were slower in pigs which were curarized than in those normally slaughtered. Hallund and Bendall (1965) and Bendall (1966) showed that an acceleration in the rate of postmortem pH fall occurred when excised segments of longissimus dorsi muscle were stimulated electrically.

In this study, pH changes were compared between longissimus dorsi muscle taken immediately after slaughter and muscle taken from the live animal under anesthesia. The effects of curarization and stimulation of muscle via its motor nerve in vivo were examined to determine the role which neural stimuli, entering the muscle at the time of death, play in the phenomenon of rapid postmortem glycolysis.

EXPERIMENTAL

Animals

Purebred pedigree Landrace pigs (26) between 5 and 6 months old and in the liveweight range 160–180 lb, from herds kept under the same conditions on 1 farm, were used. They were transported individually (17 miles) to the laboratory the day before each experiment, fed on arrival and kept in the heated laboratory overnight. Maximum care was taken to see that the animals were not subjected to stress.

Materials

1) Pigs were anesthetized, respiration maintained by means of a tracheotomy and specimens of muscle taken from the live animal.

2) Pigs were stunned by shooting through the forebrain using a captive-bolt pistol, exsanguinated and specimens of muscle removed.

3) Pigs were given 40 mg of tubocurarine chloride (Burroughs Wellcome) intramuscularly. When the paralysant effect of the curare became apparent (about 5 min after injection) the animals were slaughtered as described above.

4) The gastrocnemius muscle and sciatic nerve were exposed under anesthesia. The nerve on one side was stimulated electrically to make the muscle contract. The blood supply to the muscle was clamped off during contraction and the muscle removed. The unstimulated muscle from the other side of the animal was used as a control.

Methods

Anesthesia. The animal was held in a folding table and a polythene bag placed over the head. The bag had 2 apertures, 1 to allow a mixture of nitrous oxide and oxygen to enter; the other to let expired gas out. Nitrous oxide was delivered at a rate of 7 liters/min, oxygen at 3 liters/min. The gases were bubbled through halothane (2-bromo-2-chloro-1,1,1-trifluoroethane, Hoechst, Germany) so that the emerging gas mixture contained about 1–2% of this substance. When the animal was unconscious, an incision was made in the mid-line of the throat and the larynx and the upper part of the trachea exposed by dissection. The trachea was cut transversely but was not severed. A tracheal tube was inserted and the cuff inflated. The incision was then closed using clamping forceps. Respiration was controlled, where necessary, by means of a bag. The animal was placed on an operating table and when it had respired normally for 20–30 min, specimens of muscle were removed.

Stimulation of sciatic nerve in vivo. The semitendinosus muscle on one side was removed and the gastrocnemius muscle and sciatic nerve proximal to it exposed. The nerve was directly stimulated using an electronic stimulator which delivered a square pulse. The minimum strength of current necessary to elicit a single twitch from the gastrocnemius muscle was determined. Repetitive shocks were then given and the frequency of stimuli increased until the muscle just went into a sustained contraction.

pH/ time curves. A section of the longissimus dorsi muscle (about 10 cm below and above the level of the last rib, i.e., the lower thoracic-lumbar region) was removed, stripped of superficial fat and connective tissue and placed at 37°C in a stream of moist nitrogen. Sections were taken as soon after excision as possible and subsequently at 1-hr intervals.

pH measurements. Sections were homogenized in a Turrax Ultrablender with iodoacetate (0.005 M, neutralized to pH 7.0) to give 20% w/v suspensions. The pH was measured using a Radiometer pH meter 22 with scale expander.

RESULTS & DISCUSSION

POSTMORTEM pH changes in the longissimus dorsi muscle of 3 groups of pigs were studied (Fig. 1). The pigs in the first group were anesthetized and sections of muscle removed. Since these sections were deprived of their blood and oxygen supplies on removal, the biochemical changes which later occurred in them

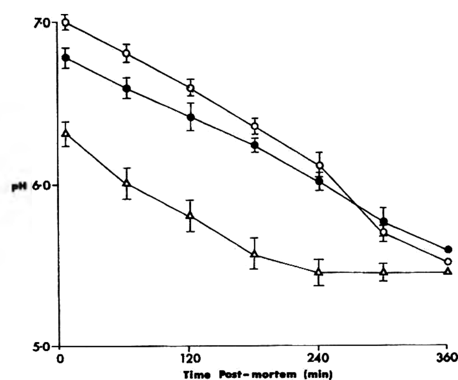

Fig. 1—Post-mortem pH changes in longissimus dorsi muscle of Landrace pigs. Muscle from:— live anesthetized pigs (6) ○ — ○ — ○; curarized pigs (7) after slaughter ● — ●; untreated pigs after slaughter (10) △ — △.

I = standard error of mean values.

under nitrogen at 37°C may be described validly as postmortem changes. These sections of muscle were not subjected to the effects of the death reaction associated with slaughter.

The pH of muscle taken from the live animal was 7.0 at 5 min after excision and fell at a rate of 0.22 unit/hr between pH 7.0 and 6.1. The rate of fall accelerated somewhat below 6.0 to give an over-all rate of 0.26 unit/hr for 6 hr.

The pigs in the second group were stunned and exsanguinated. In this instance, muscle pH had already fallen to 6.3 within 5 min after death and reached 6.0 at 1 hr post-mortem. Since the latter value had not been attained in muscle taken from the live animal until glycolysis had proceeded for 4.5 hr, clearly slaughter had had a marked accelerating influence on the rate of anaerobic glycolysis in the period immediately following death.

Animals which were stunned and slaughtered frequently struggled violently. Theoretically, struggling at death will accelerate the onset of rigor mortis and the rate of pH fall by reducing the initial levels of phosphocreatine and ATP in muscle and thus accelerating the decay of the latter substance (Bendall, 1960). The extent to which struggling contributed to the low initial muscle pH was studied by giving curare intramuscularly to the third group of pigs just before slaughter. Curare blocks transmission of motor impulses at the neuromuscular junction and thus prevents the contraction of skeletal muscle (Bernard, 1857). Consequently, curarization prevents struggling at death. The curarized pigs did not struggle, although some twitching of the musculature did occur on slaughtering. Although these pigs suffered from severe respiratory distress due to paralysis of the muscles of respiration by curare, the initial pH of the longissimus dorsi muscle was 6.8. Abolition of muscle contraction associated with death clearly raised the initial pH appreciably.

The very low initial pH values found in muscle taken from pigs after stunning and exsanguination suggest that an almost explosive burst of glycolysis occurred at or just after death. Such glycolysis might have been caused by a combination of factors such as struggling at death, anoxia following loss of the muscle blood supply or the release of adrenaline following ante-mortem stress or the shock of stunning. Struggling at death will accelerate the rate of postmortem glycolysis because under such conditions phosphocreatine is depleted and the resynthesis of ATP is primarily dependent on the anaerobic breakdown of glycogen. Loss of the blood supply to the muscle will reduce the amount of oxygen available to the muscular tissue and lead to a more rapid accumulation of lactic acid. The release of adrenaline at death will contribute to a postmortem biochemical environment conducive to an increased rate of anaerobic glycolysis because adrenaline converts the inactive (β) form of phosphorylase to the active (a) form (Cori, 1956).

The results reported here suggest that anoxia just before or at death and the release of adrenaline probably contributed appreciably less to the effect of the death reaction than did muscular contraction. The pigs slaughtered without curare were subjected to no obvious physical or psychological stress before death; they were quickly stunned as they walked from the pen in which they had been held overnight. It seems unlikely, under these circumstances, that the low initial muscle pH was caused by antemortem stress; particularly so, since the curarized pigs, unavoidably subjected to antemortem stress, had a much higher initial pH. The curarized pigs were held and given an intramuscular injection and suffered from severe respiratory difficulties before slaughter. In spite of this, the initial pH of muscle from the curarized pigs was 0.5 unit higher than that of muscle from the pigs slaughtered without curare. Similarly, it seems unlikely that the release of adrenaline following stunning and exsanguination was a major factor responsible for the low initial pH of muscle from the slaughtered animals because of the marked elevation in muscle pH which followed curarization. Curare combines specifically with sites at the neuromuscular junction which normally combine with acetylcholine and in this way prevents the depolarization of the post-junctional end plate which initiates the sequence of changes leading to the contraction of muscle. The effect of curare observed in these experiments can hardly be attributed to anything other than its characteristic action of blocking transmission at the neuromuscular junction, thus preventing the contraction of muscle.

The effect of contraction just before excision of muscle from the live animal was studied by stimulating the sciatic nerve on 1 side so that the gastrocnemius muscle contracted, and using the unstimulated muscle on the other side as a control. The results (Table 1) showed that stimulation lowered the initial pH and accelerated the subsequent rate of pH fall. The rate of fall was measured between the initial value and pH 6.0 because of differences in the final pH on the muscles studied and the retarding effects of low concentrations of glycogen substrate on the activity of the enzymes involved in anaerobic glycolysis.

Results in Table 1 show that repeated tetanic contractions had a more marked effect than a single 10-sec contraction. Repeated stimulation substantially lowered the initial pH to a level close to the average value found in the longissimus dorsi muscle of the pigs stunned and exsanguinated. The results support the conclusion drawn from the effects of curarization, i.e., that muscle contraction at death appreciably influences the course of postmortem glycolysis. The results are also in agreement with those of Hallund and Bendall (1965) and Bendall (1966), who reported that stimulation of excised muscle had an accelerating effect on the rate of postmortem glycolysis, provided the pH had not already fallen below 6.8 before stimulation, in which case the muscle failed to respond.

The extent of the fall in the initial pH of the gastrocnemius muscle following

Table 1—Effect of stimulating the sciatic nerve on post-mortem pH changes in the gastrocnemius muscle.

Animal (No.)	Contraction time (sec)	Initial pH		Rate of pH fall (units/hr)	
		Control	Simulated	Control	Stimulated
1	10	7.2	6.9	0.18	0.23
2	10	7.2	7.0	0.17	0.26
3	10	7.2	7.0	0.25	0.32
4	10 × 3[a]	7.1	6.6	0.16	0.31
5	10 × 3[a]	7.1	6.5	0.18	0.33

[a] 10-sec Tetanic contractions, repeated 3 times.

stimulation was greater than that reported by Hallund and Bendall (1965) and Bendall (1966) for longissimus dorsi muscle. This may be because these workers used excised sections of muscle, whereas in this study the contracting muscle was still attached to the skeleton and performed work by moving the limb. Cori (1956) showed there is a relationship between the rate of chemical changes in contracting muscle and the mechanical work it does. Muscles contracting under tension have a higher rate of lactic acid production and of glycogen depletion than muscles which contract unloaded. Consequently, it is likely that the death struggles of an animal will have a more profound effect on postmortem pH changes than will contraction of the excised muscle. Nevertheless, the principle holds in both cases, i.e., that contraction of pig muscle causes not only a fall in initial pH but also an acceleration in the subsequent rate of pH fall.

The observations reported here indicate that contraction of the musculature at death played a major part in the Landrace pigs studied. Stress immediately antemortem and anoxia appeared to have little effect on the pattern of pH change other than a relatively small lowering of the initial pH. The absence of any marked effect due to these 2 factors may not apply to all breeds of pig or even to strains of pig within breeds. Inherent differences in patterns of postmortem glycolysis and muscle characteristics between pigs such as the Poland China, the Hampshire and the Chester White in the U.S.A have been thoroughly documented (see a comprehensive review by Briskey, 1964). Bendall et al. (1963) found 2 distinct groups of Danish Landrace pigs on the basis of differences in rates of postmortem glycolysis. This observation was supported by the pattern of distribution of pH_1 values (pH at 45 min post-mortem) for commercially slaughtered Danish pigs (Bendall and Lawrie, 1964). Pigs in Ireland probably have a relatively low evidence of pale, soft exudative muscle (McLoughlin, 1965). In spite of this, muscle contraction has a very marked effect on the entire pattern of postmortem pH change in the musculature of these pigs. Perhaps one of the earliest changes in muscle physiology, associated with the deterioration in meat quality accompanying the improvement in pigs as meat animals, is an increased response of the muscle to neural and perhaps even to direct stimuli.

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Ms. received 12/2/68; revised 8/3/69; accepted 6/29/70.

Acknowledgment is made to Dr. B. de Britt for assistance and advice on anesthesia; and to J. Rowley and S. Byrne for technical assistance.

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EFFECT OF MICROBIAL GROWTH UPON SARCOPLASMIC AND UREA-SOLUBLE PROTEINS FROM MUSCLE

SUMMARY—A comparison of starch gel patterns of sarcoplasmic proteins from aseptic and inoculated porcine and rabbit muscles after storage for 0, 8 and 20 days at 10°C indicated that different microorganisms preferentially utilized specific proteins. Pseudomonas fragi *showed the greatest amount of proteolytic activity upon the sarcoplasmic fraction, causing extensive breakdown in both rabbit and porcine muscle.* Leuconostoc mesenteroides *caused extensive alteration in the sarcoplasmic proteins of rabbit muscle, but had less effect upon porcine muscle.* Pediococcus cerevisiae *exhibited similar action to* L. mesenteroides *upon rabbit muscle sarcoplasmic proteins, but had no effect upon pig muscle.* Micrococcus luteus *showed only minor breakdown of rabbit muscle sarcoplasmic proteins, and had no action upon porcine muscle. Both* P. fragi *and* P. cerevisiae *caused considerable breakdown of the urea-soluble proteins in pig muscle and to a lesser extent in rabbit muscle. Neither* M. luteus *nor* L. mesenteroides *exerted any measurable proteolytic effect upon the urea-soluble proteins. Possible implications concerning meat spoilage are discussed.*

INTRODUCTION

ALTHOUGH the role of bacteria in spoilage of meat has long been recognized, the mechanism of degradation and the extent of muscle protein involvement has not been elucidated. Several workers (Brown and Weidman, 1958; Ayres, 1960; Jay, 1967) have shown that *Pseudomonas* strains constitute the predominant group contributing to the spoilage of fresh meat. Alford (1960) reported that Pseudomonads and *Achromobacter* liquefy gelatin and digest litmus milk at 20°C. More recently, Jay et al. (1967) reported that there is a decrease in the quantity and number of amino acids and nucleotides as bacterial spoilage of meat proceeds. They concluded that bacteria are unable to utilize proteins per se for growth but exert their action through digestion of the nonprotein constituents of meat. On the other hand, Borton et al. (1968) have shown that bacterial growth on meat decreases its emulsifying capacity, apparently by proteolysis.

In view of the controversy and lack of information concerning the influence of microbial growth upon muscle, our study was initiated to determine the extent and nature of changes in the sarcoplasmic proteins from skeletal muscle as a consequence of microbial growth. This was accomplished by inoculating aseptically removed rabbit and porcine muscle with several specific organisms and following changes in the sarcoplasmic proteins using starch gel and disc gel electrophoresis after incubation at 10°C for 0, 8 and 20 days' storage.

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EXPERIMENTAL

Sampling procedures

7 market-weight pigs (180–230 lb) produced at Michigan State University Farms were slaughtered individually over a 7-month period in a conventional manner, except that special techniques were followed to aseptically obtain muscle samples. The animal was electrically stunned and suspended by the hind leg. The area of the neck utilized in sticking was scrubbed thoroughly with hexachlorophene bactericidal soap and sticking was accomplished with a sterilized knife. After conventional dehairing and eviscerating, the unsplit carcass was rinsed with absolute alcohol and chilled at 1–3°C. The alcohol rinse was repeated after chilling for 20 hr and the carcass laid on a kraft-paper-covered table in a room free of excessive air currents.

The shoulders section was removed and the middle portion posterior to the 4th rib (belly and loin) was positioned so that the dorsal midline was accessible. A cut was made along the dorsal midline of backfat-loin using a sterilized knife. Subsequently, cuts were made perpendicular to the midline and the backfat stripped away. Slices about 3 cm in thickness were then aseptically excised from the exposed M. longissimus and placed in sterile containers. Both longissimus muscles were excised and handled in the same manner. The excised samples were ground through a sterile, prechilled grinder and placed in presterilized sample jars. At all times the operator wore disposable sterile plastic gloves.

Rabbit carcasses were handled in essentially the same manner. The carcasses were washed with absolute alcohol immediately after skinning; the muscles were removed with a sterile knife and tweezers, minced in a presterilized grinder and placed in sterilized beakers.

Inoculation of samples

A pure culture (American Type Culture Collection) of each bacteria was made to give a 100-fold dilution in sterile buffer. The diluted culture was mixed with the minced muscle to give a concentration of approximately 10^5-10^6 organisms per gram. After thoroughly mixing, the inoculated sample was divided into 3 approximately equal portions of 50 g and each placed in sterile sample bottles (Miller and Price, 1969). Sterile caps were placed on the bottles but they were not tightly sealed. The 3 sample bottles were placed in an incubator at 10°C. The contents of 1 bottle were analyzed at 0, 8 and 20 days' storage. The organisms utilized in this study were chosen on the basis of having widely different properties and yet being commonly found on various meat products.

Sterile-control samples

Control samples were handled in the same way, except they were not inoculated. Thus, control uninoculated samples were available at each storage period so that one could differentiate between the changes due to microbial growth and those due to storage alone.

Bacterial counts

Bacterial numbers were obtained using the methods described by the American Public Health Association (1966). APT (all-purpose plus Tween) agar was used as the plating media. All plates were incubated at 25°C for 48 hr.

Extraction of sarcoplasmic proteins

The procedures for extraction and separation of the sarcoplasmic and urea-soluble proteins were similar to those outlined by Hegarty et al. (1963) and are outlined in Figure 1. All extraction procedures were carried out at approximately 3°C. 4 vol 0.3 M sucrose-0.01 M KCl-0.01 M Tris buffer at pH 7.6 were added to a weighed sample of muscle in a Waring Blendor and homogenized at high speed for 1 min. The

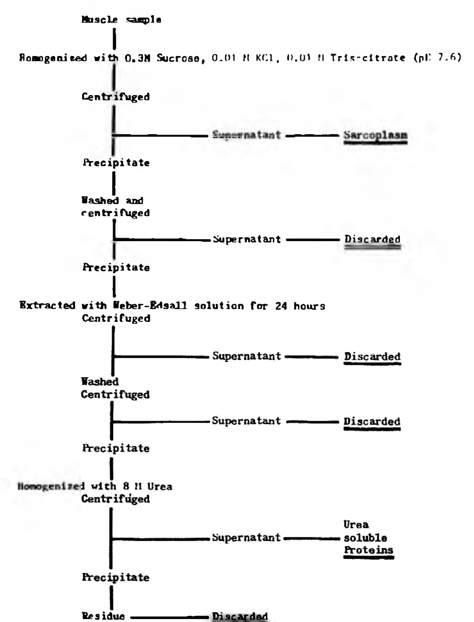

Fig. 1—Procedures utilized for extracting sarcoplasmic proteins from muscle.

homogenate was then centrifuged at 19,400 × g for 15 min. The supernatant consisting of sarcoplasm was decanted and retained for further study. The precipitate was washed with 12 vol sucrose-KCl-Tris solution (see above) and centrifuged. The supernatant was discarded and the washed precipitate washed with 6 vol Weber-Edsall solution (0.6 M KCl, 0.04 M $KHCO_3$, 0.01 M K_2CO_3) and stored for 24 hr. Then 18 vol Weber-Edsall solution were added and mixed with magnetic stirrer. The solution was then centrifuged at 28,600 × g for 30 min. The supernatant consisting of the salt-soluble proteins was discarded while the residue was washed with 12 vol Weber-Edsall extract to more completely remove the residual salt-soluble protein. Centrifugation was repeated as in the previous step and the supernatant discarded; the precipitate was then homogenized with 4 vol 8 M urea solution. Centrifugation was performed at 28,600 × g for 30 min. The supernatant was retained as the urea-soluble protein and the precipitate discarded. Isolation of the 8 M urea-soluble protein was essentially the same as outlined by Maier et al. (1966). The protein content of the samples was determined by the ninhydrin method as described by Jacobs (1959).

Starch-gel electrophoresis

A slight modification of the starch gel electrophoresis procedure of Scopes (1964) was utilized in this study. The outer solution was made up using 60 mM-Tris-50 mM-boric acid (pH 8.6 at 5°C), and the inner gel in 12 mM-Tris-2mM-diethylenetriamine pentaacetic acid (pH 8.25 at 5°C). Electrophoresis was carried out at 400 v (approx 36 v/cm) for 6.0–6.5 hr, 8–17 ma in a cold (3°C) room on a horizontal axis. After slicing, the gels were stained in 0.18% Buffalo Black (NBR-naphthol blue black), in methanol-acetic acid-water (5:5:1-v/v), then washed with glycerin-water-methanol-acetic acid (1:5:5:1-v/v) and photographed.

Disc gel electrophoresis

Acrylamide disc gel electrophoresis was performed on the 8 M urea-soluble fraction. The method used was essentially that of Davis (1964) with a few minor modifications. The 6.5% running gel and the 5.0% spacer gel contained 7.2 M urea. Gel tubes of 5 mm id were generally used and the spacer gel (7.5 mm) added upon the acrylamide running gel (50 mm). The samples were applied by a syringe directly on the surface of the spacer gel underneath the buffer.

Electrophoresis was carried out at 200 v (approx 2.5 ma/tube) in 5 mM Tris-40 mM glycine buffer solution for 2 hr at room temperature and at 13 to 34 ma. The gels were stained with a solution of 0.18% Buffalo Black (NBR-naphthol blue black) in methanol-acetate-water (5:5:1, v/v) for 1 hr. They were then kept overnight (18 to 20 hr) in 7% acetic acid solution and destained electrically. Densitometer tracings were made with a Canalco Model F Microdensitometer.

Detection of enzymes

Methods for detecting certain enzymes on the gel surface were based on the reduction of nitro-blue tetrazolium by NADH. The products of the reaction were partially fixed close to their generation point by using a sheet of filter paper soaked in the reaction mixture placed on the gel. This proved to be sufficient for normal "positive" methods involving 1 or more coupling enzymes to reduce NAD^+. It produces bands of reduced nitro-blue tetrazolium at the sites of enzyme activity on the filter paper. Use of filter paper also enables "negative" methods to be employed, in which the enzymes manifest themselves by oxidation of applied NADH. After incubation the filter paper is removed and both the paper and the gel itself can then be treated with nitro-blue tetrazolium to detect unchanged NADH. Composition of the reaction mixtures is shown in Table 1.

Table 1—Reaction mixtures for filter-paper staining of specific proteins along with the codes for their identification.

Enzyme	Abbreviation	Method (+ or –)	Substrates, co-enzymes, etc.
Phosphoglucose isomerase (EC 5.3.1.9)[a]	PGI	+	Fructose-6-phosphate, 8 mM; NADP, 0.2 mM
Phosphofructokinase (EC 2.7.1.11)[a]	PFK	+	Fructose-6-phosphate, 8 mM; ATP 5 mM; NAD^+, 2 mM; sodium arsenate, 5 mM
Aldolase (EC 4.1.2.7)[a]	ALD	+	Fructose-1,6-diphosphate, 5 mM; NAD^+, 2 mM; sodium arsenate, 5 mM
Glyceraldehyde phosphate dehydrogenase (EC 1.2.1.12)[a]	GAPDH	+	Fructose-1,6-diphosphate, 5 mM; NAD^+, 2 mM; sodium arsenate, 5 mM
α-Glycerophosphate dehydrogenase (EC 1.1.1.8)[a]	αGPDH	–	Fructose-1,6-diphosphate, 5 mM
Phosphoglycerate kinase (EC 2.7.1.31)[a]	PGAK	–	3-Phosphoglycerate, 8 mM; ATP, 8 mM
Phosphoglycerate mutase (EC 2.7.5.3)[a]	PGAM	–	2-Phosphoglycerate, 5 mM; 2,3-diphosphoglycerate, 0.1 mM; ATP, 5 mM; phosphoenolpyruvate, 5 mM; KCl, 0.1 M
Phosphopyruvate hydratase (EC 4.2.1.11)[a]	PPH	–	2-Phosphoglycerate, 8 mM; ADP, 8 mM; KCl, 0.1 M; glucose, 20 mM
Pyruvate kinase (EC 2.7.1.40)[a]	PK	–	Phosphoenolpyruvate, 8 mM; ADP, 5 mM; KCl, 0.1 M; glucose, 20 mM
Lactate dehydrogenase (EC 1.1.1.27)[a]	LDH	–	Sodium pyruvate, 10 mM
Creatine kinase (EC 2.7.3.2)[a]	CK	–	Creatine, 30 mM; ATP, 5 mM; phosphoenolpyruvate, 8 mM; KCl, 0.1 M

Solutions were buffered at pH 7.8 with Tris buffer (50 mM) including 5 mM-$MgSO_4$. All positive (+) methods also included nitro-blue tetrazolium (3 mg/ml) and phenazine methosulphate (0.5 mg/ml). All negative methods (–) included NADH at 6.0 mM. A mixture of nitro-blue tetrazolium (3 mg/ml) and phenazine methosulfate (0.5 mg/ml) was used to detect unchanged NADH. The reaction was carried out with 2 ml of the above buffer solution.

[a]E.C. = Enzyme Commission characteristic numbers.

In practice, the procedure for staining was as follows: A piece of filter paper (Whatman No. 1) was soaked with the reaction mixture (2 ml was enough for 2 papers covering the entire gel) and laid on the gel. With positive methods, purple bands appeared after a few minutes at the sites of activity. The filter paper was removed, washed with water, dried and kept for the reference.

The negative methods used removal of the filter paper which contained the reaction mixture after approximately 2 or 3 min, depending on the enzyme activity. The solution containing nitro-blue tetrazolium and phenazine methosulfate was placed on a glass pan, about 10 ml being sufficient for 6 sheets of filter paper. The paper was quickly passed through this solution and the NADH remaining caused the appearance of a purple precipitate of the reduced dye on the paper, with the clear areas indicating the location of the enzymes. Myoglobin (MB) and hemoglobin (HB) were stained with benzidine as described by Owen et al. (1958).

RESULTS & DISCUSSION

TABLE 2 shows the changes in log bacterial numbers and pH values, in both the muscle homogenate and the sarcoplasmic fractions from rabbit and pig muscle. The data show there was a rapid increase in bacterial numbers during the first 8 days. Generally, there was but little further increase between 8 and 20 days' storage. Although no growth of bacteria was evident in control rabbit muscle, there was some growth in part of the control muscles from the pig (Table 3).

The pH values were relatively stable during storage of the control samples. However, there were marked changes in the pH of the inoculated samples as a consequence of bacterial growth. There

Table 2–Log bacterial numbers and pH from control and inoculated muscle stored for 0, 8 and 20 days.

Organisms	Days at 10°C	Rabbit muscle: Bacterial counts[a] Control	Rabbit muscle: Bacterial counts[a] Inoculated	Rabbit muscle: pH Control	Rabbit muscle: pH Inoculated	Pig muscle: Bacterial counts[a] Control	Pig muscle: Bacterial counts[a] Inoculated	Pig muscle: pH Control	Pig muscle: pH Inoculated
Pseudomonas fragi, ATCC4973	0	0.00	5.40	5.63	5.50	0.00	3.30	5.45	5.41
	8	0.00	10.03	5.62	8.33	2.98	9.72	5.46	7.59
	20	0.00	6.47	5.86	8.72	3.28	8.36	5.56	8.49
Micrococcus luteus	0	0.00	5.33	5.68	5.64	0.00	4.73	5.45	5.46
	8	0.00	9.81	5.70	6.13	0.00	7.20	5.51	5.70
	20	0.00	9.86	6.04	6.91	1.88	9.18	6.37	6.09
Leuconostoc mesenteroides	0	0.00	5.51	5.57	5.78	0.00	4.39	5.45	5.49
	8	0.00	8.27	5.57	5.07	1.53	7.67	5.34	4.91
	20	0.00	8.18	6.04	5.50	3.66	7.71	5.58	4.86
Pediococcus cerevisiae	0	0.00	6.01	5.53	5.57	0.00	5.31	5.52	5.58
	8	0.00	7.97	5.65	5.45	1.44	7.63	5.54	5.46
	20	0.00	8.47	6.17	5.37	3.07	7.97	5.90	5.28

[a]Log bacterial numbers per gram of sample.

Table 3–Influence of enzymes and proteins in rabbit sarcoplasm.

Organisms	PFK	ALD	GAPDH	αGPDH	PGAK	PGAM	PPH	PK	LDH	CK	ALB	MB	HB
	Enzyme and protein patterns on starch gel electrophoresis												
Control	+	+	+	+	+	+	+	+	+	+	+	–	+
Pseudomonas fragi ATCC4973	–	+	+	–	–	–	–	+	–	–	+	–	–
Micrococcus luteus	–	+	+	–	+	–	+	+	+	+	+	–	–
Leuconostoc mesenteroides	+	–	–	–	–	–	–	–	+	–	+	–	–
Pediococcus cerevisiae	+	–	–	–	–	–	–	–	+	–	+	–	–

[+]The enzyme or protein pattern of the inoculated meat was present on starch gel electrophoresis after storage for 20 days at 10°C.
[–]The enzyme or protein disappeared upon starch gel electrophoresis under the above conditions.

Table 4–Influence of enzymes and proteins in porcine sarcoplasm.

Organisms	ALD	GAPDH	PGI	αGPDH	PGAM	PK	LDH	CK	ALB	MB	HB
	Enzyme and protein patterns on starch gel electrophoresis										
Control	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas fragi ATCC4973	–	–	–	–	–	+	–	–	+	–	–
Micrococcus luteus	+	+	+	+	+	+	+	+	+	–	+
Leuconostoc mesenteroides	+	+	–	–	–	–	+	–	+	–	–
Pediococcus cerevisiae	+	+	+	+	+	+	+	+	+	+	–

[+]The enzyme or protein pattern of the inoculated meat was present on starch gel electrophoresis after storage for 20 days at 10°C.
[–]Above pattern was not present upon starch gel electrophoresis under the same conditions.

was a gradual increase in the pH of both rabbit and porcine muscle (Table 2) after inoculation with either *P. fragi* or *M. luteus*, but there was a decrease in pH after storage following inoculation with *L. mesenteroides* and *P. cerevisiae*. Storage of both rabbit and porcine muscle inoculated with *P. fragi* resulted in some increase in the percentage of the sarcoplasmic protein fraction.

Sarcoplasmic proteins

Identification of the various sarcoplasmic proteins in rabbit and porcine muscle was made on fresh sarcoplasmic preparations. Results are shown in Figure 2. A comparison of the various proteins identified for rabbit and pig muscle shows generally good agreement with those reported earlier by Scopes (1968). In the present study with rabbit muscle, one of the LDH (lactic dehydrogenase) isozymes is much larger than the similarly located one identified by Scopes (1968). In addition, several isozymes of PK (pyruvate kinase) were also identified in rabbit muscle, whereas Scopes (1968) identified only a single PK band. Similarly, 2 PGAM (phosphoglycerate mutase) isozymes were found for rabbit muscle, whereas Scopes (1968) found only a single PGAM band.

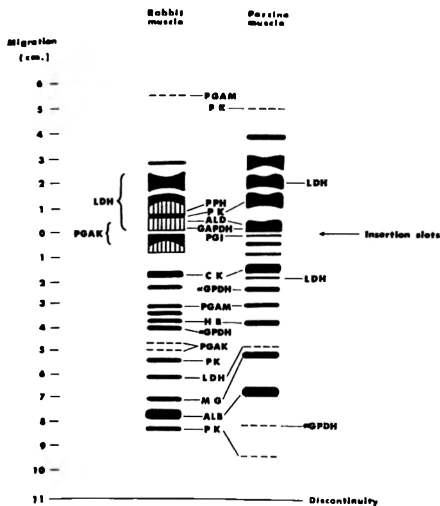

Fig. 2—Identification of the sarcoplasmic proteins by starch-gel electrophoresis of extracts from rabbit and porcine muscle. Abbreviations for the various proteins are given in Table 1, except for: HB = hemoglobin; MB = myoglobin; and ALB = albumin.

With pig muscle, Scopes (1968) identified a PGAK (phosphoglycerate kinase) band, while the corresponding band in the present study was identified as PGI (phosphoglucose isomerase). Results should not be expected to completely agree, since Scopes (1968) dialyzed the sarcoplasmic preparations and utilized vertical starch gel electrophoresis. In the present study, dialysis of the sarcoplasmic extracts was not utilized and flat-bed starch-gel electrophoresis was substituted for the vertical bed.

Although protein values were determined for all samples, the magnitude of the standard deviations for the proteins indicated that the variation between samples within treatments was greater than that due to treatments. Consequently, the protein values are not included herein. That total protein values do not change significantly should not be construed as indicating there were no marked changes within the proteins, since the earliest steps in protein breakdown involve only denaturation or partial hydrolysis, or both. Even minor changes in the properties of the proteins could affect their migration on electrophoresis and would be reflected in the disappearance or decrease in the size of certain fractions.

The effects of the different microorganisms upon the sarcoplasmic proteins of rabbit muscle are summarized in Table 3; similar information for porcine sarcoplasmic proteins is in Table 4. Results show that there was some protein breakdown of the control samples during storage at 10°C. In porcine muscle, myoglobin showed a strong band at 0 days storage, which almost completely disappeared after 20 days. Rabbit myoglobin also showed considerable breakdown during storage of the control sample, but to a lesser extent than that from the pig. On the other hand, albumin from both rabbit and pig muscle was relatively stable for storage periods as long as 20 days in the absence of microbial growth. Creatine kinase was also relatively stable in control samples of both rabbit and porcine muscle during storage at 10°C, although some breakdown was evident at 20 days.

Fig. 3—Densitometer tracings of disc-gel patterns from rabbit muscle. Top—control; center—Pseudomonas fragi; bottom—Pediococcus cerevisiae. Numbers on all tracings correspond to those of the control, while letters indicate new bands.

Fig. 4—Densitometer tracings of disc-gel patterns from porcine muscle. Top—control; center—Pseudomonas fragi; bottom—Pediococcus cerevisiae. Numbers correspond to those of the control on all tracings; letters indicate new bands.

P. fragi caused the most extensive protein degradation of any of the microorganisms studied. This is not surprising, since it is known that *P. fragi* has proteolytic activity. Examination of Table 4 shows that *P. fragi* decomposed aldolase, glyceraldehyde phosphate dehydrogenase, lactic dehydrogenase, creatine kinase and hemoglobin in the sarcoplasm from the pig. Rabbit muscle inoculated with *P. fragi* decomposed creatine kinase, phosphofructokinase, phosphoglycerate kin-

ase, phosphopyruvate hydratase and hemoglobin. Results showed that the amount of sarcoplasmic protein breakdown was almost as extensive at the end of 8 days' storage as after 20 days.

L. mesenteroides caused breakdown of aldolase, glyceraldehyde phosphate dehydrogenase, pyruvate kinase, phosphopyruvate hydratase and creatine kinase in rabbit muscle. For porcine muscle, the action of *L. mesenteroides* was less extensive, acting only upon pyruvate kinase and creatine kinase. The differences in activity upon the 2 kinds of muscle could be a consequence of pH differences, but this seems unlikely since the pH values were only about 0.10–0.15 unit higher in rabbit muscle. Apparently, the organism has the ability to produce the enzyme necessary for breaking down several other proteins but for some reason does not act upon pig muscle. Work is being initiated to elucidate the reason for the difference in action upon porcine and rabbit muscle.

M. luteus acted upon rabbit muscle to decompose creatine kinase and phosphofructokinase, whereas, with porcine muscle there was little action except for a minor effect upon myoglobin. Thus, *M. luteus* has only a limited ability to hydrolyze muscle sarcoplasmic proteins and apparently does not play a direct role in their degradation. That they have a limited effect upon sarcoplasmic proteins, however, does not necessarily mean they are not of significance in meat spoilage. Jay and Kontou (1967) have previously demonstrated that many meat spoilage microorganisms act upon nucleotides, free amino acids and other nonprotein nitrogenous constituents.

P. cerevisiae acted upon rabbit muscle to decompose aldolase, glyceraldehyde phosphate dehydrogenase, pyruvate kinase, phosphopyruvate hydratase and creatine kinase. The action of *P cerevisiae* upon porcine muscle was limited to slight breakdown of hemoglobin. Reduction in pH resulting from lactic acid production by *P. cerevisiae* could be responsible for the smaller amount of proteolysis by this microorganism, but it does not explain the differences between rabbit and porcine muscle.

Urea-soluble proteins

Disc-gel electrophoresis was utilized to follow changes in the 8 M urea-soluble proteins of inoculated rabbit and porcine muscle. Densitometer tracings of the disc gel patterns of the urea-soluble proteins from control and inoculated rabbit and porcine muscle are shown in Figures 3 and 4, respectively. Control rabbit muscle stored for 20 days at 10°C showed 15 distinct disc gel bands. After incubation with *P. fragi,* the intensity and size of most of the bands was greatly diminished, indicating considerable protein breakdown. Figure 3 shows that 2 of the bands had completely disappeared, while 4 new bands were present. Control pig muscle incubated for 20 days at 10°C contained 17 distinct protein bands. Only 8 of the original bands were still present after inoculation and incubation with *P. fragi,* although 1 new band was apparent (Fig. 4). With *P. cerevisiae*, 12 of the original bands were present following incubation of pork muscle, while 1 new band was apparent (Fig. 4). 4 new bands were apparent in rabbit muscle (Fig. 3). Neither *M. luteus* nor *L. mesenteroides* showed any measurable effect upon the 8 M urea-soluble proteins.

Interestingly enough, the 8 M urea-soluble proteins from pig muscle were affected more by growth of *Pseudomonas* and *Pediococcus* than were those from rabbit muscle. Thus, it seems likely that the proteolytic activity of different microorgansims is quite specific depending upon the type and properties of the enzymes produced by each. This suggests that meat spoilage is a highly complex process involving a number of organisms producing highly specific enzymes, which preferentially act upon only certain proteins or enzymes indigenous to muscle. A complete understanding of the specificity of enzyme activity of meat spoilage microorganisms could lead to methods of inhibiting or preventing microbial spoilage of meat.

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Ms. received 7/30/69; revised 12/1/69; accepted 12/1/69.

Journal Article No. 4818, Michigan Agricultural Experiment Station.

This investigation was supported in part by Public Health Service Research Grant No. FD-00097 from the Food and Drug Administration.

The authors acknowledge the technical assistance of Margaret Shumsky Koechlein.

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DENATURATION THERMOPROFILES OF SOME PROTEINS

SUMMARY—Denaturation thermoprofiles of raw beef muscle tissue and egg albumin were determined. Irreversible changes occurred in two stages: the first peak was observed at 65° and 73°C and the second at 82° and 83°C, respectively for beef muscle tissue and egg albumin. Pertinent calculations suggest that the second peak is due to changes in the water structures. It may be hypothesized that protective, semicrystalline water structures surrounding the nonpolar amino acid radicals of proteins were collapsed by heat, followed by the formation of hydrophobic bonding yielding aggregated denatured state. Thermodynamic information was obtained from the thermoprofiles.

INTRODUCTION

DENATURATION manifests itself most profoundly in decreased solubility of proteins and, therefore, in decreased water binding effect, since solubility and water binding are intimately associated (Hamm, 1966). Another of the most commonly recognized features of protein denaturation is the unmasking of reactive groups (Ling, 1962). Denaturation phenomena have been reviewed in several comprehensive writings (Joly, 1965; Kauzmann, 1959; Neurath et al., 1944). In the denaturation process, proteins undergo profound changes in many specific properties following very mild treatments. Hence, protein molecules must have structures which fail rather easily under certain circumstances.

Steim (1965) used a differential thermal analysis technique to study the denaturation of six isolated, purified proteins in aqueous solution. Each protein processed a characteristic denaturation endotherm, the peak temperature of which varied from 55°C–77°C. However, it is very likely that isolation and purification treatments change structures which may be present in unpurified natural proteins.

The object of this study, using differential microcalorimetry, was to investigate the denaturation thermoprofiles of two animal proteins, beef muscle tissue and egg albumin, in their natural state.

EXPERIMENTAL

RAW BEEF MUSCLE tissue and egg albumin as well as salt-soluble protein extracted with 7% saline from beef muscle tissue (Karmas, 1968) were used as sample materials. Samples weighing 18–20 mg were sealed hermetically in aluminum pans and held at 2°C, –12°C (36 hr), or –196°C (liquid N_2, 1 hr). Changes taking place when the temperature of the samples was programmed upwards in the Differential Scanning Calorimeter, Model DSC-1B (The Perkin-Elmer Corporation, Norwalk, Connecticut) were recorded on charts. The trials were performed at 0.001 cal/sec output sensitivity for a full-scale deflection at 10°C/min heating rate from 37°C–140°C. The sample pans were able to withstand internal pressures developed when the temperature was raised to 140°C maximum. After cooling, the same sample was re-run under identical conditions. In addition to this, other temperature patterns between 37°C and 140°C were used.

RESULTS & DISCUSSION

THE ENTHALPY INCREASE in the denaturation process of pure proteins is in the range of 10–200 kcal/mole (Joly, 1965; Neurath et al., 1944). Taking into consideration the molecular weights of pure proteins, the enthalpy of denaturation is in the order of only 1 cal/g of protein. This is very small compared with the enthalpy of 539 cal required to evaporate 1g of water at 100°C (Weast, 1968). In order to observe the small enthalpy change, water evaporation had to be prevented. This was accomplished by filling the sample containers practically to capacity and sealing the samples hermetically.

Representative heat denaturation thermoprofiles are presented in Figure 1. Freezing the samples at either –12°C or –196°C, prior to trials, did not affect the shape of the thermoprofiles to any significant extent.

At the first trial, the thermoprofile labeled "native state" was traced. When the same sample was cooled and re-run, the portion of the thermoprofile between the temperature range of 50°C–90°C was different for beef muscle tissue and egg albumin, while the remaining parts of the thermoprofiles were unaltered. When the same sample was run a third time, the thermoprofile of the denatured state was reproduced. When the beef muscle tissue and egg albumin samples were run first from 37°C–75°C, only the first peak appeared on the thermoprofile. When the sample was re-run from 37°C–95°C, the first peak was absent but the second peak appeared. Finally, when the samples were run once again from 37°C–140°C, the thermoprofiles for denatured proteins

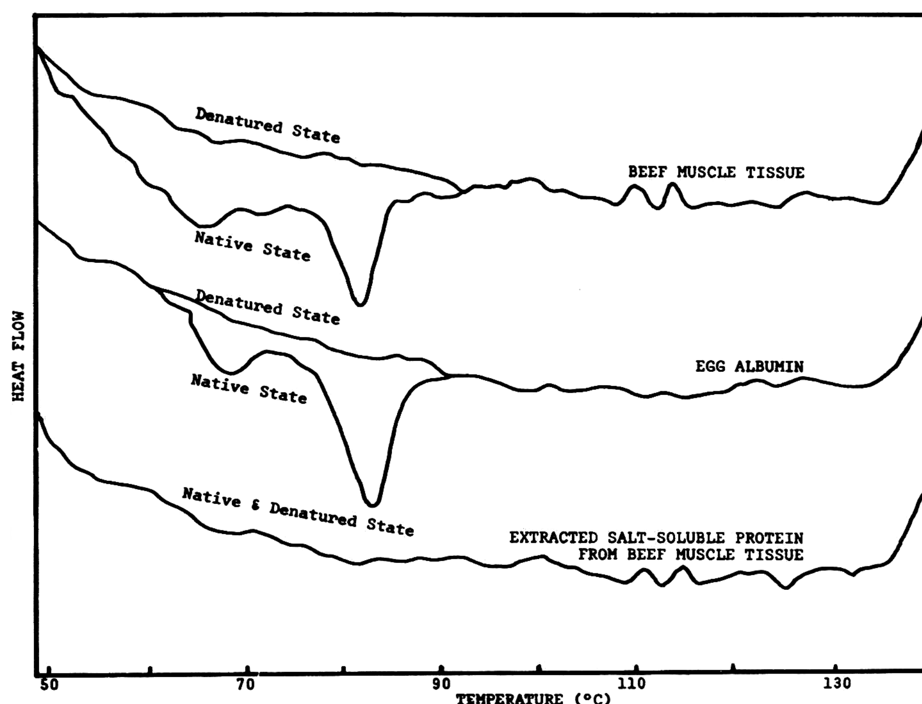

Fig. 1—Representative DSC heat denaturation thermoprofiles of various proteins subjected to a 10°C/min temperature increase. The curve labeled "native state" was traced at the first trial; the curve labeled "denatured state" was traced at the second trial with the same sample.

were obtained (Fig. 1). After each trial, the sample capsule was opened and the state of denaturation confirmed visually.

Table 1–Data on heat denaturation of proteins obtained from thermoprofiles.

Phenomenon of denaturation	Beef: Most occurrence or mean value	Beef: Range	Egg albumin: Most occurrence or mean value	Egg albumin: Range
Beginning (°C)	49	49–52	62	60–63
End (°C)	90	90–94	96	91–97
Temp. of first peak max (°C)	66	65–68	72	68–73
Temp. of inflection point (°C)	73	73–75	77	74–77
Temp. of second peak max (°C)	82	±0	83	±0
Total energy of denaturation (cal/g of sample)	0.210	±0.02	0.146	±0.01
First peak area (%)	59	58–61	30	28–32
Partial energy of denaturation per first peak (cal/g of sample)	0.124		0.044	
Protein content (%)	20		10.5	
Partial energy of denaturation in the first peak per 1% protein (cal)	0.0062		0.0042	
Second peak area (%)	41	39–42	70	68–72
Partial energy of denaturation per second peak (cal/g of sample)	0.086		0.102	
Moisture content (%)	73.8	±0.5	87.7	±0.3
Partial energy of denaturation in the second peak per 1% moisture (cal)	0.0012		0.0012	
pH before denaturation	5.65	±0.05	8.2	±0.1
pH after denaturation	5.85	±0.05	8.5	±0.1

The thermoprofiles of the extracted salt-soluble protein from beef muscle tissue indicated no significant difference between the "native" and the denatured state. It may be possible that the protein was denatured while extracted with a 7% NaCl solution.

An interesting duplication of the peak complex in beef muscle tissue and extracted salt-soluble protein was observed between 109°C and 116°C in the thermoprofiles. When a few nonpolar aqueous amino acid solutions were run under identical experimental conditions, isoleucine was the only amino acid giving peaks of similar shape in the same temperature range. No systematic follow up was performed. It should be stated that the instrument was free of any background noise at this high sensitivity level.

A sudden exothermic rise in the thermoprofile was noticed above 135°C. It was not possible to follow this change further because the hermetically sealed sample capsule burst at about 140°C, accompanied by a perpendicular endothermic drop in the thermoprofile.

A summary of data obtained from numerous denaturation trials (at least 12 replicates) with pertinent calculations has been compiled in Table 1. Although the beginning of the denaturation process for beef muscle tissue was observed at 49°C, this point was not necessarily the first reaction. The initial temperature of the instrument was 37°C, which resulted in the first undisturbed recorded temperature at 49°C. Of course, cooling of the sample holder to a lower temperature would permit a lower temperature of observation; however, this would introduce other undesirable variables. Hamm (1966) states that changes in myofibrillar proteins start at approximately 50°C, whereas denaturation of the sarcoplasmic proteins starts at lower temperatures.

The first peak maximum occurred at 66°C for beef muscle tissue and at 73°C for egg albumin (Table 1). Hamm (1966) states that most of the globular muscle proteins are coagulated at 65°C, while collagen shrinks at temperatures around 63°C, gelatinizing at higher temperatures. According to Ling (1967), on heating in a near-neutral solution, the α-helical structure rather abruptly disappears between 60°C and 70°C, and the denatured protein molecule assumes a form referred to as a random coil. This information implies that the first peak of the denaturation thermoprofile may be strongly related to the protein portion of the water-protein system of the sample. Calculations indicated that the energy of denaturation per 1% protein was 1.5 times larger for beef muscle tissue than for egg albumin (Table 1). This difference may be due to the nature of the proteins and maybe greater alkalinity of egg albumin.

The second peak, however, occurred at almost the same temperature for both proteins, 82°C and 83°C, with no significant deviation. The shape of this peak was a rather narrow normal distribution curve, indicating that a single reaction could have taken place over a rather narrow temperature range. The peak size was almost the same for both proteins. This observation directed attention to the presence of water. Beef muscle tissue as well as egg albumin both contain large amounts of water (Table 1). Drost-Hansen (1965) has repeatedly pointed out that the temperatures at which the "kinks" occur in aqueous solutions are rather insensitive to both the nature and concentration of the solute, i.e., protein in living systems. Due to this implication, calculations were performed for the second denaturation peak with respect to the water content of the samples. The partial energy of denaturation per 1% moisture in the second peak for both beef muscle tissue and egg albumin was 0.0012 cal (Table 1). This fact suggests that the second denaturation peak is mainly due to a change in the water structures of the water-protein system with a peak occurring at 82°C–83°C.

It is difficult to interpret these data. According to Klotz (1960), nonpolar side chains of proteins form crystalline hydrates with water and the coalescence of these hydration "icebergs" through hydrogen bonding procedures stable ice lattices in which the nonpolar amino acid residues, rather than being on the interior of the protein molecules, are on the exterior in contact with water. Although the entire subject matter of protein denaturation has been (Schachman, 1963) and still is subject to much controversy, it is reasonable to hypothesize that the nonpolar residues may be encapsulated by semicrystalline polyhedral water cages which stabilize them in vivo. With an increase in enthalpy, the thermodynamic barrier is overcome, and the protective polyhedrons surrounding the nonpolar amino acid residues collapse. As a consequence of that the nonpolar residues are unmasked and aggregate through formation of hydrophobic bonds. Maybe through this transformation transparent soluble egg albumin becomes an opaque insoluble coagulate. As another argument, it is known that coagulation of myofibrillar proteins through heat produces toughness (Lawrie, 1966). It could be that when the water structures collapse, the formation of cross-linked structures in proteins (through hydrophobic bond-

ing) may produce toughness in myofibrillar proteins.

Whatever is happening, the structure of water in water-protein system probably plays an important role. Ling (1967) states that each native protein must possess a specific complex water structure surrounding it; hence, heat denaturation involves not just protein but the entire water-protein system, for without water, protein cannot undergo denaturation. Based on the limited data of this study it may be hypothesized that denaturation of the two proteins investigated takes place in two irreversible steps: (a) a region mainly related to the protein portion and (b) a region mainly related to the water structures of the water-protein system. It was surprising that these two steps occurred similarly in two entirely different proteins: beef muscle tissue (with highly-developed structural bioengineering features), and egg albumin (a solution of proteins).

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Ms. received 10/28/68; revised 7/1/70; accepted 7/14/70.

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FLAVOR QUALITY IN EXPLOSION PUFFED DEHYDRATED POTATO. 1. A Gas Chromatographic Method for the Determination of Aldehydes Associated with Flavor Quality

SUMMARY—Explosion puffed dehydrated potatoes may develop a characteristic off-flavor during puffing. An objective method, based on the analysis of headspace vapor by GLC, was developed to measure peak heights of components associated with this off-flavor. Ground potatoes and an internal standard, ethylbutyrate, were added to boiling water, and the mixture was equilibrated for 10 min at 98°C. A vapor sample was then withdrawn and analyzed chromatographically on a Carbowax 20M packed column at 100°C using an inlet splitter. Heights of peaks corresponding to 2-methylpropanal plus acetone, 2-methylbutanal plus 3-methylbutanal, hexanal, and ethylbutyrate were determined and peak ratios calculated. The method was found to be precise, accurate, and rapid. Comparisons of a number of potato samples by sensory evaluation and the objective method showed that the intensity of the off-flavor is associated with the heights of the 2-methylpropanal plus acetone and 2-methylbutanal plus 3-methylbutanal peaks.

INTRODUCTION

EXPLOSION PUFFED dehydrated potatoes may develop a characteristic off-flavor during puffing which has at least two major flavor notes, one being described as scorched and the other as resembling wet fur or laundry. Both are distinct from the rancid flavors which may also develop in this product on storage. The intensity of the "puffing" off-flavor varies with the processing conditions and can be reduced by various modifications of the process (Cording et al., 1968).

Research was undertaken to determine the identity and origin of the "puffing" off-flavor and to eliminate this defect through process modification. To accomplish these aims, an objective method for the determination of flavor quality in explosion puffed dehydrated potatoes was required.

Investigations of the flavor of boiled potatoes by Self et al. (1963) resulted in the identification of 11 volatile compounds, a number of which may arise from the Strecker degradation of amino acids (Casey et al., 1965). Buttery and Teranishi (1963), using direct vapor injection gas-liquid chromatography, found that three of these volatile compounds, 2-methylpropanal (2MP) and 2- and 3-methylbutanal (2MB and 3MB) were present in the vapor above reconstituted potato granules which had been stored under conditions favoring nonenzymatic browning. Using a modification of the direct vapor injection technique, Filipic (1967) demonstrated that these same compounds were present in explosion puffed dehydrated potatoes and that their concentration was related to the intensity of the "puffing" off-flavor.

The present work describes the development and application of this objective method for the determination of flavor quality in explosion puffed dehydrated potatoes.

EXPERIMENTAL

Preparation of potato samples

15g of ground (20 mesh) dehydrated potatoes were added to 300 ml preboiled distilled water at 98–100°C in a 500 ml Erlenmeyer flask which was immersed to a constant depth in a boiling water bath, mixing in the flask being provided by a magnetic stirrer. Immediately after the addition of the potato sample, the flask was stoppered with a No. 7 rubber stopper through which was inserted a 90 × 21 mm glass tube closed at the top with a serum cap. The glass tube was heated to a wall temperature of approximately 105°C by a heater tape to minimize condensation. The flask-stopper assembly was vented to the atmosphere with a 24 gauge 3/4 in. hypodermic needle inserted in the serum cap.

Exactly 1 min after the flask was stoppered, 1 ml of a freshly prepared aqueous solution containing 40–50 ppm ethylbutyrate, used as an internal standard, was injected through the serum cap into the potato slurry using a 1 ml syringe and a 20 gauge 6 in. hypodermic needle.

Within 5 min the potato slurry attained a constant temperature of 98°.

Exactly 10 min after the flask was stoppered, a vapor sample was removed for analysis.

Syringe technique

A 2.5 ml Hamilton Gas Tight syringe, insulated with asbestos tape and fitted with a Hamilton 2 in. chromatograph type needle (KF 72822), was heated to approximately 80°C in a vacuum oven. The syringe was removed from the oven 15 sec before the vapor sample was taken, and the needle was inserted through the serum cap in the stopper assembly. Approximately 2.2 ml of vapor was withdrawn, held in the syringe for 5 sec and then injected back into the flask without removing the needle. A second 2.2 ml vapor sample was then withdrawn and held in the syringe for 10 sec. The volume was adjusted to 2.0 ml, the needle was withdrawn from the serum cap, and the vapor was injected immediately into the gas chromatograph.

Gas-liquid chromatography

Vapor samples were analyzed with a Perkin-Elmer Model F-11 Gas Chromatograph using a 9 ft 1/8 in. stainless steel column containing 20% Carbowax 20M on 60/80 acid washed Chromosorb W. The oven temperature was set to 100°C and the injection temperature to a setting of 5 (265°C). An inlet splitter (Perkin-Elmer Split Needle Assembly N 72822) was used to give a split ratio of approximately 3 and a helium flow rate through the column of 22 ml/min; air and hydrogen flow rates to the flame ionization detector were more than 200 and 24.5 ml/min, respectively. The flame ionization amplifier was set to a range of 10. The amplifier output was recorded using a Clevite Brush Mark 250 Recorder set at 100 mv with a chart speed of 0.5 min/division. Chromatograms were recorded for approximately 10 min after which no further peaks were observed.

With this procedure it was possible to perform as many as 18 analyses in an 8-hr day.

Mass spectral analysis of headspace vapor components

The previously described procedure for sample preparation and analysis were modified as described below to facilitate the mass spectral analysis of headspace vapor components. A slurry consisting of 50g ground explosion puffed dehydrated potato in 300 ml H_2O in a 500 ml Erlenmeyer flask was held at room temperature for 3 hr, then saturated with anhydrous Na_2SO_4 and equilibrated in a boiling water bath for 1 hr. A 2 ml headspace sample was injected into an F & M 810 Gas Chromatograph and separated on a 12 ft 1/8 in. stainless steel column containing 15% Carbowax 20M on 80/100 Chromosorb W at 100°C using a helium flow rate of 20 ml/min and a thermal conduc-

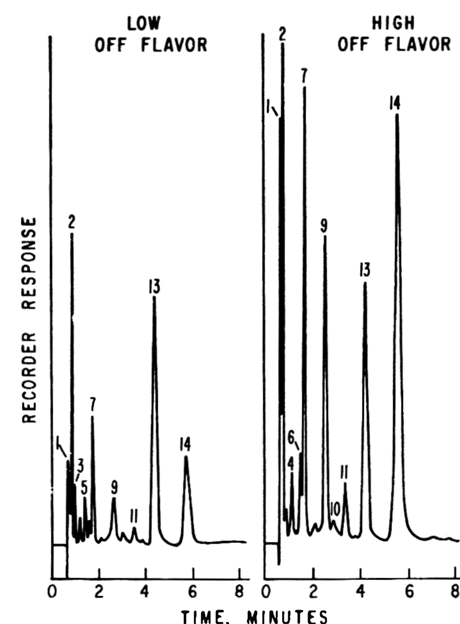

Fig. 1—Headspace vapor analysis of explosion puffed dehydrated potatoes having low and high off-flavor levels.

tivity detector at maximum sensitivity. The gas chromatograph was coupled to a CEC 103 Mass Spectrometer using a stainless steel helium separator at the interface. Chromatograms obtained by this procedure were found to be similar to those obtained by the standard procedure.

Analysis of aldehyde solutions

Standard curves of peak height vs aldehyde concentration in aqueous solution were obtained by adding different volumes of freshly prepared standard 2MP, 2MB, and 3MB solutions to 300 ml of preboiled distilled water at 98–100°C in place of the potato sample and then following the procedure described previously without exception. Concentrations were chosen to produce peak heights similar to those obtained with potatoes having an intense "puffing" off-flavor.

Similar curves of peak height vs. aldehyde concentration in potato were obtained by adding different volumes of the same aldehydes to boiling slurries of dehydrated potato having a low level of the puffing off-flavor immediately after the slurry (15g/300 ml distilled water) had been prepared. The previously described procedure was then followed.

All analyses were performed in triplicate or quadruplicate.

Sensory evaluations

Samples of dehydrated potatoes were reconstituted in boiling water (30g dice /500 ml H_2O) in covered saucepans; explosion puffed products were simmered for 5 min while conventionally dried potatoes were simmered for 20 min. Reconstituted samples were examined in the cooking water by a panel of three expert tasters who are experienced in the evaluation of potato products and sensitive to the off-flavors produced by puffing. Members of the panel independently evaluated coded samples for aroma and flavor and then discussed their observations to arrive at an over-all judgment of off-flavor intensity.

A trained panel of 15 tasters were also used to validate the objective headspace vapor analytical procedure. Tasters were screened for sensitivity to the off-flavor; those selected for the panel were trained by repeated tasting of fresh boiled potato and dehydrated potato samples varying in off-flavor intensity. Panelists were asked to assess the off-flavor level in coded samples of puffed and conventionally dried potatoes using a nine point scale ranging from 0 (no off-flavor) to 8 (very high off-flavor level).

Table 1—Precision of potato headspace vapor analysis.

	Peak height (cm) or ratio					
	Good potato			Poor potato		
Component[a]	Mean	Std. Dev.	Coeff. Var.	Mean	Std. Dev.	Coeft. Var.
2MP + acetone	2.75	.42	15.2	9.11	.651	7.15
2MB + 3MB	1.02	.15	14.5	5.92	.474	8.00
Hexanal	1.98	.36	18.3	7.56	1.06	14.00
(2MP + acetone)/EB[b]	.504	.054	10.7	2.04	.247	12.1
(2MB + 3MB)/EB[b]	.187	.020	10.8	1.32	.122	9.24
Hexanal/EB[b]	.360	.018	5.09	1.68	.056	3.33
Number of replicates		5			5	

[a]2MP = 2-methylpropanal; 2MB = 2-methylbutanal; 3MB = 3-methylbutanal; EB = ethylbutyrate.

[b]Ratio of individual peak heights to the height of the ethylbutyrate peak for each analysis.

Table 2—Regression analysis of peak heights and peak ratios vs aldehyde concentrations in aqueous solutions and in potato slurry.[a]

		Regression coefficient and standard error[b]					
		Peak Height			Peak Ratio		
Aldehyde	Sample	a	b	Sy	a	b	Sy
2-Methylpropanal	Aqueous soln.	0.26	1.16	0.417	0.13	0.782	0.312
	Potato slurry	.99	1.28	.250	.52	.695	.096
3-Methylbutanal	Aqueous soln.	.06	1.02	.341	.07	.627	.166
	Potato slurry	.55	1.01	.463	.35	.499	.068
2-Methylbutanal	Aqueous soln.	–.03	.933	.538	0	.592	.140
	Potato slurry	.68	1.06	.357	.32	.609	.118

[a]Prepared from potato sample having low off-flavor level, 2-MP peak height = 0.97 cm; 2-MB plus 3-MB peak height = 0.69 cm.

[b]Regression coefficients and the standard error of the estimate in y (Sy) were calculated for the equation $y = a + bx$ where y = peak height (or ratio); a = y intercept; b = slope; and x = aldehyde conc. (μg/ml).

RESULTS & DISCUSSION

Analysis of potato samples

Headspace vapor analysis of potato samples differing in flavor quality produced chromatograms showing at least 15 peaks (Fig. 1). Peak 7, identified by retention time and mass spectroscopy as 2MP plus acetone, and peak 9, identified by these procedures as 2MB and/or 3MB, were found to be major components in potato samples having the "puffing" off-flavor. Peak 14, identified by retention time and mass spectroscopy as n-hexanal, was present as a major component in rancid potato samples. These results are in agreement with those of Buttery and Teranishi (1963). The size of other peaks showed little or no relationship to flavor defects in the potato samples.

Effect of water vapor

Preliminary headspace vapor analyses, carried out without the inlet splitter, frequently produced chromatograms in which the hexanal peak was greatly sharpened; in addition, the retention time of this and nearby peaks was slightly reduced. Experiments with wet and dry samples of potato headspace vapor and the vapor above various aldehydes demonstrated that this effect was due to the injection of large and variable amounts of water vapor in headspace samples. Only those compounds having retention times close to that of water were subject to the effect. Similar results were reported by Perry (1966) for n-alkanes in the presence of water and by Wills and Palmer (1967) for esters and some aldehydes and 2-alkanones in the presence of water.

Since the water vapor effect was sufficiently great and unpredictable to impede any quantitative peak height measurements of hexanal or other subject compounds, attempts were made to minimize the effect by changing the injection and/or chromatographic techniques. The water vapor effect could be completely eliminated by introducing the inlet splitter into the chromatographic system, thereby decreasing the amount of water vapor reaching the column, and by concurrently decreasing the attenuation of the flame ionization amplifier to compensate for the smaller sample size.

Precision of headspace vapor analysis

To obtain satisfactory precision, it was necessary to standardize the procedure rigorously, especially the method of vapor sampling, and to introduce an internal standard. Ethylbutyrate was selected as the internal standard since it was found to be sufficiently water soluble and stable for this purpose and since its retention time under the conditions of the analysis corresponded to a gap in the potato headspace vapor chromatograms.

Examples of the precision of the method are given in Table 1. The conversion of peak heights to peak ratios, using the internal standard, was found to be especially advantageous with the 2MB

Table 3–Comparison of objective and subjective evaluations of off-flavor level in dehydrated potatoes.

			Peak ratio[a]		
			2MP + acetone	2MB + 3MB	Sensory evaluation
Panel	Sample	Process	EB	EB	of off-flavor level[b]
Expert 1	334 wo	Puffed	2.03	1.41	Moderate-strong
	338 wo	Puffed	1.66	2.00	Moderate-strong
	334 w	Puffed	0.50	0.19	Low
	337 w	Puffed	0.83	0.58	Low
Expert 2	338 wo	Puffed	1.66	2.00	Moderate-strong
	342 wo	Puffed	2.24	1.89	Moderate
	344 wo	Puffed	1.60	0.63	Moderate
	344 w	Puffed	0.54	0.20	Low
	344	Conventional	0.48	0.10	Absent
Expert 3	359 wo	Puffed	1.32	1.15	Moderate-strong
	359 w	Puffed	1.13	0.94	Moderate
	358 wo	Puffed	0.82	0.56	Low-moderate
	359	Conventional	0.32	0.11	Absent
Trained	359 wo	Puffed	1.32	1.15	3.25 (Moderate)
	359 w	Puffed	1.13	0.94	2.07 (Slight)
	359	Conventional	0.32	0.11	0.14 (Absent)

[a] 2MP = 2-methylpropanal, 2MB = 2-methylbutanal, 3MB = 3-methylbutanal, EB = ethylbutyrate.

[b] Numerical scores obtained from the trained panel of 15 tasters are on a 9 point scale ranging from 0 (no off-flavor) to 8 (very high off-flavor).

plus 3MB peak and the hexanal peak since coefficients of variation for these ratios tended to be substantially lower than for the individual peak height.

Accuracy of headspace vapor analysis

An indication of the accuracy of the headspace vapor analysis was obtained from the results of analyses of aqueous aldehyde solutions and potato slurries to which aldehydes had been added. In all cases, a linear relationship was found between peak height and aldehyde concentration over the range of aldehyde concentrations employed: 0–0.5 μg/ml of solution or slurry (Table 2).

The slopes of these curves, calculated by linear regression, were similar for aqueous solutions and potato slurries. Slopes for 2MB and 3MB were similar and slightly lower than those obtained with 2MP. Calculated intercepts (aldehyde concentration – 0) for potato slurries to which aldehydes were added corresponded well to independent measurements of peak heights in slurries containing no added aldehydes.

Another aspect of the accuracy of the procedure is the rate of equilibration of the headspace vapor composition. This is determined by the rate of rehydration of the potato sample, the geometry of the system, the temperature of the potato slurry, the extent of condensation in the system and the degree to which air in the system is vented. Analyses of potato headspace vapor sampled after 5, 10, 15, 30, 45, and 60 min showed that equilibration occurred within 5–10 min after the addition of the ground potato to the flask; little or no change occurred in the peak heights thereafter.

Application of headspace vapor analysis

The headspace vapor analysis was applied to many samples of explosion puffed and conventionally dehydrated potatoes which were also subjected to sensory evaluation by the expert panel. These samples represented a number of different experimental batches in which the raw material and processing conditions were varied. The sensory and analytical data summarized in Table 3 were obtained at three different panel sessions held during a 1 yr period. Also included in Table 3 are the results of an evaluation of 3 samples by the larger trained panel.

These data show a positive association between the intensity of the off-flavor produced by puffing and the peak heights of the branched chain aldehydes. Differences reported by the trained panel are significant at $P < .05$ using Duncan's multiple range test (Le Clerg, 1957). The association appears to be more consistent with 2 MB + 3MB than with 2MP, probably since the latter peak also contains acetone, a normal volatile component of fresh potato (Self et al., 1963). No attempt has been made to quantify the association between peak height and off-flavor intensity because of the difficulty in comparing sensory data obtained at different panel sessions. This might be feasible if a standard source of the off-flavor could be prepared and evaluated by the panel at each session.

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Ms. received 3/23/70; revised 7/14/70; accepted 7/14/70.

Presented in part at the 30th annual meeting of the Institute of Food Technologists in San Francisco.

The authors wish to thank Miss Oksana Panasiuk for her assistance in the gas chromatographic studies and Dr. Stanley F. Osman for the mass spectral analyses.

Mention of company or trade names does not imply endorsement by the USDA over others not named.

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FLAVOR QUALITY IN EXPLOSION PUFFED DEHYDRATED POTATO. 2. Flavor Contribution of 2-Methylpropanal, 2-Methylbutanal and 3-Methylbutanal

SUMMARY—Headspace vapor components previously associated with the intensity of an off-flavor in explosion puffed dehydrated potatoes were added to potatoes lacking the off-flavor to determine the flavor contribution of these compounds. 2-Methylpropanal produced a characteristic wet fur flavor note while 2- and 3-methylbutanal modified this flavor and contributed burnt flavor notes. While these flavor notes resembled some elements of the puffing off-flavor, the total off-flavor at its normal intensity could not be simulated by combinations of these compounds in potato at realistic concentrations. Acetone, which is also present in the headspace vapor of explosion puffed dehydrated potatoes, was found as a major headspace component of fresh boiled potatoes. This compound and smaller amounts of 2- and 3-methylbutanal were produced in overcooked fresh potatoes which lacked the puffing off-flavor.

INTRODUCTION

PREVIOUS STUDIES of an off-flavor produced by puffing in explosion puffed dehydrated potatoes showed that the intensity of this flavor was associated with the heights of peaks corresponding to 2-methylpropanal (2 MP) plus acetone and 2-methylbutanal (2 MB) plus 3-methylbutanal (3 MB) obtained by GLC analysis of potato headspace vapor (Sapers et al., 1970). The significance of this association with regard to the flavor contribution of these compounds is examined in the research reported herein.

EXPERIMENTAL

Addition of aldehydes to dehydrated potatoes

The contribution of 2 MP, 2 MB and 3 MB to the flavor of dehydrated potatoes was determined by adding these compounds (City Chemical Corporation) at realistic concentrations, individually and in combination, to conventionally dehydrated potatoes after reconstitution and by comparing their flavor with that of explosion puffed dehydrated potatoes. Both dehydrated products were prepared from the same lot of Idaho Russet potatoes using the same process except for the omission of the puffing step with the conventional potato. The explosion puffed and conventional products were reconstituted in boiling water (30g/500 ml) for 5 min and 20 min, respectively. After reconstitution, the puffed sample was found to have a moderate level of the "puffing" off-flavor while the conventional sample had a normal flavor. Immediately after reconstitution, aliquots of freshly prepared aqueous aldehyde solutions were added to samples of conventionally dehydrated potatoes to produce concentrations comparable to those calculated from headspace vapor analyses of potatoes having the off-flavor. Standard curves of peak height vs aldehyde concentration, described by Sapers et al. (1970), were used for this purpose. GLC analyses showed that the aldehydes contained only trace quantities of impurities.

The aroma and flavor of samples to which aldehydes had been added were compared with that of a conventionally dried control and the corresponding puffed product, all samples being examined hot by a three-member expert panel.

Aldehyde concentrations in fresh and dehydrated potatoes

Samples of fresh and dehydrated Idaho Russet potatoes were subjected to different thermal treatments and were then analyzed for 2 MP + acetone and 2 MB + 3 MB by the headspace vapor procedure described previously (Sapers et al., 1970) to determine the relationship between these compounds and potato flavor.

Raw potato dice (65g) were added to 250 ml of preboiled water in the headspace vapor flask and were "cooked" under the same conditions as those used for generating headspace vapor. A vapor sample was taken after 25 min and analyzed by the standard GLC procedure modified by the omission of the inlet splitter and by the use of a lower attenuation (× 5).

Raw potatoes were wrapped in aluminum foil, baked for 60 min at 225° in an oven, diced, added to preboiled H_2O (65g/250 ml) in the headspace vapor flask, heated for 25 min and analyzed as described above.

Raw potato dice (65g) were pressure cooked for 45 min at 15 psi, placed in 250 ml preboiled H_2O in the headspace vapor flask, heated for 25 min and analyzed as described above.

A sample of explosion puffed dehydrated potato prepared from the same raw material as used elsewhere in the experiment was also analyzed by the headspace vapor procedure.

The aroma of each sample was noted after the completion of the headspace vapor analysis.

Mass spectral analyses of fresh boiled potato headspace vapor

A mass spectral analysis of the 2 MP + acetone component of fresh cooked potato headspace was obtained using the same GLC mass spectrometer system described previously (Sapers et al., 1970). The sample was prepared by adding 65g of diced raw Idaho Russet potatoes to 250 ml of boiling saturated Na_2SO_4 solution in a 500 ml Erlenmeyer flask, equilibrating the mixture in a boiling water bath for 80 min and then withdrawing 2 ml of headspace vapor for analysis.

Resolution of aldehyde peaks

The headspace vapor analytical procedure was modified by the substitution of a capillary column for the 1/8" Carbowax 20M packed column to improve the resolution of the aldehyde peaks and to locate additional peaks which might be associated with the "puffing" off-flavor.

The capillary column (Perkin Elmer No. 2) was stainless steel, 50 ft × .02 in., coated with

Table 1—Flavor contribution of 2-methylpropanal (2 MP), 2-methylbutanal (2 MB) and 3-methylbutanal (3 MB) to dehydrated potatoes.

Sample	Added Aldehyde Conc. (ppm)			Aroma	Flavor
	2 MP	2 MB	3 MB		
Puffed	0	0	0	Typical off	Typical off
Conventional	0	0	0	Normal	Normal
Conventional	0.16	0	0	V. Sl. abnormal	Sl. wet fur
Conventional	0.32	0	0	Sl. abnormal	Wet fur
Conventional	0	0.16	0	V. Sl. burnt	Baked potato
Conventional	0	0.32	0	Sl. burnt	Aldehyde
Conventional	0	0	0.16	Sl. burnt	Sl. burnt
Conventional	0	0	0.32	Weak off	Weak off
Conventional	0.16	0.16	0	Weak raw peas	Sl. wet fur
Conventional	0.16	0.32	0	Wet fur	Wet fur
Conventional	0.32	0.16	0	Wet fur	Strong wet fur
Conventional	0.32	0.32	0	Raw peas	Burnt – weak off
Conventional	0.16	0	0.16	Wet fur	Wet fur
Conventional	0.16	0	0.32	Raw peas	Burnt
Conventional	0.32	0	0.16	Aldehyde	Wet fur
Conventional	0.32	0	0.32	Strong raw peas	Wet fur
Conventional	0	0.16	0.16	Raw peas	Burnt – aldehyde
Conventional	0.16	0.16	0.16	Raw peas > wet fur	Wet fur
Conventional	0.32	0.16	0.16	Raw peas > wet fur	Weak off

Table 2–Headspace vapor composition of fresh and dehydrated potatoes.

Sample	Peak height (cm)[a]		Aroma
	2 MP + acetone	2 MB + 3 MB	
Explosion puffed dehydrated	11.40	11.70	Typical off
Conventional dehydrated	4.35	1.40	Normal
Fresh boiled potato (25 min at 98°)	9.65	0.60	Sl. raw boiled potato
Fresh baked potato (60 min at 225°)	12.00	1.55	Baked potato
Fresh pressure cooked potato (45 min at 15 psi)	25.00	7.80	Over-baked potato

[a]No splitter; headspace vapor analyzed after 25 min equilibration; attenuation 5×.

Table 3–Partial resolution of potato headspace vapor components with capillary column.[a]

Sample	Peak height (cm)[b]				Aroma
	2 MP	Acetone	2 MB	3 MB	
Explosion puffed	5.35	3.05	3.10	3.10	Off
Explosion puffed	2.90	2.30	1.80	1.30	Sl. off
Conventional dehydrated	0.85	2.30	0.60	0.45	Normal
Fresh "boiled" (25 min at 98°)	Trace	8.75	0.60	0.50	Boiled
Fresh pressure cooked (45 min at 15 psi)	Trace	11.60	2.50	2.10	Over-baked

[a]Headspace vapor sampled after 25 min; attenuation 5×.
[b]Peak heights measured directly from partially resolved peaks.

Carbowax 1540. An inlet splitter (Perkin Elmer Split Needle Assembly N 72822) was used to give a split ratio of approximately 3 and a helium flow rate through the column of 12 ml/min. A column temperature of 30° was used. Other operating conditions were the same as those described previously (Sapers et al., 1970).

Headspace vapor analyses were performed on explosion puffed, conventionally dehydrated and fresh cooked potatoes using the capillary column and previously described techniques for sample preparation.

Headspace vapor analyses were also performed on aqueous solutions of acetone, 2 MP, 2 MB and 3 MB and on fresh boiled and explosion puffed potatoes to which these compounds had been added.

RESULTS & DISCUSSION

Contribution of aldehydes to the flavor of dehydrated potatoes

The flavor contribution of 2 MP, 2 MB and 3 MB added to conventionally dehydrated potato after reconstitution is summarized in Table 1. The typical off-flavor found in explosion puffed dehydrated potatoes can be resolved into three major flavor notes: the first a scorched or burnt note resembling the flavor developed by non-enzymatic browning during storage of a dry food; the second resembling wet fur or laundry; and the third a toasted flavor similar to that of nuts or dry cereal products and suggestive of pyrazines.

The addition of 2 MP, alone and in combination with the aldehydes, produced the wet fur note of the "puffing" off-flavor. This flavor did not occur when 2 MP was omitted from the mixture. The presence of the other aldehydes with 2 MP modified the wet fur note, in some cases producing an aroma reminiscent of raw peas and burnt flavors, the latter being similar to a weak "puffing" off-flavor at the highest level of 2 MP + 3 MB.

The addition of 2 MB and 3 MB individually produced burnt flavors which in the case of 3 MB at the higher concentration resembled the "puffing" off-flavor at a very low level. 2 MB and 3 MB added together at the lower concentration (this combination using higher concentrations would not be representative of potato headspace vapor composition) produced a raw pea aroma and a burnt flavor similar in some respects to the flavor of the aldehyde solutions.

The most realistic "puffing" off-flavor was produced by the three component mixture, 2 MP being at the higher concentration and 2 MB and 3 MB being at the lower concentration. As with all other samples containing added aldehydes, the off-flavor intensity of this sample was substantially lower than that of the explosion puffed potato. This was true, even though the headspace vapor aldehyde peak heights corresponding to the three component mixture would resemble chromatograms obtained with explosion puffed potatoes having high levels of the off-flavor.

It is apparent, therefore, that important elements of the "puffing" off-flavor (i.e., the toasted flavor note) are due to the presence of compounds other than 2 MP, 2 MB and 3 MB even though these compounds contribute some wet fur and burnt notes to potato.

Headspace vapor composition of fresh and dehydrated potatoes

Headspace vapor analyses were performed on fresh cooked and dehydrated potatoes using the packed Carbowax 20M column which was not capable of resolving 2-methylpropanal and acetone or 2-methylbutanal and 3-methylbutanal. Peak heights for these headspace vapor components are summarized in Table 2.

Fresh boiled and baked potatoes having typical cooked potato aromas were found to have a 2 MP + acetone peak comparable in height to explosion puffed potato having the off-flavor. Mass spectral analysis of the headspace vapor above boiled potato showed this peak to be acetone; previously reported analyses of explosion puffed potato showed the corresponding peak to be a mixture of 2 MP and acetone (Sapers et al., 1970). Consequently, 2 MP rather than acetone is associated with the off-flavor.

Fresh potato, pressure cooked at 15 psi until a brown color and an over-baked flavor were produced, developed a significantly higher 2 MP + acetone peak and a 2 MB + 3 MB peak comparable to that obtained with the puffed sample.

These relationships were further clarified by headspace vapor analyses of fresh

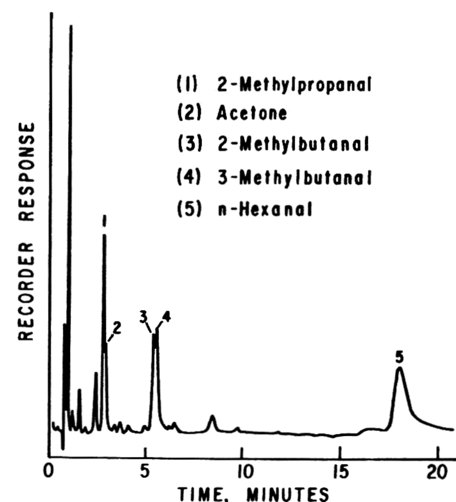

Fig. 1–Headspace vapor analysis of explosion puffed dehydrated potato using Carbowax 1540 capillary column.

cooked and dehydrated potatoes using a Carbowax 1540 capillary column which was capable of partially resolving the 2 MP + acetone and 2 MB + 3 MB peaks (Fig. 1).

Peak heights obtained from these analyses (Table 3) were subject to error because of the mutual contribution of the partially resolved components to each peak and could not be used for quantitative determinations; however, large differences between samples could be established without difficulty.

It can be seen from Table 3 that 2 MP was associated with the off-flavor in dehydrated potatoes and was present only in trace quantities in the headspace vapor above fresh cooked potatoes. The acetone peak was found to be smaller than the 2 MP peak and was not associated with the off-flavor in dehydrated potatoes. Acetone was a major headspace vapor component of fresh boiled potatoes and increased with over cooking.

The 2 MB and 3 MB peaks were approximately equal in height in explosion puffed potatoes having the off-flavor and were both associated with the intensity of the off-flavor. These components were present at low levels in the headspace vapor of boiled potato and were increased to levels comparable to those from puffed potatoes by pressure cooking.

These results show that the association between headspace vapor components and the puffing off-flavor in dehydrated potatoes is meaningful but subject to several limitations. Unless separations are carried out with columns capable of resolving 2 MP and acetone, some confusion may result from the coincidence of these components, the former compound being associated with elements of the off-flavor and the latter component being a normal constituent of fresh cooked potatoes (Self and Swain, 1963). Furthermore, the association should not imply a cause and effect relationship between the off-flavor and the headspace vapor aldehydes. Other compounds, not detected by this method, certainly contribute to the off-flavor. No conclusions can be drawn regarding the cause and means of elimination of the off-flavor until these compounds are identified. Research along these lines is continuing.

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Ms. received 3/23/70; revised 7/14/70; accepted 7/14/70.

Read by title only at the 30th annual meeting of the Institute of Food Technologists in San Francisco.

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FLAVOR VOLATILES OF SOME COOKED VEGETABLES

SUMMARY—Using previously described sampling techniques, the flavor volatiles of a number of cooked vegetables were collected and analyzed by gas chromatography. The vegetables investigated were Brussels sprouts, cauliflower and runner beans and, in addition to the fresh vegetables, some preserved samples were examined. Quantitative measurements were made of the individual flavor components and the results were compared with those for fresh cabbage.

INTRODUCTION

MOST of the flavor volatiles produced from cabbage during cooking have been identified and their relative percentage abundances calculated (MacLeod et al., 1968). Using the same sampling and analysis techniques, 2 other vegetables (closely related botanically to the cabbage) were studied. These were members of the Brassica family, namely Brussels sprouts and cauliflower. It was intended to assess how closely these related vegetables resembled the cabbage in flavor. 1 other green vegetable which was not a Brassica was also studied to discover how this differed from the selected vegetables of the Brassica family. This was the runner bean.

Considering the undesirable results obtained for dehydrated cabbage (Jones, 1967, Part III), some preserved samples of the 3 vegetables also were examined.

Very little work has been carried out on the flavor volatiles of these 3 vegetables. Self et al. (1963) have studied the low-boiling volatiles only of a large number of cooked vegetables including cauliflower, sprouts, and French beans. These cooked vegetables gave the same pattern of low-boiling volatiles, varying only in the relative quantities produced. The higher-boiling volatiles were, however, not considered and some of these may well be specifically present in 1 particular vegetable and completely absent from another. The determination of such higher-boiling volatiles as well as the relative proportions of the low-boiling components thus could be of considerable importance in distinguishing the flavors of cooked vegetables.

EXPERIMENTAL

COOKING, sampling and analysis in all cases were carried out in exactly the same way as for cooked cabbage (MacLeod et al., 1968), although cooking was limited to 10 min. The experiments conducted are summarized in Table 1.

RESULTS & DISCUSSION

Brussel sprouts

Table 2 lists the relative percentage abundances of all the components of the 4 sprout experiment samples and also quoted for comparison are results for an equivalent cooked cabbage sample. The cabbage values quoted here differ slightly from those previously reported (MacLeod et al., 1968), since in this case, as for all other experiments, the cabbage was cooked for 10 min only. This was more representative of normal cooking time than the 30 min originally used, where the intention had been to obtain a large sample for qualitative analysis and not necessarily completely to simulate normal cooking.

Figures 1 and 2 give typical gas chromatograms obtained for fresh chopped cabbage and fresh chopped sprouts, respectively. The original peaks for the 10-min cooked cabbage are numbered in sequence and any new components produced in the experiments described here are shown with the number of the peak immediately preceding and an appended letter (e.g., 14a). These new components were identified where possible by relative retention times as previously described (MacLeod et al., 1968).

2 experiments were carried out on fresh sprouts using 1) whole sprouts; 2), sprouts chopped before cooking. The latter experiment was done to obtain a direct comparison with the other vegetables which are normally chopped or sliced before cooking, although sprouts, of course, are normally cooked whole. It was also considered that the flavor volatiles of the sprouts could well vary as a result of chopping before cooking.

Comparing the results for the fresh chopped sprouts with those of the fresh chopped cabbage, an obvious difference noticed is that the total amount of volatiles liberated by the sprouts is far greater (about 4 times) than that evolved by the cabbage, weight for weight. This might imply that the sprouts have a stronger flavor although, of course, this depends on the nature of these volatiles and the relative amount of each individual component liberated. Chopped sprouts, for example, produce slightly more dimethyl sulfide in absolute terms than the cabbage, although the important point is that relative to the total volatiles liberated in each case, the amount of sulfide formed by the sprouts is only about 1/3 the percentage produced by the cabbage. On the other hand, acetone is present to twice the extent of that produced from cabbage and is by far the most abundant volatile. The methanol content is greatly decreased and ethanol is completely absent.

Both trans-hex-2-en-1-al and cis-hex-3-en-1-ol are present to a lesser extent and so too is the cis-pent-2-en-1-ol. The allyl isothiocyanate content is slightly decreased but the allyl cyanide (3-butene nitrile) is present to an extent of 5 times the cabbage value.

Table 1

Expt. series	Vegetable		Nature of vegetable
A	Brussels sprouts	1.	Fresh–whole
		2.	Fresh–chopped
		3.	Frozen–whole
		4.	Frozen–chopped
B	Cauliflower	5.	Fresh–inflorescence plus outer leaves
		6.	Fresh–inflorescence only
		7.	Fresh–outer leaves only
C	Runner beans	8.	Fresh–sliced
		9.	Frozen
		10.	Dehydrated–"Surprise" beans[1]

[1] It was calculated that on rehydration, "Surprise" beans attained 14 times their own weight. Therefore, to simulate the standard cooking methods, 57 g (equivalent to 800 g fresh vegetable) were added to 1,300 ml of boiling water. Cooking, etc., was then as for the fresh beans and other vegetables.

Table 2.

		Sample peak relative retention time (min)[2]	Reference compound relative retention time (min)	Approximate percentage relative abundance										
					Sprouts				Cauliflower			Runner beans		
Peak No.	Identification			Cabbage	Fresh whole	Fresh chopped	Frozen whole	Frozen chopped	Whole	Inflore-scence only	Outer leaves only	Fresh	Frozen	"Surprise"
Z	Diethyl ether	1.45	1.45	–	0.01	0.001	–	–	–	–	–	–	–	–
1	Methanethiol	1.72	1.72	0.5	0.05	0.05	0.1	0.5	0.5	0.1	0.5	0.1	0.1	0.5
1a	Formaldehyde	2.15	2.15	–	–	–	–	1.0	1.5	2.0	1.0	–	–	–
2	Acetaldehyde[1]	2.35	2.35	3.5	8.0	5.0	4.5	5.5	8.0	6.0	6.0	7.5	27.0	8.0
3	Dimethyl sulfide[1]	2.65	2.65	26.5	11.5	9.0	20.5	14.5	26.5	23.5	16.5	4.5	5.0	–
4	Propionaldehyde[1]	3.08	3.08	0.5	0.5	0.5	0.1	0.1	0.5	1.5	1.0	1.0	4.0	0.5
5	Acetone[1]	3.45	3.45	14.5	30.0	28.0	32.0	28.5	20.0	19.5	25.5	14.5	18.0	57.0
6	Acrolein	4.25	4.25	1.0	–	–	0.1	0.05	–	1.0	1.0	–	1.5	–
7	n-Butyraldehyde	4.75	4.75	1.0	1.0	1.0	0.5	0.5	0.5	1.0	1.0	0.5	2.0	3.5
8	Ethyl methyl ketone[1]	5.30	5.30	1.0	1.5	1.0	0.5	0.5	0.5	0.5	1.5	1.5	0.5	1.0
9	Methyl alcohol[1]	5.75	5.75	12.0	1.5	0.5	0.5	0.5	1.0	1.0	1.0	0.5	2.0	9.0
10	Ethyl alcohol	6.65	6.65	3.5	2.5	2.0	1.0	0.5	0.5	0.5	4.0	17.0	1.5	9.5
11	?	7.25		2.5	–	–	–	–	–	1.0	2.0	–	–	–
12	Diethyl ketone[1] plus diacetyl[1]	8.30	8.30	6.0	5.5	2.5	8.0	4.0	1.0	2.5	4.5	10.5	16.0	4.0
12b	?	10.25		–	0.5	0.5	0.5	–	3.5	2.5	–	–	–	–
13	Methyl alcohol/water profile[4]	~11.2	~11.2	–	–	–	–	–	–	–	–	–	–	–
14	But-2-en-1-al	11.90	11.90	1.0	1.0	0.5	1.0	1.0	0.5	1.0	1.0	2.0	2.5	2.0
14b	Dipropyl sulfide	12.60	12.60	–	–	–	0.05	0.01	0.05	–	0.01	0.1	1.0	0.1
15	Dimethyl disulfide[1] plus *n*-Hexanal[1]	12.95	12.95	1.0	1.5	1.0	1.0	0.5	0.5	1.5	2.0	5.5	12.0	4.0
16	Allyl alcohol	13.75	13.75	0.1	0.1	0.1	0.1	0.05	0.5	0.5	1.0	1.5	0.5	0.1
17	Dipropyl ketone	14.4	14.4	0.1	–	–	–	–	–	–	–	2.5	0.5	–
18	Trans-pent-2-en-1-al[1]	15.0	15.0	0.5	0.5	0.5	0.5	0.5	2.0	0.5	0.5	2.0	0.5	1.0
19	?	15.4		1.5	0.05	1.0	0.1	0.05	0.5	0.5	1.0	0.1	0.1	0.1
19a	Trans-crotononitrile	16.2	16.2	–	–	–	–	0.01	–	0.01	–	–	–	–
20	Allyl cyanide[1]	16.8	16.8	3.0	7.0	15.0	13.5	22.5	24.5	23.0	9.5	6.5	0.1	0.5
21	Trans-but-2-en-1-ol	17.9	17.9	0.5	0.5	0.1	–	–	–	–	0.5	3.0	–	–
21a	?	18.4		–	–	–	–	–	–	–	–	7.0	0.5	–
22	Trans-hex-2-en-1-al[1]	19.1	19.1	1.5	0.05	0.1	0.01	0.05	0.5	0.1	0.5	–	0.1	–
23	Methyl propyl disulfide	20.0	20.0	1.0	1.0	0.5	1.0	1.0	0.5	1.0	0.5	0.1	0.1	–
24	Methyl isothiocyanate?	20.7	20.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	–	–	–
24a	?	21.3		–	–	–	–	–	–	–	–	4.0	1.0	–
25	Cis-pent-3-en-1-ol	22.5	22.5	0.5	16.0	15.0	12.0	17.5	0.1	0.5	–	1.5	1.0	–
25a	Acetoin or n-octanal	23.6	23.5 acetoin 23.7 octanal	–	0.1	0.05	0.05	0.05	0.1	0.1	0.5	–	–	–
26	?	24.6		0.1	0.5	0.05	0.5	0.1	0.5	0.5	0.5	–	1.0	–
26a	Butyl methyl disulfide or propyl isothiocyanate	25.6	25.6 disulfide 25.7 isothiocyanate	–	–	1.0	–	–	0.5	–	4.0	0.5	1.5	–
27	Cis-pent-2-en-1-ol ?[3]	26.6	26.6	2.0	0.1	0.05	–	–	0.01	0.05	–	0.5	–	–
27a	?	27.6		–	0.1	0.1	–	–	–	–	–	2.5	–	–
28	n-Hexanol	27.9	27.9	0.5	–	–	–	–	0.05	0.1	0.1	–	–	–
29	Allyl isothiocyanate[1]	30.5	30.5	6.5	2.0	4.0	0.5	0.1	4.5	5.5	10.0	–	–	–
30	Cis-hex-3-en-1-ol[1]	31.6	31.6	4.5	–	1.5	–	–	0.5	0.5	2.0	1.5	–	–
30a	Dipropyl disulfide	33.2	33.2	–	–	–	–	–	–	–	–	–	0.5	0.5
31	Butyl isothiocyanate	33.8	33.8	1.0	0.5	0.5	0.5	0.5	0.5	1.5	1.0	1.0	–	–
32	Trans-hept-2-en-1-ol	38.6	38.6	1.0	0.1	0.1	–	–	–	0.1	0.5	–	–	–
32a	Trans-hept-3-en-1-ol	39.8	39.8	–	–	–	–	–	–	–	–	0.5	0.1	–
33	?	43.8		1.0	6.5	8.5	0.1	0.5	–	–	–	0.5	–	–
	Total volatiles (sq mm)			15,760	31,315	65,905	20,630	57,775	41,015	56,150	32,385	14,060	12,535	7,190

[1] Identified by mass spectrum also.
[2] Internal standards were used (MacLeod et al., 1968).
[3] Tentative identification only, due to a mass spectrum being available which could not be identified positively and which does not completely confirm the identification given.
[4] See MacLeod et al., 1968, for details.

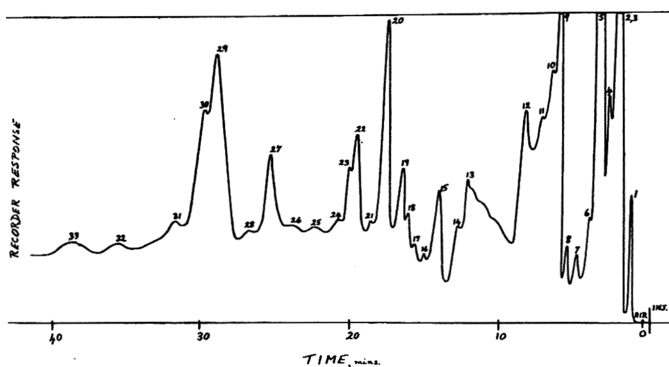

Fig. 1–Typical gas-liquid chromatogram of the flavor volatiles obtained from fresh cabbage. Conditions of analysis as previously given (MacLeod et al., 1968), with an attenuation setting of 50 (5 × 10^{-11} A.) and a 1-µliter sample.

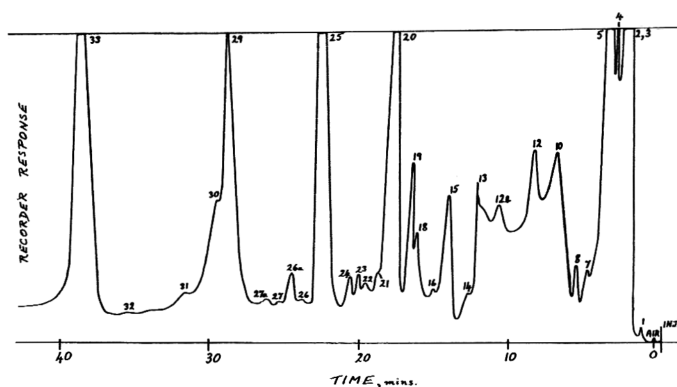

Fig. 2–Typical gas-liquid chromatogram of the flavor volatiles obtained from fresh chopped Brussels sprouts. Conditions of analysis as previously given (MacLeod et al., 1968), with an attenuation setting of 50 (5 × 10^{-11} A.) and a 1-µliter sample.

2 most important facts: the unidentified peak 33 is present to the large extent of 8.5% and there is also a great increase in the relative abundance of cis-pent-3-en-1-ol (from 0.5 to 15%). Peak 33 for the sprout is probably one of the important higher-boiling components which, although present in small quantities in the other vegetables also, in this case in its increased amount contributes to the characteristic sprout flavor distinguishing this vegetable from others.

In conclusion, therefore, the flavor profile of sprouts is quite different from that of cabbage. The sulfur content of the sprouts' volatiles is much lower (about 15% as against 36%) and the lack of unsaturated alcohols and aldehydes in general is compensated for by a large increase in only 1 of these, i.e., cis-pent-3-en-1-ol. Thus, the results imply that many of the flavor precursors and enzymes are common to both sprouts and cabbage, but that their amounts present are probably vastly different. Either that, or the reactions leading to the release of the volatiles from their nonvolatile precursors are catalyzed or inhibited by other natural constituents which may vary between the 2 vegetables.

As might be expected, fresh chopped sprouts produce far more volatiles than fresh whole sprouts. Other differences between these 2 sets of results is that chopping tends to reduce the carbonyl content slightly and increase the products of the thioglucoside sinigrin (i.e., the allyl cyanide and the allyl isothiocyanate). Apart from these differences, the results are remarkably similar and the implication is that the flavor of chopped sprouts differs from that of whole sprouts only in being slightly stronger and more bitter (the latter due to the higher isothiocyanate content).

The only preserved Brussels sprouts available on the retail market are frozen ones, and these were treated in exactly the same way as the fresh vegetable. It had to be accepted that the frozen sprouts and the fresh sprouts were almost certainly of a different variety. It is possible, therefore, that any slight differences in flavor volatiles between the fresh and preserved samples could be due to varietal differences. However, such effects would be comparatively slight and were overshadowed by the marked changes brought about by the use of preserved products as against fresh. The same reasoning applies to the experiments on fresh and preserved runner beans, as described later.

To assess the value of freezing as a method of preservation, the results for fresh and frozen whole sprouts are best compared. There are differences in the dimethyl sulfide and acetaldehyde contents, but the most important variation is, again, in the amount of allyl isothiocyanate produced. This is noticeably reduced and nearly absent in frozen sprouts, whereas the allyl cyanide is considerably increased. The decrease in isothiocyanate is most likely due to inactivation of the enzyme myrosinase (which causes its liberation from the thioglucoside sinigrin) during the blanching stage of the preservation process. The increased amount of cyanide formed is probably a result of thermal as against enzymatic decomposition of the sinigrin during this blanching stage. Other important effects of freezing are an increase in sulfur volatiles and a decrease, or a complete loss, of unsaturated alcohols and aldehydes. The same over-all trend is apparent when comparing the results for fresh and frozen chopped sprouts. On the whole, deep-freezing as a method of preservation is a reasonably mild process and does not drastically affect the volatiles produced on cooking, particularly when compared with other methods of preservation (Jones, 1967, Part III).

Cauliflower

Results for the cauliflower are quoted in Table 2. Figure 3 is of a typical gas chromatogram of the cauliflower volatiles (inflorescence only). The experiments using inflorescence only and outer leaves only were performed because a comparison of the volatiles from the inner and outer leaves of the cabbage had produced some very interesting results (Jones, 1967, Part II). Preserved cauliflower is not available on the retail market and, therefore, only the fresh vegetable was considered here.

The total amount of volatiles liberated from the cooked cauliflower was far greater than obtained from the cooked cabbage, weight for weight. In fact, most of this was produced by the inflorescence rather than by the outer leaves. When comparing the flavor profile of the complete cauliflower with that of the cabbage, some interesting points arise. Not only is the acetaldehyde content increased (doubled) relatively, but formaldehyde, not produced by fresh cabbage, is also present to a large extent (1.5%). The relative amount of dimethyl sulfide produced was exactly the same as for the cabbage. The over-all alcohol production from the cauliflower (1.5%) was much less than from the cabbage (24%).

The allyl isothiocyanate content is slightly reduced but the relative percentage of allyl cyanide formed is much greater than from the cabbage. About 25% of the total volatiles was accounted for by this compound, it being nearly the most abundant component.

The volatiles liberated from the outer leaves of the cauliflower showed a fair similarity to the results obtained for the cabbage outer leaves. However, the inflorescence understandably showed a lesser similarity to the heart volatiles of the cabbage. The most interesting differences between the inflorescence and the outer leaves of the cauliflower were again in the 2 main sinigrin products (i.e., allyl cyanide and allyl isothiocyanate). The outer leaves produced rather more of both

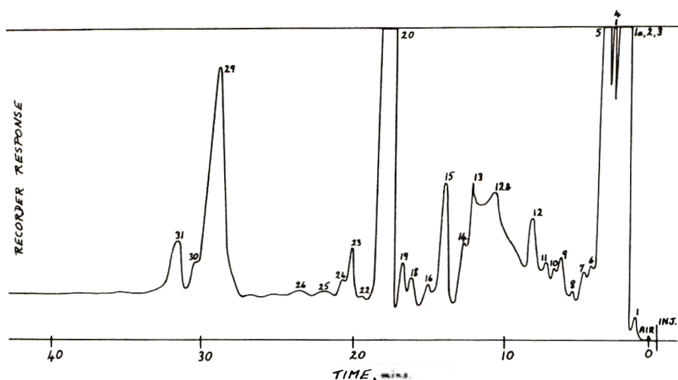

Fig. 3–Typical gas-liquid chromatogram of the flavor volatiles obtained from cauliflower (inflorescence only). Conditions of analysis as previously given (MacLeod et al., 1968), with an attenuation setting of 50 (5 × 10^{-11} A.) and a 1-μliter sample.

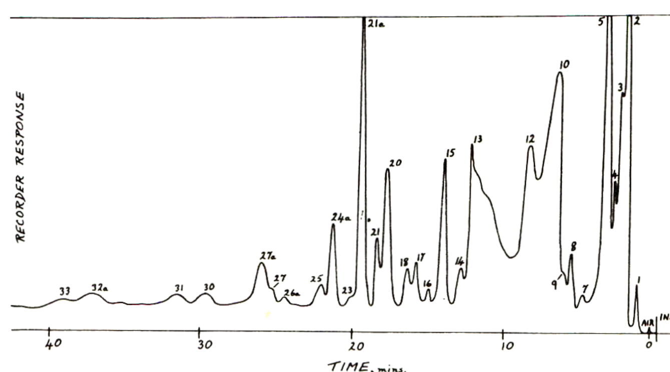

Fig. 4–Typical gas-liquid chromatogram of the flavor volatiles obtained from fresh runner beans. Conditions of analysis as previously given (MacLeod et al., 1968), with an attenuation setting of 50 (5 × 10^{-11} A.) and a 1-μliter sample.

products than the cabbage outer leaves and, as in the cabbage, the isothiocyanate content was greater than the nitrile. However, from the inflorescence alone, the nitrile content was greatly increased to 23%, whereas the isothiocyanate percentage abundance was lower and more normal at 5.5%. This bears no similarity to the much increased value of 16.5% isothiocyanate and the normal value of 3.5% nitrile for the cabbage inner leaves. This difference between the cabbage and the cauliflower may be due to 1 or more of the following reasons. Assuming that sinigrin is thermally decomposed to give the cyanide (Jones, 1967, Part III), then a lack of the enzyme myrosinase in the inflorescence would leave the sinigrin substrate relatively free for thermal decomposition to the cyanide. Alternatively, the nitrile may be produced from some other precursor or the inflorescence may contain a specific enzyme for nitrile production. Another factor which affects the cyanide: isothiocyanate ratio is the pH of the medium, which again could be different for the cauliflower.

The difference in flavor between cabbage and the inflorescence of the cauliflower must be due, at least in part, to the large percentage of allyl cyanide produced by the latter, as well as to the comparative lack of alcohols and increased aldehyde content also shown by the cauliflower.

Considering all 3 members of the Brasicca family studied, it is noticeable that they all produce the same volatiles although varying in their relative proportions. This implies that in a closely related group such as the Brassica family, a great similarity exists in their biochemistry. The same precursors and enzymes are present and the same reaction pathways are available. The differences in the relative percentages of the volatiles formed are probably due either to the presence of the precursors and enzymes and any catalysts or inhibitors in varying amounts or to different degrees of availability of these.

Runner beans

Table 2 also lists the results obtained for the runner bean experiments. Figure 4 shows a typical gas chromatogram of the volatiles from fresh runner beans.

Comparing the results of cooked fresh beans with those obtained from the cooked cabbage is not a very realistic proposition since, as to be expected, the differences are considerable. However, the most important are the following: The dimethyl sulfide, which is characteristic of the cabbage (MacLeod et al., 1968), was greatly decreased from 26.5 to 4.5%. There was a loss of methanol to 0.5%, with an accompanying increase in ethanol content to 17%. Allyl isothiocyanate, again characteristic of the cabbage and of the Cruciferae in general, was absent. However, allyl cyanide surprisingly was present at 6.5%. Thus its precursor in beans, or possibly even in both beans and cabbage, may be a compound other than sinigrin. This is probably a more likely explanation than to suggest that sinigrin is present in beans in the absence of the enzyme myrosinase.

Also evident was the presence of crotonaldehyde (but-2-en-1-al) and dipropyl ketone to a large extent. A large amount of trans crotyl alcohol (trans-but-2-en-1-ol) also was formed. The hexenal was absent and the hexenol much decreased.

6 peaks were obtained on the gas chromatogram of the bean volatiles not given by the conventionally cooked fresh cabbage. However, 3 of these (14b, 24a and 26a) were produced by the cabbage when it was submitted to variations in cooking technique (Jones, 1967, Part II), and one (27a) was given by samples of dehydrated cabbage (Jones, 1967, Part III). Of these 4 peaks, 24a(4%) and 27a(2.5%) were both present in reasonable amounts in the bean volatiles, but unfortunately both were unidentified. The remaining 2 peaks were the only new ones, obtained only from the bean and not found in any of the other vegetables investigated. 1 of these (peak 32a) was present to a small extent (0.5%) and was identified as trans-hept-3-en-1-ol. The other new peak (21a) was present at 7% and may well be one of the important specific higher-boiling volatiles characteristic of the runner bean. Unfortunately, this component too was unidentified.

The trends shown by these results are not obvious, but the methyl compounds are certainly present to a lesser extent than for cabbage and sprouts, although the propyl compounds are present to a larger extent. This implies that some precursors in the bean might be merely higher homologues of those in the Brassicas. The rather surprising loss of cis-hex-3-en-1-ol and trans-hex-2-en-1-al for the bean is accompanied by a corresponding and intriguing increase in trans-but-2-en-1-ol (crotonol) and but-2-en-1-al (crotonaldehyde). This effect is unexplained.

The appreciable difference between the flavors of fresh beans and of fresh cabbage is thus well exemplified by the considerable differences in the quoted results. In summary, the chief contributing factors to the difference in flavor between the runner bean and the cabbage are the presence or absence of allyl isothiocyanate, the presence or absence of the unidentified component 21a and the variations in the relative proportions of the more standard lower-boiling components.

The major difference between the volatiles of fresh beans and frozen beans is the increased aldehyde content of the latter. Acetaldehyde was increased nearly 4-fold, to 27.5% in frozen beans and propionaldehyde similarly to 4%. Peak 15 was increased to 12% from 5.5%, presumably due to its hexanal content. Most interesting is that acrolein and 2-hexenal were formed only by the frozen vege-

table, being completely absent from the fresh bean. This effect of increased aldehyde content must be due to the nature of the bean itself and not due to the freezing process, since the same trends were not shown by frozen sprouts.

In the case of "Surprise" beans, the aldehyde content was roughly consistent with the fresh product, although this was more than compensated for by the vast increase in the acetone produced from 14.5 to 57%. Thus, both samples of preserved beans liberate exceptional amounts of carbonyl compounds, presumably formed by oxidative methods. Such changes are most undesirable as far as flavor is concerned. Although the frozen beans retained the correct dimethyl sulfide content, the "Surprise" beans' volatiles contained none at all. The new unidentified peak 21a (7% of the fresh bean volatiles) also suffered great loss and was completely absent from "Surprise" beans. This indicates the possibility of an enzymatic mode of formation for this unknown component.

The volatiles from both preserved samples produced a new peak, namely 30a. This was identified as dipropyl disulfide and is yet another example of a propyl compound liberated from the bean. Although not present to a great extent, it is of interest in that it was absent from the fresh bean and also from all other vegetables investigated. An interesting difference between the gas chromatograms obtained from the volatiles of the preserved beans as compared with the fresh was the severe lack of higher-boiling components. For example, the "Surprise" beans liberated no higher boiling components than peak 20 with the 1 exception of the already-mentioned new peak 30a.

In conclusion, the results obtained from the frozen beans showed them to be more similar to the fresh product than were the "Surprise" beans.

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Ms. received 6/9/69; revised 8/12/69; accepted 4/22/70.

Our thanks are due to the Science Research Council for a grant covering the gas chromatography equipment and for a Research Studentship (to G.M.). We also appreciate the interest and advice of Dr. P.F.G. Praill.

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THE FLAVOR VOLATILES OF DEHYDRATED CABBAGE

SUMMARY—Using previously described sampling techniques, the flavor volatiles of a number of dehydrated cabbage samples were collected and then analyzed by gas chromatography. Quantitative measurements were made of the individual components and results compared with those for fresh cabbage. Many differences were observed, in general indicating the poor nature of the preserved product as a substitute for the fresh vegetable. In particular allyl cyanide (3-butene nitrile) was much increased in the dehydrated samples, whereas the important allyl isothiocyanate was virtually lost altogether. Keeping qualities of the dehydrated samples were also investigated.

INTRODUCTION

ONE OF THE more interesting aspects of flavor science is the comparison of the flavors of fresh and preserved foodstuffs. The main purpose of any preservation technique is to retain the foodstuff at its maximum palatability and nutritive value instead of allowing it to undergo natural changes (due to the presence of enzymes and microorganisms) which make it unsuitable for consumption. Maximum palatability under ideal circumstances can only be achieved if the natural flavor of the foodstuff is completely retained. Thus in addition to minimizing the undesirable effects caused by enzymes in the food and by contaminating microorganisms, it is also essential that the processing steps taken to inactivate or inhibit these agents, amongst other things, should not cause any chemical changes to take place which would detract seriously from the flavor of the food. Although it is true that most preserved foods do not have the perfect flavor of the fresh food, it is most important that such flavor defects are kept as far as possible at a minimum.

For this reason, it is of some importance to be able to compare the flavor volatiles of fresh and preserved foodstuffs and to assess how closely the preserved product resembles the fresh. The loss of certain components and/or the accumulation of other products as indicated by such an investigation might well lead to suggested modifications of the existing processing and/or storage methods to give a product with a composition and flavor more similar to that of the fresh foodstuff. Furthermore, a survey can be carried out comparing the results for foodstuffs preserved by different methods and the type of product which most closely resembles the fresh foodstuff determined.

In the case of the cabbage there is no great scope in this latter type of study, since, unlike certain foodstuffs, there are only a few methods of preservation of cabbage. Dehydration is the most common technique used and this may be achieved by normal air drying or by accelerated freeze drying. Four different samples of dehydrated cabbage were obtained and these were examined and compared with fresh cabbage.

Since many of the flavor volatiles are produced enzymatically from nonvolatile precursors, an obvious case of "changed" flavor in preserved foods is by the destruction of these enzymes during the actual preservation process. Unfortunately the methods used for preservation do not distinguish between the enzymes which cause destruction and those which are required for the liberation of the flavor volatiles. Inactivation of the enzymes is commonly achieved by blanching, when the food is treated with boiling water or steam. The blanched food is then processed further (e.g., by freezing, canning, dehydration, etc.) to prevent or retard chemical changes and bacterial growth on storage.

At the same time as the enzymes are destroyed by processing, some volatile components are also directly lost. However, nonvolatile, relatively heat stable compounds survive processing and these include flavor precursors. Unfortunately, in some cases enzymes are responsible for the release of flavor volatiles from these precursors and since the enzymes have been destroyed by processing such precursors will remain dormant and useless and that particular flavor volatile will be missing. However, addition of an appropriate enzyme preparation after processing will regenerate the flavor compounds from their precursors.

Much work has been carried out on the restoration of natural flavor to a preserved food by treatment with enzyme preparations, although this is not, of course, the complete answer to the problem. The enzyme is best obtained from that particular food, although enzymes from closely related foodstuffs have been used. Weurman (1961) discovered that the odor and gas chromatograph pattern of the volatiles of processed raspberries were changed for the better by the addition of a raspberry enzyme preparation. Furthermore, a similar, although lesser, effect was observed when using enzymes from other sources. Similar work has been carried out on other foods (Hultin and Proctor, 1962).

Cabbage has also been investigated, particularly by Schwimmer (1963); MacKay and Hewitt (1959); Hewitt et al. (1957) and Konigsbacher et al. (1959). In all cases the action of the enzyme myrosinase on the glucosinolate sinigrin was studied and it was shown that the allyl isothiocyanate produced by such action was vital to the natural flavor of cabbage.

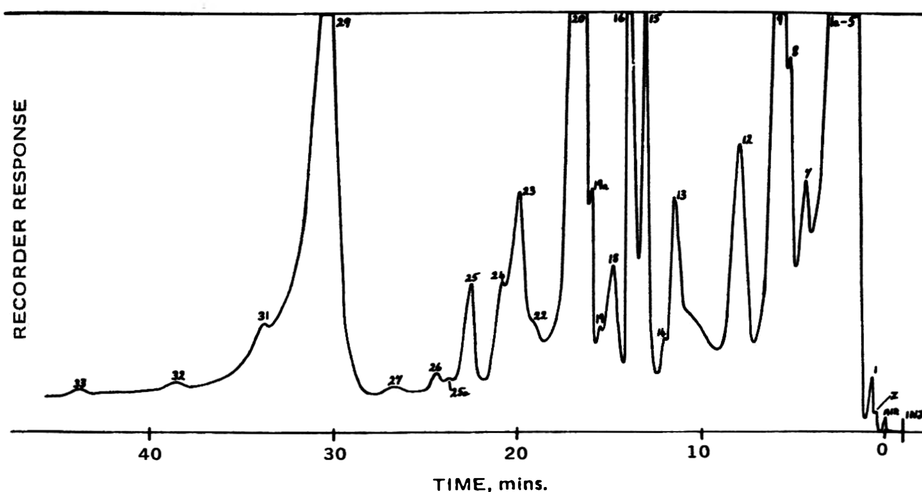


Fig. 1—Typical gas-liquid chromatogram of the flavor volatiles obtained from dehydrated cabbage sample A. Conditions of analysis as previously given (MacLeod and MacLeod, 1968), with an attenuation setting of 50 (5 × 10^{-11} A) and a 1 μl sample.

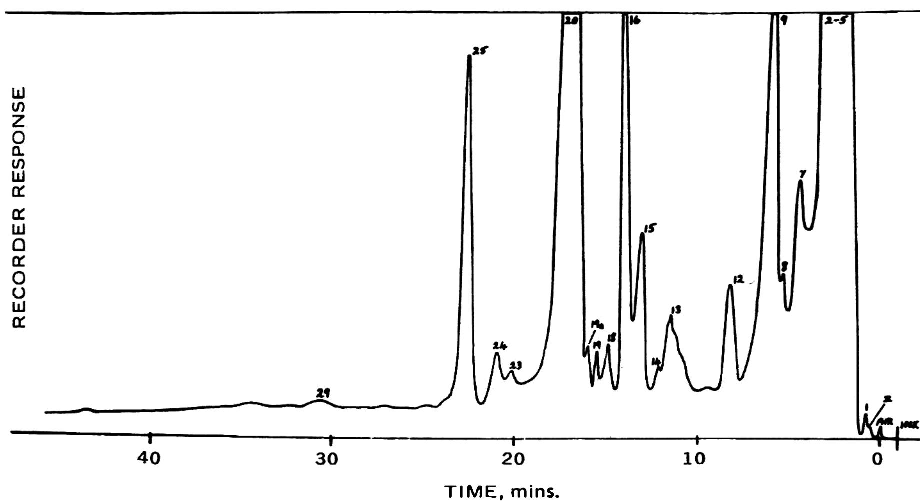


Fig. 2–Typical gas-liquid chromatogram of the flavor volatiles obtained from dehydrated cabbage sample B. Conditions of analysis as previously given (MacLeod and MacLeod, 1968), with an attenuation setting of 50 (5×10^{-11} A) and a 1 μl sample. (At this medium sensitivity some of the minor components listed in Table 1 were not detectable.)

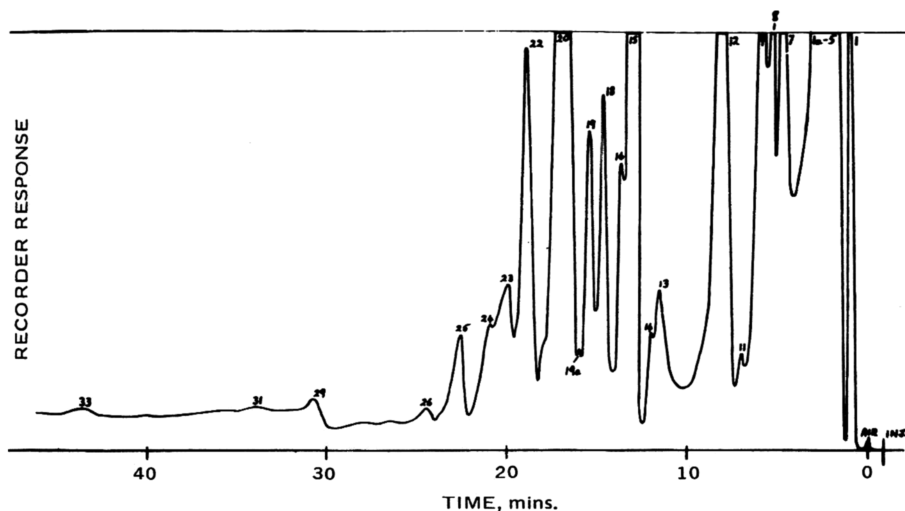


Fig. 3–Typical gas-liquid chromatogram of the flavor volatiles obtained from dehydrated cabbage sample C. Conditions of analysis as previously given (MacLeod and MacLeod, 1968), with an attenuation setting of 50 (5×10^{-11} A) and a 1 μl sample. (At this medium sensitivity some of the minor components listed in Table 1 were not detectable.)

Bailey et al. (1961) compared allyl isothiocyanate production from different cabbage samples. It was shown that fresh cabbage produced an appreciable quantity of the isothiocyanate, but a sample of dehydrated cabbage produced none. If, however, the same dehydrated cabbage was reconstituted as before but in the presence of myrosinase enzyme, then allyl isothiocyanate was produced again and in similar large quantities. This indicates that, all other things being equal, although the glucosinolate precursor is unharmed by processing, the natural myrosinase is completely destroyed or inactivated. This of course is to be expected.

Schwimmer (1963) carried this work on dehydrated cabbage further by using enzymes from different sources, but merely using tasting panels to assess the results. As expected the addition of cabbage enzyme preparations during reconstitution gave the best results and the panel considered the product to be quite close to the flavor of fresh cabbage. However, the use of a mustard enzyme preparation (mustard is also a brassica and as such is quite closely related to the cabbage) did give a better flavor than that obtained from a simple water-rehydrated control. However, the flavor was described as "lachrymatory," "pungent," etc., and this was due presumably to the production of excess of isothiocyanates. The flavor obtained using an onion enzyme preparation was slightly reminiscent of fresh onions. This was assumed to have been caused by the action of the onion enzyme on S-methylcysteine sulphoxide in the cabbage.

From these sorts of results it can be suggested that the time may not be far distant when a dehydrated food may be supplied with its own packet of "enzymes" to be added to the food during reconstitution, hence providing a product similar in flavor to the fresh.

EXPERIMENTAL

4 SAMPLES of dehydrated cabbage were obtained from suppliers in this country. Of these samples only one (A) was actually manufactured in Britain, the others being imported, although sample D was manufactured in and imported from the Republic of Ireland. All 4 samples were produced, to the best of our knowledge, by air drying. Only sample D is available on the retail market, the others being supplied to the catering market.

The appearance of the 4 samples was different and the color varied from deep, dirty green in one case to light green in another. In some cases the cabbage was finely chopped and in others it was shredded. The samples were supplied with full instructions for rehydration and these were carefully followed in such a way that the reconstituted cabbage plus water was equivalent in weight and volume to that of the fresh cabbage plus water used as a standard for comparative purposes. The percentage of water required for rehydration was not the same in all cases, indicating that the products had been dehydrated to slightly varying extents.

The fresh cabbage standard was cooked and sampled exactly as previously described by MacLeod and MacLeod (1968), except that the cabbage was heated for 10 min only as against the original 30 min. This made very little difference to the flavor volatiles produced and was more representative of the normal cooking time for cabbage. The sample obtained was separated and analyzed by gas chromatography again exactly as previously described.

Rehydration, cooking and sampling of the dehydrated samples were carried out as follows:

Sample A

According to the rehydration instructions this sample produced 7 times its own weight of cabbage when reconstituted. Thus 115g was used (equivalent to the 800g fresh cabbage used in the standard experiment). The instructions also recommended the use of 2½ gal of water per 2 lb of dehydrated cabbage for rehydration. Therefore 115g required approximately 1,250 ml of water. It is interesting to note in confirmation that the 115g dehydrated cabbage plus 1,250 ml water (= 1,365g) is approximately the same total as for the fresh cabbage experiments using 800g cabbage plus 600 ml water (= 1,400g).

The 1,250 ml of water was brought to a boil and the 115 g of sample A was added. There was obviously very little cooling effect here (compared with fresh cabbage) and the water very quickly returned to a boil. The cabbage was then cooked and sampled for 10 min exactly as in the standard experiment described for fresh cabbage.

Sample B

This was presumably more dehydrated, since, according to the instructions, it formed 8 times its own weight of cabbage when reconstituted. Therefore, only 100g were used in this

instance. However, the amount of water recommended for rehydration was the same as for A and therefore the quantity used in this experiment was again 1,250 ml. The total of 1,350g is again confirmation of the recommended figures. The method of cooking, etc., was exactly as described for sample A.

Sample C

This required 110g per 1,250 ml of water (total 1,360g) in order to simulate as closely as possible the amounts used in the fresh cabbage experiments. Again the cooking, etc., was as described for sample A.

Sample D

This required 100g per 1,250 ml of water and again cooking and sampling were as for sample A.

It was pointed out by the manufacturers and suppliers of these products that, in common with most dehydrated green vegetables, the dried cabbage was subject to oxidative deterioration and thus should be used within 1 month unless stored in an oxygen-free environment. To test for this deterioration on storage the products were kept in a normal (oxygen present) atmosphere for just over 3 months and then rehydrated, cooked and sampled exactly as described above.

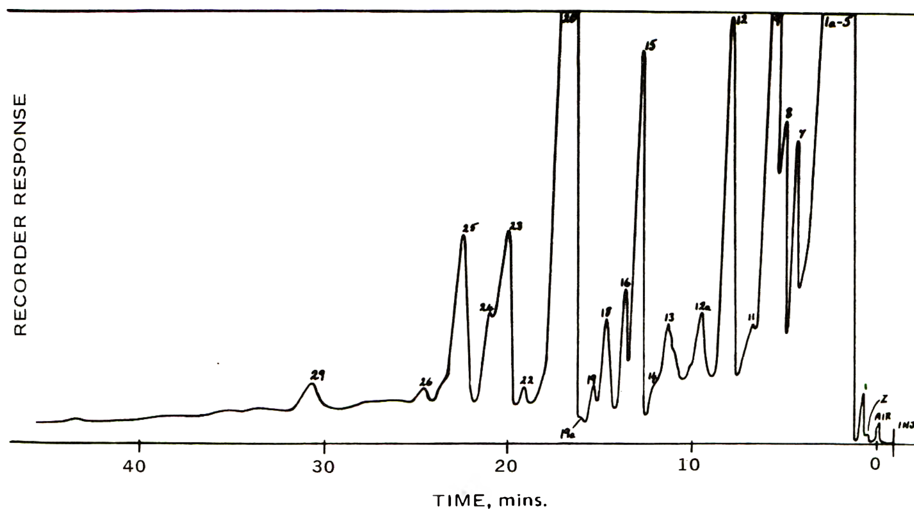


Fig. 4–Typical gas-liquid chromatogram of the flavor volatiles obtained from dehydrated cabbage sample D. Conditions of analysis as previously given (MacLeod and MacLeod, 1968), with an attenuation setting of 50 (5 × 10^{-11} A) and a 1 μl sample. (At this medium sensitivity some of the minor components listed in Table 1 were not detectable.)

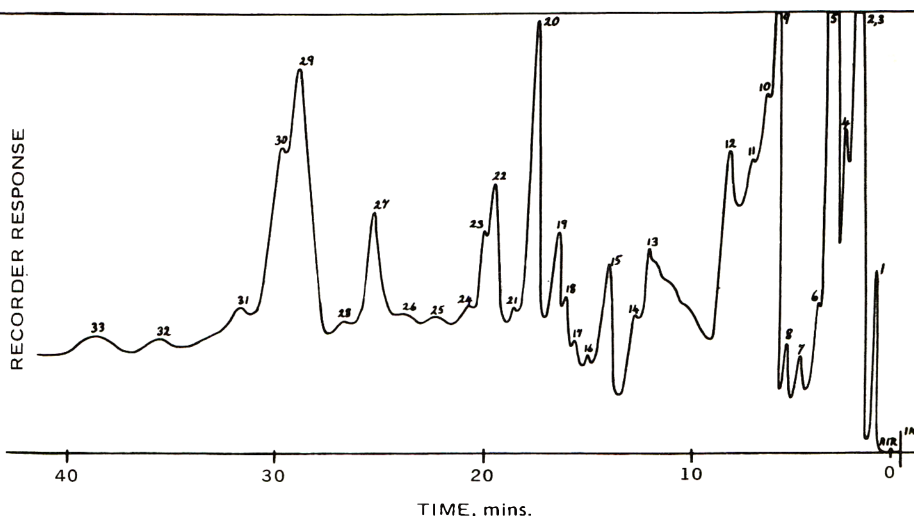


Fig. 5–Typical gas-liquid chromatogram of the flavor volatiles obtained from fresh cabbage. Conditions of analysis as previously given (MacLeod and MacLeod, 1968) with an attenuation setting of 50 (5 × 10^{-11} A) and a 1 μl sample.

RESULTS & DISCUSSION

IT HAD TO BE accepted that the 4 samples of dehydrated cabbage were possibly all prepared from different varieties of cabbage and that none was of the same variety as the fresh cabbage used as a standard. It is possible, therefore, that any slight differences in flavor volatiles between the fresh cabbage and the 4 dehydrated samples could be due to varietal differences. However, such variations would be slight and were in fact overshadowed by the marked changes brought about by the use of dehydrated products as against fresh.

The dehydrated cabbage was found to be a poor substitute for the fresh vegetable. After cooking, all 4 samples not only looked most unappetizing but also possessed a rather strong objectionable odor. Simple tasting tests confirmed that the cooked dried cabbage samples were very poor and there seemed little to choose between the four on this basis.

Figures 1 to 4 give medium sensitivity gas chromatograms obtained for the 4 dehydrated cabbage samples, and Figure 5 gives that of fresh cabbage for comparison. Table 1 lists the relative percentage abundances of all the components of the 4 dried cabbage samples and the results for the standard cooked fresh cabbage are also quoted for comparative purposes. Certain new components were produced by the dehydrated samples that had been absent from the fresh cabbage volatiles and these were tentatively identified as far as possible by relative retention times as previously described (MacLeod and MacLeod, 1968). The original peaks for the fresh cabbage standard had been numbered in sequence and the new components are shown with the number of the peak immediately preceding and a letter following (e.g.,14a). This system shows at once those components which were not produced by the standard fresh cabbage.

All 4 samples produced far more volatiles overall than the fresh cabbage. Samples B and D liberated 8 times the normal total; sample C 11 times; and sample A 16 times as much. This liberation of large quantities of volatiles explains the strong odor after cooking.

Since the results obtained for the dehydrated samples are so different from those for the fresh cabbage standard, some of the major groups of compounds will be discussed separately. Important individual compounds within these groups will also be considered.

Alcohols

The dehydrated samples all produced far less of the saturated alcohols than did the fresh cabbage, and in some cases the alcohol was completely absent (e.g., ethanol and n-hexanol).

Cis-hex-3-en-1-ol was interesting in that it was absent from the flavor volatiles of all 4 dehydrated samples although normally present to about 4.5% in the fresh cabbage volatiles. Apart from agreeing with the results for the saturated alcohols this does imply an enzymatic mode of formation for this important compound. At present little detail is known regarding the origin of cis-hex-3-en-1-ol or the related aldehyde, trans-hex-2-en-1-al, although the common pre-

cursor is probably linolenic acid, or ester.

Aldehydes

In samples A, B and D the aldehyde content was roughly the same as for fresh cabbage, except for formaldehyde and acetaldehyde. The latter was increased from a normal value of 3.5% to approximately 13% in all three cases.

In the volatiles liberated from sample C, the amount of acetaldehyde was further increased to 21% and the other aldehydes were also increased considerably. For example, propionaldehyde was raised from a normal 0.5% for fresh cabbage to 5.5% and n-butyraldehyde from 0.5% to 4.0%. Thus approximately 40% of all volatiles liberated from sample C were saturated aldehydes compared with a normal value for fresh cabbage of 6%. This is a most significant difference.

Formaldehyde was not produced by fresh cabbage and although also absent from sample B it was present in quite

Table 1–Relative percentage abundances of all components of dried cabbage samples compared with cooked fresh cabbage.

Peak No.	Identification	Sample peak relative retention time (min)[a]	Reference compound relative retention time (min)	Approximate percentage relative abundance: Fresh cabbage	Sample A	Sample B	Sample C	Sample D	Sample A after 3 mo	Sample B after 3 mo	Sample C after 3 mo
Z	Diethyl ether	1.45	1.45	–	0.005	0.005	0.0005	0.005	0.001	0.001	–
1	Methanethiol	1.72	1.72	0.5	0.01	0.01	0.5	0.05	0.05	0.05	0.1
1a	Formaldehyde	2.15	2.15	–	2.5	–	7.5	3.0	5.0	–	2.0
2	Acetaldehyde[b]	2.35	2.35	3.5	12.0	13.5	21.0	14.0	17.0	19.0	12.5
3	Dimethyl sulphide[b]	2.65	2.65	26.5	10.5	23.0	5.0	9.5	4.5	11.5	4.5
4	Propionaldehyde[b]	3.08	3.08	0.5	0.1	0.5	5.5	0.5	0.5	0.5	5.0
5	Acetone[b]	3.45	3.45	14.5	6.5	10.0	9.0	27.0	7.5	13.0	26.5
6	Acrolein	4.25	4.25	1.0	–	–	–	–	–	–	–
7	n-Butyraldehyde	4.75	4.75	1.0	0.5	0.5	4.0	1.0	0.5	1.0	5.0
8	Ethyl methyl ketone[b]	5.30	5.30	1.0	1.0	0.5	3.0	1.5	0.5	0.5	0.5
9	Methyl alcohol[b]	5.75	5.75	12.0	7.5	5.5	4.0	8.5	6.5	5.5	5.0
10	Ethyl alcohol	6.65	6.65	3.5	–	–	–	–	–	–	–
11	?	7.25		2.5	–	–	0.1	0.1	–	–	0.5
12	(Diethyl ketone[b] plus Diacetyl[b])	8.30	8.30	6.0	1.0	1.0	5.0	3.0	1.0	1.5	8.5
12a	Acrylonitrile	9.45	9.45	–	–	–	–	0.5	–	–	–
12b	?	10.25		–	–	–	–	0.05	–	–	–
13	Methyl alcohol/water profile[c]	~11.2	~11.2	–	–	–	–	–	–	–	–
14	But-2-en-1-al	11.90	11.90	1.0	0.1	0.1	0.5	0.1	0.5	0.5	0.5
15	(Dimethyl disulphide[b] plus n-Hexanal[b])	12.95	12.95	1.0	0.5	0.5	4.0	1.0	1.0	0.5	9.0
16	Allyl alcohol	13.75	13.75	0.1	4.5	4.0	1.5	1.0	3.5	2.5	1.0
17	Dipropyl ketone	14.4	14.4	0.1	–	–	–	–	–	–	–
18	Trans-pent-2-en-1-al[b]	15.0	15.0	0.5	0.1	0.1	0.5	0.1	0.1	0.1	1.0
19	?	15.4		1.5	0.01	0.1	0.5	0.05	0.1	0.1	0.5
19a	Trans-crotononitrile	16.2	16.2	–	0.1	0.1	0.05	0.01	0.1	0.05	0.05
20	Allyl cyanide[b]	16.8	16.8	3.0	48.5	33.5	25.0	23.5	47.5	34.5	13.5
21	Trans-but-2-en-1-ol	17.9	17.9	0.5	–	–	–	–	–	–	–
22	Trans-hex-2-en-1-al[b]	19.1	19.1	1.5	0.01	0.01	0.5	0.05	0.01	0.01	0.5
23	Methyl propyl disulphide	20.0	20.0	1.0	1.0	0.5	1.0	2.0	0.5	0.5	1.0
24	Methyl isothiocyanate?	20.7	20.5	0.5	0.5	0.5	0.5	0.5	0.5	1.0	–
25	Cis-pent-3-en-1-ol	22.5	22.5	0.5	1.0	5.5	1.0	3.5	1.0	7.5	0.5
25a	Acetoin or n-octanal	23.6	23.5 acetoin 23.7 octanal	–	0.01	0.05	0.01	0.01	0.005	0.05	0.05
26	?	24.6		0.1	0.05	0.05	0.1	0.1	0.05	0.1	0.1
27	Cis-pent-2-en-1-ol?[d]	26.6	26.6	2.0	0.01	0.01	0.05	0.01	0.005	0.01	0.1
27a	?	27.6		–	–	–	0.05	0.01	–	–	0.1
28	n-Hexanol	27.9	27.9	0.5	–	–	–	–	–	–	–
29	Allyl isothiocyanate[b]	30.5	30.5	6.5	1.5	0.1	0.1	0.1	1.5	0.1	0.1
30	Cis-hex-3-en-1-ol[b]	31.6	31.6	4.5	–	–	–	–	–	–	–
31	Butyl isothiocyanate	33.8	33.8	1.0	0.5	0.05	0.05	0.01	0.1	0.1	0.1
31a	?	35.4		–	–	–	–	0.05	–	–	–
32	Trans-hept-2-en-1-ol	38.6	38.6	1.0	0.05	–	–	–	–	–	–
33	?	43.8		1.0	0.01	0.01	0.05	0.05	0.01	0.05	0.1
	Total Volatiles (sq. mms.)			15,760	248,310	131,500	186,390	127,980	253,000	92,920	38,760

[a]Internal standards were used (MacLeod and MacLeod, 1968).

[b]Identified by mass spectrum also.

[c]MacLeod and MacLeod (1968).

[d]Tentative identification only, due to a mass spectrum being available which could not be identified positively and which does not completely confirm the identification given.

Table 2—Allyl compounds produced in dehydrated samples compared with standard fresh cooked cabbage.

	Std. %	Sample A %	Sample B %	Sample C %	Sample D %
Alcohol	0.1	4.5	4.0	1.5	1.0
Nitrile	3.0	48.5	33.5	25.0	23.5
Isothiocyanate	6.5	1.5	0.1	0.1	0.1

Table 3—Comparison of the relative percentage abundances of trans-hex-2-en-1-al and "peak 19" in volatiles of dehydrated samples and standard fresh cooked cabbage."

	Std. %	Sample A %	Sample B %	Sample C %	Sample D %
Hexenal	1.5	0.01	0.01	0.5	0.05
Peak 19	1.5	0.01	0.1	0.5	0.05

large quantities in the other 3 samples (2.5% in A, 3% in D and 7.5% in C).

In all 4 dehydrated samples the percentages of unsaturated aldehydes were reduced (e.g., acrolein, normally 1% was absent and but-2-en-1-al, also normally 1%, was reduced to 0.1%). However, in agreement with the findings for saturated aldehydes, in all cases the values for sample C were higher than for the other samples. For example, trans-hex-2-en-1-al, normally 1.5% for fresh cabbage, was found in approximately 0.05% quantities for the 3 samples but 0.5% in sample C. Incidentally, it is interesting that the hexenal is decreased as well as the cis-hexenol and this gives further evidence for the relationship between the 2 and their enzymatic formation.

Sulphides

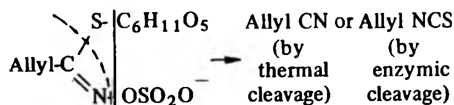
Dimethyl sulphide was the most abundant volatile of fresh cabbage (26.5%), but only sample B produced the sulphide in similar quantities. Samples A and D formed far less (approximately 10%) and sample C formed very little indeed (5%). These quantities probably indicate a direct loss of a very volatile compound during processing, rather than a reduction due to inactivation of a possible enzyme. It is to be noted that again sample C gives very poor results.

Allyl compounds

These include the important allyl isothiocyanate, allyl cyanide (3-butene nitrile) and allyl alcohol. The results obtained for the dehydrated samples show considerable differences from the more normal values obtained for the fresh cabbage. Table 2 summarizes the results for these components only.

Although the isothiocyanate content is drastically reduced in 3 cases, for some unknown reason it is not decreased to such an extent in sample A. However, more serious than this loss is the extraordinary increases in nitrile percentages. The lowest value of 23.5% is surprising enough, but the highest value of 48.5% for sample A is more than 16 times the normal amount and it comprises just about half of the total volatiles.

It is interesting to note that as far as alcohol and nitrile are concerned, as the percentage of one increases or decreases for a dehydrated sample so also does the value of the other. This presumably means that the extent of their formation is dependent on the amount of precursor freely available. Since the decrease in the isothiocyanate content is due to inactivation of the myrosinase enzyme during processing, then the increased production of nitrile and alcohol cannot be enzymatic. It is possible that heat treatment during processing causes decomposition of the sinigrin precursor to give exclusively the nitrile plus some alcohol. These accumulate and are liberated in these comparatively vast amounts on cooking. The minimal amounts of isothiocyanate produced may be formed enzymatically during the period between harvesting and processing. Having been produced, this relatively involatile compound could withstand the processing treatment and be present in the resultant product. It can be suggested therefore that sinigrin can be decomposed either enzymatically to give the isothiocyanate or thermally to give the nitrile, these reactions normally being in competition:



For fresh cabbage, in the presence of myrosinase, much isothiocyanate is formed and less nitrile. During dehydration processing the enzyme is inactivated and on later cooking the nitrile is the main product, in excess because of the previous heat treatment during processing, and only a minute quantity of isothiocyanate is obtained.

Other compounds

A few compounds were produced by the dehydrated samples which were not obtained at all from fresh cabbage. These include diethyl ether, which was formed in very small amounts by all 4 samples; trans-crotononitrile (trans-2-butene nitrile), again produced by all samples; and acrylonitrile (propene nitrile) which was only given by sample D. These 2 latter compounds were probably formed from the excess of allyl cyanide.

Peak 19, which was unidentified and which was normally obtained from fresh cabbage, was present to a much lesser extent in dried cabbage. By its increased presence in the volatiles of sample C compared with the others, it resembles very closely the behavior of aldehydes and in particular that of trans-hex-2-en-1-al. This agreement is very good and peak 19 may well be an unsaturated aldehyde.

Results of experiments to determine the keeping quality of the dehydrated cabbage (samples A, B and C only) after storage for three months are also given in Table 1. Samples A and B showed only a few minor changes due to storage, the total volatiles liberated being much the same. The most important difference is that the dimethyl sulphide content, which was lower than for fresh cabbage anyway, was approximately halved. Sample C did not keep as well as the others. The total volatiles produced was much less and acetone became a major volatile with a 3-fold increase to over 25%. Rather interesting is the fact that the allyl cyanide content was halved.

Therefore, although sample C did change quite considerably on storage, it is remarkable how little difference the 3 months' storage made to samples A and B.

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Ms. received 1/28/70; accepted 3/19/70.

Thanks are due the Science Research Council for a grant covering the gas chromatography equipment and for a Research Studentship to G.M. We are grateful to the suppliers who gave samples of their dehydrated cabbage products and we appreciate the interest and help of Dr. P.F.G. Praill and Miss J. Walley, Queen Elizabeth College, Campden Hill Road, London, W. 8.

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EFFECTS OF VARIATIONS IN COOKING METHODS ON THE FLAVOR VOLATILES OF CABBAGE

SUMMARY—Cabbage was cooked by a number of different methods and the flavor volatiles liberated were collected and analyzed by gas chromatography. The relative percentage abundance of each volatile was calculated and the values compared for the different cooking variations. The fates of certain individual volatiles were particularly interesting and are described. The cooking variations considered include cooking for different lengths of time, using microwave radiation instead of conventional heating and using different parts of the plant.

INTRODUCTION

IN RECENT years much work has been carried out on the nature of the flavor volatiles of foodstuffs, in most cases using gas chromatography as the basic tool. Nearly every possible foodstuff has been investigated in this way, although the emphasis seems to have been mainly on fruit and fruit products [e.g., apricots (Tang et al., 1967), pears (Giannone et al., 1967), pineapples (Creveling et al., 1968), oranges (Wolford et al., 1967), apples (Bertrand et al., 1967), etc.], alcoholic beverages [e.g., beer (Powell et al., 1966), wine (van Wyk, 1967), Scotch whisky (Duncan et al., 1966), etc.] and more recently nonalcoholic beverages such as tea (Bondarovich et al., 1967), coffee (Stoffelsma et al., 1968) and cocoa (van der Wal et al., 1968). Less attention has been paid to vegetables, but there is still ample information concerning, for example, potatoes (Self, 1967), onions (Bernhard, 1968), peas (Shipton et al., 1966).

One of the major difficulties in investigating the flavor volatiles of vegetables is that in most cases the vegetable is normally cooked in some way before being consumed; thus it is necessary to examine the flavor volatiles of the cooked and not the raw vegetable. This has been done for potatoes (Gumbmann et al., 1964), rutabaga (Hing et al., 1964) and others (Self et al., 1963), but in nearly all cases the cooking technique has been standardized. In actual practice this could never be the case and the cabbage can be submitted (deliberately or accidentally) to a wide range of cooking methods, depending on many circumstances. It is to be expected that the flavor volatiles would be different, depending on the method of cooking. It was for this reason we decided to cook cabbage in a number of different ways; to determine the flavor volatiles both qualitatively and quantitatively and to attempt to assess from the results how such variations in cooking had affected the chemical basis of the vegetable flavor.

Cooked cabbage itself has not been investigated in great detail, although before the advent of gas chromatography Dateo et al. (1957) studied the volatile sulfur compounds using a series of precipitation traps. They wrongly concluded the major flavor components to be H_2S and dimethyl disulfide (Dateo et al., 1957). S-Methyl-L-cysteine sulfoxide, isolated from cabbage by Synge et al. (1956), accounts for 25% of the free sulfur in cabbage and this has been shown to be the main precursor of the dimethyl disulfide (Dateo et al., 1957). It does seem likely, however, that this may produce other volatiles also. Bailey et al. (1961) and Clapp et al. (1959) also further investigated cabbage volatiles, although concentrating on the sulfur compounds, mainly the isothiocyanates. More recently, we carried out a more complete examination of the total organic flavor volatiles of cooked cabbage (MacLeod et al., 1968); the cooking variations are based on these results.

It is most important that not only should the nature of the flavor volatiles of a cooked foodstuff be determined completely but, except in very rare cases, all the more common methods of cooking of that particular food must also be comparatively studied.

EXPERIMENTAL

A PREVIOUS paper described the collection and identification of the flavor volatiles liberated from cooking cabbage (MacLeod et al., 1968). The cooking procedure used and described there was intended to simulate normal cooking conditions as far as possible. However, to ensure that a large enough sample was produced for accurate analysis, the cabbage was somewhat overcooked (30 minutes' boiling) in the expectation that a larger, more concentrated sample would be obtained than by cooking for a more usual time. In all other respects the cooking method used was perfectly normal.

Consequently, before any investigation could be carried out into how cooking variations affect normal cabbage flavor, it was necessary to determine the correct cooking time. Cabbages were cooked normally as before (MacLeod et al., 1968) except that the length of cooking time was varied. Tasting tests were carried out on the products and cooking for 10 min only produced by far the best results. It was concluded that an ideally cooked cabbage was one treated as previously described, except that the heating was discontinued after 10 min.

Using these basic, ideal cooking conditions and specially grown F_1 hybrid Summer Monarch cabbages, gas chromatograms of the flavor volatiles were obtained as before (MacLeod et al., 1968). Maximum variations in amounts present were calculated for each component over a number of chromatograms. In all but 3 cases, such variations were very small indeed and even these 3 components (methyl alcohol, ethyl alcohol and but-2-en-1-al) did not vary to a great extent. It was thus possible to carry out a range of cooking variations and to ascribe any differences, both qualitative and quantitative, in the nature of the volatiles to the cooking variations as such and not to other factors.

Methods of sampling and analysis were the same as previously described, although obviously slight modifications in technique were necessary to accommodate the particular cooking variation. The variations considered, and any necessary modifications, were as follows:

1. Variations in cooking time

a) Complete sampling. Cooking was stopped after different times (5, 20, 30, 60 and 90 min) and results compared with the normally cooked cabbage (10 min). When cabbage in reasonably large amounts is added to boiling water, there is a cooling effect and the water goes off the boil. It takes only a few minutes for the water to reach boiling again and all experiments were timed from the point when the water reboiled. One final time variation experiment is referred to as "0 minutes" and this was when sampling ceased immediately the water reboiled (i.e., "blanching").

b) Interval sampling (i.e., sampling only at set intervals). Volatiles were collected during periods of 10 min only, at the end of a certain cooking time. For example, cabbage was cooked for 20 min, but no sample was collected during the first 10 min. At the end of that time the collecting U-tube was added to the apparatus and a sample collected between 10 and 20 min. Other interval samples were obtained at 20–30, 30–40 and 40–50 min.

2. Using microwave radiation

a) Microwave radiation was used to heat the cabbage instead of the usual, more conventional methods. The microwave oven used was a Philips prototype model operating at a frequency of 2,450 Mcps. This had a large enough oven to hold the 2-liter flask used for all experiments. It was necessary for the reflux condenser to be outside the oven, not only because of space restrictions but because of the heating effects on the cooling water flowing through it. The volatiles were led from the top of the flask through polythene tubing via the oven ventilation holes to the water condenser outside. A receiver was connected to the bottom of the condenser to collect the condensed water that distilled from the cooking flask. Otherwise the apparatus was the same as usual.

It is uncertain to what extent these unavoidable variations in apparatus affected the results.

Firstly, the use of any tubing except glass should be avoided due to the risk of adsorption or contamination of volatiles. However, polythene is better than rubber tubing and even after repeated use there was no detectable odor. Secondly, water was actually removed from the reaction flask by distillation rather than being retained by simple refluxing as usual. Under certain conditions reasonably large volumes of water were distilled off (up to 250 ml). This water was repeatedly checked, but in no case did it contain more than minute traces of certain volatiles normally present in quite large amounts (e.g., acetone, methyl alcohol). This, then, did not affect the quantitative validity of the experiments. Cabbage was cooked, otherwise normally, by microwave radiation for 5, 10, 20, 30 and 40 min.

b) Foods subjected to microwave radiation are cooked as a result of the transformation of electrical energy into heat energy on absorption of the microwaves by the food material. The higher the water content of the food, the greater the absorption of microwave radiation by that food and this, of course, shortens the cooking time. A large volume of additional water used for boiling vegetables, etc., in a microwave oven preferentially absorbs the microwave radiation (Bilbrough, 1966). This prolongs the cooking time of the vegetable, which is cooked more by conduction and convection from the cooking water than by actual penetration of the radiation into the cabbage tissues themselves.

It is possible, therefore, that in experiments a) the microwave radiation was merely serving to heat the water, the cabbage being cooked virtually as in conventional cooking with very little penetrative heating. Conseqently, to overcome this problem, a series of experiments was carried out in exactly the same way except that no cooking water was used.

Cabbage was cooked dry, as described, for 5, 10, 20 and 30 min. Cooking for 40 min proved impossible due to the great pressure developed by this length of time; a safety valve incorporated in the apparatus repeatedly blew. Even cooking for 20 min resulted in an obviously overcooked, rather charred mass. On the other hand, after 5 min the vegetable was still undercooked.

3. Using different parts of the plant

Old or outer leaves were cooked normally and compared with young or inner leaves (including heart) from the same plant. In addition, the experiments were carried out using microwave radiation (in the presence of cooking water).

RESULTS & DISCUSSION

ON SUBMITTING the cabbage to variations in cooking technique, certain new flavor components were produced that had not been formed when the cabbage had been cooked normally (for 10 min). These new components were identified as far as possible purely by relative retention times, as previously described (MacLeod et al., 1968). Since the original peaks for the 10-min cooked cabbage were numbered in sequence, the new components are shown with the number of the peak immediately preceding with a letter following (e.g., 14a, 14b, 15a, etc.).

Table 1 gives the complete list of all the components identified and their relative retention times. Also shown are results obtained from the 10-min normally cooked cabbage of the approximate percentage abundance of the components in the sample relative to the total organic volatiles present. These latter values were determined by peak area measurements and corrected to allow for response factors of the detector. Not all of the components listed were detected for every cooking variation and certain new components were obtained only in 1 or 2 instances.

A few of the new components not formed when cabbage is cooked normally are worthy of brief mention. Most were obtained only in very small amounts and

Table 1—Identifications and retention times of cabbage sample components.

Sample Peak No.	Identification	Sample peak relative retention time (min)[2]	Reference compound relative retention time (min)	Approximate percentage relative abundance[4]
Z	Diethyl ether	1.45	1.45	–
1	Methanethiol	1.72	1.72	0.5
2	Acetaldehyde[1]	2.35	2.35	3.5
3	Dimethyl sulfide[1]	2.65	2.65	26.5
4	Propionaldehyde[1]	3.08	3.08	0.5
5	Acetone[1]	3.45	3.45	14.5
6	Acrolein	4.25	4.25	1.0
7	n-Butyraldehyde	4.75	4.75	1.0
8	Ethyl methyl ketone[1]	5.30	5.30	1.0
9	Methyl alcohol[1]	5.75	5.75	12.0
10	Ethyl alcohol	6.65	6.65	3.5
11	?	7.25		2.5
12	Diethyl ketone[1] plus Diacetyl[1]	8.30	8.30	6.0
13	Methyl alcohol/water profile (MacLeod et al., 1968)	~11.2	~11.2	–
13a	Butyl methyl sulfide	11.50	11.50	–
14	But-2-en-1-al	11.90	11.90	1.0
14a	?	12.40		–
14b	Dipropyl sulfide	12.60	12.60	–
15	Dimethyl disulfide[1] plus n-Hexanal[1]	12.95	12.95	1.0
15a	Cis-crotononitrile	13.50	13.50	–
16	Allyl alcohol	13.75	13.75	0.1
17	Dipropyl ketone	14.4	14.4	0.1
18	Trans-pent-2-en-1-al[1]	15.0	15.0	0.5
19	?	15.4		1.5
19a	Trans-crotononitrile	16.2	16.2	–
20	Allyl cyanide[1]	16.8	16.8	3.0
21	Trans-but-2-en-1-ol	17.9	17.9	0.5
22	Trans-hex-2-en-1-al[1]	19.1	19.1	1.5
23	Methyl propyl disulfide	20.0	20.0	1.0
24	Could be methyl isothiocyanate	20.7	20.5	0.5
24a	?	21.3		–
25	Cis-pent-3-en-1-ol	22.5	22.5	0.5
26	?	24.6		0.1
26a	Butyl methyl disulfide or propyl isothiocyanate	25.6	25.6 Disulfide 25.7 Isothio-cyanate	–
27	Could be cis-pent-2-en-1-ol[3]	26.6	26.6	2.0
28	n-Hexanol	27.9	27.9	0.5
29	Allyl isothiocyanate[1]	30.5	30.5	6.5
30	Cis-hex-3-en-1-ol[1]	31.6	31.6	4.5
31	Butyl isothiocyanate	33.8	33.8	1.0
31a	?	35.4		–
32	Trans-hept-2-en-1-ol	38.6	38.6	1.0
33	?	43.8		1.0
33a	Trans-oct-2-en-1-ol	49.8	49.9	–

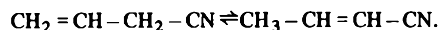
[1] Identified by mass spectrum also.

[2] Internal standards were used (MacLeod et al., 1968).

[3] Tentative identification only, due to mass spectrum being available which could not be identified positively and which does not completely confirm the identification given.

[4] Values given are for the 10-min, normally cooked cabbage.

drastic conditions were usually necessary for their production, e.g., diethyl ether, cis- and trans-crotononitrile, butyl methyl sulfide. The cis- and trans-crotononitrile were always produced together and it is possible that they were formed, under vigorous conditions, by rearrangement of the isomeric allyl cyanide (3-butene nitrile)-



$$CH_2 = CH - CH_2 - CN \rightleftharpoons CH_3 - CH = CH - CN.$$

Some of the new components had to remain unidentified, or only tentatively identified, basically because they were merely trace components.

1. Variations in cooking time

a) Complete sampling. One of the major causes of badly cooked cabbage is overcooking. It is well known that of all foodstuffs, when overcooked, the flavor and odor of the cabbage can be most objectionable. A range of experiments was, therefore, carried out, cooking for different lengths of time to discover if possible which volatiles might cause the bad flavor and odor on progressive cooking.

Table 2–Effect of variation in length of cooking time on the flavor volatiles of cabbage (complete sampling).

Total volatiles[1] (sq mm)	744	13,760	15,760	19,390	35,840	41,420	46,890
Peak No.	0 min	5 min	10 min	20 min	30 min	60 min	90 min
Z	–	–	–	–	–	–	–
1	1.5	0.5	0.5	1.5	0.5	1.0	0.5
2	–	1.5	3.5	3.5	5.0	4.0	7.0
3	2.0	17.5	26.5	28.0	34.0	29.0	38.5
4	–	0.1	0.5	0.5	0.5	0.5	0.1
5	93.5	15.0	14.5	9.5	11.5	11.5	13.0
6	–	–	1.0	–	0.5	–	–
7	–	0.5	1.0	1.0	0.5	1.0	1.5
8	–	0.5	1.0	0.5	0.5	1.0	0.5
9	–	11.0	12.0	11.0	12.0	29.5	9.0
10	–	3.5	3.5	3.5	4.0	–	–
11	–	10.0	2.5	7.0	1.5	–	3.0
12	–	6.5	6.0	5.5	5.0	4.0	4.0
13	–	–	–	–	–	–	–
13a	–	–	–	–	–	–	–
14	–	2.0	1.0	0.5	0.5	–	0.5
14a	–	0.01	–	–	–	–	–
14b	–	–	–	–	–	0.1	–
15	–	1.0	1.0	1.5	1.5	1.0	0.5
15a	–	–	–	–	–	–	0.5
16	–	0.5	0.1	0.1	0.1	0.1	–
17	–	–	0.1	0.1	0.1	–	–
18	–	0.5	0.5	1.0	0.5	0.5	0.5
19	–	1.5	1.5	1.0	1.0	0.5	1.0
19a	–	–	–	–	–	–	0.1
20	1.0	2.5	3.0	4.5	3.0	3.5	8.5
21	–	0.5	0.5	–	0.1	–	0.1
22	–	2.5	1.5	1.0	2.0	1.0	1.5
23	–	0.5	1.0	0.5	0.5	0.5	–
24	–	–	0.5	0.5	0.5	0.5	1.5
24a	–	–	–	–	–	1.5	–
25	–	0.5	0.5	0.5	0.5	0.5	0.5
26	–	0.1	0.1	0.05	0.1	0.5	0.1
26a	–	0.5	–	–	–	–	0.1
27	–	1.5	2.0	0.5	1.5	1.0	1.0
28	–	0.5	0.5	0.1	0.1	0.1	0.1
29	1.5	7.0	6.5	15.5	6.0	5.5	3.0
30	–	5.0	4.5	–	4.0	–	2.0
31	–	2.0	1.0	–	1.0	1.0	0.5
31a	–	–	–	–	–	–	–
32	–	0.5	1.0	1.0	0.5	0.5	0.1
33	–	1.0	1.0	1.0	0.5	1.0	0.5
33a	–	–	–	–	–	–	–

[1] An arbitrary value, being in effect an indication of the total amount of all volatiles obtained. This is calculated by adding together the areas (in sq mm) enclosed by all the peaks of the cabbage sample chromatogram. Such measurements were, of course, always made under the same chromatographic conditions.

Table 2 gives the approximate relative percentage abundances of all the individual volatiles after different cooking times.

The cabbage cooked for 5 min can best be considered as an intermediate to the 10-min cooked cabbage since the volatiles, in most respects, approach the 10-min percentage compositions. In fact, the cabbage cooked for 5 min was only slightly inferior in taste to the 10-min one.

Most information from these experiments was obtained from a consideration of the ways in which groups of compounds and important individual components varied with time. The saturated aldehydes as a group illustrate this quite well, in that their relative percentages increase with time. This implies that the reactions causing the formation of these aldehydes from their precursors (e.g., the Strecker degradation of the corresponding α-amino acids) proceed slowly and that their precursors are present in reasonably large amounts. Acetaldehyde shows this effect best.

The sulfides also increase with time, although dimethyl sulfide itself is not greatly increased at 60 min (29%), but after 90 min it is excessive at 38.5%. Dipropyl sulfide (relative retention time 12.6 min), not normally obtained, is formed in small quantities only after 60 min.

Allyl isothiocyanate shows a different behavior and the amount formed increases to a maximum of 15% at 20 min and then decreases. Similarly, the same effect is shown for allyl cyanide, this increasing to 4.5% at 20 min and then falling again. This suggests that after 20 min the common precursor of these compounds (the thioglucoside sinigrin) is slowly exhausted.

b) Interval sampling. The complete sampling method just described is a total, quantitative method only and gives no indication of the actual percentage of volatiles being produced at any particular time. In the so-called interval sampling method, volatiles were collected during a number of set 10-min periods and results were obtained which indicate the amounts of volatiles being formed at a specific time during the cooking period.

The results (see Table 3) show well how the total amount of volatiles produced during any single period decreases with time, a maximum being found at 10–20 min.

The sample collected between 10 and 20 min shows an increased amount of allyl isothiocyanate (16.5%); otherwise the chromatogram obtained was much the same as for the normally cooked cabbage (i.e., sample collected between 0 and 10 min). Roughly the same amount of allyl isothiocyanate is produced between 20 and 30 min also, but after 30 min of cooking its concentration is much

less. This confirms the findings in a), that the amount of allyl isothiocyanate formed reaches a maximum at about 20 min and most is formed between 10 and 30 min. Thus, it can be seen that a presumably normally desirable volatile can be present in possibly detrimentally excessive amounts, since the ideal quantity of allyl isothiocyanate is 6.5% at 10 min and the slightly overcooked cabbage at 20 min (which by tasting tests is inferior) possesses more than twice this amount. This is the major difference between properly cooked and slightly overcooked cabbage.

2. Using microwave radiation

a) In the presence of cooking water. Use of microwave radiation for the cooking of raw food as opposed to reheating of precooked food is comparatively new. Although conduction of heat aids in equalizing the temperature throughout the food material once the temperature has risen, the initial heat production is a result of the penetration of the microwaves from all directions into the foodstuff. The generation of heat within the food is a result of molecular friction mainly within the water content (Billbrough, 1966). Thus, in general, cooking by microwaves is a far more rapid process than conventional cooking. This would seem to be highly advantageous, but the technical and practical difficulties involved in cooking by microwave radiation are great and possibly insurmountable. For example, different phases in the raw food absorb the radiation to different extents and this can result in charring of certain parts while others are still raw.

In this work the main purpose of using microwaves for cooking was to deduce if possible whether the production of certain flavor volatiles was purely thermal or whether this was to some extent depend-

Table 3—Effect of variation in length of cooking time on the flavor volatiles of cabbage (interval sampling).

Total volatiles (sq mm)	15,760	17,130	5,015	2,230	1,575
Peak No.	0–10 min	10–20 min	20–30 min	30–40 min	40–50 min
Z	–	–	–	–	–
1	0.5	1.0	1.0	0.5	1.0
2	3.5	6.0	3.0	–	–
3	26.5	35.0	21.5	27.0	28.5
4	0.5	0.1	0.5	0.1	–
5	14.5	17.0	24.5	46.0	35.0
6	1.0	–	–	–	–
7	1.0	1.0	1.0	1.0	1.0
8	1.0	0.5	0.5	0.5	1.0
9	12.0	5.0	8.5	9.5	13.5
10	3.5	1.5	5.5	6.0	4.5
11	2.5	–	2.5	–	–
12	6.0	4.5	3.5	4.0	5.5
13	–	–	–	–	–
13a	–	–	–	–	–
14	1.0	–	–	–	–
14a	–	0.1	–	–	–
14b	–	–	0.5	–	–
15	1.0	0.5	1.0	–	–
15a	–	–	–	–	–
16	0.1	0.05	–	–	–
17	0.1	0.05	0.1	–	–
18	0.5	0.5	0.5	–	–
19	1.5	0.5	0.5	0.1	–
19a	–	–	–	–	–
20	3.0	3.5	3.0	2.0	6.5
21	0.5	0.1	0.5	–	–
22	1.5	2.5	2.0	0.5	0.5
23	1.0	–	–	–	–
24	0.5	0.1	0.5	–	–
24a	–	–	–	–	–
25	0.5	0.5	0.5	–	–
26	0.1	0.05	0.1	–	–
26a	–	–	–	–	–
27	2.0	1.0	0.5	–	–
28	0.5	0.05	–	–	–
29	6.5	16.5	16.0	2.0	3.0
30	4.5	–	–	–	–
31	1.0	1.0	1.5	–	–
31a	–	–	–	–	–
32	1.0	0.5	0.5	–	–
33	1.0	1.0	1.0	–	–
33a	–	–	–	–	–

Table 4—Effect of the use of microwave cooking on the flavor volatiles of cabbage.

Total volatiles (sq mm)	7,200	5,780	8,020	69,295	502,765
Peak No.	Wet microwave 5 min	Wet microwave 10 min	Wet microwave 20 min	Wet microwave 30 min	Wet microwave 40 min
Z	–	–	–	0.001	0.005
1	0.1	0.05	0.5	0.1	0.01
2	2.0	1.0	2.0	10.0	2.5
3	5.0	8.5	15.0	34.5	5.0
4	0.1	0.1	0.1	0.1	0.05
5	25.5	14.0	11.5	13.0	3.5
6	3.5	1.0	1.5	0.5	–
7	0.5	0.5	0.5	0.5	0.1
8	1.0	1.0	0.5	0.5	0.05
9	12.0	11.0	10.5	16.5	84.5
10	–	3.5	–	–	–
11	5.5	11.5	8.5	–	–
12	10.5	9.5	9.5	3.0	0.5
13	–	–	–	–	–
13a	–	–	–	–	–
14	1.0	1.5	1.0	–	–
14a	–	0.5	0.5	–	–
14b	0.05	0.5	0.5	–	–
15	1.5	1.5	2.0	0.5	0.1
15a	–	–	–	–	–
16	–	–	0.1	0.1	0.05
17	–	0.5	–	–	–
18	3.5	7.0	5.0	1.5	0.1
19	2.5	3.0	1.5	0.5	0.1
19a	–	–	–	–	–
20	2.5	3.5	7.5	9.5	1.0
21	0.5	–	–	–	–
22	2.0	1.0	1.0	1.0	0.1
23	0.5	1.0	0.5	–	0.01
24	–	–	0.5	0.1	0.05
24a	–	2.0	–	–	–
25	–	0.5	0.5	0.5	0.05
26	–	0.5	0.5	–	0.005
26a	–	–	–	0.1	0.005
27	4.0	3.5	2.0	0.1	0.05
28	0.5	0.1	0.05	0.1	0.01
29	5.5	3.5	11.0	3.5	1.0
30	7.5	6.0	4.0	2.0	0.5
31	1.5	1.0	1.5	0.5	0.05
31a	–	–	–	–	–
32	1.5	–	0.5	0.1	0.05
33	0.5	0.1	0.5	0.1	0.05
33a	0.1	–	–	–	0.005

ent on time. If the liberation of a particular volatile was a result of the energy supplied by the heat, then it should be produced far more rapidly by microwave radiation than during conventional cooking. However, if time were the more important factor then microwave energy would achieve little in the shorter time. It was also of interest to compare the 2 methods of cooking as a whole.

Table 4 lists the percentage abundances of the various components when cooked by microwave radiation in the presence of water.

To compare microwave and conventional cooking, the 10-min cooking period is best considered. Of particular interest are the increases in certain compounds which have been little affected in the variations considered so far. For example, using microwave cooking the percentage composition of *trans*-pent-2-en-1-al (peak 18) is raised from its normal value of 0.5% to 7%. Furthermore, certain new components were produced. An unidentified peak (14a) appeared at a relative retention time of 12.4 min (1.5%) and some dipropyl sulfide (peak 14b) was also produced (0.5%). Dipropyl ketone (relative retention time 14.4 min) contributes 0.5% and a further unknown compound (peak 24a, relative retention time 21.3 min) is present to the extent of 2%.

The 40-min sample shows a large increase in the amount of methyl alcohol formed (84.5%). This possibly indicates the complete breakdown of the system at this stage after such drastic treatment.

In conclusion, it seems that the use of microwave radiation in this way does not achieve more rapid cooking.

b) In the absence of cooking water. Table 5 gives the percentage abundances of the cabbage components produced during microwave cooking in the absence of water.

It is not surprising to find that the 2

Table 5—Effect of the use of microwave radiation, in the absence of cooking water, on the flavor volatiles of cabbage.

Total volatiles (sq mm)	11,290	35,260	64,205	79,000
Peak No.	Dry microwave 5 min	Dry microwave 10 min	Dry microwave 20 min	Dry microwave 30 min
Z	–	–	0.005	0.001
1	0.01	0.5	0.1	0.1
2	1.5	4.5	9.0	8.0
3	4.0	17.0	17.0	29.0
4	0.1	0.1	0.1	0.1
5	14.5	12.0	14.0	18.0
6	1.5	1.0	–	0.5
7	0.5	0.5	0.5	0.5
8	0.5	0.5	0.5	–
9	41.5	37.5	14.5	24.5
10	–	–	–	–
11	–	–	2.0	–
12	11.5	5.0	3.0	2.5
13	–	–	–	–
13a	–	–	0.1	0.5
14	1.5	0.5	0.5	–
14a	–	–	–	–
14b	–	–	–	–
15	1.0	1.0	0.5	0.5
15a	–	–	–	–
16	–	–	0.5	0.5
17	0.5	–	–	0.1
18	2.0	3.0	2.5	2.0
19	2.5	1.5	0.5	0.5
19a	–	–	–	–
20	3.5	5.5	26.5	7.5
21	–	–	–	–
22	1.0	0.5	0.1	0.1
23	–	0.5	0.5	0.05
24	–	0.1	1.0	0.5
24a	–	0.1	–	–
25	0.5	0.5	1.0	0.1
26	0.1	0.1	–	0.05
26a	–	0.1	0.1	0.01
27	3.0	0.5	0.5	0.1
28	0.1	0.1	0.1	0.05
29	2.0	2.5	1.5	2.0
30	5.5	2.5	2.5	1.5
31	0.5	0.5	0.5	0.5
31a	–	0.1	–	–
32	–	0.5	0.1	0.1
33	0.5	0.5	0.1	0.1
33a	0.1	0.1	0.05	0.05

Table 6—Comparison of the flavor volatiles of cabbage produced from inner leaves only with those produced from outer leaves only, by both conventional and microwave cooking.

Total volatiles (sq mm)	9,815	20,485	253,620	76,065
Peak No.	Conventional inner leaves	Conventional outer leaves	Microwave inner leaves	Microwave outer leaves
Z	–	–	–	0.05
1	0.5	0.5	0.05	0.1
2	1.0	8.0	2.0	6.0
3	36.5	9.5	14.0	23.5
4	0.1	0.5	0.05	0.1
5	13.5	24.0	2.0	14.5
6	–	–	–	–
7	2.0	2.5	0.05	0.5
8	1.0	1.5	0.1	0.5
9	4.5	2.0	72.0	31.0
10	2.5	0.5	–	–
11	7.0	6.5	–	–
12	5.5	9.0	1.0	5.0
13	–	–	–	–
13a	–	–	–	–
14	–	1.0	0.5	1.5
14a	–	–	–	–
14b	0.1	–	–	–
15	0.5	2.5	0.1	1.0
15a	–	–	–	–
16	–	0.1	0.05	–
17	0.1	–	–	–
18	0.5	1.0	0.5	1.0
19	0.5	4.5	0.05	1.0
19a	–	–	–	–
20	3.5	1.0	4.0	4.0
21	–	0.5	–	0.1
22	0.05	8.5	0.005	3.5
23	0.5	–	0.05	–
24	0.5	0.1	0.1	0.1
24a	–	–	–	–
25	0.5	0.1	0.1	0.1
26	0.1	0.5	0.005	0.1
26a	0.5	–	–	0.1
27	0.5	3.5	0.01	1.0
28	0.05	0.5	0.05	0.1
29	16.5	3.0	3.0	1.5
30	–	6.5	–	2.0
31	–	1.0	0.5	0.5
31a	–	–	–	–
32	0.5	0.1	0.1	0.1
33	1.5	0.5	0.1	0.1
33a	–	–	0.01	0.05

Table 7

	Conventional cooking	Microwave cooking
Percentage abundance of methyl alcohol	4.5 (Inner leaves) 2.0 (Outer leaves)	72 (Inner leaves) 31 (Outer leaves)

sets of microwave results resemble each other to a far greater extent than either resembles the results for the normally cooked cabbage. For example, the amount of trans-pent-2-en-1-al formed during microwave cooking (whether in the presence or absence of added water) is greater than that produced while the cabbage is cooked conventionally. This increase is specifically an effect of the microwave radiation, since the amount of this component does not usually vary to a great extent. This indicates that the length of time of cooking is not so important in this instance, the heat energy supplied probably being the main factor in the compound's formation.

The increased effect of the microwaves in the absence of water can be well illustrated by a consideration of 3 components. By the wet microwave method, the methyl alcohol content for the first 20 min of cooking is little different from conventional cooking (10.5%). At 30 min it is slightly higher (16.5%) but at 40 min it is greatly increased to 84.5%. In the absence of water and after only 5 min of exposure to microwave radiation, the alcohol is an extremely large component at 41.5%, dropping to 37.5% at 10 min and to 24.5% at 30 min. Therefore, in the absence of water the increased production of methyl alcohol by microwave radiation occurs much earlier. A similar effect is observed for both allyl isothiocyanate and allyl cyanide. When water is present, the cyanide increases to a maximum at 30 min (9.5%), whereas the isothiocyanate rises to a maximum of 11% at 20 min. However, in the absence of water, although the same basic effect is observed, the maximum is attained sooner and the actual value of the maximum is very different. The cyanide increases to a maximum at 20 min (26.5%), whereas the isothiocyanate increases only to 2.5% but after 10 min. The fact that in the absence of water both the cyanide and the isothiocyanate reach their maximum earlier, again supplies evidence for the more rapid action of microwaves when no added water is present. It is also of interest that when water is present, nitrile and isothiocyanate reach similar percentage maxima, but in the absence of water much less isothiocyanate is formed and far more cyanide. Allyl cyanide and allyl isothiocyanate are both formed from a common precursor, namely the thioglucoside sinigrin; the latter is produced by the action of the enzyme myrosinase and the former probably by simple thermal degradation of the precursor (Jones, 1967). The "dry" microwave results tend to support this theory of cyanide production, since presumably the greater energy supplied by the microwaves in the absence of added water increases the efficiency of the thermal degradation reaction to produce more of the nitrile than usual (i.e., more than in the presence of cooking water).

3. Using different parts of the plant

It is generally agreed that the heart of the cabbage has a much stronger flavor (not necessarily better) than the outer leaves when both are cooked in exactly the same way. To discover to what extent the 2 parts differed in this respect, experiments were conducted on both inner leaves only and outer leaves only. These were cooked in the same way as the properly cooked cabbage, and also by microwave radiation. Results are given in Table 6.

These results show well the appreciable differences in the nature of the volatiles produced from the different parts of the plant. During conventional heating, the most important differences were that 36.5% dimethyl sulfide and 16.5% allyl isothiocyanate were obtained from the heart as against only 9.5 and 3%, respectively, from the outer leaves. Apart from these compounds, the other volatiles liberated mainly from the young leaves were ethyl alcohol, dipropyl sulfide, allyl cyanide and methyl propyl disulfide. These are mostly sulfur compounds, which tends to stress the contribution of these (especially dimethyl sulfide and allyl isothiocyanate) to the characteristic cabbage flavor, which is much more evident in the heart than in the outer leaves. The compounds found mainly in the outer leaves were acetaldehyde, acetone, ethyl methyl ketone, diethyl ketone, diacetyl, trans-hex-2-en-1-al, cis-pent-2-en-1-ol and cis-hex-3-en-1-ol. The first 2 of these are particularly important, since the outer leaves produce 8 times as much acetaldehyde as the inner leaves and acetone is the most abundant volatile obtained from the older leaves.

In general, then, the sulfur volatiles are produced mainly from the young leaves, while the older leaves liberate a greater proportion of aldehydes, ketones and alcohols. A whole cabbage cooked normally forms about 36% sulfur volatiles, whereas the heart cooked in the same way gives 46% and the outer leaves only 15%. In other words, although the increase in sulfur content from the heart is not too great, the decrease in the outer leaves is quite appreciable and must detract from their flavor. This can be easily confirmed by tasting tests.

Certain individual volatiles were found to be liberated only from either the heart or from the older leaves. Some examples which illustrate this point and also the preponderance of sulfur compounds in the heart are mentioned below.

But-2-en-1-al (crotonaldehyde, peak 14) was produced only from the outer leaves whereas dipropyl sulfide (peak 14b) was formed only from the heart. Peak 19, unidentified, was present to a far greater extent in the outer leaves (implying that this is not a sulfur compound). Trans-but-2-en-1-ol (peak 21) also was found only in the outer leaves and although trans-hex-2-en-1-al was formed in both, there was very little from the young leaves (0.05% as against 8.5% from the outer leaves). Another sulfide, methyl propyl disulfide, was present only in the heart. Cis-hex-3-en-1-ol (normally 4.5%) was found only in the outer leaves (6.5%). The complete absence of certain compounds from certain parts of the plant is very surprising, since such a sharp, clear-cut division would not have been expected.

That cis-hex-3-en-1-ol and trans-hex-2-en-1-al were found to be almost exclusively in the outer leaves is of interest. It is possible that their precursors may be formed only at a later stage in the maturation of the leaves and, therefore, they will be present only after a certain stage of growth (i.e., in the older leaves). Autoxidation could well be an important consideration here. Since the 2 components have such a similar behavior and structure, they might either have a common precursor or even perhaps be interconvertible. Considering that the hexenol and hexenal are such common components of foodstuffs (particularly vegetables) and plants, it is rather surprising that little is known regarding their origins.

The results obtained when using microwave radiation were similar to those described for conventional heating. Here, however, in agreement with the previous microwave work, the amount of methyl alcohol produced was greatly increased, although the ratio of the amounts formed from the inner and outer leaves was the same as for conventional cooking.

Many other cooking variations were also investigated. These included cooking at a range of different pH values, using old cabbage up to 4 weeks old, cabbage chopped and allowed to stand for some time before cooking, reheated cooked cabbage; a large-scale experiment (5 times normal) was also carried out. However,

the results obtained were not very conclusive and, therefore, are not reported here.

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Ms. received 8/26/68; revised 8/8/69; accepted 10/3/69.

Our thanks are due to the Science Research Council for a grant covering the gas chromatography equipment and for a Research Studentship (to G.M.). We are grateful to the London University Botanical Supply Unit for growing the cabbages and we much appreciate the interest and help of Dr. P.F.G. Praill and Miss J. Walley.

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VARIANT AND INVARIANT PROPERTIES OF THE MITROCHONDRIAL FRACTION ISOLATED FROM RIPENING BANANA FRUIT

SUMMARY—The mitrochondrial characteristics of oxidation of Krebs cycle intermediates, respiratory control by ADP, succinate-linked oxidative phosphorylation, and overall morphology, and the amount of protein in the fraction did not vary as the ripening of banana fruit progressed. However, stimulation of succinoxidase activity by calcium and succinate-driven calcium accumulation underwent striking changes as terminal senescence ensued. The results are consistent with observations of the controlled nature of terminal senescence and to the possible involvement of ancillary work performances, such as active transport, with climacteric respiration.

INTRODUCTION

THE ONSET OF senescence in certain detached fruit is accompanied by a rapid upsurge in respiration. The burst in carbon dioxide evolution and oxygen consumption has been referred to as the "climacteric rise" (Kidd and West, 1931) and fruit which possess this characteristic as climacteric fruit (e.g., Biale and Young, 1962). The regulatory influence of environmental factors such as temperature, oxygen and carbon dioxide tension, and moisture level on the onset of climacteric respiration have been studied (Loesecke, 1950 and Haard and Hultin, 1969), as have respiratory initiators and inhibitors (Abdul-Baki et al., 1965). Climacteric respiration in banana fruit is accompanied by metabolic changes-driven by a host of enzymes including emergence of chlorophyllase in the peel (Looney and Patterson, 1967), pectin methyl esterase in the pulp (Hultin and Levine, 1963), changes in organic acid pools (Wyman and Palmer, 1964) and phenolic constituents (Goldstein and Swain, 1963). The possibility that climacteric respiration is central to ripening and senescence has led us to investigate the nature of mitochondrial-linked reactions in ripening banana fruit. Understanding control mechanisms intrinsic to climacteric respiration would make possible new avenues of approach to extend the food use of such fruit.

Available evidence points to the conclusion that the CO_2 evolution and O_2 consumption associated with climacteric respiration derives from cytochrome oxidase, the terminal step in mitochondrial electron transfer; other oxidases do not appear to be involved (see Haard, 1967 for review). The relationship between climacteric respiration and mitochondrial-linked oxidations can be of two modes, "coupled" or "uncoupled." In brief, a "coupled theory" would view all of the energy-conserving processes of the mitochondria as intact and the climacteric rise in respiration simply as the manifestation of increased cellular work performance. The alternative view, or "uncoupled mechanism," would explain the climacteric rise as a result of a severing of the link between electron transfer and work performance. The route of "coupled electron transfer" had previously been thought to be limited to energy conservation via oxidative phosphorylation (ADP + Pi → ATP). Numerous recent studies have demonstrated that mitochondrial electron transfer may be linked to other work functions including ion transport (Brierley et al., 1964); solute transport (Jackson and Brown, 1963); membrane configurational change (Hackenbrock, 1966); transhydrogenation (Ernster and Lee, 1964); energized swelling (Chappell and Crofts, 1966); and perhaps others.

In this communication we describe certain constant and changing properties of the mitochondrial fractions isolated from the pulp of banana fruit at preclimacteric, climacteric and postclimacteric stages of ripening. Stimulation and succinoxidase activity by calcium and succinate-driven calcium accumulation were found to vary in banana mitochondria prepared from fruit at different stages of ripeness.

MATERIALS & METHODS

Materials

Green, preclimacteric banana fruit (*M. cavendishii*, Valery type) were purchased from a local distributor of the United Fruit Company and ripened at a temperature of 24°C; relative humidity was maintained in the range of 90–100%. Reagents and equipment employed in preparation and assay of mitochondria were

Table 1—Influence of stage of fruit ripeness on succinoxidase activity and protein of mitochondrial fractions.[a]

Days ripening (24°C)	Protein extracted (mg)	Succinoxidase [QO_2 (N)]	Whole fruit respiration (mg CO_2/Kg-Hr)
0	27.0	465	4.81
1	30.4	430	—
3	29.5	455	—
5	28.5	450	13.9
7	34.0	465	—
9	33.2	440	—
11	26.5	445	29.5
13	27.0	480	—
15	27.5	490	21.2

[a]Assay medium contained sucrose, 0.4 M; ATP, 0.5 M; total magnesium chloride, 83 mM; bovine serum albumin, 1 mg; potassium phosphate, pH 7.3, 13 mM; cytochrome *c*, 2 × 10^{-5}M; succinate, 10 mM; mitochondrial preparation, approximately 3 mg and water to 5 ml.

Table 2—P/O ratios typical of mitochondria from pre- and postclimacteric fruit.

Δ Assay medium[a]	P/O Preclimacteric fruit	P/O Climacteric fruit
———	0.37	0.35
- BSA[b]	0.08	0.09
+ 0.56 mM DNP[c]	0.11	0.11
+ 0.07M Sucrose	0.24	0.29
+ 40 mM Phosphate	1.75	0.77
+ 40 mM Phosphate, 0.74M sucrose	2.20	1.90

[a]Changes indicate variation in assay medium from that containing sucrose, 0.4M; ATP, 0.5 mM; $MgCl_2$, 83 μM; 1 mg BSA; succinate, 13 mM; potassium phosphate, 13 mM, pH 7.3; and cytochrome c, 2 × 10^{-5} μM and water to 5 ml.

[b]BSA = bovine serum albumin (Fraction V), Sigma Chemical Co.

[c]DNP = 2,4-dinitrophenol.

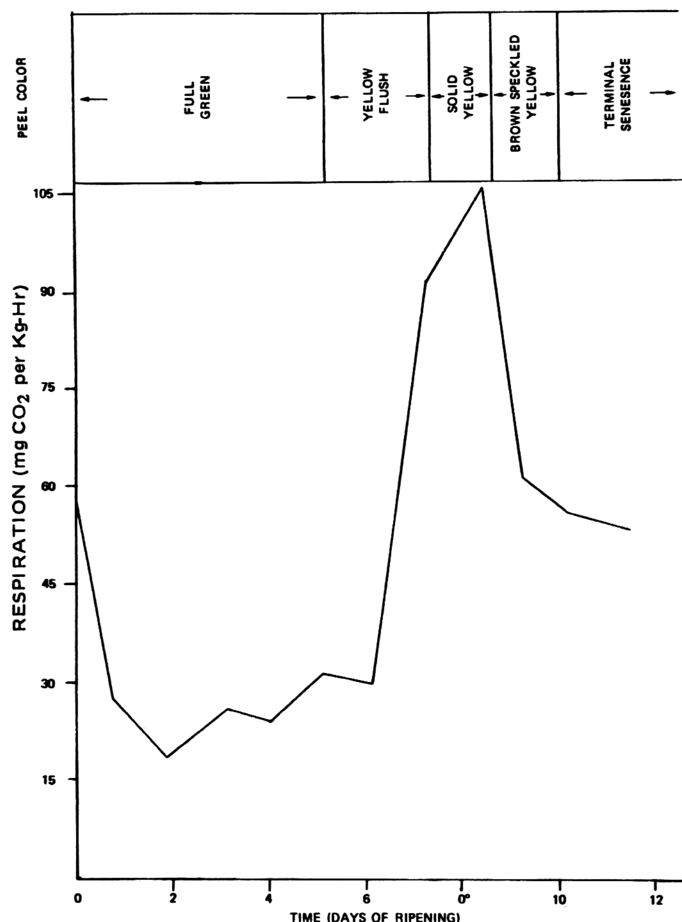

Fig. 1–Respiration and peel color of ripening "Valery" banana fruit.

Table 3–Influence of stage of fruit ripeness on succinate-driven accumulation of calcium ions by mitochondria.

Description of fruit extracted	Calcium accumulation[a]
Green, preclimacteric	0.488
Yellow-green, climacteric	0.726
Full yellow with brown spots, postclimacteric	1.625

[a]µmoles of calcium actively accumulated by 1 mg of mitochondrial protein nitrogen in 15 min under conditions described in text.

Table 4–Influence of potassium phosphate on succinate-driven and passive calcium accumulation by mitochondria.

Pi (mM)	Fruit ripeness	Passive Ca^{++} uptake[a]	Active Ca^{++} uptake[a]
0.0	Post-	1.40	0.00
0.8	climacteric	1.41	1.02
1.6		1.38	1.35
4.0		1.35	1.69
8.0		1.20	1.65
0.0	Pre-	1.24	0.062
0.8	climacteric	1.42	0.292
2.4		1.46	0.345
4.0		1.25	0.495
8.0		1.20	0.670

[a]µmoles of calcium activity accumulated by 1 mg of mitochondrial protein nitrogen in 15 min under conditions described in text.

as described elsewhere (Haard and Hultin, 1968). $^{45}CaCl_2$ and $H_3{}^{32}PO_4$ were purchased from New England Nuclear Corporation.

Measurement of intact fruit respiration

The carbon dioxide evolved from intact banana fruit was collected and estimated as bicarbonate ion with an apparatus and method constructed analagous to the design described by Braverman (1963).

Preparation of mitochondria from the pulp of banana fruit

Mitochondria were prepared from 150g of pulp by extracting frozen tissue (Haard and Hultin, 1968) and were assayed within 4 hr after preparation. Mitochondrial fractions were suspended and stored in a solution containing sucrose, 0.4M; Tris-chloride, (tris = tris-(hydroxymethyl)aminomethane), 0.01M; ATP, 2.5 mM; magnesium chloride, 5.0 mM; and bovine serum albumin, 3 mg/ml.

Tricarboxylic acid cycle intermediate utilization

The oxidation of succinate, fumarate, citrate, α-ketoglutarate, pyruvate and malate by isolated mitochondrial fractions was measured with a differential manometer (Gilson Respirometer) by the technique outlined by Umbreit et al. (1959). Specific conditions of assay are indicated in the Results section.

Oxidative phosphorylation

Phosphate esterification was estimated by the method of Lindberg and Ernster (1960) as modified by Peniall (1966), using a Nuclear Chicago liquid scintillation counter. Counting vials contained 0.2 ml of sample in 10.8 ml of scintillation solution and 4.0 ml of absolute ethanol (after Rapkin, 1963). The ratio of phosphate esterified to oxygen consumed (P/O) was calculated according to Umbreit et al. (1959).

Protein determination

Protein was estimated by the modified Folin procedure described by Lowry et al. (1951).

Calcium accumulation

The uptake of calcium ion by isolated mitochondria was measured by the following procedure. Mitochondria were suspended in an assay medium containing cytochrome *c*, 3×10^{-5} moles; succinate, 20 µmoles; potassium phosphate, pH 7.4, 20 µmoles; sucrose, 1 mM; calcium chloride, 1.5 µmoles; approximately 3 mg of mitochondrial protein; water to 5 ml. Each reaction flask contained approximately 2,500,000 counts/min of $^{45}CaCl_2$. The reaction mixture was incubated for 5 min in a Dubnoff metabolic shaker in 10 ml Erlenmeyer flasks at a shaker speed of 100 rotations per minute and a bath temperature of 30°C. Reaction was started by addition of the mitochondrial suspension and after an appropriate time, a 0.2 ml aliquot was removed, blown into 3 ml of cold 1M sucrose, and immediately filtered through a 0.8 micron filter (Millipore Filter Company). Suction was applied for 1 min using a Duo-Seal vacuum pump, and the filtrate was collected in a test tube inside of a 125 ml vacuum flask. The mitochondrial fraction (on filter paper) was placed in a counting vial with the same solvent and scintillator described for ^{32}P estimation. An aliquot of the filtrate was pipetted into another vial, and each was counted for 80 min in the scintillation counter at the optimal gate and data voltages for ^{45}Ca.

Electron microscopy

Samples for electron microscopic examination were suspended in sucrose (0.4M) and Tris-chloride (0.01M), pH 7.3 at a protein concentration of 1 mg/ml, and an equal volume of fixative (2% glutaraldehyde; 2% acrolein; sodium cacodylate, 50 mM; sucrose, 0.25M) was added. Samples were fixed and stained with uranyl acetate and potassium permanganate and embedded in Luft's Epon (Luft, 1961).

RESULTS & DISCUSSION

Respiration of intact banana fruit

Bananas were purchased at stage 1 or 2 [solid green peel, see Loesecke (1950)] and ripened at 24°C; the relative humidity was maintained in the range of 90–100%. Figure 1 shows the respiratory pattern typical of these fruit, indicating the carbon dioxide evolved and peel color as a function of time in days of ripening. The peel color was found to be a useful index of respiratory stage.

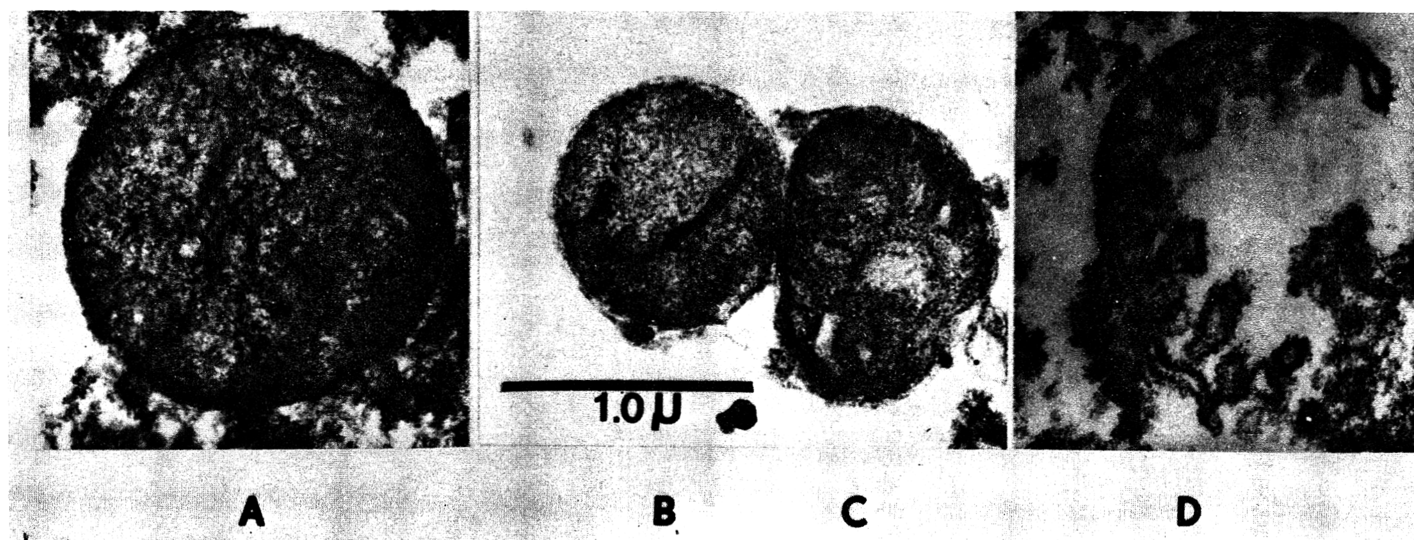

Fig. 2—Electron micrographs of mitochondria from ripening banana fruit: (A) most common configurational state observed in all mitochondrial preparations; (B—D) other less common configurational states observed.

Yield of mitochondria as a function of respiratory stage of fruit

Mitochondrial fractions were isolated from the pulp of ripening bananas at different stages of the respiratory spectrum illustrated in Figure 1. The yield of protein in the mitochondrial fractions are reported as mg of protein extracted from 100g of pulp. The data averaged from two experiments are shown in Table 1. The variation in protein extracted from fruit at progressive stages in ripeness (Table 1) was not significantly different from that obtained when replicates were run on fruit of the same ripeness (see Haard and Hultin, 1968). Examination of these preparations in the phase-contrast microscope showed that they were similar in homogeneity with respect to Janus Green B positively stained particles. Contaminating particles included occasional starch granules and some cell wall fragments. Succinoxidase activity was used as a mitochondrial enzyme marker and was found to be similar throughout ripening.

Other mitochondrial properties invariant with ripeness of fruit

The mitochondrial fractions isolated from ripening banana fruit were remarkably similar in oxygen consumption when intermediates of the tricarboxylic acid cycle were used as substrates (Haard and Hultin, 1968), in P/O ratio when succinate was substrate (Table 2), and respiratory control by ADP when succinate was substrate (approaching 4.0 under optimal conditions, Haard, 1967). While these mitochondrial functions had some unique peculiarities and were extremely sensitive to slight modifications of assay conditions (Haard, 1967), there was no indication of different response with mitochondria isolated from preclimacteric, climacteric, and postclimacteric fruit at any one standardized condition of isolation and/or assay.

The point should be emphasized that the properties described above of succinate-linked oxidation and succinate-linked phosphorylation have been those parameters typically used to characterize mitochondria. These studies do show that the basic mitochondrial functions of oxidation and coupling to phosphorylation remain intact throughout ripening of the fruit.

Electron microscopy

Samples of isolates were observed in the electron microscope to determine whether any gross change in ultrastructure was discernible in mitochondria isolated from preclimacteric, climacteric, and postclimacteric fruit. Although this study was not extensive enough to definitively conclude on such comparisons, the preliminary results reported here gave no indication of change in general morphology, size, inner membrane, or overall integrity. Few damaged mitochondria were observed in any preparation, and there was striking heterogeneity in size and inner membrane configuration. Figure 2 illustrates examples of morphological types observed in all of these preparations; the mitochondrion exemplified by Figure 2A was the dominant form in all preparations. Figures 2B—2D represent other forms observed in these preparations. The configurational form in Figures 2A—2C bear a similarity to those recently equated to the energized states of certain mammalian mitochondria (Harris et al., 1968).

Stimulation of succinoxidase by $CaCl_2$

Addition of calcium chloride to succinate-oxidizing mitochondria caused stimulation of succinoxidase activity: the magnitude of stimulation was dependent on both the amount of added $CaCl_2$ and the stage of fruit ripeness (Figure 3). Mitochondria isolated from green, preclimacteric fruit showed maximal stimulation of succinoxidase at 1—2 mM $CaCl_2$; higher concentrations of this cation led to eventual inhibition of mitochondrial electron transfer. Calcium-stimulated respiration was only slightly depressed when exogenous ATP was included in the assay medium, suggesting that if such calcium-stimulated respiration was coupled to work performance, it was only slightly competitive with ATP-supported work performance. Added ATP, in the absence of $CaCl_2$, had no influence on succinoxidase activity under these experimental conditions, so that the slight depression of calcium-stimulated succinoxidase activity by ATP was possibly competitive with the calcium-stimulated function.

Unlike mitochondrial fractions from preclimacteric fruit, those from postclimacteric fruit did not respond to added calcium chloride. Control rates of succinoxidase were identical for these two systems; no response to calcium was evident in the absence of succinate with any of these preparations.

Active calcium accumulation

Mitochondria from banana fruit at all stages of ripeness were found to be

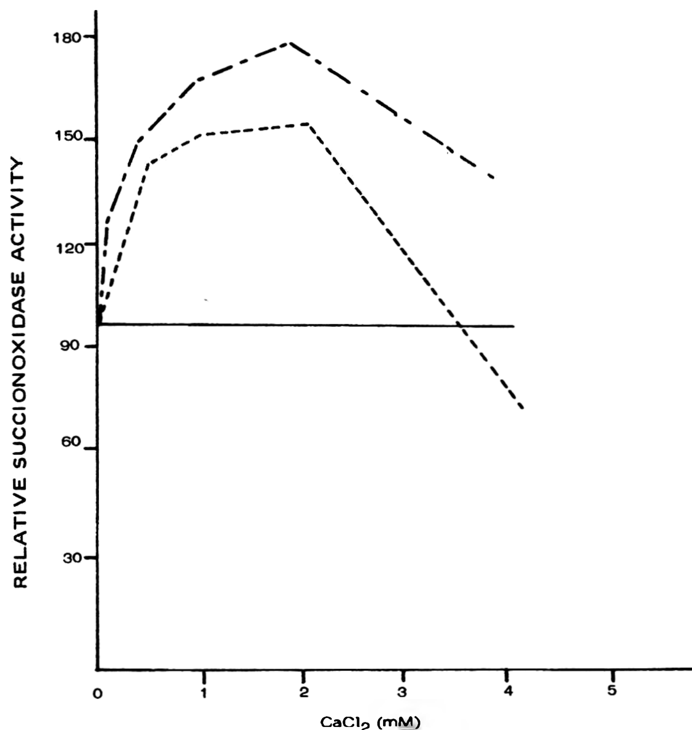

Fig. 3–Stimulation of mitochondrial succinoxidase by calcium: postclimacteric mitochondria, with or without ATP (—); preclimacteric mitochondria without ATP (– –); preclimacteric mitochondria with ATP, 0.6 mM (- - -). Assay media were as described in the text.

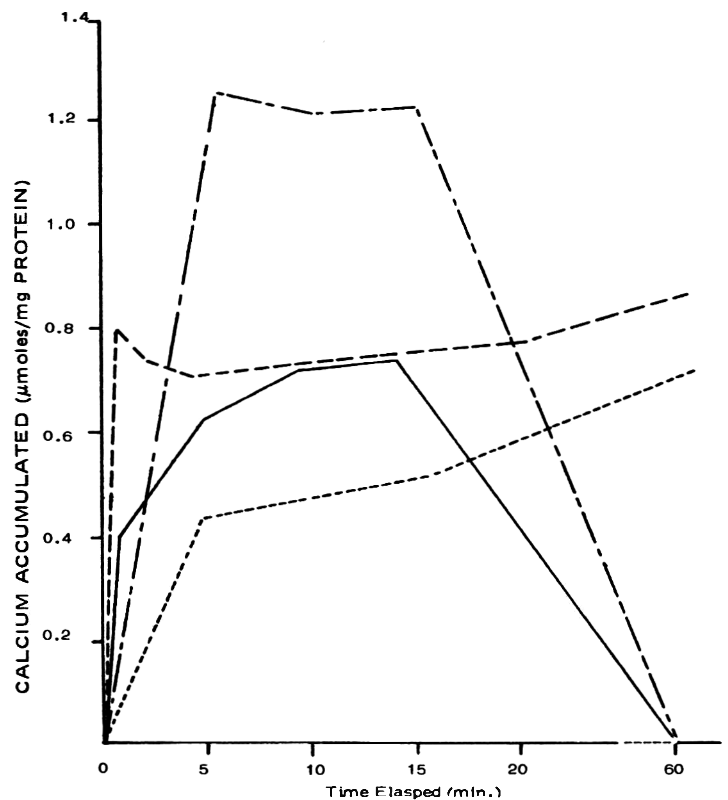

Fig. 4–Substrate mediated and passive calcium uptake by mitochondria; active uptake by postclimacteric mitochondria (— -); active uptake by preclimacteric mitochondria (—); passive uptake by preclimacteric mitochondria (— —); passive uptake by postclimacteric mitochondria (- - -). Assay media were as described in text.

capable of actively accumulating calcium ions with succinate as substrate. The maximal levels of calcium accumulated by preclimacteric, climacteric and postclimacteric mitochondria differed; more calcium was accumulated by mitochondria from ripe fruit (Table 3, Figure 4). Mitochondria isolated from postclimacteric fruit accumulated approximately two-fold more calcium than preparations from climacteric fruit and in excess of three-fold that accumulated by preclimacteric mitochondrial fractions. Active calcium accumulation, was corrected for passive uptake (without added substrate or ATP); passive uptake was generally of the order of 0.5 µmoles of calcium accumulated per mg protein-nitrogen in 15 min. In all cases, the amount of actively accumulated calcium was maximal after 5–15 min of reaction time and decreased to zero after 1 hr; the passively accumulated calcium showed no tendency to efflux over this period of time (Fig. 4). Active calcium transport was 100% sensitive to the electron transfer inhibitor cyanide (10 mM) and the uncoupler 2,4-dinitrophenol (50 mM); passive uptake was not altered when these inhibitors were included in the assay medium.

Phosphate requirement

Active calcium accumulation was strictly dependent on the presence of phosphate in the reaction medium. This requirement was for mitochondria from fruit at all stages of ripeness; the influence of phosphate concentration on the magnitude of calcium uptake of pre- and postclimacteric mitochondria is indicated in Table 4. Total calcium accumulation at 15 min rose with phosphate concentration to 8 mM; similar requirements for relatively high levels of inorganic phosphate were found for high efficiency of succinate-coupled oxidative phosphorylation (Haard, 1967). Increased phosphate had the influence of slightly diminishing passive uptake of calcium. The absolute increase in active calcium uptake between 0.8 and 8.0 mM phosphate was similar in the mitochondria from pre- and postclimacteric fruit, but this represented a

Table 5–Influence of $MgCl_2$ on succinate-driven calcium accumulation by mitochondria from preclimacteric fruit.[a]

$MgCl_2$ (mM)	(µmoles/15 min./mg protein N) Passive Ca^{++} uptake	Active Ca^{++} uptake
0.000	1.24	0.481
0.012	1.28	0.490
0.024	1.20	0.694
0.048	1.15	0.520
0.120	0.93	0.526
0.240	1.04	0.616

[a]Except for $MgCl_2$ additions where indicated, assay conditions were as described in the text.

Table 6–Inhibition of succinate-driven Ca^{++} uptake by ADP.

Fruit ripeness	ADP conc. (mM)	Active Ca^{++} uptake[a]	Percent inhibition
Preclimacteric	0	0.57	—
	0.4	0.50	12.6
	2.0	0.37	35.0
Postclimacteric	0	1.67	—
	0.4	1.10	34.2
	2.0	0.00	100.0

[a]Assay conditions as described in text except with ADP additions indicated. Calcium uptake reported as µmoles/15 min./mg Protein-N.

Table 7–Influence of ATP on calcium uptake by mitochondria from preclimacteric fruit not prewashed with BSA.[a]

Succinate (8 mM)	ATP (0.3 mM)	µmoles/15 min/mg Protein-N Passive accumulation	Active accumulation
+	+	—	0.28
+	–	—	0.02
–	+	—	0.04
–	–	0.513	—

[a]Results were similar with mitochondria prepared from postclimacteric fruit.

Table 8–Influence of ATP on calcium uptake by mitochondria from postclimacteric fruit prewashed with BSA.

Succinate 8 mM	ATP (mM)	µmoles/15 min/mg Protein-N Active accumulation
+	0.0	1.25
–	0.0	—
+	0.3	0.45
–	0.3	0.18
+	0.6	0.22
–	0.6	0.16

much greater percentage increase for the mitochondria from preclimacteric fruit. The requirement of phosphate for calcium uptake is in general accord with studies of other systems (Brierley et al., 1964; Hodges and Hanson, 1965), although phosphate-independent calcium accumulation by mitochondria has been observed (Rasmussen et al., 1965).

Specificity–influence of competitive levels of magnesium chloride

Inclusion of equimolar levels of magnesium chloride in the reaction system did not repress calcium uptake but actually caused slight stimulation of the calcium accumulated actively (Table 5) by mitochondria from preclimacteric fruit. The concurrent passive accumulation declined when magnesium was present in the reaction system; this is consistent with the likely non-specific nature of the passive process (diffusion, osmotic, Donnan forces). Passive uptake by postclimacteric mitochondria was depressed in similar fashion; however, there was no measurable effect of magnesium or succinate-driven calcium uptake in these studies. The observation that competitive levels of $MgCl_2$ facilitated stimulation of calcium accumulation by mitochondria from preclimacteric, but not from postclimacteric fruit should be emphasized; this represents another variant property associated with this ancillary work performance.

Effect of ADP on calcium accumulation

ADP stimulated both respiration and phosphorylation of banana mitochondria (Haard, 1967). Oxidative phosphorylation was shown to be responsive to a number of cofactors in addition to ADP; however, mitochondria from fruit at all postharvest stages were indistinguishable with respect to such responses.

The influence of added ADP on succinoxidase-coupled calcium accumulation varied with the ripeness of the fruit extracted. ADP caused inhibition of succinate-driven calcium uptake by mitochondria from both pre- and post-climacteric pulp (Table 6). This inhibition was considerably more extensive with the postclimacteric preparations. Active calcium uptake by postclimacteric mitochondria was completely suppressed when 2 mM ADP was included in the reaction system; the same levels of ADP decreased active uptake in preclimacteric mitochondria by only 35%. Suppression of calcium uptake by ADP (energy acceptor during oxidative phosphorylation) suggests that a competitive relationship between calcium translocation and oxidative phosphorylation exists in banana mitochondria; moreover, it raises the possibility that the preferable outlet of coupling might change from calcium translocation to oxidative phosphorylation as the ripening process ensues.

Table 9–High amplitude calcium accumulation[a] *by banana mitochondria with succinate as substrate.*

Fruit	µmoles Ca/mg Protein-N/5 min[b] Passive Ca^+ uptake	Active Ca^+ uptake
Preclimacteric	23.3	5.75
Climacteric	16.0	6.00

[a]Refer to Brierley et al. (1965) for discussion of high amplitude calcium accumulation.

[b]Assay conditions were as described in Experimental except that calcium chloride was 4 mM. Results are averages on duplicate preparations.

Effect of ATP on calcium accumulation

Previous studies demonstrated that mitochondria which were not prewashed in a solution containing bovine serum albumin (BSA), showed no coupling of succinate oxidation to phosphorylation (Haard, 1967). Similarly, such mitochondria from all postharvest stages did not accumulate calcium ions actively with either ATP or succinate as energy source. However, when both of these substrates were present, significant levels of calcium were accumulated in mitochondria from all fruit at similar levels (Table 7). The magnitude of calcium accumulation was significantly lower than that of mitochondria prewashed with BSA. In these "uncoupled" preparations, then, ATP and succinate synergistically facilitated the energized coupling of succinoxidase to calcium accumulation.

The influence of ATP on mitochondrial preparations prewashed with BSA was different. ATP alone supported low levels of calcium accumulation in both pre- and postclimacteric mitochondria (Table 8). ATP caused marked inhibition of succinate-driven calcium accumulation in postclimacteric preparations but did not alter uptake by preclimacteric mitochondria. Brierley et al. (1964) demonstrated that freshly-prepared beef heart mitochondria accumulate approximately 40% more calcium when ATP was included with mitochondria oxidizing succinate, while Hodges and Hanson (1965) observed that ATP decreased succinate-driven calcium uptake by soybean root mitochondria.

Massive calcium accumulation

When high levels of $CaCl_2$ (4 mM) were present in the reaction system massive levels of calcium were accumulated by banana mitochondria (Table 9). Both passive and active accumulation were very high. Unlike low amplitude calcium uptake, there were no clearly demonstrable differences between mitochondria from unripe and ripe fruit. Low amplitude and massive accumulation differ in the mechanism by which calcium accumulates (Lehninger, 1964). The similarity in levels of massive uptake by

mitochondria from pre- and postclimacteric fruit suggest that these mitochondria have similar compartments for calcium phosphate deposition (presumably the matrix space).

The findings reported and discussed here are consistent with the notions advanced by Frenkel et al. (1968) whose study of ripening pear fruit led them to view ripening as an organized and genetically controlled process rather than a result of a loss in cellular control, maintenance and organization. An essential element of this view is that energy transducing processes are maintained during terminal senescence since the dynamic state of living matter requires a continual supply of available energy.

Within the control of the experimental work reported here, it is clear that the mitochondria from all stages of postharvest banana fruit, as isolated, are remarkably similar with respect to the primary functions of electron transfer and oxidative phosphorylation. Isolated mitochondria from all stages of ripening fruit were also remarkably similar in morphology and configurational state, a fact compatible with the similarities in biochemical properties. This was demonstrable only because the technique employed in isolating mitochondria disallowed anomalies usually occurring in banana pulp (Haard and Hultin, 1968). We have demonstrated that the ancillary function of electron transfer-linked calcium accumulation exists in banana mitochondria. Further, it is shown that stimulation of succinoxidase activity by calcium and succinate-driven calcium accumulation, as well as the response of these functions to various metabolites such as magnesium ions, inorganic phosphate, and ADP, varied with stage of ripeness.

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Ms. received 10/31/69; revised 5/15/70; accepted 5/15/70.

Paper presented in part at the 27th Annual Meeting of the Institute of Food Technologists in Minneapolis.

During the course of part of this study, N.F. Haard was supported by a N.D.E.A. Title IV Fellowship. We thank and acknowledge the assistance of Dr. G. Vail (Institute of Enzyme Research, University of Wisconsin) with the electron microscopy.

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ALCOHOL-INSOLUBLE CONSTITUENTS OF JUICE VESICLES OF CITRUS FRUIT

SUMMARY—The texture of the edible portion of citrus fruit is determined by the characteristics of the juice vesicles and their insoluble constituents. Alcohol-insoluble solids (AIS) of the juice vesicles of several varieties of oranges, grapefruit and tangerines were studied at different stages of maturity. The protein content of the AIS as determined by the Kjeldahl method was found to decrease with fruit maturity, although total amount per fruit tended to increase. Polysaccharides were separated by extracting the AIS with alkali of various concentrations. Each fraction after hydrolysis was analyzed for the monomers both by paper chromatography and gas chromatography of the silylated derivatives. Galactose, arabinose and traces of glucose were found in fractions associated with galacturonic acid, and xylose, arabinose and glucose in different proportions were found in the hemicellulose and cellulose fractions. Traces of rhamnose and other uronic acids also were detected.

INTRODUCTION

THE TEXTURE of the edible portion of citrus fruit is determined by the condition of the juice vesicles and their cell walls. In canned or chilled citrus fruit sections, the texture of the juice vesicles is a determining factor of their quality. The pulp content of citrus juices is derived entirely from the insoluble constituents of the juice vesicles and is important in its influence on the viscosity and turbidity of the juice.

In citrus juice manufacturing, the excess coarse pulp is discharged from the finisher and combined with the peel residue dried for animal feed. In some instances the pulp is recovered as a valuable by-product to be used in orange-flavored or orange juice drinks. Recently, it has been proposed as a substitute for milk solids as a filler for certain meat products (Kesterson, 1969).

The importance of the juice vesicles warrants a more thorough knowledge of their chemical composition. Morphologically, a juice vesicle is an emergence from the carpellary walls and contains an epiderm of longitudinally elongated cells, a subepiderm of large elongated cells and numerous large, isodiametric cells towards the center (Winton and Winton, 1935). As fruit ripens, the vacuoles of the cells of the subepidermal and the central parenchyma cells enlarge and the epidermal layers stretch and become thinner. During juice extraction or crushing, the epidermal layer of the juice vesicles is ruptured, releasing the parenchyma cells. Walls of these paranchyma cells are extremely fragile and are broken during the crushing process.

Little is known about the chemical constituents of the juice vesicle cell walls, which include the walls of epidermal, subepidermal and central parenchyma cells. The study of the alcohol-insoluble cell constituents will include mainly those derived from the cell walls and the cytoplasm. However, the most abundant component of the plant cells besides water is the polysaccharides of the cell walls, generally isolated by alcohol precipitation. The alcohol precipitate also includes protein and some inorganic substances.

By extracting the alcohol-insoluble solids (AIS) with alkali of different concentrations, polysaccharides can be separated into relatively distinctive fractions, such as pectic substance, hemicellulose and cellulose (Jermyn and Isherwood, 1956). Each of these fractions contains a mixture of different polysaccharides, but 1 or more predominate. Upon hydrolysis of each fraction, an admixture of monomers which are readily separated by paper chromatography is obtained (Jermyn and Isherwood, 1956; Ting and Deszyck, 1961; Tavakoli and Wiley, 1965).

Simple sugars have also been separated as their trimethylsilyl ether (TMSE) derivatives (Sweeley et al., 1963). Brower et al. (1966) determined the sugars of the hydrolysates of wood constituents by gas chromatography. Many of these sugars are also found in the hydrolysate of citrus peel AIS (Ting and Deszyck, 1961). A similar class of monomers is also found in the hydrolysate of juice vesicle AIS. The hexuronic acids of fruit were determined by gas chromatography (Wiley et al., 1966). In the present study, paper chromatography was generally used in a quantitative estimation, and gas chromatographic separations were made on the monomers in the hydrolysates of various fractions to ascertain the possibility of using gas chromatography as an alternate method of analysis.

MATERIALS & METHODS

MATURE and immature fruit samples of 3 varieties of oranges, 2 of grapefruit and 1 of tangerine were obtained from groves of the Citrus Experiment Station in Lake Alfred. 1,000 g of juice vesicles were randomly excised from each sample of 40 fruit and placed in a muslin bag. Most of the juice was removed by kneading, and the pulp was pressed in a "Pexton" press. The 2 liquids were combined.

Maturity index

The maturity index of each sample was determined by measuring the juice for soluble solids on an Abbé refractometer. Readings were corrected for temperature but not for citric acid. Total acidity was determined by titrating 25 ml of the juice with standard alkali to a phenolphthalein end point and expressing as percent anhydrous citric acid. The Brix-acid ratios were calculated.

Preparation of alcohol-insoluble solids (AIS)

The pulp was washed once with about 1,000 ml of cold distilled water and the liquid removed through the muslin cloth filter and the "Pexton" press as for the juice. The washing was combined with the juice and concentrated in a rotary evaporator to about 25° Brix. The pulp was emptied into a large Waring Blendor and sufficient 95% ethyl alcohol added to obtain a final concentration of 80%. The contents were blended and filtered. 3 or more such extractions were necessary to render the insoluble materials sugar-free as tested by the anthrone procedure (Jacob, 1958). The AIS was washed with 95% ethanol and acetone and air dried. It was then dried in an oven at 90°C, ground in a Wiley mill to pass a 40-mesh screen and stored in a tightly closed jar.

The juice AIS was obtained by adding 95% ethyl alcohol to the concentrated juice to a final alcohol concentration of 80%. The alcohol was removed by centrifugation and the contents in the centrifuge tube repeatedly washed with 80% alcohol until the supernatant was free of sugar. The AIS was washed with 95% ethanol and acetone, air dried and finally dried at 90°C. The dried samples were stored in tightly closed containers until used.

Soluble solids and total solids

The combined alcohol extracts remaining from the preparations of pulp and juice AIS were concentrated under vacuum to 500 ml. An aliquot was dried at 90°C and weighed for the calculation of alcohol-soluble solids. The combined weight of the pulp and juice AIS plus the dry weight of the alcohol-soluble solids was considered as total solids.

Protein and ash content of the AIS

Total nitrogen was determined with a semimicro-Kjeldahl procedure using 0.5 g of the peel AIS and 0.2 g of the juice AIS, and the protein values obtained by using the factor 6.25. For ash determination, 1.0 and 0.5 g of the peel AIS and juice AIS, respectively, were ignited and heated in a muffle furnace at 600°C.

Fractionation of the AIS

2 g of the pulp AIS were shaken twice, each time with 200 ml of 0.05 N NaOH. After stand-

Table 1–General composition of juice vesicles of citrus fruit at different stages of maturity.

Fruit	Stages of maturity	Maturity indices: Soluble solids (° Brix)	Titratable acidity (%)	Brix acid ratios	Total solids (%)	Alcohol-insoluble solids (%): Juice	Pulp	Juice vesicle
Hamlin orange	Immature	9.4	0.85	10.6	9.3	0.29	0.70	0.99
	Mature	10.6	0.72	14.7	12.5	0.27	0.80	1.07
Pineapple orange	Very immature	8.5	1.88	4.5	9.5	0.27	2.34	2.61
	Immature	8.4	0.80	10.5	8.7	0.25	1.04	1.29
	Mature	10.6	0.84	12.6	10.5	0.27	0.73	1.00
Valencia orange	Immature	8.0	1.20	6.7	8.4	0.30	1.02	1.32
	Mature	10.9	0.90	12.1	11.2	0.30	0.89	1.19
Duncan grapefruit	Immature	8.0	2.00	4.0	8.4	0.14	1.20	1.34
	Mature	9.0	1.20	7.5	9.3	0.15	0.58	0.73
Marsh Seedless grapefruit	Immature	8.4	2.10	4.0	8.4	0.17	1.39	1.56
	Mature	8.4	1.30	6.1	8.6	0.17	0.75	0.92
Dancy tangerine	Immature	7.8	1.10	7.1	11.4	0.26	0.86	1.12
	Mature	10.4	1.00	10.4	17.1	0.39	0.88	1.27

Table 2–Composition of alcohol-insoluble solids (AIS) of juice vesicles of citrus fruit.

Fruit	Stages of maturity	Alcohol-insoluble solids: Juice (% dry weight)	Pulp (% dry weight)	Protein: Juice (AIS) (%)	Pulp (AIS) (%)	Ash: Juice (AIS) (%)	Pulp (AIS) (%)	Carbohydrate[a]: Juice (AIS) (%)	Pulp (AIS) (%)
Hamlin orange	Immature	3.1	7.5	29.2	11.4	4.6	1.4	66.2	87.2
	Mature	2.2	6.4	28.4	11.2	4.1	1.7	67.5	87.1
Pineapple orange	Very immature	2.8	24.6	27.3	11.5	4.3	1.2	68.4	87.3
	Immature	2.9	11.9	28.2	10.6	5.6	1.4	66.2	87.2
	Mature	2.6	6.9	32.7	10.9	3.7	1.8	63.6	87.3
Valencia orange	Immature	3.6	12.1	31.3	11.2	4.2	1.4	64.5	87.4
	Mature	2.7	7.9	28.2	13.4	6.6	1.4	65.2	85.2
Duncan grapefruit	Immature	1.7	14.3	30.1	10.3	5.9	1.3	64.0	87.9
	Mature	1.6	6.2	27.0	11.9	6.4	2.0	66.6	85.2
Marsh Seedless grapefruit	Immature	2.0	16.6	28.9	10.3	4.6	1.6	66.5	88.1
	Mature	2.0	8.7	26.6	8.6	4.4	1.8	69.0	89.6
Dancy tangerine	Immature	2.3	7.5	21.9	11.3	8.1	1.3	70.0	87.4
	Mature	2.3	5.1	25.2	12.5	4.2	1.3	70.6	86.2

[a]By difference.

ing for 30 min, the residue was separated each time by centrifugation. The AIS was finally washed once with 200 ml of hot water followed by 95% alcohol and acetone. For juice AIS, 500 mg was used in the extraction, which consists of 150 ml of 0.05 N NaOH and 100 ml of hot water. The combined NaOH and water extracts were neutralized with glacial acetic acid to pH 5.5. 5 ml of filtered extracts of Pectinol 100 D (10 g to 10 ml water) were added. The mixture was allowed to stand overnight at room temperature with a few drops of toluene. Each sample was then evaporated under vacuum and reduced in volume to 100 ml.

The residue from the 0.05 N NaOH extraction was dried and weighed, the difference in weight being that extracted. Hemicellulose was extracted from the residue once with 4 N KOH (Jermyn and Isherwood, 1956). The acidified extract was precipitated with alcohol and the precipitate washed with alcohol and acetone and recovered by centrifugation. The residue after the 4 N KOH extract was considered as crude cellulose.

This extraction was omitted with the juice AIS because over 90% of it was soluble in 0.05 N NaOH, and the hemicellulose and the crude cellulose fractions were combined.

Hydrolysis of AIS fractions for chromatography

The pectic fraction was chromatographed directly. The hemicellulose fraction was hydrolyzed in a sealed tube with 0.5 N H_2SO_4 for 6 hr in a boiling-water bath. The crude cellulose was hydrolyzed by solubilizing 0.1 g of the residue with 0.5 ml of 72% H_2SO_4 by letting the mixture stand for 2 hr. The acid concentration was then adjusted to 0.5 N and the mixture heated in a sealed tube for 8 hr in a water bath at 100°C. The sulfuric acid was removed by adding barium carbonate, heating on a hot plate and filtering. After filtration, the hydrolysate was concentrated for chromatography.

Paper chromatography

Whatman No. 1 paper was used and the irrigating solvent was butanol-pyridine-benzene-water (50:30:4.5:30) Approximately 18 hr were required to separate glucose and galactose. The aniline phthalate was prepared by dissolving 1.6 g of phthalic acid and 0.9 g of aniline in 100 ml of 98% ethanol.

5 μliters of the hydrolysate of each fraction were applied to a paper, together with a series of standards containing known quantities of various sugars expected in each sample. Triplicate chromatograms were run for each sample with the standards. After the paper was developed and dried, it was sprayed with aniline phthalate. The spots produced by the sample were compared with the standards and an estimation made. The results thus obtained were semiquantitative but they do give the approximate magnitude of each sugar present.

Preparation of trimethylsilyl ether (TMSE) derivatives

TMSE derivatives were prepared for the standard sugars and hydrolysates of the fractions by the method of Brower et al. (1966). An aliquot of 1% solutions of L-arabinose, D-xylose, D-galactose, D-glucose, D-rhamnose or D-galacturonic acid singly or in combinations was pipetted into a 1/2-dram vial together with 0.1 ml of a 1% myo-inositol solution. The total amount of carbohydrate was less than 5 mg. The water in the vial was completely removed by evaporation on a rotary evaporator and finally by a vacuum pump. Pyridine (0.7 ml), hexamethyldisilbenzene (HMDS, 0.2 ml) and trimethylchlorosilene (TMCS, 0.1 ml) were added with a syringe. The vial was stoppered with a serum cap and shaken vigorously for several minutes. The prepared sample was ready for injection within 10–15 min.

For hydrolysates of the various fractions, 0.1–0.5 ml containing less than 5 mg of total sugars and 0.1 ml of 1% myo-inositol were placed in a vial and treated in a manner similar to that used for the standard sugars. The inositol was added as an internal reference which can be used both as a qualitative and quantitative standard.

Gas chromatography

An Aerograph Model 600-C equipped with a hydrogen flame ionization detector and Model 328 temperature programmer was used. The column was a 6-ft 1/8-in. stainless steel tubing packed with 90–100 mesh Anakrom ABS with 6% SE 30 silicone gum rubber as the stationary phase. 1-μliter sample of the TMSE derivatives was injected and the column temperature programmed from 100–215°C at approximately 5° per minute. The temperature rise had a ballistic character. As the maximum was approached, the rate was very slow. The injection port was maintained at 200°C. Nitrogen was used as the carrier gas at 30 ml/min. The flow rates for hydrogen and oxygen were approximately 20 ml and 60 ml per minute, respectively.

RESULTS

Relationship of fruit maturity and AIS content of juice vesicles

The maturity differences in fruit of

Table 3–Percentage distribution by weight of various fractions of the citrus juice vesicle AIS.

Fruit	Stages of maturity	Pectic substance		Hemicellulose fraction		Cellulose fraction	
		Juice	Pulp	Juice[a]	Pulp	Juice	Pulp
Hamlin orange	Immature	89.1	65.0	10.9	8.2	Not separated from the hemicellulose	26.7
	Mature	88.7	68.0	11.3	6.8		25.1
Pineapple orange	Very immature	76.2	58.0	23.8	6.6		38.1
	Immature	95.0	62.0	5.0	5.8		30.0
	Mature	92.0	59.5	7.0	6.2		33.7
Valencia orange	Immature	92.0	62.5	8.0	5.7		31.2
	Mature	93.0	62.0	7.0	7.0		30.0
Duncan grapefruit	Immature	94.0	65.5	6.0	6.5		27.3
	Mature	94.0	62.5	6.0	6.6		28.6
Marsh Seedless grapefruit	Immature	83.2	60.0	16.8	6.2		33.3
	Mature	92.6	63.0	7.4	6.7		29.0
Dancy tangerine	Immature	91.3	63.5	8.7	8.6		28.1
	Mature	91.7	64.5	8.3	8.6		26.7

[a]Combined hemicellulose and cellulose fraction.

Table 4–Percentage distributions by weight of monosaccharides found in various fractions of juice vesicle AIS. (All varieties and maturities combined.)

Alcohol-insoluble solids	Fractions	Arabinose	Xylose	Rhamnose	Galactose	Glucose	Galacturonic Acid	Other uronic acids
In pulp	Pectic substance	17.2	Trace	2.8	4.2	Trace	76.6	0
	Hemicellulose	16.0	49.8	0	21.1	5.7	5.6	1.7
	Cellulose	21.6	2.5	Trace	13.9	58.1	0	3.6
In juice	Pectic substance	11.4	0.4	5.2	14.4	0	68.8	0
	Combined hemicellulose and cellulose	38.2	6.4	0	44.6	0	10.6	0

each variety harvested at different stages were quite marked, as shown by their maturity indices (Table 1). The immature fruit was relatively low in juice content. With the exception of Hamlin oranges and Dancy tangerines, these immature fruit samples had coarse to "ricey" texture; and the very immature sample of Pineapple oranges had ricey texture as defined by Harding et al., 1940, and Harding and Fisher, 1945. The immature fruit of Hamlin oranges and Dancy tangerines were much more advanced in maturity and their textures not as coarse as the others. In general, the dry weight (total solids) was higher in mature fruit. With the exception of Hamlin orange and Dancy tangerine, immature fruit was higher than mature fruit in pulp AIS. The juice AIS did not show such differences. Pulp AIS was about 3 to 4 times as great as juice AIS and grapefruit has much lower juice AIS than either the orange or tangerine.

Differences in AIS between the immature and mature samples were much more pronounced when compared on a dry weight basis (Table 2). Very immature Pineapple oranges contained about 4 times as much AIS in pulp as did the mature fruit. Smaller differences were found in other cases.

Composition of juice vesicle AIS

Between 22 and 33% of the juice AIS but only 9–12% of the pulp AIS were proteins (Table 2). Although only a small fraction of the total AIS was from the juice, a large portion of the protein of the mature juice vesicles was found in the juice AIS. Most of the cell organelles of the juice cells and other cytoplasmic contents are probably contained in the juice AIS protein. Although no consistent differences were found in protein of juice vesicles between the mature and immature fruit as percentage of AIS, protein content was considerably lower in the mature fruit than in the immature fruit when calculated on the dry weight basis. The juice AIS is also much higher in ash content than that of pulp.

Besides the mineral and protein contents, the AIS of juice vesicles contains a large amount of polysaccharides. Since the AIS was extracted essentially free of lipids, the difference between the total AIS and the combined weight of protein and ash may be considered as carbohydrates, with the possible presence of some glycosides. On this basis, nearly two-thirds of the juice AIS and five-sixths of the pulp AIS can be considered as carbohydrates.

Fractions of juice vesicle AIS

Upon fractionation by extraction, it was found that between 58 and 68% of the pulp AIS and an average of about 90% of the juice AIS make up the pectic substance fraction (soluble in 0.05 N NaOH) (Table 3). Only a small fraction, averaging about 7% of the AIS, was extracted as hemicellulose. Both the immature Marsh Seedless grapefruit and the very immature Pineapple orange left slightly higher residue after the 4 N KOH extraction of the pulp AIS. About 1/3 to 1/4 of the total AIS of all samples could be considered as cellulose, which is generally known as the principal cell wall constituent of plants (Whistler and Smart, 1953).

Sugar monomers in the hydrolysate of various fractions

Since no apparent differences were found in the individual monomers in the hydrolysates of various fractions either due to maturity or variety, the values were averaged in calculating the percentage distributions (Table 4). Upon hydrolysis by Pectinol, nearly 70% of the accountable monomers of the hydrolysate of the pectic substance of the juice AIS and an average of 77% of that of the pulp AIS were galacturonic acid. Arabinose was also found in large amounts (about 17%) of the total monomers (Table 4). Other monomers in that fraction were galactose, rhamnose and traces of xylose. Pectic substances in cell walls and the middle lamella usually contain a triad of polysaccharides (Whistler and Smart, 1953); namely, galacturonans, galactans and arabans. The proportions of these 3 components vary since it is not certain that they are chemically bonded. Some cell wall pectic compound may also be present in the form of calcium pectate which would be extracted by the dilute NaOH.

Rhamnose and glucose are known to be in the disaccharide rutinose of the hesperidin and the neohesperidose of naringin (Horowitz, 1964). Enzymes which will remove rhamnose alone from naringin (Griffith and Lime, 1959) have been isolated from commercial pectic enzymes (Thomas et al., 1958). This may explain why rhamnose and glucose did not occur in about equal amounts in the hydrolysate. Hesperidin is relatively insoluble and could be present in the alcohol-insoluble solids during the alcohol pre-

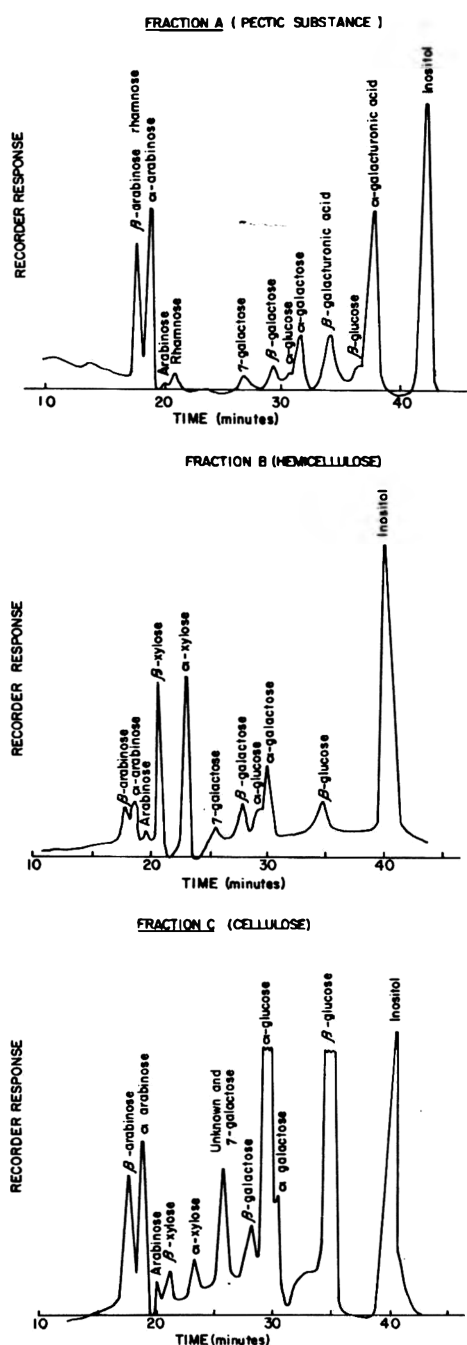

Fig. 1—Chromatograms of TMSE derivatives of monosaccharides in hydrolysates of the 3 fractions of juice vesicle AIS of mature Hamlin orange and TMSE of inositol added as an internal standard. A) Pectic substance fraction. B) Hemicellulose fraction. C) Cellulose fraction.

cipitation.

The monomers in the hemicellulose fraction of the pulp AIS were xylose, arabinose, galactose, glucose and galacturonic acid as determined by paper chromatography (Table 4). Xylose alone was nearly 50% of the total monomers found in the hydrolysate of that fraction.

The total sugars in the hydrolysate of the cellulose fraction of the pulp AIS contained nearly 60% glucose, with arabinose and galactose constituting almost 35%. Xylose and some uronic acids other than galacturonic acid also were detected. In most wood cell walls (Whistler and Smart, 1953) and in the cell wall polysaccharides of citrus peel (Ting and Deszyck, 1961), xylose was usually the major pentose complement of the glucose. In the juice vesicle cellulose fraction, however, arabinose was found to be nearly 1/3 of the glucose present and xylose was present in only small amounts.

The hydrolysate of the residues of the juice AIS after the 0.05 N NaOH extraction yielded only 3 sugars: arabinose, galactose and xylose, and 1 uronic acid, galacturonic. Arabinose and galactose combined constituted about 83% of the total monomers.

GLC of TMSE derivatives of sugar monomers from various fractions

The chromatograms of the TMSE derivatives of the monomers in the pectic substance fractions (Fig. 1: fraction A) showed 11 distinct peaks from 6 components contained in that fraction. The peaks were identified by retention time of authentic samples run singly and in combinations of 2 or more in different proportions. 1 of the rhamnose peaks was completely masked by 2 of the arabinose peaks. The minor arabinose peak was consistently observed in standard arabinose samples. Alpha-galacturonic acid is the major peak of that fraction. Traces of glucose could be detected by the presence of a shoulder on α-galactose and 1 on α-galacturonic acid. Inositol as appeared in this chromatogram and in the 2 other chromatograms was added as an internal reference.

The hydrolysate of the hemicellulose fraction showed distinct, characteristic peaks for TMSE derivatives of xylose. Arabinose, glucose and galactose peaks also were detected. Alpha-glucose appeared as a shoulder on α-galactose but β-glucose was free from other components in this fraction (Fig. 1: fraction B).

In the hydrolysate of the cellulose fraction, the 2 glucose peaks were dominant (Fig. 1: fraction C). On the chromatogram they were not attenuated to the scale. In addition to the sugars so far identified, a large peak at the same retention time as γ-galactose was noted. Alpha-mannose has a retention time similar to that of γ-galactose, and β-mannose could be masked by the large peak of β-glucose. With the solvent system used in paper chromatography in this experiment, mannose could not be separated in the presence of large amounts of glucose. The occurrence of rhamnose, as detected on paper chromatograms as trace from this fraction, was not noted on the gas chromatogram. The anomers of rhamnose have retention times similar to those of arabinose and xylose and were completely masked by these 2 other pentoses.

DISCUSSION

THE PROTEINS play an important role in the plant cells in general (Brown, 1960) and the juice cells are probably no exceptions. The metabolic system of the cells is more related to the amount of protein than to either the fresh or dry weight. The amount of protein in the juice vesicles on the AIS basis is considerably greater than that found in the peel (Ting and Deszyck, 1961), indicating that the juice vesicle is a site of high metabolic activity.

The ash of the insoluble juice constituents could derive either from the pectic substances, as calcium pectate in cell wall or from protein. Brown (1960) suggested that there may be reactions of protein with inorganic ions. Different carboxyl groups may be linked together through divalent cations such as calcium. The higher ash content in the juice AIS than in pulp AIS would indicate this possibility.

The polysaccharides of juice vesicle cell walls contain pectins and related polysaccharides. The walls of the central parenchyma cell are so fragile that most of them could be broken and passed through the muslin filter. They were not cellulosic in nature since little glucose was found in the hydrolysate of the juice AIS.

Use of gas chromatography as a quantitative method for determining the sugar monomers in the hydrolysate presents good possibilities. Unfortunately, due to mutarotation, each sugar gives several peaks and many of them overlap. The main sugars in each fraction, however, were separated sufficiently for quantitative estimations. Recently, Crowell and Burnett (1967) suggested that the sugars in the hydrolysates of wood pulp may be determined by gas chromatography as their alditol acetate derivative to give a single peak for each sugar. Galactose and galacturonic acid could not be separated in this manner because both would be reduced to the same alcohol. However, uronic acid could be first removed by an ion-exchange resin which would also remove the acid used in hydrolysis. The uronic acid could then be eluted and treated separately.

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Ms. received 6/25/70; accepted 7/27/70.

Journal Series Number 3611, Florida Agricultural Experiment Stations.

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POLYPHENOLS IN MONTMORENCY CHERRIES

SUMMARY—An examination of the polyphenols of Montmorency cherries resulted in the separation and identification of six isomers of caffeoyl-quinic acid, four isomers of p-coumaryl-quinic acid, caffeic acid, and p-coumaric acid. In addition two flavonols, kampferol-3-rhamnoglucoside and kampferol-3-monoglucoside were also identified. Four additional polyphenols were separated but could not be identified.

INTRODUCTION

POLYPHENOLS are important sources of astringent flavor and dark discoloration in foods (Swain, 1962). Their identification has been the subject of many studies. Sondheimer (1958) reported caffeic and chlorogenic acids in pears, grapes, peaches, prunes, plums, and sweet cherries. Hulme (1958) studied the polyphenols of apples and pears, El-Sayed and Luh (1965) of apricots, and Luh et al. (1967) reported the polyphenols of cling peaches. Studies of sour cherries have been limited to the polyphenols in leaves. Schenk, as reported in Olden and Nybom (1968), found quercetin and kampferol glycosides in leaves of several varieties of sour cherries. Olden and Nybom (1968) separated the polyphenols from the leaves of *Prunus avium*, *Prunus cerasus*, and *Prunus fructosa*. These authors compared the chromatograms without identifying the compounds involved. Since cherries and cherry products are subject to browning during processing and storage, knowledge of the polyphenols is of interest. Efforts to isolate and identify these compounds in Montmorency cherries are presented here.

MATERIALS & METHODS

MONTMORENCY CHERRIES (*Prunus cerasus* L., var, Montmorency) used in this work were obtained from the University of Wisconsin Branch Experiment Station. The cherries were pitted, individually frozen at −34°C and stored at −23°C until used.

To obtain a crude extract of polyphenols, 2 kg of cherries were thawed and ground with 3l of boiling methanol in a Waring Blendor. The mascerate was filtered with suction through Whatman No. 1 filter paper using celite as a filter aid. The filter pad was extracted twice with 3l of boiling methanol, the filtrates combined, and evaporated under vacuum at 30°C. The residue was extracted twice with hexane to remove carotenoids, then saturated with NaCl and extracted with an equal volume of ethyl acetate. After separation of the organic phase, the lower aqueous phase was reextracted four times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried with anhydrous Na_2SO_4 under N_2 overnight. The water-free ethyl acetate solution was concentrated to 10 ml under vacuum at 30°C.

Separation and purification of individual polyphenols were carried out by descending chromatography in 63 × 65 × 85 cm chromatography tanks. One ml portions of crude extract were applied as streaks to each of ten 46 × 57 cm sheets of Whatman 3MM filter paper. The chromatograms were developed with 2% acetic acid (2%HOAc) for 4 hr. The developed papers were scanned with ultraviolet light and divided into 9 sections corresponding to 8 bands of fluorescent compounds plus the origin. The band with the highest R_f value was labeled Band 1 and others were numbered in order of decreasing R_f value. The bands were eluted with 80% methanol and concentrated to small volumes under vacuum at 35°C. Each band was rechromatographed by two dimensional chromatography on 46 × 57 cm sheets of Whatman 3MM filter paper. The chromatograms were developed with n-butanol-acetic acid-water 6:1:2 v/v/v (BAW 6:1:2) in one direction for 18 hr and 2% HOAc in the other direction for 3.5 hr. The spots were located with ultraviolet light before and after fuming with NH_3, cut out, eluted with 80% methanol and concentrated to small volumes under vacuum at 30°C. The compounds were further purified by paper chromatography using water and 15% v/v acetic acid as solvents.

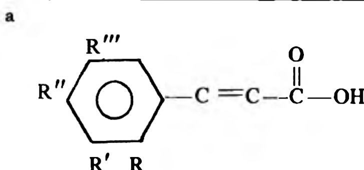
Final purification was accomplished by thin layer chromatography which was carried out by layering a 1 mm thickness of 80% ethanol washed cellulose powder on 20 × 20 cm glass plates and developing with 2% HOAc in 21 × 21 × 8.5 cm glass tanks. The spots were located by ultraviolet fluorescence before and after fuming with NH_3. The compounds were eluted with 80% ethanol and dried under vacuum at 30°C.

For identification, ultraviolet spectra of individual compounds were obtained in 95% ethanol with a Beckman DK-2 recording spectrometer. The presence of an $AlCl_3$ shift was determined by the addition of a 5% solution of $AlCl_3$ in ethanol (w/v). Anhydrous sodium acetate and H_3BO_3 were used to analyze spectral shifts of flavonols. To obtain R_f values for polyphenols, descending paper chromatography was used. All chromatograms were al-

Table 1—Fluorescence and color reactions of hydroxy-cinnamic acid esters.

Compound	Substitution[a]	Fluorescence[b] in U.V.	Fluorescence[b] in U.V. + NH_3	Color[b] Hoepfner	Color[b] with Hoepfner base
p-coumaric acid esters	R=R′=R‴=H; R″=OH	Cl	BW	Cl	Cl
m-coumaric acid esters	R=R″=R‴=H; R′=OH	Cl	M	—	—
o-coumaric acid esters	R′=R″=R‴=H; R=OH	Y	Or	—	—
caffeic acid	R=R‴=H; R′=R″=OH	BW	BW	R	OR
caffeic acid esters	R=R‴=H; R′=R″=OH	BW	BG	Y	R
ferulic acid esters	R=R‴=H R′=OCH_3; R″=OH	BW	BG	Y	Y
sinapic acid esters	R=H; R′=R‴=OCH_3; R″=OH	DB	G	—	—

a



[b]Cl=colorless, B=blue, W=white, M=mauve, Y=yellow, Or=orange, R=red, G=green, D=dark.

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lowed to equilibrate 12 hr and were developed at 20°C. The air-dried chromatograms were observed under ultraviolet light before and after fuming with NH_3. Polyphenols were visualized with diazo-p-nitroaniline (Swain, 1953). The Hoepfner reagent was used to differentiate among hydroxy-cinnamic acid derivatives (Walker, 1952). For the detection of quinic acid the reagent of Cartwright and Roberts (1955) was used.

Acid hydrolysis of flavonols and other polyphenols was carried out in 1.5N HCl for 1 hr under N_2 in an oil bath at 105°C. The hydrolyzate was extracted with diethyl ether. After separation and concentration of the two phases each phase was spotted on Whatman No. 1 filter paper for the determination of R_f values. The chromatograms of flavonol aglucons were developed with n-butanol-acetic acid-water 4:1:5 (v/v/v) upper phase (BAW 4:1:5) and Forrestal solvent (water-acetic acid-concentrated HCl, 10:30:3 v/v/v) with quercetin as a standard. The chromatograms of sugar moities were developed with BAW, 4:1:5 and n-propanol-ethyl acetate, 7:1:2 v/v/v (PEW) with glucose and rhammose as standards. Spots were visualized with the spray reagent by Partridge (1949). The chromatograms spotted with the organic phase of the acid hydrolysis of the polyphenols, and p-coumaric acid, caffeic acid, and gallic acid as standards, were developed with BAW 4:1:5 and 2% HOAc and examined under ultraviolet light before and after fuming with NH_3. The chromatograms spotted with the aqueous phase and quinic acid as standard were developed with BAW 4:1:5 and 2% HOAc and the spots were visualized with the spray reagent of Cartwright and Roberts (1955).

RESULTS & DISCUSSION

A TWO-DIMENSIONAL chromatogram of Montmorency cherry polyphenols (Fig. 1) when examined under ultraviolet light, fumed with NH_3, and reexamined

Table 2—R_f values, color reactions and absorption spectrum of polyphenols in Montmorency cherries.

Spot No.	R_f Values Solvent System[a] I	II	Spray Reagent[b] I[c]	II[c]	Color in UV Without NH_3^c	With NH_3^c	Spectrum in 95% ethanol Max ± 1nm	Min ± 1nm	$AlCl_3$ shift Band I	Band II	NaOAc shift Band I	NaOAc H_3BO_3 shift Band II	Identification
1	.82	.74	NR	NR	U	BW	314,290[d]	255	neg	—	—	—	analog of cis chlorogenic acid
2	.64	.73	NR	NR	U	BW	314,300[d]	255	neg	—	—	—	analog of cis neochlorogenic acid
3	.84	.63	NR	NR	U	BW	312,295[d]	254	neg	—	—	—	analog of trans chlorogenic acid
4	.66	.64	NR	NR	U	BW	314,300[d]	249	neg	—	—	—	analog of trans neochlorogenic acid
5	.71	.65	Y	R	BW	BG	322,300[d]	264	pos	—	—	—	cis chlorogenic acid
6	.57	.66	Y	R	BW	BG	324,300[d]	265	pos	—	—	—	cis neochlorogenic acid
7	.55	.58	Y	R	BW	BG	328,300[d]	266	pos	—	—	—	trans neochlorogenic acid
8	.84	.54	—	—	P	P	—	—	—	—	—	—	unidentified
9	.80	.49	—	—	U	BW	—	—	—	—	—	—	p-coumaric ester
10	.65	.56	Y	R	BW	BG	328,302[d]	266	pos	—	—	—	trans chlorogenic acid
11	.81	.45	NR	NR	U	B	310,300[d]	249	neg	—	—	—	p-coumaric acid
12	.66	.42	Y	R	BW	BG	324,300[d]	264	pos	—	—	—	isochlorogenic acid
13	.58	.35	—	—	D	YG	352,266	—	—	pos	pos	neg	kampferol-3-rhamnoglucoside
14	.70	.33	—	—	B	B	—	—	—	—	—	—	unidentified
15	.68	.24	R	OR	BW	B	328,300[d]	266	—	—	—	—	caffeic acid
16	.14	.26	—	—	U	Y	—	—	—	—	—	—	unidentified
17	.59	.13	—	—	D	YG	353,267	—	—	pos	pos	neg	kampferol-3-monoglucoside
18	.63	.09	Y	R	BW	BG	322,300[d]	266	pos	—	—	—	isochlorogenic acid
19	.56	0	—	—	BW	BW	—	—	—	—	—	—	unidentified
20	.28	0	—	—	BW	BW	—	—	—	—	—	—	unidentified
aglucon #13							366,267	—	—	pos	pos	neg	
caffeic acid							327,300[d]	266	—	—	—	—	
quercetin							370,256	—	—	pos	pos	pos	
p-coumaric acid							310,300[d]	248	—	—	—	—	
kampferol							368,266	—	—	pos	pos	neg	
kampferol-3-monoglucoside							353,267	—	—	pos	pos	neg	

[a]Solvent systems: I BAW, 6.1:2 v/v/v; II 2% HAc v/v.
[b]Spray reagent: I Hoepfner; II Hoepfner and base overspray.
[c]U=colorless, B=blue, G=green, P=purple, R=red, D=dark, W=white, Y=yellow NR=no reaction, O=orange.
[d]inflection.
[e]Jurd, 1962.
[f]Harborne and Hall, 1964.

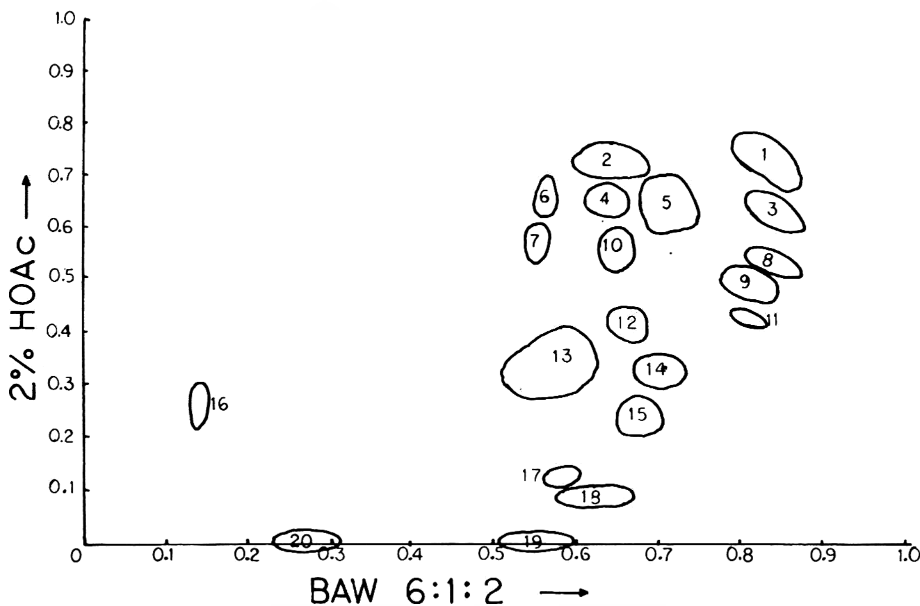

Fig. 1—Two-dimensional chromatogram of the polyphenols in Montmorency cherries.

under ultraviolet light, shows several groups of spots that behave similarly and each reacted with diazo-p-nitroaniline. The pattern obtained was similar to those obtained in other studies (e.g., Roberts, 1962; El-Sayed and Luh, 1965).

Caffeic acid esters

Spots 5, 6, 7, 10, 12, and 18 (Fig. 1) change from a blue-white color to a blue-green color when fumed with NH_3 and viewed under ultraviolet light. This color reaction is characteristic of caffeic and ferulic acid esters (Table 1). The reaction of these spots with the Hoepfner reagent and overspray with base indicated that these are caffeic acid esters. Spot 15 gave a red color with the Hoepfner reagent and an orange color when oversprayed with base which is an indication that this compound is caffeic acid. All other hydroxy-cinnamic acids give the same color reactions as their esters with this spray reagent (Harborne and Simmonds, 1964). All of these compounds, after purification, gave absorption spectra characteristic of hydroxy-cinnamic acid derivatives (Table 2). Compound 15 was indistinguishable from caffeic acid. (Tables 2, 3). Compounds 5, 6, 7, 10, 12, and 18 were hydrolyzed and all gave quinic acid and caffeic acid as hydrolysis products (Table 3).

The presence of hydroxy-cinnamic acid derivatives is to be expected. They have been shown to occur in virtually every higher plant examined (Harborne and Hall, 1964). These authors reported that the most common hydroxy-cinnamic acid esters are isomeric forms of caffeoyl-quinic acid (chlorogenic acids). The various caffeoyl-quinic acid isomers are positional and cis, trans isomers of mono-caffeoyl-quinic acid, di-caffeoyl-quinic acid, etc. Chlorogenic acid has been identified as 3-o-caffeoyl-quinic acid and is present as cis and trans isomers (Williams, 1955; Roberts, 1956) These authors reported that the cis isomer has a higher R_f value in dilute acid systems on paper chromatograms. Neochlorogenic acid has been thought to be a positional isomer of chlorogenic acid (Williams, 1955). It also is present as cis and trans isomers that have R_f values similar to chlorogenic acid in weak acid systems, but lower R_f values in butanol systems than chlorogenic acid. Isochlorogenic acids are taken to be all other observed caffeoyl-quinic acid isomers and have R_f values in weak acid systems that are less than those of either chlorogenic acid or neochlorogenic acid.

According to this data, spot 5 (Fig. 1) would correspond to cis-chlorogenic acid and spot 10 to trans-chlorogenic acid. Spots 6 and 7 would correspond to cis- and trans-neochlorogenic acid respectively and spots 12 and 18 to isochlorogenic acids.

p-Coumaric acid esters

Another group of similar compounds was noted which included spots 1, 2, 3, 4, 9, and 11 (Table 2; Fig. 1). The ultraviolet absorption spectra of compounds 1, 2, 3, 4, and 11 (Table 2) and their

Table 3—R_f values of aglucons and the acid hydrolysis products of polyphenols in Montmorency cherries.

Compound		R_f Values 2%HOAc	BAW 4:1:5	PEW	Forrestal	Identification
1	e[a]	.45	.89	—	—	p-coumaric acid
	a[b]	.82	.30	—	—	quinic acid
2	e	.44	.90	—	—	p-coumaric acid
	a	.82	.30	—	—	quinic acid
3	e	.44	.88	—	—	p-coumaric acid
	a	.80	.28	—	—	quinic acid
4	e	.35	.89	—	—	p-coumaric acid
	a	.83	.30	—	—	quinic acid
5	e	.24	.80	—	—	caffeic acid
	a	.82	.29	—	—	quinic acid
6	e	.20	.79	—	—	caffeic acid
	a	.84	.30	—	—	quinic acid
7	e	.23	.81	—	—	caffeic acid
	a	.80	.31	—	—	quinic acid
10	e	.23	.80	—	—	caffeic acid
	a	.82	.30	—	—	quinic acid
11		.45	.89	—	—	p-coumaric acid
12	e	.26	.82	—	—	caffeic acid
	a	.82	.30	—	—	quinic acid
13	e	—	.83	—	.56	kampferol
	a	—	.20/.38	.30/47	—	glucose/rhamnose
15		.24	.80	—	—	caffeic acid
18	e	.22	.81	—	—	caffeic acid
	a	.82	.30	—	—	quinic acid
caffeic acid		.23	.80	—	—	
p-coumaric acid		.45	.90	—	—	—
quinic acid		.84	.30	—	—	
kampferol[c]		—	.83	—	.55	
glucose		—	.22	.27	—	
rhamnose		—	.37	.47	—	

[a]e = ethereal layer of acid hydrolysate.
[b]a = aqueous layer of acid hydrolysate.
[c]Harborne, 1959.

reactions with Hoepfner spray reagent and overspray are characteristic of p-coumaric acid esters. Compounds 1, 2, 3, and 4 gave p-coumaric acid and quinic acid upon hydrolysis (Table 3). Compound 11 was indistinguishable from p-coumaric acid (Tables 2 and 3) and therefore identified as p-coumaric acid. Compound 9 was not present in sufficient quantity to identify, but it appears to be a p-coumaric acid ester from its appearance under ultraviolet light (Table 2).

Levy and Zucker (1960) proposed that hydroxy-cinnamic acid derivatives in plants are formed by enzymes that first form esters of cinnamic acid, then hydroxylate these esters to form hydroxy-cinnamic acid esters (e.g., p-coumaric acid). Only then does further hydroxylation or methoxylation take place to form other derivatives (e.g., caffeic acid). This reasoning is substantiated by the structure of the naturally occurring hydroxy-cinnamic acids (Table 1). In no case is the para position (R″ in footnote "a" of Table 1) substituted with anything other than an hydroxyl group. Levy and Zucker (1960) also noted that p-coumaric acid appears to be the parent compound for all hydroxy-cinnamic acid derivatives other than m- or o-coumaric acid.

According to these theories and the data obtained from the p-coumaryl-quinic acid compounds in sour cherries, spots 1 and 3 would be analogs of cis- and trans-chlorogenic acid respectively. Spots 2 and 4 would be analogs of cis- and trans-neochlorogenic acid respectively.

Flavonols

Spots 13 and 17 (Fig. 1) showed the same color under ultraviolet light when fumed with NH_3 and were dark under ultraviolet light without NH_3 (Table 2).

Compound 13 was identified as a flavonol from its absorption spectrum (Table 2). Band II of its absorption spectrum shifted with $AlCl_3$ toward longer wave lengths indicating a free 3- or 5-hydroxyl group. Since it does not fluoresce under ultraviolet light (except after fuming with NH_3) it does not have a free 3-hydroxyl group; therefore, it must have a free 5-hydroxyl group. Band I shifted upon addition of sodium acetate indicating a free 7-hydroxyl group. From its R_f values (Table 2), products upon hydrolysis (Table 3), ultraviolet spectrum and ultraviolet spectrum of its aglucon (Table 2), it was identified as kampferol-3-rhamnoglucoside. Compound 17 was present in small amounts and had the same absorption spectra as kampferol-3-monoglucoside. Its absorption spectrum and the presence of Compound 13 led to the tentative identification of Compound 17 as kampferol-3-monoglucoside.

Glycosides of kampferol have already been reported in the leaves of sour cherries (Schenk as reported in Olden and Nybom, 1968).

Unidentified polyphenolic compounds

Four other compounds were separated, but they could not be identified. Compound 8 fluoresced purple in ultraviolet light and in ultraviolet light plus NH_3 fumes (Table 2). It did not react with the Hoepfner reagents and it could not be hydrolyzed with 1.5N HCl. Its characteristics could not be matched with any known phenolic compound.

Compounds 16, 19, and 20 were also not identified. Compound 16 proved to be labile when isolation was attempted. Compounds 19 and 20 appear to be polymeric in nature because of their low R_f value in 2% HOAc.

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Ms. received 1/12/70; revised 6/15/70; accepted 7/3/70.

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RELATIVE RECOVERY AND IDENTIFICATION OF CARBONYL COMPOUNDS FROM CELERY ESSENTIAL OIL

SUMMARY—The carbonyl compounds in celery essential oil obtained from celery leaves and stalks by two essence recovery methods were separated by gas chromatography and identified by chemical and spectroscopic methods. Two epoxides, five ketones, five esters, three acids and three phthalides, 3,n-butylphthalide, sedanolide, and 3,n-butylhexahydrophthalide, were reported as constituents of the essential oil from fresh celery. 3,n-Butylphthalide and sedanolide possess the characteristic odor and flavor of celery. Sixteen of the 18 compounds have not been reported as constituents of the essential oil from fresh celery. Semiquantitative relationships were established for each carbonyl and the carbonyl fractions.

INTRODUCTION

GOLD AND WILSON (1961, 1963a, 1963b) reported the identity of flavoring constituents in celery essential oil that was recovered from celery juice by a vacuum essence recovery technique. The amount of oil available for analysis was extremely small and some identifications were made on the basis of gas chromatographic retention times and functional group analysis. Since some of these identifications were tentative, positive identifications based on chemical and spectroscopic analysis were needed.

Investigations on methods for recovering celery essential oil in high yield provided enough essential oil to conduct an extensive investigation on the chemical composition of celery (Wilson et al., 1967). This paper is the third in a series on the identification and quantitative estimation of the constituents in celery essential oil that was recovered by two different atmospheric steam distillation methods. The first two papers covered the terpene and sesquiterpene hydrocarbons, and alcohols (Wilson 1969a, 1969b). This report covers the isolation, identification and quantitative estimation of the carbonyl compounds in the essential oil recovered from celery stalks and leaves.

EXPERIMENTAL

Isolation of essential oils

The essential oil was recovered by two different methods. In the first, batches of celery puree were steam distilled and the vapors rectified in a packed distillation column (Wilson et al., 1967). In the second, celery puree was pumped into a Turba-Film evaporator and the vapors rectified in a distillation column of different design from the first (Wilson 1969a).

Separation of carbonyl fraction

The essential oils were separated into functional groups by column chromatography on neutral alumina (Fisher A-90, 80-200 mesh) (Wilson 1969a, 1969b). The carbonyls were eluted from the column in three 150 ml fractions with 1:1 v/v hexane-diethyl ether, and the solvent removed in vacuo on a rotary evaporator.

Gas chromatographic analysis

Each carbonyl fraction was separated into individual components by gas-liquid chromatography using the equipment described by Wilson (1969a, 1969b). Stainless steel columns, 15 ft × 0.25 in., packed with 30% Carbowax 20M or 25% Silicone 200 (2,500,000 cstks) on 60-80 mesh Gas Chrom P were used. The helium flow rate was 70 ml/min. Samples were temperature programmed from 100-225°C at 0.5°C/min. Components of the column effluent were collected by trapping in liquid nitrogen cooled glass capillary tubes. Identifications were based on comparisons of GLC retention times with known compounds and by comparison of infrared, NMR and mass spectra with known compounds. The compounds used for comparison purposes were either commercially available or snythesized in the laboratory.

Saponification and synthesis of esters

The esters were saponified by a microsaponification technique, and the alcohol and acid moieties were identified. Saponification was accomplished by placing 25 μl of ethanolic sodium hydroxide (prepared by dissolving one pellet of sodium hydroxide in 0.1-0.2 ml of 50% ethanol) in the glass capillary collection tube with the ester and the mixture was allowed to stand overnight. The reaction mixture was neutralized with 25 μl of concentrated hydrochloric acid, extracted twice with 50 μl of diethyl ether, and then chromatographed. The esters, except for pinocarvyl acetate, were synthesized for comparison purposes by adding acetic anhydride and pyridine 1:2 v/v to the alcohol in a glass capillary collection tube. The mixture was allowed to stand overnight and then chromatographed. Pinocarvyl acetate was synthesized by lead tetraacetate oxidation of

Table 1—Identity and quantitative estimation[a] of major carbonyl constituents in celery essential oil recovered by two different methods.

Column fraction	Constituent	% of Carbonyls: Batch distillation	Continuous distillation[b]
1	t-Limonene oxide	0.50	5.0
	c-Limonene oxide	10.00	5.0
	Dihydrocarvone[c]	5.0	1.5
	Carvone	10.0	5.0
2	α-Ionone	1.0	2.0
	n,Butyl-phenyl ketone	1.0	—
	c,3-Hexenyl-1-acetate	0.25	—
	Pinocarvyl acetate	0.50	5.0
	Dihydrocarvyl acetate	—	1.0
	t-Carvyl acetate	20.0	25.0
	c-Carvyl acetate	2.0	3.0
	c-p-1(7)8-Menthadienyl-2-acetate	2.0	3.0
	Acetic acid	1.0	2.0
	Tiglic acid	0.5	1.0
	Angelic acid	—	0.5
3	3,n-Butylhexahydrophthalide	0.25	0.25
	3,n-Butylphthalide	20.0	20.0
	Sedanolide	5.0	5.0
	Unidentified	21.0	15.75

[a]Quantitative extimations were done by column chromatography and gas-liquid chromatography.
[b]Turba-film evaporator used.
[c]A mixture of cis- and trans-dihydrocarvone.

β-pinene (Gruenewald and Johnson, 1965).

Reduction of phthalides

Identification of 3,n-butylhexahydrophthalide was confirmed by reducing 3,n-butylphthalide and sedanolide to the parent compound. Hydrogenation was done on a Parr pressure-reaction apparatus. 1 ml of 3,n-butylphthalide and sedanolide were each added to 25 ml portions of glacial acetic acid and 50 mg of platinum oxide catalyst. Each mixture was shaken for 24 hr at 80 psi hydrogen pressure.

Pure limonene oxide was processed over neutral alumina, activity 2 (Fisher A–90, 80–200 mesh) to determine if isomerization was occurring (Nigam and Levi, 1968). Limonene oxide was synthesized from limonene and peracetic acid (Newhall, 1959). 5 ml (4.5g) of purified limonene oxide was dissolved in about 25 ml of n-hexane and transferred to a column containing 100g of neutral alumina. The column was eluted with about 150 ml of n-hexane, 200 ml of 1:1 v/v n-hexane-diethyl ether, and then stripped with approximately 150 ml of absolute ethanol. The solvent was removed in vacuo and each fraction was examined by GLC.

Limonene oxide was removed from celery essential oil by high vacuum, low-temperature distillation and the limonene oxide free celery essential oil was subjected to column chromatography. The alcohol fraction was analyzed by GLC (Wilson, 1969b).

Spectrophotometric methods

Infrared spectra were obtained neat on a Perkin-Elmer, Model 137, spectrophotometer. Ultraviolet spectra were obtained on a Cary-14 recoding spectrophotometer. Mass spectra were determined on a Bendix Time-of-Flight, Model 3012, mass spectrometer. Nuclear magnetic resonance spectra (NMR) were taken on a Varian A–60 instrument equipped with a time-averaging computer; carbon tetrachloride was the solvent and tetramethylsilane was the internal standard.

Quantitative comparisons of the two different essential oils were made by estimating the volume of each carbonyl fraction from the neutral alumina column. Quantitative relationships of the individual constituents in each fraction as determined by gas chromatography were established by comparing each constituent with internal standards of identical or similar structure.

RESULTS & DISCUSSION

COLUMN CHROMATOGRAPHY indicated that the carbonyls comprised about 5.0–10.1% (1 and 2 ml respectively) of the two essential oils that were recovered in the continuous and batch distillation units, respectively. Table 1 shows the identity and quantitative estimation of the individual carbonyl compounds. The first fraction eluted from the neutral alumina column consisted of cis- and trans-limonene oxide, cis- and trans-dihydrocarvone, and carvone. Limonene oxide, although it is not a carbonyl, was accounted for because it was isolated with the carbonyls. It is estimated that the first carbonyl fraction accounts for about 25.5% of the total carbonyls in the essential oil obtained by batch distillation, and accounts for about 16.5% of the total carbonyls in the essential oil recovered from the continuous distillation unit.

The second carbonyl fraction of the essential oil recovered by batch distillation contained cis-3-hexenyl-1-acetate, pinocarvyl acetate, cis- and trans-carvyl acetate, cis-1(7)8-p-menthadienyl-2-acetate, α-ionone, n-butyl-phenyl-ketone, and acetic and tiglic acid. These 9 compounds accounted for about 28.5% of the total carbonyls. The second carbonyl fraction of the essential oil recovered by the continuous recovery process contained pinocarvyl acetate, dihydrocarvyl acetate, cis- and trans-carvyl acetate, cis-1(7)8-p-methadienyl-2-acetate, α-ionone; and acetic, tiglic, and angelic acid. These eight compounds accounted for about 42.5% of the total carbonyls.

The third carbonyl fraction consisted primarily of 3 compounds: 3,n-butylphthalide, sedanolide, and 3,n-butylhexahydrophthalide. The ratio of 3,n-butylphthalide to sedanolide was about 4:1 and both of these compounds possessed a strong celery aroma. 3,n-butylhexahydrophthalide was present in trace amounts and its aroma was not as strong as that of 3,n-butylphthalide or sedanolide. The phthalide compounds reported in this study were previously found in celery seed oil and oleo resin (Barton and De Vries, 1963), and have been isolated from other plant sources in the umbelliferae family (Mitsuhashi and Muramatsu, 1964). These compounds have not been reported as constituents of the essential oil from celery stalks and leaves. The third carbonyl fraction accounted for approximately 25.0% of the total carbonyls.

Gold and Wilson (1963) reported the identification of 4 compounds that possessed a characteristic celery aroma and flavor. The previously reported compounds, 3,isobutylidene-3a,4-dihydrophthalide, 3-isovalidene-3a,4-dihydrophthalide, 3-isobutylidenephthalide, and 3-isovalidenephthalide, were not found during the current study. Comparison of chemical and spectral data for the three phthalides reported in the current study with the 4 reported by Gold and Wilson affirms the difference in structure. A possible explanation of the previously reported dihydrophthalides is that in the research reported by Gold and Wilson (1963) large quantities of celery were blanched in flowing steam for 3–4 min and considerable amounts of the steam volatile materials were lost. The quantity of the dihydrophthalides found in the essential oil was extremely small. During the current investigation less celery was used to obtain larger quantities of essential oil. It is conceivable that the dihydrophthalides were present in such small quantities that their presence was not detected in the current study.

The main difference between the oil recovered by batch distillation and that recovered by continuous distillation was in the amount of the more volatile compounds (i.e., the volatile compounds were considered to have gas chromatographic retention times of less than 45 min and the high boiling compounds eluted from the column after 90 min). The oil recovered by continuous distillation had a higher percentage of the more volatile materials. The volatile materials in the oil recovered by continuous distillation accounted for about 10.0% of the unidentified carbonyls. The oil recovered by batch distillation contained very little of the more volatile materials, and the high boiling constituents in this oil accounted for about 20.0% of the unidentified carbonyls.

Column chromatography showed that limonene oxide was readily isomerized by neutral alumina, activity 2. Fractions consisting of 0.1g of cis- and trans-limonene oxide, 0.1g of carbonyls, and 3.5g of alcohols were recovered from the 4.5g of limonene oxide that was placed on the column. The majority of the carbonyl fraction consisted of cis- and trans-dihydrocarvone and carvone. The major constituents in the alcohol fraction were 1-methyl-3-isopropenylcyclopentyl-1-carbonol, cis- and trans-1(7)8-p-menthadiene-2-ol, cis- and trans-carveol, 1,8(10)menthadiene-9-ol, and 8,9-p-menthene-1,2-diol.

Some of the alcohols from limonene oxide isomerization were found in celery essential oil. However, comparison of results obtained from analysis of the alcohols obtained from limonene oxide free celery essential oil and that reported by Wilson (1969b) showed virtually no significant difference.

The organoleptic properties of the carbonyls from the oil recovered by batch distillation and that recovered by continuous distillation were considerably different. The carbonyl fractions from batch distillation possessed a pleasing celery aroma but lacked the notes attributed to the more volatile compounds that were present in the oil recovered by continuous distillation. During the analysis, the odor of each compound was examined as they emerged from the gas chromatograph. All of the carbonyls had a pleasant aroma but there was not any single compound, other than the phthalides, that had an odor charactertistic of celery.

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Ms. received 11/10/69; revised 3/6/70; accepted 3/8/70.

Mention of brand names is for identification only and does not imply recommendation by the U.S. Department of Agriculture.

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IDENTIFICATION OF CARBONYL AND SULFUR COMPOUNDS FROM NONENZYMATIC BROWNING REACTIONS OF GLUCOSE AND SULFUR-CONTAINING AMINO ACIDS

SUMMARY—Odor evaluations, chemical analyses and the effect of pH on the nonenzymatic browning reaction products between equimolar concentrations of glucose and each of the sulfur-containing amino acids, methionine, cysteine and cystine, were conducted. None of the 3 mixtures emitted an odor associated with meat. The cystine-glucose mixture produced no significant aroma, the cysteine-glucose mixture an odor resembling over-boiled egg and the methionine-glucose mixture an objectionable odor associated with boiled potato, the most interesting of the 3 mixtures. The characteristic odor of the last-named mixture was attributed to mercaptans. Only the methionine-glucose mixture was affected by an increase in pH.

INTRODUCTION

IT HAS frequently been suggested that the reactions between amino acids and sugars contribute to the production of flavor in food upon processing or cooking. Heating of naturally occurring amino acids and sugars has resulted in production of flavors described as typical of many foods (Barnes and Kaufman, 1947; Keeney and Day, 1957; Herz and Shallenberger, 1960 and El'odé et al., 1966). Only a small number of investigators, however, have identified the compounds responsible for the flavors (Keeney and Day, 1957; El'odé et al., 1966; Obata, 1964 and Obata and Tanaka, 1965).

Our study was conducted on the Maillard reaction of the sulfur-containing amino acids with sugar. Particular emphasis was placed on the sulfur-containing amino acids because they are undoubtedly a source of a very important group of flavor compounds in meat. Glucose was used as the sugar because it has been found in greatest quantity in beef, pork and lamb, as compared with fructose and ribose (Macy et al., 1964). Our work was undertaken to 1) confirm the characteristic aromas emitted from these mixtures; 2) determine which volatile reaction products could account for these characteristic aromas and 3) to study the effect of pH on these reactions.

EXPERIMENTAL

Heating procedures

An equimolar amount (0.0011 M) of glucose and each amino acid was dissolved in 8 ml of distilled water in a 20-ml test tube. Concentrated HCl was used to dissolve the amino acid when necessary. The mixture was heated for 2 hr at 98 (± 2)°C in a water bath with the top of the test tube covered with a marble. Contents of the 3 test tubes were reacted for each reaction mixture. The same procedure was used for the variable pH study, except that the pH of the mixture was adjusted to ca. 6.5, 7.0, 7.5 and 8.0 before heating.

Odor evaluations

Contents of each of the 3 test tubes of pH-controlled heated mixtures were poured separately into 3, 250-ml Erlenmeyer flasks designated as Flasks A, B and C. Flask A contained no reagent, Flask B, 50 ml of 4% mercuric cyanide solution (mercuric cyanide reacts with mercaptans) and Flask C, 50 ml of 3% mercuric chloride solution (mercuric chloride reacts with mercaptans and sulfides). By comparing the aroma evolved from Flasks A, B and C, the volatile product (or products) mainly responsible for the aroma of the heated mixture could be determined.

Contents of the 5 test tubes (Control, pH 6.5, 7.0, 7.5 and 8.0) of the pH-varied heated mixtures were separately poured into 5, 250-ml Erlenmeyer flasks and their odors evaluated.

An odor panel consisting of 7–10 members was used to evaluate the odors of the flask. The judges were asked to characterize and score the intensity of the odors.

Mercaptans, sulfides and carbonyls

The precipitates formed in Flasks B and C were filtered, washed and dried. If no precipitate was found, the reaction mixture was extracted with ethyl acetate.

The filtrate from Flask C was mixed with an equal volume of saturated 2,4-dinitrophenylhydrazine-HCl solution (0.5 N), stoppered and allowed to stand overnight. The resulting precipitate was filtered and the filtrate extracted with chloroform. The precipitate and the residue of the chloroform extract were combined for carbonyl analysis.

Identification

The mercaptan or mercaptan and sulfide derivatives (2–3 mg) were placed in a 25-ml Erlenmeyer flask and the flask stoppered with a previously boiled and dried rubber serum cap. 1.5 ml of 6 N HCl was injected through the cap and approximately 2 ml of the regenerated volatiles removed with a syringe for gas-chromatographic analysis. Silicone-rubber and carbowax columns were employed. The gas chromatograph was an F & M Model 810 with dual flame ionization detectors. Temperature of the injection port was 130°C for the silicone-rubber column and 180°C for the carbowax columns. The detector temperature was 236°C for all columns, and the oven temperature was 48°C for the silicone-rubber columns and 58°C for the carbowax columns. The flow rate was 60 ml per minute.

The carbonyl 2,4-dinitrophenylhydrazones were identified by thin-layer chromatography and spectrophotometric analysis. The thin-layer chromatography procedure of Libbey and Day (1964) was modified by using 5% mineral oil instead of 10% to coat the thin-layer chromatographic plates for reverse-phase chromatography.

RESULTS & DISCUSSION

TABLE 1 presents the odor evaluations of the 3 mixtures before and after treatment with reagents.

The intensity of the aromas, the browning, weight of the mercuric chloride derivatives and carbonyls increased with pH. These findings agree with the conclusions made by Underwood et al. (1959), who concluded that the browning of α-amino acids increased with pH, with the largest increase at pH 7–9. The volatile compounds obtained from the

Table 1—Odor evaluation of the amino acid-glucose browning reaction mixtures before and after treatment with reagents.

Flask	Methionine-glucose (10 members)	Cysteine-glucose (7 members)	Cystine-glucose (7 members)
A[a]	Potato-like	Overboiled or rotten egg	Odor undescribable
B[b]	Potato-like Odor eliminated	Odor eliminated	Odor different from that of Flask A
C[c]	Potato-like Odor eliminated	Odor eliminated	Odor different from that of Flask A

[a]Amino acid-glucose mixture.
[b]Amino acid-glucose mixture plus 4% mercuric cyanide solution.
[c]Amino acid-glucose mixture plus 3% mercuric chloride solution.

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Table 2–Sulfur compounds identified from methionine, cysteine and cystine-glucose mixtures.[a]

Methionine-glucose	Cysteine-glucose	Cystine-glucose
Methyl mercaptan	Methyl mercaptan	Methyl mercaptan
Ethyl mercaptan	Propyl mercaptan	Ethyl mercaptan
Propyl mercaptan	Pentyl mercaptan	Propyl mercaptan
Dimethyl sulfide		
Dimethyl disulfide		

[a]Compounds were identified by comparing retention times of known and unknown compounds on silicone-rubber and carbowax columns.

Table 3–The carbonyls identified from the 3 amino acid-glucose mixtures.

Identified carbonyls	TLC[a]	λ max[b]	λ max of Standard
Methionine-glucose			
Ethanal	+	358	353
Propanal	+	358	358–361
Furfural	+	379	368–372
Cysteine-glucose			
Propanal	+	358	358
Cystine-glucose			
Propanone	+	363	363

[a]Identified by comparing the R_f of known compounds to unknown.
[b]λ max were determined in chloroform.

mercuric chloride derivatives of the 5 methionine-glucose mixtures were qualitatively identical according to gas chromatographic data.

The intensity of the odor did not differ in the cysteine-glucose mixtures at various pH levels. In fact, no browning was observed with this mixture in any of the experiments. Formation of the brown pigment may have been prevented by the mercaptans produced in the reaction (Song and Chichester, 1967a; 1967b).

Since concentrated HCl was used to dissolve the cystine for the cystine-glucose mixture, addition of 0.1 N ammonium hydroxide to increase the pH caused the cystine to precipitate. Therefore, the effect of pH on browning was not determined for this mixture.

Tables 2 and 3 show the mercaptans, sulfides and carbonyls identified from the 3 mixtures.

The methionine-glucose mixture

The identification of methyl mercaptan was expected, since this sulfur volatile was identified by Casey et al. (1965), Self et al. (1963) and Ballance (1961) as a nonenzymatic browning reaction product of methionine. Casey et al. (1965) concluded that dimethyl sulfide and dimethyl disulfide were produced when methionine was degraded by compounds such as glucose. These compounds could also be produced from the decomposition of methional. When methionine was degraded by dehydroascorbic acid, Self et al. (1963) found dimethyl disulfide in the volatiles from the mixture. Dimethyl disulfide may have been derived from methyl mercaptan, since it is known that dimethyl disulfide is produced from methyl mercaptan in the presence of oxygen. The origin of ethyl and propyl mercaptans remains to be elucidated.

Ethanal and propanal were also identified by El'odé et al. (1966). In fact, propanal was shown by Bayer (1958) to be the major volatile product of the hypochlorite breakdown of methionine. The Strecker degradation of this amino acid would produce propanal. The identification of furfural agreed with the theory of the Maillard reaction.

The cysteine-glucose and the cystine-glucose mixture

The identification of some sulfur volatiles in these mixtures did not agree with the results obtained by Self et al. (1963) in their model cysteine and cystine-dehydroascorbic acid reaction mixtures. Possibly a more complex reaction is involved when glucose is used than when dehydroascorbic acid is used as the degrading agent.

Following the mode of action of the Strecker degradation and according to Kobayasi and Fujimaki (1965), ethanal should be produced when cysteine (and possibly cystine) is degraded by such compounds as ninhydrin and dehydroascorbic acid. Degradation of this amino acid by glucose resulted in propanal and propanone.

The egg-like aroma of the cysteine-glucose mixture was probably due to hydrogen sulfide. Kazeniac (1961) described the egg-like aroma of this inorganic sulfide.

Kobayasi and Fujimaki (1965) stated that hydrogen sulfide was produced when cystine was boiled with α-diketones. The cystine-glucose mixture did not have an egg-like aroma. Hydrogen sulfide may have been produced without emitting a distinct egg-like odor, which Kazeniac (1961) indicated becomes less pronounced in the presence of ammonia.

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Ms. received 12/22/69; revised 7/1/70; accepted 7/28/70.

Journal paper No. J-6426 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project 1264; Center for Agriculture and Economic Development Cooperation. This investigation was supported by Public Health Service research grant UI-00263-02 from the Division of Urban and Industrial Health, National Institutes of Health.

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COLORIMETRY OF FOODS. 3. Carrot Puree

SUMMARY—A series of 10 carrot purees was prepared with added FD&C Green No. 1 food color in increments of 0.015 ppm. The samples were evaluated visually for color differences under illumination of 7400°K and measured with a G.E. spectrophotometer with D&H tristimulus integrator and a Hunterlab D_{25} colorimeter in cells varying from 2–8 mm in thickness against both a white and black background. The instrumental data were calculated in conventional and K/S ratios and correlated with visual and theoretical rankings. The panelists could reasonably well rank samples differing by 0.10–0.15 delta E units, calculated as $[(\text{delta } L)^2 + (\text{delta } a)^2 + (\text{delta } b)^2]^{1/2}$. Highest correlations with the theoretical order were obtained with a black background and sample thicknesses of 6 mm or more. Adequate rankings with either instrument required a multiple correlation with all 3 color parameters (X Y Z or L a b). Optimum separation of samples with the G.E. spectrophotometer was obtained with a white background and a sample thickness of 5 mm or more. The Hunterlab D_{25} colorimeter was superior to the G.E. spectrophotometer for separation of samples. Optimum separation was with a 2-in.-diameter aperture and a cell 4 mm or more in thickness against a white background. Correlations of Hunter data with theoretical order were higher than those with visual vs. theoretical ranking. Individual K and S values may be useful in characterizing purees for colorimetry.

INTRODUCTION

A PREVIOUS paper (Huang et al., 1970) described the application of the Kubelka-Munk equations to squash puree. This turbid media theory application was found by Mackinney et al., 1966 and Little, 1964 to be effective in separating the colors of appleberry-applesauce mixtures in color space. They were attempting to find a mathematical transformation of conventional color data which would better portray differences in color their panelists actually could see. With samples of high chroma, such as squash puree, the calculation of reflectance data, in terms of absorption (K) and scattering (S) coefficients, was no more effective in separating samples in color space than conventional tristimulus data using a 3-component regression equation (Huang et al., 1970).

This work was undertaken with 3 aims: 1) to test the turbid media theory with another product with high K and S values for separation of samples in color space; 2) to develop the optimum method of obtaining instrumental color data with carrot puree; 3) to test several visual methods for adequacy of separation of samples in color space.

MATERIALS & METHODS

THE CARROT puree used in this work was commercial junior babyfood puree. A series of samples was colored with a green food color (FD&C Green No. 1). Carrot puree is so highly colored that it was impractical to add sufficient red color (canthaxanthin) to produce the required color change. It was simpler to add a green dye to cause a small color change in the reverse order. 10 samples of colored carrot puree were prepared for each series by adding stock solution (0.3 mg/ml) of dye such that the mixtures contained from 0–0.135 ppm dye in increments of 0.015 ppm.

The samples were placed in circular 3-in.-diameter Plexiglas cells with sample thicknesses from 2–8 mm. The samples were measured with a General Electric Recording Spectrophotometer (GERS, General Electric Co., Lynn, Mass.) and a Hunterlab D_{25} Colorimeter (Hunterlab Associates, Inc., Fairfax, Va.). The visual judgments were performed in a MacBeth Lablite booth at 7400°K (MacBeth Daylighting Corp., Newburgh, N. Y.). The panelists were shown 10 samples simultaneously with the same cell thickness arranged such that the viewing angle was 45° to the illumination. The panelists could tilt the samples if they wished and were asked to rank them in terms of redness. The visual and instrumental methods as well as the calculations were the same as those used by Huang et al. (1970).

The K and S values were calculated from reflectance data obtained with a black (Munsell N 2.25/) and a white (Munsell N 9/) background. The equations were as follows:

$$a = \tfrac{1}{2}\left[\frac{(R + Ro - R - Rg)}{RoRg}\right] = \frac{S + K}{S}$$

$$b = (a^2 - 1)^{1/2}$$

$$R_\infty = a - b$$

$$K/S = \frac{(1 - R_\infty)^2}{2 R_\infty}$$

$$K = S(a - 1)$$

$$SX = {}^{1}/_{b}\ \text{Arctgh}\left[\frac{(1 - aRo)}{bRo}\right]$$

where R = reflectance of sample with white background
Ro = reflectance of sample with black background
Rg = reflectance of white background
R_∞ = reflectance of infinitely thick sample
K = coefficient of absorption
S = coefficient of scattering
X = thickness of sample
Arctgh = inverse hyperbolic cotangent.

Simple and multiple correlations between instrumental data and visual judgments or theoretical order were calculated to test the ability of the panelists or the instruments to place the samples in correct order. To predict fiducial statements about small sample correlations, the correlation coefficients were transformed to a quantity z (Snedecor, 1956). A paired "t" test was used to detect differences in z values and an F test for thickness effects.

Table 1—Relationship between theoretical ranking and visual ranking for samples with a white and black background.

Correlation	Cell thickness (mm) 2	3	4	5	6	7	8
	Correlation coefficient (r × 1000)[a]						
Theor.[b] vs. V_B[c]	491	544	540	449	423	552	655
Theor. vs. V_W	327	397	530	439	348	618	544
	Index of confusion[d]						
V_B	31	30	29	30	32	28	29
V_W	34	34	30	32	32	28	30

[a]All correlation coefficients are the averages of r values from 3 experiments using 4 trained judges.

[b]Theoretical ranking.

[c]Visual ranking for samples with a black (V_B) and a white (V_W) background, respectively.

[d]Index of confusion = sum of differences between theoretical rank and visual rank.

Table 2–Relationship between added pigment concentration and delta E for different cell thicknesses.

Cell thickness (mm)	Conc of green pigment (ppm) 0.015	0.045	0.075	0.105	0.135
	ΔE^a				
Small-illumination area and white background					
2	1.0	1.4	1.8	2.2	2.7
3	0.8	1.1	1.4	1.7	2.0
4	0.6	1.1	1.5	1.9	2.4
6	0.6	0.9	1.2	1.5	1.8
8	0.3	0.7	1.0	1.3	1.6
Large-illumination area and black background					
2	0.9	1.0	1.1	1.3	1.4
3	0.5	0.6	0.8	1.0	1.2
4	0.2	0.4	0.5	0.7	0.9
6	0.2	0.5	0.7	0.9	1.1
8	0.3	0.6	0.9	1.1	1.4

[a]Data obtained with a Hunterlab D_{25} colorimeter and calculated as $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$ = difference between samples with added pigment and controls.

Table 3–Absorption and scattering coefficients for carrot and squash samples.

Cell thickness (mm)	Carrots K × 1000	Carrots S × 1000	Squash K × 1000	Squash S × 1000
		Hunterlab L data		
2	195[a]	432	174	487
5	159	297	140	326
8	145	266	129	291
		GERS X data		
2	255	163	245	179
5	216	131	199	139
8	198	122	185	129
		GERS Y data		
2	324	163	300	183
5	270	125	242	140
8	241	116	221	128
		GERS 585 nm data		
2	202	190	185	209
5	202	165	182	172
8	201	163	185	170

[a]Each datum is the average from 10 pigment concentrations.

All statistical analyses and color indices were programmed on a CDC 3600 computer. Only a small sampling is presented here; the remainder may be found in the original thesis (Huang, 1969).

RESULTS & DISCUSSION

Visual data

Table 1 presents data on the correlations of visual panel results with the theoretical order of samples. The correlations are approximately 0.5, indicating that the differences between the samples were getting close to the detectable limit. The panelists could separate the samples better with the samples against a black background for all thicknesses of cells. The judgments were better with cell thickness of 7 mm or higher. The indices of confusion in Table 1 show the same conclusions. This is in general agreement with the conclusions on squash puree.

The differences in color between the samples as influenced by method of measurement are shown in Table 2. Since the color differences were close to the limit of measurement capability of the instrument, a large measurement error as compared with the real color difference was present in the raw data. To obtain color readings closer to the true values for correlation with visual data, the differences in Table 2 were actually calculated from regression equations relating pigment concentration to color readings. 10 pigment concentrations were used for each cell thickness and a regression equation calculated for each color parameter (L, a, b). The data in Table 2 represent differences twice as large as those presented to the panels since, in the interests of brevity, only half of the data is presented in Table 2. Using the thicker cells with both methods, the actual color differences judged by the panelists were between 0.10 and 0.15 delta E units. The panelists had more difficulty separating carrot puree samples with delta E differences of 0.15 than for squash samples with a delta E of 0.2. The higher chroma values for the carrot puree may also have contributed to this effect.

The most effective visual presentation for separation of samples was with a cell of 6 mm or more in thickness and a black background.

K and S values

The expansion in color space with the thin cells was again noted (Table 2), but it was not nearly so pronounced as with the squash samples. The effect is more obvious with small-area illumination, which suggests that the scattering effect may have something to do with this phenomenon. To test this, individual values for K and S were calculated (Table 3) and compared with those from squash samples used in previous work (Huang et al., 1970).

Values for K and S in Table 3 are the averages for values for each of 10 pigment concentrations. Only 3 of the 8 cell thicknesses and 4 of a number of ways of calculating these values are reported. Actually, the K values do vary with pigment concentration but in this work the added pigment was so small, as compared with the carotenoids present in the carrots, that the K values varied little over the range of samples. K and S values for carrot and squash puree were similar but they do show the expected differences. The K values for carrots were higher than those for squash, reflecting the higher chroma and natural pigment content of the carrots. The S values for carrots were lower and this would be expected because the particle sizes in squash were smaller, creating more internal reflection. The higher S values of the squash sample probably contributed to the greater space expansion with the thin cells. This may be a general phenomenon and will be investigated in more detail. However, in spite of the apparent expansion in color space, the panelists could not separate the carrot samples more effectively with the thinner cells. Paradoxically, they could do better with the thicker cells (Table 1).

Measurement of K and S values may be very useful in classifying purees by both pigment content and particle size to predict the success of color measurement for sample separation. This concept is being studied with 2 other aqueous carotenoid-containing purees with different K and S values.

GERS correlations

A sampling of the theoretical and visual rankings with GE data is presented in Table 4. All correlations of theoretical ranking with a single color parameter were low. The same instrumental data correlated with visual rankings were higher. Correlations of 2 instrument parameters (e.g., X Y) with visual or theoretical rankings were higher. Correlations using all 3 color parameters were higher still and were similar for both theoretical and visual rankings. Both theoretical and visual rankings with 3 color parameters were higher for the white background than for the black, for samples less than 5 mm in thickness. Calculation of X Y Z data in terms of K/S values provided slightly higher correlations but not enough to be

Table 4–Correlation of visual and theoretical rankings with data from a GE Spectrophotometer.

Correlation[a]	Cell thickness (mm) 2	4	6	8
	Correlation coefficient (r or R × 1000)			
Th. vs. X_B	489	485	486	482
V_B vs. X_B	760	683	784	736
Th. vs. $X_B Y_B$	537	559	887	703
V_B vs. X_B Y_B	789	719	816	755
Th. vs. X_B Y_B Z_B	601	627	894	758
V_B vs. X_B Y_B Z_B	796	741	832	760
V_W vs. X_W Y_W Z_W	781	823	834	861
Th. vs. X_W Y_W Z_W	704	895	878	831
Th. vs. (K/S) X	490	460	465	477
V_B vs. (K/S) X	718	674	768	739
Th. vs. (K/S) X (K/S) Y	513	815	716	698
V_B vs. (K/S) X (K/S) Y	774	812	829	771
Th. vs. (K/S) X (K/S) Y (K/S) Z	681	888	873	810
V_B vs. (K/S) X (K/S) Y (K/S) Z	862	822	830	826
Th. vs. (K/S) 585	499	481	473	477
V_B vs. (K/S) 585	719	687	773	738

[a]X, Y, Z are tristimulus values obtained with a GERS. The subscripts B and W refer to black and white backgrounds; 585 refers to the wavelength of reflectance measurement. The prefix (K/S) refers to K/S values calculated from GE X Y Z data by the Kubelka-Munk equations. Other symbols same as in Table 1.

Table 5–Correlation of visual and theoretical rankings with data from Hunterlab D_{25} colorimeter.

Correlation[a]	Cell thickness (mm) 2	4	6	8
	Correlation coefficient (r or R × 1000)			
Th. vs. a_{Bl}	454	474	470	450
V_B vs. a_{Bl}	764	685	779	732
Th. vs. a_{Bl} b_{Bl}	602	739	840	939
V_B vs. a_{Bl} b_{Bl}	782	743	798	780
Th. vs. a_{Bl} b_{Bl} L_{Bl}	663	842	870	943
V_B vs. a_{Bl} b_{Bl} L_{Bl}	837	815	831	798
Th. vs. a_{Wl} b_{Wl} L_{Wl}	623	951	937	978
Th. vs. a_{Bs} b_{Bs} L_{Bs}	634	661	883	957
Th. vs. a_{Ws} b_{Ws} L_{Ws}	699	952	792	944
Th. vs. (K/S) a_l	472	445	425	442
V_B vs. (K/S) a_l	768	673	769	726
Th. vs. (K/S) a_l (K/S) b_l	519	856	911	976
V_B vs. (K/S) a_l (K/S) b_l	778	804	804	808
Th. vs. (K/S) a_l (K/S) b_l (K/S) L_l	619	933	928	979
V_B vs. (K/S) a_l (K/S) b_l (K/S) L_l	810	830	857	815
Th. vs. (K/S) a_s (K/S) b_s (K/S) L_s	665	957	911	936
V_B vs. ($\tan^{-1}a/b$) Bl	761	688	781	739
Th. vs. ($\tan^{-1}a/b$) Bl	480	489	485	479
V_B vs. (K/S) al (K/S) bl	771	680	774	730

[a]Subscripts l and s, respectively, refer to large- (2-in.-diameter) and small- (3/8-in.-diameter illumination in a 2-in.-diameter aperture) area illumination. Other symbols same as in Table 4.

of much importance. The experiment was repeated using black specular cups in the GERS to minimize the gloss component, but the results were similar to those with normal operation including the specular component. The most effective way to use a GE spectrophotometer to separate these samples in color space is to use a sample thickness of 5 mm or more against a white background and a multiple regression involving X, Y and Z. A single set of readings would be superior in accuracy to results with a trained panel of 4 judges.

Hunterlab D_{25} correlations

A sampling of the theoretical and visual rankings with Hunter data is presented in Table 5. Simple correlations of theoretical ranking with single-color parameters were all low. Correlations of visual rankings with 1 color parameter were higher, as previously observed with the GE data. This is surprising but probably unimportant. Correlations of visual or theoretical rankings with 2 instrument parameters were higher and similar. Correlations using 3 color parameters were even higher, and correlations of theoretical rankings were higher than those with visual rankings. This is significant, since the average visual rankings were used in the correlations; thus, this does not show the variability effects usually seen with panel judgments when all the data are used in the calculation of R. The error in instrumental readouts is usually less and this is reflected in the higher correlations. With a larger panel the error in the average visual ranking would be less.

Calculations of Hunter data in terms of K/S ratios with the theoretical rankings were higher than those obtained with conventional L, a, b data, but both sets were above 0.9 for all but the 2 mm thickness. Correlations of K/S Hunter data with the theoretical order were higher than the same data correlated with visual rankings. Again, this probably reflects the higher error associated with the visual panels. With thicker samples, the improvement in the correlation of theoretical ranking with Hunter a vs. b by adding the L value was fairly small. This suggested that the panelists might be judging mainly by hue but correlations of $\tan^{-1} a/b$ with theoretical rankings were low (0.48). Correlations of $\tan^{-1} a/b$ with visual rankings were approximately 0.7 and the same calculations in terms of K/S were similar (Table 5).

The most efficient way of separating these samples using the Hunterlab D_{25} colorimeter would be to use a cell 4 mm or more in thickness against a white background with large-area illumination and all 3 color parameters. This system would be superior to that using the GE spectrophotometer.

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Ms. received 3/27/70; revised 7/17/70; accepted 7/20/70.

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DISC GEL ELECTROPHORESIS OF PROTEINS IN NATIVE AND HEAT-TREATED ALBUMEN, YOLK, AND CENTRIFUGED WHOLE EGG

SUMMARY—Polyacrylamide disc gel electrophoresis was an effective technique for resolving the proteins of albumen, yolk, and centrifuged whole egg into distinct bands. Albumen proteins including ovalbumins, conalbumins, and globulins were separated into 12 bands. Ovomucoid could not be detected and lysozyme did not migrate into the gel. With yolk, 19 bands including livetins and lipovitellins but not low-density lipoproteins were formed. When centrifuged whole egg was subjected to electrophoresis, livetin and albumen protein bands were detected, but no evidence was obtained for the formation of new protein complexes. Alterations of gel patterns were observed when albumen, yolk, and centrifuged whole egg were heated to pasteurization temperatures of 61.7°C and above.

INTRODUCTION

SEVERAL ELECTROPHORETIC methods have been used to separate the proteins of albumen and yolk. According to Longsworth et al. (1940) and Forsythe and Foster (1949), albumen proteins were resolved by moving boundary electrophoresis into 7 major peaks. Ovalbumins (A_1 and A_2), globulins (G_2 and G_3), ovomucoid and conalbumin were identified in both of these studies. The moving boundary technique for yolk could not be used successfully to resolve the proteins and lipoproteins (Clegg et al., 1955; Young and Phinney, 1951). Recently zone electrophoresis using paper and starch gel stationary phases has become a useful method for the study of egg proteins. Evans and Bandemer (1956) showed that distinct bands of ovalbumin, ovomucoid plus ovoglobulin, conalbumin, and lysozyme were formed on paper during electrophoresis with albumen. However, much better resolution of albumen proteins has been demonstrated with starch gel electrophoresis. Lush (1961), Steven (1961), and Feeney et al. (1963) succeeded in separating the albumen proteins into as many as 19 bands. Variation in the number of bands for albumen from various strains and inbred lines of hens has been attributed to genetic polymorphism (Feeney et al., 1963). Globulins, in particular, were found to be genetically controlled.

Separation of yolk proteins and lipoproteins has been achieved with the use of paper electrophoresis (Evans and Bandemer, 1957; McCully et al., 1962; Powrie et al., 1963). The high-density lipoprotein portion of the yolk granules was immobile and remained at the origin, whereas the low-density lipoproteins, livetins, and phosvitin migrated on the paper during electrophoresis. Judging from the starch gel pattern of Steven (1961) for egg yolk, the proteins were separated into well-defined bands, but none of the yolk proteins in the bands were identified.

Within recent years, polyacrylamide gel electrophoresis has been used extensively for the separation of a variety of proteins into small distinct bands, but no report has been found on the effectiveness of this technique for resolving the proteins and lipoproteins in egg products. The purpose of this study was to obtain polyacrylamide gel patterns for native albumen, yolk, and centrifuged whole egg as well as for these systems heated to pasteurization temperatures. Whole yolk was centrifuged prior to experimentation for removal of particulate granules.

EXPERIMENTAL

Materials

Yolk with no albumen contamination was prepared by the method of Powrie et al. (1963) from infertile eggs not more than 24 hr old. Homogeneous albumen was obtained by combining the thin and chalazae-free thick albumen, mixing mechanically to break down the gel masses, and filtering through cheesecloth. About 33% yolk and 67% homogeneous albumen were combined to produce a whole egg mixture. After centrifuging this mixture (7°C) at 78,000 × G for 8 hr, the supernatant (centrifuged whole egg) was removed from the sedimented granules.

Plasma was obtained by ultracentrifugation of yolk (10°C) for 10 hr at 78,000 × G. Livetin fractions and low-density lipoproteins (LDL) were obtained from plasma by the centrifugation method of Suvansatit (1965). In this method, plasma diluted with 15% NaCl (1:4 dilution) was centrifuged for 15 hr at 78,000 × G. Centrifugation resulted in a floating pellicle, a cloudy area immediately below the pellicle, a clear colorless subnatant and a precipitate at the bottom of the tube. The LDL fraction in the pellicle was purified by dispersing the pellicle in 10% NaCl (1:10 dilution) and recentrifuging to float up the LDL. The clear subnatant, containing the livetins, was dialyzed against distilled water at 7°C for about 24 hr. γ-livetin precipitated out whereas the α- and β-livetins remained in solution. The mixed α,β-livetin fraction was freeze dried.

Isolation of the lipovotellin fraction from yolk was carried out by the method of Joubert and Cook (1958a). The phosvitin fraction was isolated from yolk by the method of Joubert and Cook (1958b).

Ovalbumin (2x crystallized) was purchased from Nutritional Biochemicals Corporation,

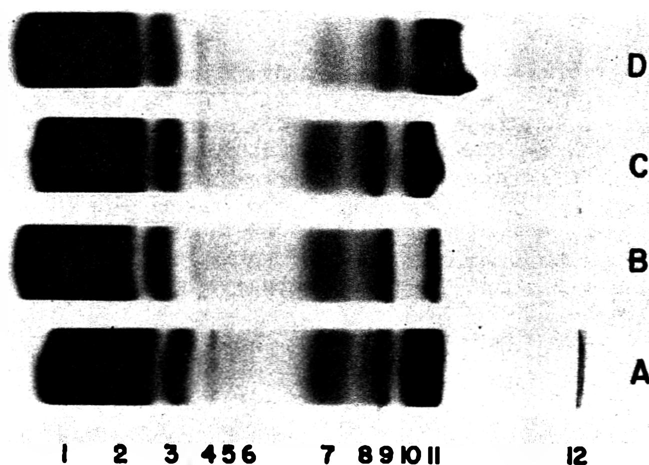

Fig. 1—Electrophoretic patterns of native and heated (61.7°C for 3 min) albumen. A: native (pH 8.5); B: heated albumen (pH 7.0); C: heated albumen (pH 8.5); D: heated albumen (pH 9.0). Gels prepared with Tris-glycine buffer, pH 8.1 and stained with amido black.

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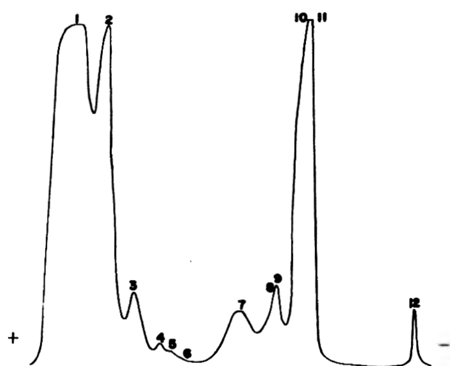

Fig. 2–Densitometric tracing of native albumen (pH 8.5).

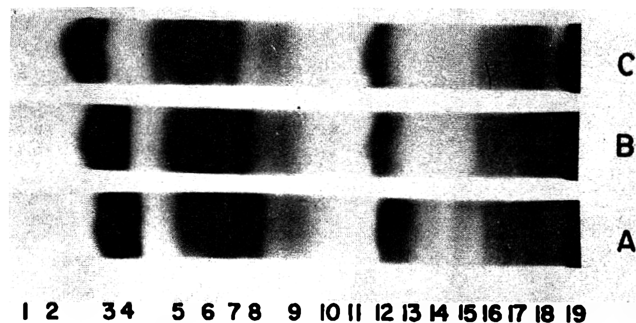

Fig. 3–Electrophoretic patterns of yolk. A: native; B: heated at 61.7°C for 3 min; C: heated at 65.6°C for 3 min. Gels prepared with Tris-glycine buffer, pH 8.1 and stained with amido black.

Cleveland, Ohio. Conalbumin (iron-free) was obtained from Sigma Chemical Co., St. Louis, Mo. Lysozyme (3x crystallized) and ovomucoid (trypsin inhibitor) were purchased from Pierce Chemical Co., Rockford, Ill.

Heat treatment

Albumen, yolk, and centrifuged whole egg were heated in glass tubes (sealed at one end), each 7.5 cm long and 0.6 cm I.D. Samples were introduced into the tubes with the aid of a hypodermic syringe. Each filled tube was immersed in a constant-temperature water bath for heat treatment. Copper-constantan thermocouple wires were inserted through a rubber septum to the center of each tube and the temperature was measured by means of a Leeds and Northrup potentiometer. The desired temperatures were reached in less than 45 sec and each sample was held at the heating temperature for 3 min. Each egg sample was cooled immediately after heating by placing the glass tubes in ice water at 0°C.

Disc gel electrophoresis

A disc gel electrophoresis method, described by Davis (1964), was used to separate the egg proteins. This procedure involved the preparation of tubes each with 0.2 ml of large-pore gel layered on 1.8 ml of small-pore gel (7.5% acrylamide). The polyacrylamide gels were prepared with a Tris-glycine buffer, pH 8.1. For electrophoresis, a current of 4 ma per tube was applied for about 1.5 hr while the tubes were held in a refrigerator at 7°C. The protein concentration of each solution was about 1%. An aliquot of protein solution containing about 0.2 mg protein was applied to the upper gel. The protein bands in the gels were stained by immersing the gels in a 1% amido black (Schwarz) solution containing 7% acetic acid. Destaining was carried out electrophoretically in 7% acetic acid. A Beckman Analytrol, Model RB, with an adapter for holding the gel, was used to obtain absorbance tracings. An interference filter with a maximum transmission of 660 mμ was used. The lipid portion of the lipoproteins was stained for 2–3 days with a solution containing Oil Red O-trichloroacetic acid (Smithies, 1959), then destained in 40% methanol-water solution.

RESULTS & DISCUSSION

Albumen protein

As shown in Figure 1 (pattern A), the proteins in native albumen (pH 8.5) separated into 12 distinct bands during electrophoresis. Figure 2 shows the densitometric tracing of the albumen pattern A in Figure 1. The polyacrylamide gel pattern for native albumen was similar to the starch gel pattern of Feeney et al. (1963). Bands 1, 2, and 3 in Figure 1 were ovalbumins as demonstrated by comparison of the albumen pattern with that for crystalline ovalbumin. The proportion of 66, 28, and 6% for the 3 albumins was determined from the areas under the curves in the tracing (Fig. 2). Feeney et al. (1963), using starch gel electrophoresis, obtained 3 albumen bands in roughly the proportion of 75, 20, and 5%. The proteins leading in their starch gel patterns were also ovalbumins. In our study, the proteins in bands 1, 2, and 3 may be regarded as ovalbumins A_1, A_2, and A_3, respectively, as designated previously by Perlmann (1948). The proteins in bands 4 to 7 could not be positively identified, but can be considered tentatively as unidentified globulins (Feeney et al., 1963). In their starch gel electrophoretic patterns of albumen, Feeney et al. (1963) noted 2 minor bands near the conalbumin bands and assigned the names globulin A_1 and A_2 to these proteins. Since the minor bands 8 and 9 in our electrophoretogram (Fig. 1) are close to the conalbumins, possibly the proteins in these bands are globulins A_1 and A_2, respectively. Globulins A_1 and A_2, with molecular weights in the region of 35,000, are governed by one genetic locus (Feeney et al., 1963). The presence of these globulins in albumen will be dependent on the genetic background of the hen.

Bands 10 and 11 were definitely conalbumins since crystallized conalbumin, when subjected to gel electrophoresis, migrated to the same positions as these bands. The minor band 12 was not identified, but undoubtedly this protein has a high-molecular weight since it did not migrate appreciably into the small-pore gel. Feeney et al. (1963) had suggested that the band close to the origin in the starch gel is a high-molecular weight compound. More recently, Miller and Feeney (1966) isolated this protein and estimated the molecular weight to be about 800,000. Lysozyme, having an isoelectric point of about 10, did not migrate into the small-pore gel because the pH of the Tris-glycine buffer was 8.1. Purified ovomucoid, when subjected to polyacrylamide gel electrophoresis, could not be detected after staining. Feeney et al. (1963), also unable to detect this protein in his starch gel electrophoretogram, suggested that ovomucoid may be washed out of the gel during the destaining period.

Polyacrylamide gel electrophoresis was found to be an effective technique for assessing the influence of heat treatment of albumen on the alteration of proteins. As shown in Figure 1 (pattern B), the dye intensities of some bands were reduced when albumen at pH 7 was heated to 61.7°C for 3 min. In particular, the conalbumin band 10 and band 12 were absent whereas bands 6 and 11 were greatly reduced in intensity. The other bands in pattern B did not appear to decrease in intensity to any appreciable extent due to the heat treatment of the albumen. When albumen at pH 8.5 was heated at 61.7°C, both of the conalbumin bands 10 and 11 were present with moderate intensities in pattern C of Figure 1. Moreover, the intensity of band 6 was almost comparable to the intensity of band 6 in pattern A for native albumen, while band 12 was barely visible. Heat treatment of albumen at pH 9 did not cause any drastic changes in the electrophoretic pattern. The only bands which diminished in intensity were 7 and 12. Lineweaver et al. (1967), considering band 7 as ovomucoid, observed a loss of band intensity in a starch gel pattern when albumen (pH 9) was heated to 60°C for 5 min. It is of interest to note that no new bands were present in the patterns for heated albumen samples. Presumably,

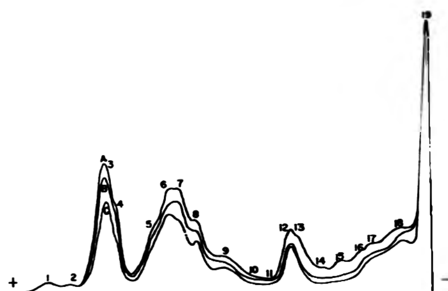

Fig. 4—Densitometric tracings of yolk. A: native; B: heated at 61.7°C for 3 min; C: heated at 65.6°C for 3 min.

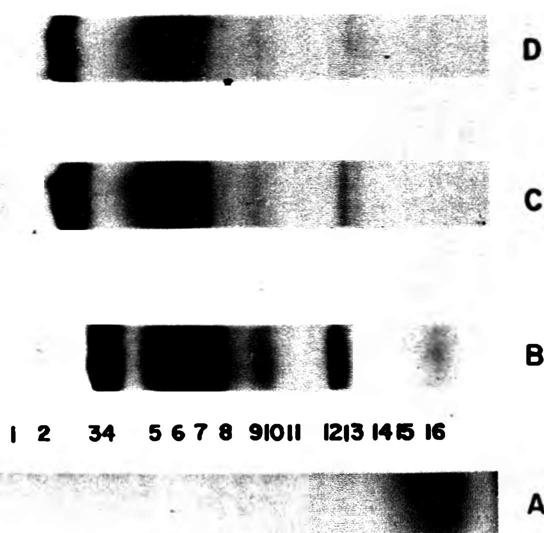

Fig. 5—Electrophoretic patterns of livetins. A: native γ-livetin fraction; B: native α,β-livetin fraction; C: α,β-livetin solution heated to 61.7°C for 3 min; D: α,β-livetin solution heated to 65.6°C for 3 min. Gels prepared with Tris-glycine buffer, pH 8.1 and stained with amido black.

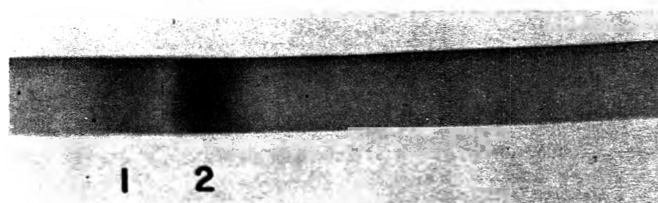

Fig. 6—Electrophoretic pattern of phosvitin. Gel prepared with a Tris-glycine buffer, pH 8.1 and stained with amido black.

the proteins aggregated during heat treatment to very high-molecular weight complexes which were unable to migrate into the small-pore gel.

Judging from our results and those of others (Seideman et al., 1963; Cunningham and Lineweaver, 1965; and Lineweaver et al., 1967), the heat stability of conalbumin is pH dependent. In our study, the gel patterns and densitometric tracings indicated that the heating of albumen at pH values below 9 caused conalbumin aggregation causing electrophoretic immobility. However, at pH 9, no conalbumin loss from the gel was evident. Lineweaver et al. (1967) estimated that more than 50% of conalbumin would be altered when pH 9 albumen was heated for 3.5 min at 62°C. Their estimate was presumably based on studies with purified conalbumin in buffer rather than with the naturally-occurring protein in albumen. Regardless of albumen pH, heating did not cause any apparent change on the intensity of the ovalbumin bands as assessed by the calculation of the areas under the curves in the densitometric tracings. According to Lineweaver et al. (1967), heating of pH 9 albumen at 62°C for 3.5 min altered only 3 to 5% of the ovalbumin whereas negligible amounts of ovalbumin were changed during the heat treatment of albumen at pH 7. Due to the lack of lysozyme migration in the gel, the heat sensitivity of this protein in albumen at various pH levels could not be assessed in our study.

Yolk protein

Preliminary experiments with the gel electrophoresis of yolk indicated that a maximum number of bands were obtained when the diluted yolk contained about 10% NaCl. At this salt concentration, the granules were decomposed. Figure 3 shows the electrophoretic pattern (gels stained with amido black) of proteins and lipoproteins in native and heated yolk. Tracings of these patterns are presented in Figure 4. The pattern for native yolk contained 19 bands. Bands 12 and 13 had a slight affinity for Oil Red O and bands from 16 to 19 were stained strongly with this lipid-soluble stain. Comparison of pattern A in Figure 3 for native yolk with the patterns in Figure 5 for the α,β-livetin fraction and γ-livetin revealed that bands 1 to 16 in pattern A for native yolk are livetin proteins. The diffuse band 16 in the yolk pattern had the same mobility as γ-livetin (pattern A in Fig. 5). Phosvitin bands could not be detected in the pattern A (Fig. 3) for native yolk. However, when isolated phosvitin was used for gel electrophoresis, two diffuse, purple-colored bands (as shown in Fig. 6) moved to the positions of livetin bands 1 and 2 in the yolk pattern A of Figure 3. Using moving boundary electrophoresis, Bernardi and Cook (1960) obtained 3 peaks with isolated phosvitin when the ionic strength of the buffer was 0.3, but only 2 components were detected when the buffer strength was 0.1. McCully et al. (1962) reported that a complexing agent such as EDTA in diluted yolk was responsible for the appearance of a well-defined phosvitin band on paper electrophoretograms. Upon the addition of 1% of this sequestering agent to our dilute yolk solution, faint purple phosvitin bands overlapping on livetin bands 1 and 2 were observed in our gel pattern.

The components in bands 17, 18, and 19 in the yolk pattern A (Fig. 3) were considered to be lipovitellins of the granules. With gel electrophoresis of yolk plasma, a pattern similar to that for livetin (Fig. 5) was obtained. Apparently, the low-density lipoproteins (LDL) in the plasma did not migrate into the small-pore gel. Moreover, migration of isolated LDL was not evident. LDL with an averge molecular weight of 4.8×10^6 (Martin et al., 1959) would not be expected to move into the small-pore gel. On the other hand, lipovitellins with molecular weights of 4.0×10^5 (Bernardi and Cook, 1960b) ought to be able to move slowly through the small-pore gel during electrophoresis.

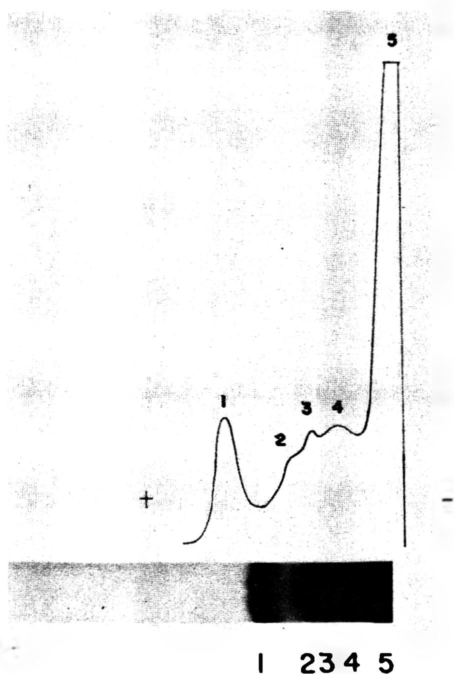

Fig. 7–Electrophoretic pattern and tracing of lipovitellin. Gels prepared with Tris-glycine buffer, pH 8.1 and stained with Oil Red O.

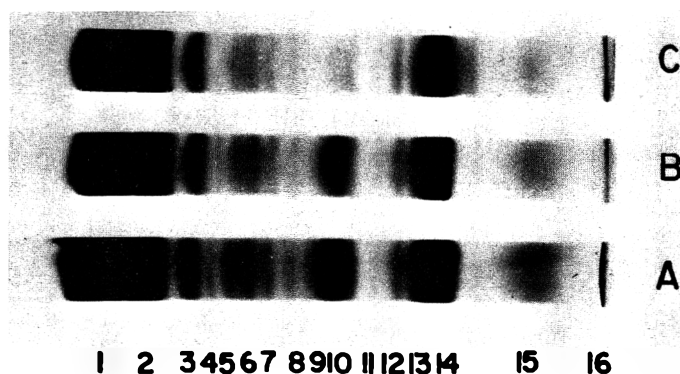

Fig. 8–Electrophoretic patterns of centrifuged whole egg. A: native; B: heated at 61.7°C for 3 min; C: heated at 62.8°C for 3 min. Gels prepared with Tris-glycine buffer, pH 8.1 and stained with amido black.

Figure 7 shows the gel pattern with 5 bands for the isolated lipovitellin fraction and the tracing of the gel. To ascertain the positions of lipid-containing proteins, the gel was immersed in Oil Red O for lipid staining. Major bands 1 and 5 in Figure 7 as well as intermediate bands were stained with the dye. The broad band 1 in Figure 7 corresponds in location to bands 12 and 13 in the yolk pattern A (Fig. 3). Thus the livetin bands 12 and 13 in yolk pattern A were overlapped with a lipovitellin. Immunoelectrophoresis was not carried out to determine whether or not ovalbumin was present in the yolk.

The electrophoretic patterns for yolk heated to temperatures of 61.7°C and 65.6°C for 3 min are shown in Figure 3 B and C. Densitometric tracings of these gels are presented in Figure 4 to illustrate more quantitatively the loss of band intensities upon the heat treatment of yolk. All of the proteins and lipoproteins, with the exception of those in bands 1, 2, and 19 in Figure 4, were heat sensitive to some degree. As shown in patterns B and C of Figure 4, proteins of the α,β-livetin fraction were very susceptible to heat damage. Bands 13, 14, and 15 in Figure 3 B and C were absent, whereas the intensities of bands 3, 4, 6, and 7 were markedly reduced particularly with the 65.6°C treatment. The formation of high-molecular weight aggregates unable to migrate electrophoretically in the small gel may explain the intensity reduction or the disappearance of bands for the heated yolk samples. It was also of interest to compare the electrophoretic patterns of the heated solutions of α,β-livetin with the pattern of the unheated α,β-livetin fraction to assess thermal damage to these proteins in the absence of lipoproteins and phosvitin. As shown in Figure 5 C and D, all of the bands in the patterns for heated livetin solutions were reduced as occurred in heated yolk.

Centrifuged whole egg

The mixing of egg albumen with yolk may not be merely an additive system of proteins and lipoproteins, but certain complexes may be formed. According to Parkinson (1967), comparison of the ion-exchange chromatograms of soluble proteins in albumen and yolk with that for whole egg revealed additional peaks representing new complexes in the whole egg mixture. Since Inoue (1957) reported the interaction of ovalbumin with phospholipid in a buffer system at pH 7, it may be speculated that free phospholipid in yolk (Vincent et al., 1966) may interact with albumen proteins. Cunningham and Lineweaver (1965) mentioned that conalbumin of albumen may react with iron ions of yolk upon the mixing of these egg fractions. Parkinson (1967) noted that a soluble fraction of whole egg from his chromatographic column was colored red, indicating a conalbumin-iron complex. In our study on centrifuged whole egg, gel electrophoresis was employed to detect the presence of any protein complexes which otherwise would not be present in either yolk or albumen. A total of 16 bands were observed in pattern A in Figure 8. Since the granules were removed from whole egg by centrifugation, lipovitellins in the upper part of the gel were absent. Comparison of the bands for albumen (Fig. 1) and for yolk (Fig. 3) with those for centrifuged whole egg in pattern A of Figure 8 indicated that albumen bands were dominant. On the basis of mobility, several bands in Figure 8 A were identified: bands 1, 2, and 3 as ovalbumins (livetin bands 3 and 4 shown in pattern A of Fig. 5 were undoubtedly present in the area of bands 1 and 2 of the whole egg pattern); bands 5, 6, 7, and 8 as α,β-livetin proteins; bands 13 and 14 as conalbumin; band 15 as γ-livetin; band 16 probably is the very high-molecular weight protein of band 12 in the albumen pattern (Fig. 1). The conalbumin bands 13 and 14 in Figure 8 A had about the same mobility rate as the bands of conalbumin in albumen. Presumably the conalbumin in centrifuged whole egg was combined with iron ions of yolk.

When centrifuged whole egg was heated to either 61.7 or 62.8°C for 3 min, the intensities of all the bands except ovalbumin bands 1, 2, and 3 were reduced (Fig. 8). The tracings in Fig. 9 provide a rough quantitative estimation of thermal damage to the proteins. Proteins in bands 9 and 10 were particularly heat sensitive. The greater thermal stability of the conalbumins in heated centrifuged whole egg as contrasted to the almost complete conalbumin damage in heated albumen (Fig. 1) may be attrib-

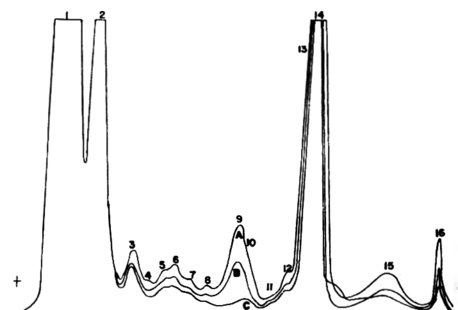

Fig. 9–Densitometric tracings of centrifuged whole egg: A: native; B: heated at 61.7°C for 3 min; C: heated at 62.8°C for 3 min.

uted to the complexing with the yolk iron. Azari and Feeney (1958) have shown that iron ions increased the heat stability of conalbumin. The high-molecular weight albumen protein (band 16 in Fig. 8) was less heat labile in centrifuged whole egg than in albumen. A new band next to conalbumin band 14 was noted in pattern C for 62.8°C-heated centrifuged whole egg.

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Ms. received 12/2/68; revised 5/11/70; accepted 5/12/70.

This investigation was supported in part by Public Health Service Grant No. UI-136-05 from the National Center for Urban and Industrial Health.

Approved by the Director of the Research Division of the College of Agricultural and Life Sciences, University of Wisconsin.

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EFFECTS OF FOUR SPECIES OF BACTERIA ON PORCINE MUSCLE. 1. Protein Solubility and Emulsifying Capacity

SUMMARY—A technique was used to obtain aseptic porcine muscle, portions of which were inoculated with cultures of Pediococcus cerevisiae, Micrococcus luteus, Leuconostoc mesenteroides *and* Pseudomonas fragi. *The inoculated samples were compared with aseptic controls throughout a 20-day storage period at temperatures of 2 and 10°C. All 4 organisms grew at 10°C, but only* P. fragi *and* L. mesenteroides *grew at 2°C. The solubilities of the various protein fractions were affected by inoculation treatment. This was exemplified by correlation coefficients ranging from −.37–0.50. The coefficients indicated the interrelationships affected by storage conditions and bacterial growth. Protein solubility studies revealed a loss in the water-soluble fraction during storage of the controls and the* M. luteus- *and* L. mesenteroides-*treated samples. Samples inoculated with* P. fragi *evidenced an initial loss, followed by an increase. The solubility of meat proteins in a salt solution increased during the first 8 days of storage, then decreased or remained relatively constant for all samples. In comparison with controls, samples inoculated with* P. fragi *increased in salt-soluble protein solubility during the first 8 days, whereas those inoculated with* L. mesenteroides *decreased during the latter part of storage. Insoluble protein generally increased except for* P. fragi-*inoculated samples, which decreased. Nonprotein nitrogen (NPN) increased for all treatments and controls during the 20-day storage period. NPN extracted from the samples inoculated with* P. fragi *increased greatly. The pH increased with growth of* M. luteus *and* P. fragi *and decreased with growth of* P. cerevisiae *and* L. mesenteroides. *The emulsifying capacity was not influenced by the growth of* M. luteus *or* P. cerevisiae. *However, the emulsifying capacity of samples inoculated with* L. mesenteroides *decreased, whereas that of samples inoculated with* P. fragi *increased.*

INTRODUCTION

RECENT studies concerning the effects of microorganisms on muscle during storage have used general inocula in which the species have not been controlled. The purpose of this study was to inoculate porcine muscle with specific cultures and determine the effects of these organisms on some of the properties of the muscle.

Borton et al. (1968a) found that inoculation of porcine muscle with a mixed culture decreased emulsifying capacity, whereas Ockerman et al. (1969) reported an increase in emulsifying capacity of bovine muscle inoculated with a mixed culture. The reason for such discrepancies in results seemed to be due to differences in the predominant types of organisms present in the inocula.

Kirsh et al. (1952) found that nonpigmented *Pseudomonas* and *Aerobacter* microorganisms dominated the flora of fresh ground beef. They also found lactic acid-producing organisms and some cocci organisms. Their findings have been substantiated by numerous other reports including those of Ayres (1955), Wolin et al. (1957) and Halleck et al. (1958). Kitchell (1962) indicated 39% of the microorganisms on fresh pork were micrococci, whereas in products with a high salt content such as hams or bacon, 89–100% of the organisms were micrococci. Shank and Lundquist (1963) found lactic acid bacteria were the primary spoilage agents of vacuum-packaged processed meat products. Such organisms were also found when meat was stored under aerobic conditions.

In this study, the organisms used were chosen to represent the groups normally found on meat and meat products. *Pediococcus cerevisiae* and *Leuconostoc mesenteroides* represented the lactic acid-producing organisms. *Micrococcus luteus* was chosen as representative of the salt-tolerant micrococci and has also been found on fresh beef as reported by Stringer et al. (1969). After preliminary investigations with 2 or 3 species of *Pseudomonas* organisms, *Pseudomonas fragi* was chosen to represent this psychrophilic, proteolytic group, as it grew more readily at the pH of porcine muscle.

MATERIALS & METHODS

Sample procurement

8 180–230-lb hogs were slaughtered at monthly intervals with conventional procedures, except the sticking area of the neck was thoroughly scrubbed with a hexachlorophene bactericidal soap. The scalding, dehairing and evisceration were accomplished normally with additional sanitary precautions (i.e., frequent knife sterilization and hand washing). The unsplit carcass was then rinsed with alcohol and flamed before being placed in a 1–3°C cooler. After cooling for 24 hr, the shoulders were removed, the remaining portion of the carcass placed on a kraft-paper-covered table so that the dorsal midline was easily accessible, and rinsed with alcohol.

Using sterilized equipment, the longissimus dorsi muscle was removed following a procedure similar to that reported by Borton et al. (1968b). A cut was made along the dorsal midline of the backfat cover and 2 cuts made perpendicularly to it, 1, 5–8 cm from the scapula and the second over the tuber coxae. The backfat was stripped and rolled back to expose the longissimus dorsi muscle which was sliced into 3-cm slices, with 1 slice being removed at a time and placed in 1 of 3 containers. The 2 longissimus dorsi muscles were excised in this manner and divided equally into the 3 containers.

Inoculation of the sample

The sample from 1 container was ground through a 2-mm grinder plate with 10 ml of sterilized water being added. The sample was reground and designated as the control. The muscle slices from a second container were treated the same as the control sample except 10 ml of a 1/100 dilution of a 48-hr culture of either *P. cerevisiae, L. mesenteroides, M. luteus* or *P. fragi* were used to inoculate the sample. After regrinding the first inoculated sample, the grinder was cleaned and resterilized. The inoculation procedure was repeated for the second culture. After grinding and inoculation, each of the samples was divided aseptically into 13 jars each containing 60–70 g of sample. 1 jar of each sample was used as the day 0 sample, with 6 of the remaining jars being stored at 2°C and 6 at 10°C. The samples were then analyzed after 2, 4, 8, 12, 16 and 20 days of storage.

Bacterial numbers

The method outlined by the American Public Health Association (1958) was used for determination of the number of bacteria per gram of sample. 11 g of sample were blended in a sterile blender with 99 ml of sterilized water. After appropriate dilution, 1.0 or 0.1 ml was pipetted into sterile disposable petri dishes to which APT agar was added. The number of colonies which had grown on each plate was counted after incubation at 25°C for 48–72 hr.

Protein solubility

The extraction procedure outlined by Helander (1957) was used. It included extraction of the water-soluble protein with 0.03 M K PO_4 buffer, pH 7.4, extraction of the salt-soluble protein with 1.1 M KI, 0.1 M K PO_4 buffer, pH 7.4 and precipitation of the water-soluble protein with 10% trichloracetic acid to obtain a nonprotein nitrogen (NPN) fraction. The micro-Kjeldahl procedure outlined by the American Instrument Company (1961) was used to determine the nitrogen content of the samples and the extraction aliquots.

RESULTS & DISCUSSION

Bacterial growth

The amount of microbial growth which took place in each treatment group is shown in Figures 1–4. The samples were not sterile, as growth was noted on

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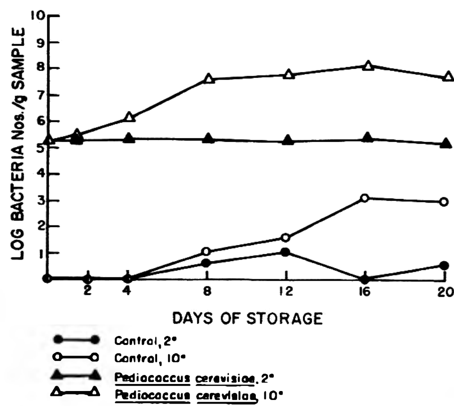

Fig. 1–Log of bacterial numbers per gram of control and Pediococcus cerevisiae-inoculated porcine samples stored at 2 and 10°C for 20 days.

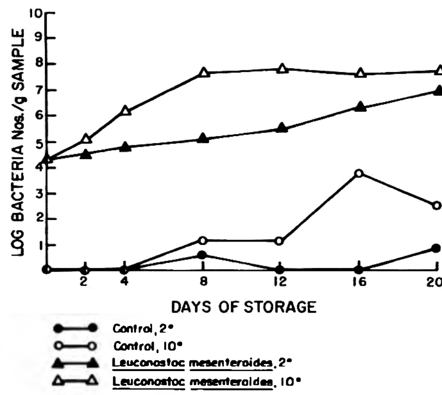

Fig. 2–Log of bacterial numbers per gram of control and Leuconostoc mesenteroides-inoculated porcine samples stored at 2 and 10°C for 20 days.

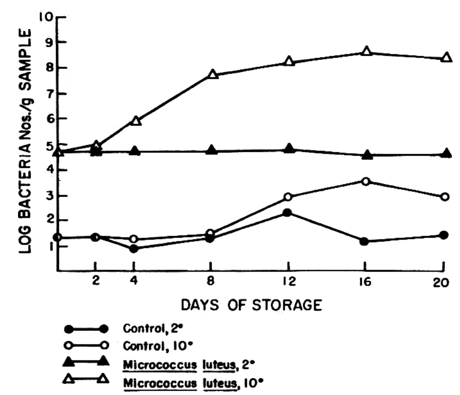

Fig. 3–Log of bacterial numbers per gram of control and Micrococcus luteus-inoculated porcine samples stored at 2 and 10°C for 20 days.

control samples during the 20-day storage period. However, counts were seldom over 10,000 organisms per gram, which was quite low, as counts of 1–95 million organisms per gram of fresh ground beef were reported by Kirsh et al. (1952). Growth for the control samples was slightly higher on those stored at 10°C than on those stored at 2°C.

P. cerevisiae and *M. luteus* organisms grew on porcine muscle when incubated at 10°C but not when incubated at 2°C (Fig. 1 and 3). In both cases the amount of growth did not exceed 100 million organisms per gram when stored at 10°C and there was no loss of viability at 2°C. *L. mesenteroides* grew at both storage temperatures, with the growth at 10°C being more rapid than that at 2°C (Fig. 2). Growth of these organisms at 10°C reached a peak of approximately 50 million after 8 days of storage and remained at that level throughout the remainder of the storage period. There was a continual increase in the number of organisms found on samples stored at 2°C during the 20-day storage period. *P. fragi* grew the fastest and showed the greatest increase in number of organisms of any species studied. The growth of this organism at 2°C was about 4 days slower than that recorded on the samples stored at 10°C (Fig. 4). The amount of growth approached 10 billion organisms per gram for samples stored at both temperatures. There was some decrease in bacterial counts for this organism during the latter days of storage at 10°C (days 12–20).

pH

Since the pH of meat has been reported to influence the extractability of meat proteins (Scopes, 1964) and emulsifying capacity (Swift and Sulzbacher, 1963), the influence of the 4 organisms on the pH of the samples is shown in Figure 5. The samples inoculated with the acid-producing organisms *(P. cerevisiae* and *L. mesenteroides)* and stored at 10°C had a lower pH than the control samples. However, at 2°C, growth of *L. mesenteroides* did not affect the pH. The samples inoculated with *M. luteus* and stored at 10°C evidenced an increasing pH but not to the same extent as found for the samples inoculated with *P. fragi* and stored at 2 and 10°C.

Protein solubility

The solubility of the various protein fractions of 2 control samples is shown in Figures 6 and 7. There was a significant decrease ($P \leqslant .01$) in the amount of water-soluble protein nitrogen during the 20-day storage period at both storage

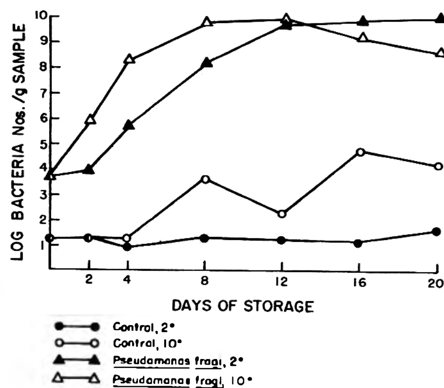

Fig. 4–Log of bacterial numbers per gram of control and Pseudomonas fragi-inoculated porcine samples stored at 2 and 10°C for 20 days.

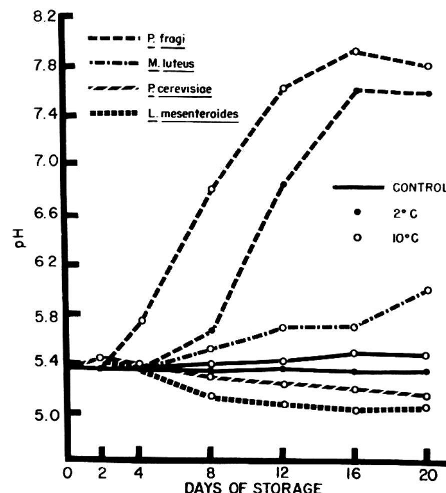

Fig. 5–pH of control and inoculated porcine samples stored at 2 and 10°C for 20 days.

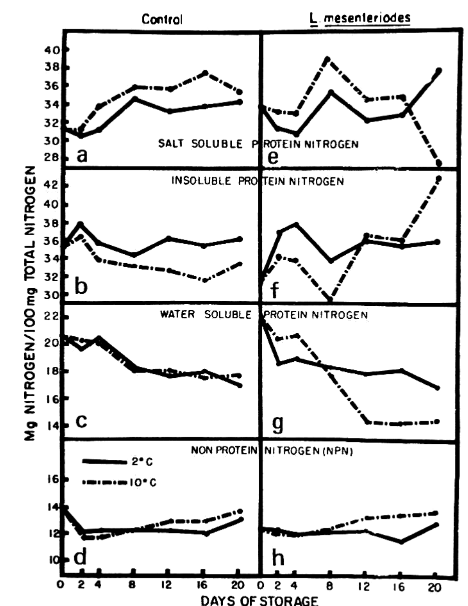

Fig. 6–Solubility of the various protein fractions of Leuconostoc mesenteroides-inoculated (e–h) and related control (a–d) porcine muscle stored at 2 and 10°C for 20 days.

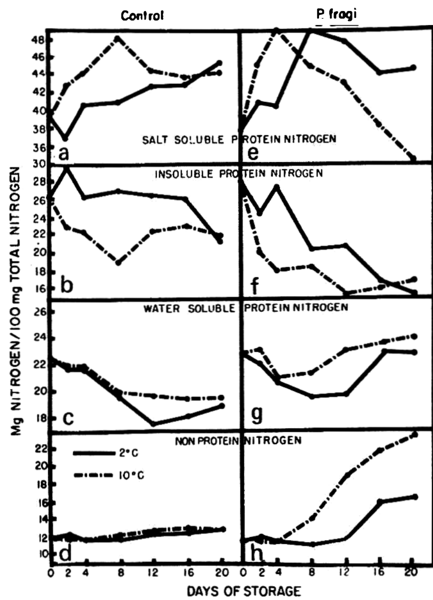

Fig. 7–Solubility of the various protein fractions of *Pseudomonas fragi* (e–h)-inoculated and related control (a–d) porcine muscle stored at 2 and 10°C for 20 days.

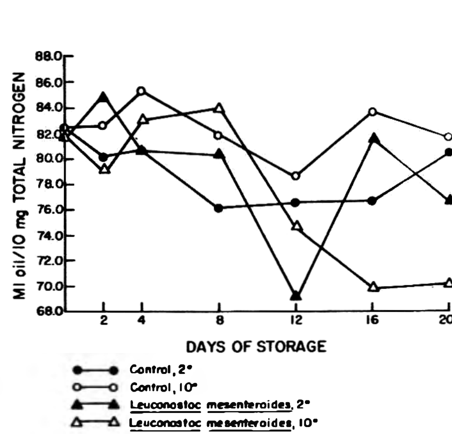

Fig. 8–Emulsifying capacity of control and *Leuconostoc mesenteroides*-inoculated porcine muscle samples stored at 2 and 10°C for 20 days.

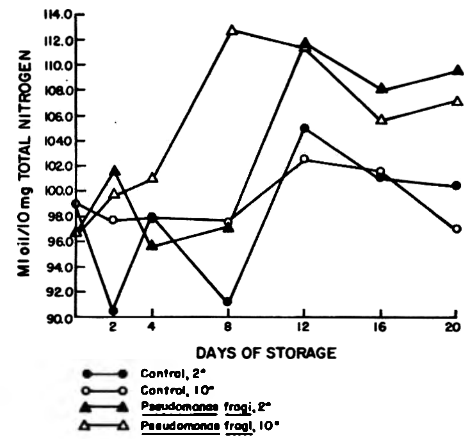

Fig. 9–Emulsifying capacity of control and *Pseudomonas fragi*-inoculated porcine muscle samples stored at 2 and 10°C for 20 days.

temperatures. The solubility decreased during the first 8 or 12 days and then remained relatively constant during the remainder of the storage period. These results are in agreement with those of Sayre and Briskey (1963) and McLoughlin (1963). The amount of extractable salt-soluble protein nitrogen increased during the first 8 days of storage, after which it remained constant or decreased for the control sample stored at 10°C, whereas that of the sample stored at 2°C increased or remained constant throughout the storage period. These results were in general agreement with those reported by McIntosh (1967). The amount of insoluble protein nitrogen decreased significantly ($P \leqslant .01$) during storage of the control samples. The mean over-all non-protein nitrogen (NPN) found in control samples stored at 10°C was significantly higher ($P \leqslant .01$) than that found in those stored at 2°C, with the difference between the 2 means being 0.5 mg NPN/100 mg of total nitrogen. An increase ($P \leqslant .01$) in NPN was also noted during the 20-day storage period. The samples inoculated with *P. cerevisiae* and *M. luteus* evidenced protein solubility patterns similar to those of the control samples.

The samples inoculated with *L. mesenteroides* and stored at 10°C did have different protein solubilities from those of the controls, whereas those stored at 2°C were not significantly different from the controls (Fig. 6). The amount of water-soluble protein nitrogen was significantly lower ($P \leqslant .05$) at days 12, 16 and 20 (14.47, 14.98 and 15.27 mg/100 mg total nitrogen) than the amount found in the control samples (all $\geqslant$ 17.0 mg/100 mg total nitrogen), which can be seen by comparing Figures 6c and 6g. There was little difference in the amount of salt-soluble protein nitrogen of the *L. mesenteroides*-inoculated samples when compared to controls, except after 20 days of storage at 10°C, at which time a considerable decrease was noted (Fig. 6e). There was no difference between the NPN content of the *L. mesenteroides*-inoculated samples and controls. The insoluble fraction increased greatly at day 20,

Table 1–Emulsion stability as measured by oil and water separation of control and inoculated samples at day 0 and after 12 days of storage at 2 and 10°C.

Sample/ time/hr emulsion held	Control		*Pediococcus cerevisiae*		*Leuconostoc mesenteroides*		*Micrococcus luteus*		*Pseudomonas fragi*	
	Oil[a]	Water[a]	Oil[a]	Water[a]	Oil[a]	Water[a]	Oil[a]	Water[a]	Oil[a]	Water[a]
Day 0										
0	0	0	0	0	0	0	0	0	0	0
.25	0	0	0	0	0	0	0	0	0	0
.50	0	0	0	0	T	0	0	0	0	0
.75	0	0	0	0	T	0	0	0	0	0
1	T	0	T	0	1	0	0	0	0	0
2	T	0	T	0	1	0	0	0	T	0
24	T	0	T	0	1	0	T	T	T	0
48	1	T	1	0	2	0	T	T	T	T
Day 12–2°C										
0	0	0	0	0	0	0	0	0	0	0
.25	0	0	0	0	0	0	0	0	0	0
.50	0	0	0	0	0	0	0	0	0	0
.75	0	0	0	0	T	0	0	0	0	0
1	T	0	0	0	T	0	0	0	T	T
2	T	0	T	0	1	0	0	0	T	2
24	T	1	1	0	1	0	T	T	T	4
48	T	1	1	0	1	0	T	T	1	5
Day 12–10°C										
0	0	0	0	0	0	0	0	0	0	0
.25	0	0	0	0	0	0	0	0	0	0
.50	0	0	0	0	0	0	0	0	0	0
.75	T	0	0	0	T	0	0	0	T	T
1	T	0	0	0	T	0	T	0	T	T
2	T	0	T	0	1	0	T	0	T	1
24	1	0	1	0	1	0	T	0	1	4
48	1	0	1	0	1	0	T	0	1	5

[a] Ml of separation–oil at top of graduated cylinder, water at bottom. T = Trace amount (definite separation but not 1 ml).

the result of the loss of solubility of the water and salt-soluble proteins. The loss of solubility of these fractions was significantly related ($P \leqslant .01$) to the decreased pH, with correlation (r) values of 0.50 and 0.39 for water and salt-soluble fractions, respectively.

The amount of nitrogen found in the various fractions of the *P. fragi*-inoculated samples is compared to the controls in Figure 7. Inoculation of porcine muscle samples with this organism and storing at 2 and 10°C for 20 days caused the greatest change in the amounts of the various nitrogen fractions when compared to control samples. The *P. fragi* over-all water-soluble protein nitrogen mean of 22.14 mg/100 mg total nitrogen was significantly higher ($P \leqslant .01$) than the 20.28 mean of the controls. The water-soluble protein of the *P. fragi*-inoculated samples decreased until 4 days at 10°C and until 8 days at 2°C and then increased throughout the remainder of the storage period (Fig. 7g). The salt-soluble protein nitrogen extracted from the *P. fragi*-inoculated samples stored at 10°C was the highest at day 4, whereas it was highest at day 8 for those stored at 2°C (Fig. 7e). After this time, there was a marked decrease in the amount of salt-soluble protein nitrogen, especially in the samples stored at 10°C. There was a decrease in the amount of insoluble protein nitrogen (Fig. 7f) from the *P. fragi*-inoculated samples, the result of the increasing solubility of the water and salt-soluble protein nitrogen fractions and the NPN fraction. The NPN fraction of samples inoculated with this organism increased considerably during the 20-day storage period, with a greater increase in the samples stored at 10°C (Fig. 7h). The increase in NPN content indicates proteolysis had occurred, which also could explain the decrease in salt-soluble fraction. The increase in the water-soluble fraction was correlated ($P \leqslant .01$) with increased pH ($r = 0.37$) and partial proteolysis of the salt-soluble protein ($r = -.37$). Results of the protein solubility study of the *P. fragi*-inoculated samples are in general agreement with those reported by Ockerman et al. (1969).

Emulsifying capacity

The emulsifying capacity did not seem to be influenced by storage temperature or time, as with protein extractability. The samples inoculated with *M. luteus* and *P. cerevisiae* had almost the same emulsifying capacity as related controls. The emulsifying capacities of control and *L. mesenteroides*-inoculated porcine samples stored at 2 and 10°C are shown in Figure 8. Disregarding the result of the inoculated sample stored at 2°C for 12 days, the control samples stored at 2 and 10°C and the inoculated samples stored at 2°C had emulsifying capacities in the same general range. The inoculated sample stored at 10°C exhibited a decrease in emulsifying capacity from day 8 to days 16 and 20 similar to the decrease in pH noted in Figure 5. These results are similar to the results reported by Borton et al. (1968a). The relationship of the emulsifying capacities of control and *P. fragi*-inoculated samples is shown in Figure 9. The mean over-all emulsifying capacity of 103.9 ml oil/10 mg total nitrogen for the *P. fragi*-inoculated samples was significantly higher ($P \leqslant .01$) than the over-all mean of 98.5 ml oil/10 mg total nitrogen for the control samples. This type of relationship can be seen for the latter days of storage in Figure 9.

The higher emulsifying capacity was related ($P \leqslant .05$) to increased pH ($r = 0.33$), which may increase protein extractability and thereby affect emulsion formation (Hansen, 1960). Results obtained in this study with *P. fragi*-inoculated samples were in general agreement with those reported by Ockerman et al. (1969).

Emulsion stability

The stability results are shown in Table 1. It should be noted that even though samples inoculated with *P. fragi* had a greater emulsifying capacity, such emulsions were very unstable and separated easily when they were allowed to set at room temperature. The other inoculated samples produced emulsions as stable as the control samples.

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Ms. received 2/18/70; revised 6/15/70; accepted 7/3/70.

Journal Article 4996, Michigan Agricultural Experiment Station.

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EFFECTS OF FOUR SPECIES OF BACTERIA ON PORCINE MUSCLE
2. Electrophoretic Patterns of Extracts of Salt-Soluble Protein

SUMMARY—Electrophoresis of 0.6 M KCl extracts of porcine longissimus dorsi muscle revealed little change in the type or number of protein bands found either by starch-urea or disc-urea gel electrophoresis. The 0.6 M KCl extracts of muscle samples inoculated with Pediococcus cerevisiae, Leuconostoc mesenteroides *and* Micrococcus luteus *and stored at 2 and 10°C for 20 days did not differ electrophoretically from control samples. Extracts of samples inoculated with* Pseudomonas fragi *showed a loss in the number of protein bands on starch-urea gel and disc-urea gel electrophoresis, indicating this organism exhibited some proteolytic effect upon the myofibrillar proteins.*

INTRODUCTION

INTEREST in the types of protein found in muscle, especially the salt-soluble, has been considerable in recent years. In most cases the researcher has been interested in isolation and purification of a particular type of protein. Rampton (1969) used a disc-urea gel electrophoretic method in an attempt to identify the various salt-soluble or myofibrillar proteins. Awad et al. (1968) used a similar method for studying the effect of frozen storage on beef muscle. Rampton (1969) studied the effect of microbial growth on the myofibrillar proteins but found no evident differences. The purpose of this study was to determine if growth of specific microorganisms on porcine muscle altered the electrophoretic behavior of the salt-soluble extract using 2 methods of electrophoresis.

MATERIALS & METHODS

Sample procurement, bacterial counts and pH

The procedures for sampling, inoculation, bacteria counts and pH were the same as those reported by Borton et al. (1970).

Extraction of salt-soluble proteins

10 g of the muscle sample were blended in 20 ml of deionized distilled water, and extracted for 30 min, centrifuged at 10,000 × g for 20 min and the residue resuspended in 80 ml of water and extracted for 1 hr. After centrifugation, the extraction with 80 ml of water was repeated. The residue was suspended in 60 ml of Weber-Edsall solution (0.6 M KCl, carbonate buffer, pH 9.2) and gently agitated for 20–24 hr. The mixture was centrifuged 1 hr at 25,000 × g and the supernatant filtered through cheese cloth. The filtrate was dialyzed against 8.0 M urea for 16–18 hr, after which it was ready for electrophoresis. The extraction procedure was similar to that reported by Rampton (1969). Extraction was done on samples after 0, 8 and 20 days of storage.

Starch-urea gel electrophoresis

The method used was a modification of one reported by Neelin and Rose (1964) for myogen extracts. The gel was formed by adding 30 g of starch to 200 ml of a buffer composed of 0.076 M Tris and 0.005 M citric acid, pH 8.6. This mixture was heated to a temperature of approximately 60°C, then 72 g of urea were added and the gel heated to approximately 86°C. Immediately after heating, the mixture was deaerated under vacuum and poured into a 2-layered gel tray. The slot former, which formed 6 slots, was positioned about 6 cm from 1 end of the tray. After sufficient hardening, a polyvinyl film was placed over the gel to prevent dehydration while the gel was allowed to set overnight. After removal of the film and slot former, the salt-soluble extracts were placed in the slots and then covered with vaseline. The gel tray was placed horizontally between 2 buffer tanks with each end of the tray resting on the inside edge of a buffer tank. The tanks contained a solution made up of 0.6 M boric acid and 0.2 M sodium hydroxide, pH 8.9. Filter-paper bridges served as conductors between the tank solutions and the gel. The slots were positioned nearest the cathode and 350 v were applied. Electrophoresis was done at 2–6°C and continued until the leading citrate boundary had moved 10 cm beyond the slots. After electrophoresis, the gel was sliced and ½ was stained with a solution of 1% Buffalo Black NBR dye in a 5:4:1 solution of methanol, water and acetic acid for 20 min. The gel was destained in fresh portions of the same solution.

Disc-urea gel electrophoresis

The method outlined by Davis (1964) and modified by Rampton (1969) was used. The running gel contained a final concentration of 6.5% cyanogum which replaced the acrylimide-bis-acrylimide used by Davis (1964). The spacer gel contained 5.0% cyanogum. Both the running and spacer gels contained 7.0 M urea purified over MB-3 resin. The gels were placed in glass tubes and polymerized by fluorescent light for 20 min. The tank buffer used for electrophoresis was a Tris-glycine buffer, pH 8.5. 3 drops of bromothymol blue were added to the buffer and 0.05 ml of the salt-soluble protein extract applied with a pipette to the surface of the gel beneath the tank buffer. A current of 2 ma per gel was maintained for protein electrophoresis. Electrophoresis was completed when the leading bromothymol blue band reached the end of the gel. The gel was removed from the glass tube by sliding a hypodermic needle along the internal surface and forcing water along the side of the gel. The gel was placed in a test tube and stained 20 min in a 0.5% Buffalo Black NBR dye solution of water, methanol and acetic acid (5:5:1). The gel was held overnight in a destaining solution of water, methanol, acetic acid and glycerol (5:5:1:1) and then electrophoretically destained in the same solution at 200 v for approximately 4 hr. The gel was then stored in a 7.5% acetic acid solution.

RESULTS & DISCUSSION

THE GROWTH of the microorganisms at 2 and 10°C was discussed fully in a previous report (Borton et al., 1970). The results of starch-urea gel electrophoresis of 0.6 M KCl extracts of control porcine samples and samples inoculated with *Pediococcus cerevisiae* and *Leuconostoc mesenteroides* stored for 8 and 20 days at 2 and 10°C are shown in Figure 1. The results are representative of all 4 replications. At day 0 there was no difference between samples, and 6–9 bands were generally evident, with 7 bands present in the results shown. In this group there was no difference between the electropherograms of the inoculated and control samples nor were there any differences due to storage temperature. There were some changes as the length of storage increased. At day 8 the first, second and third bands were more distinct than at day 0, and at day 20 the first band was less diffuse than at day 8. These differences were probably associated with changes occurring with the resolution of rigor.

The electropherograms of 0.6 M KCl extracts of control porcine samples and samples inoculated with *Micrococcus luteus* and *Pseudomonas fragi* stored 8 and 20 days at 2 and 10°C are shown in Figure 2. At day 0, 9 bands were evident but no differences were found between the control and inoculated samples. The number of bands increased to 14 by day 8. These electropherograms exhibited the largest number of bands of all the samples studied. However, the general trend was typical of the various replicates of samples inoculated with these organisms. At day 8, as at day 0, there were no differences in the number or pattern of the bands due to treatment or storage temperature. 1 or 2 bands disappeared by day 20 for all of the samples, but those inoculated with *Pseudomonas fragi* had the least number of bands. This loss of protein was probably due to proteolytic action of the organisms.

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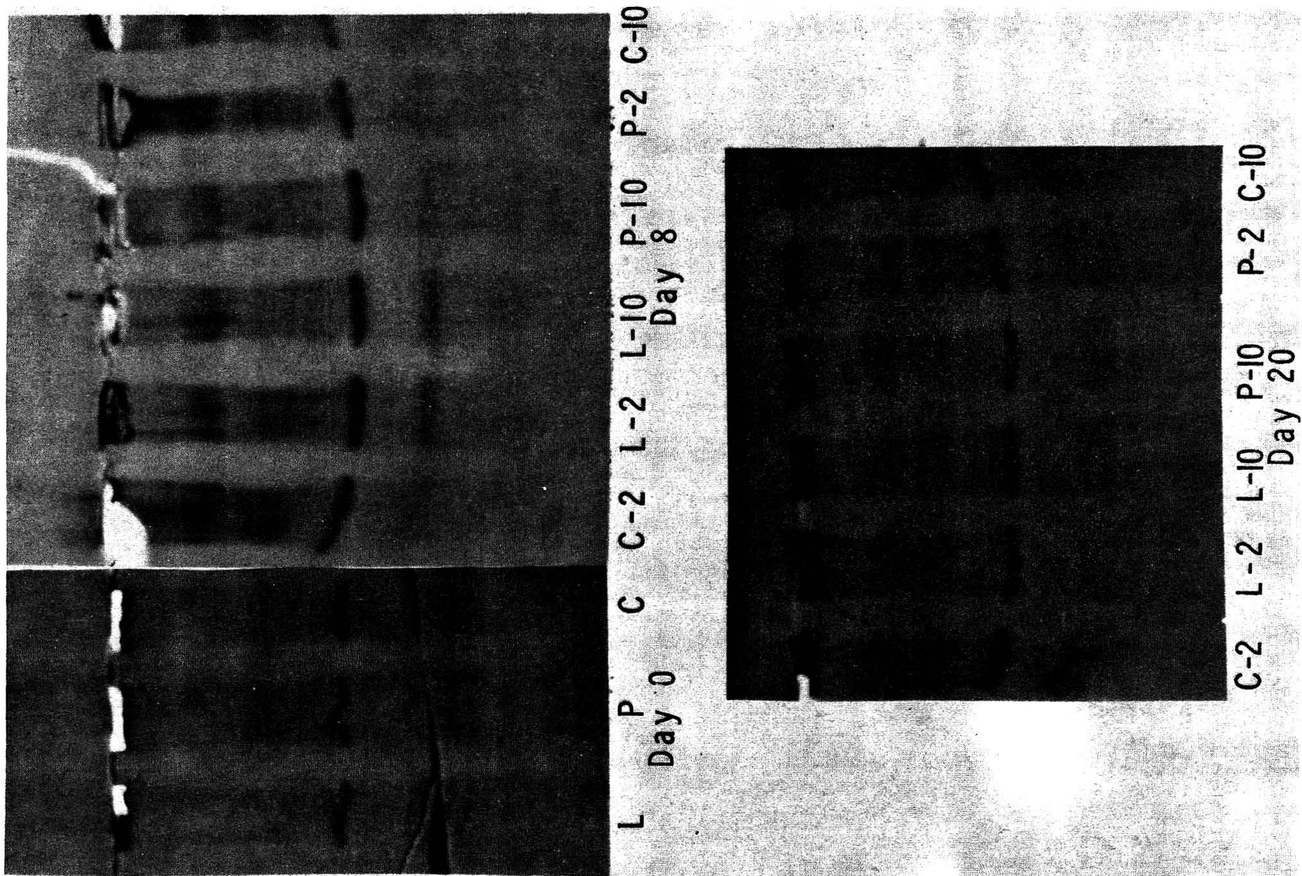

Fig. 1—Starch-urea gel electropherograms of 0.6 M KCl extracts of control porcine muscle samples (C) and samples inoculated with Pediococcus cerevisiae (P) and Leuconostoc mesenteroides (L) stored 0, 8 and 20 days at 2 and 10°C.

Fig. 2—Starch-urea gel electropherograms of 0.6 M KCl extracts of control porcine muscle samples (C) and samples inoculated with Micrococcus luteus (M) and Pseudomonas fragi (F) stored 0, 8 and 20 days at 2 and 10°C.

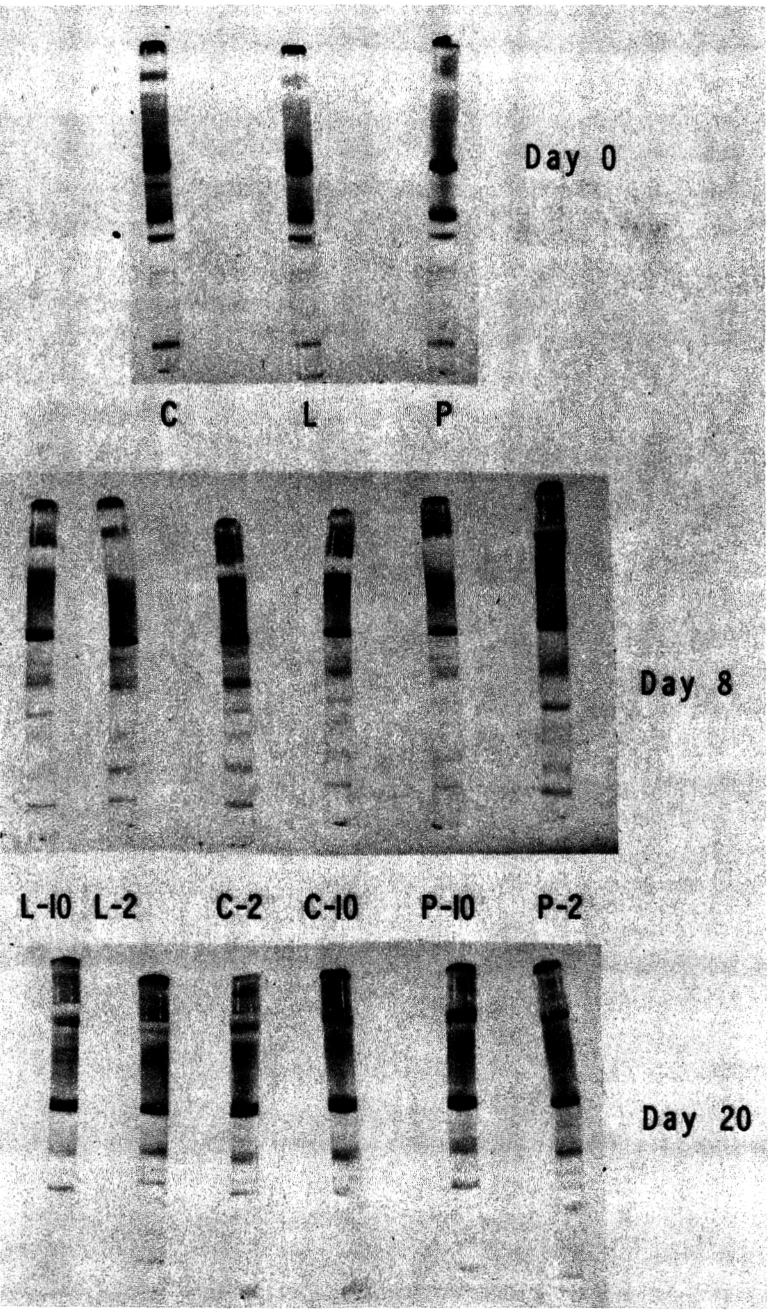

Fig. 3—Disc-urea gel electropherograms of 0.6 M KCl extracts of control porcine muscle samples (C) and samples inoculated with Pediococeus cerevisiae (P) and Leuconostoc mesenteroides (L) stored 0, 8 and 20 days at 2 and 10°C.

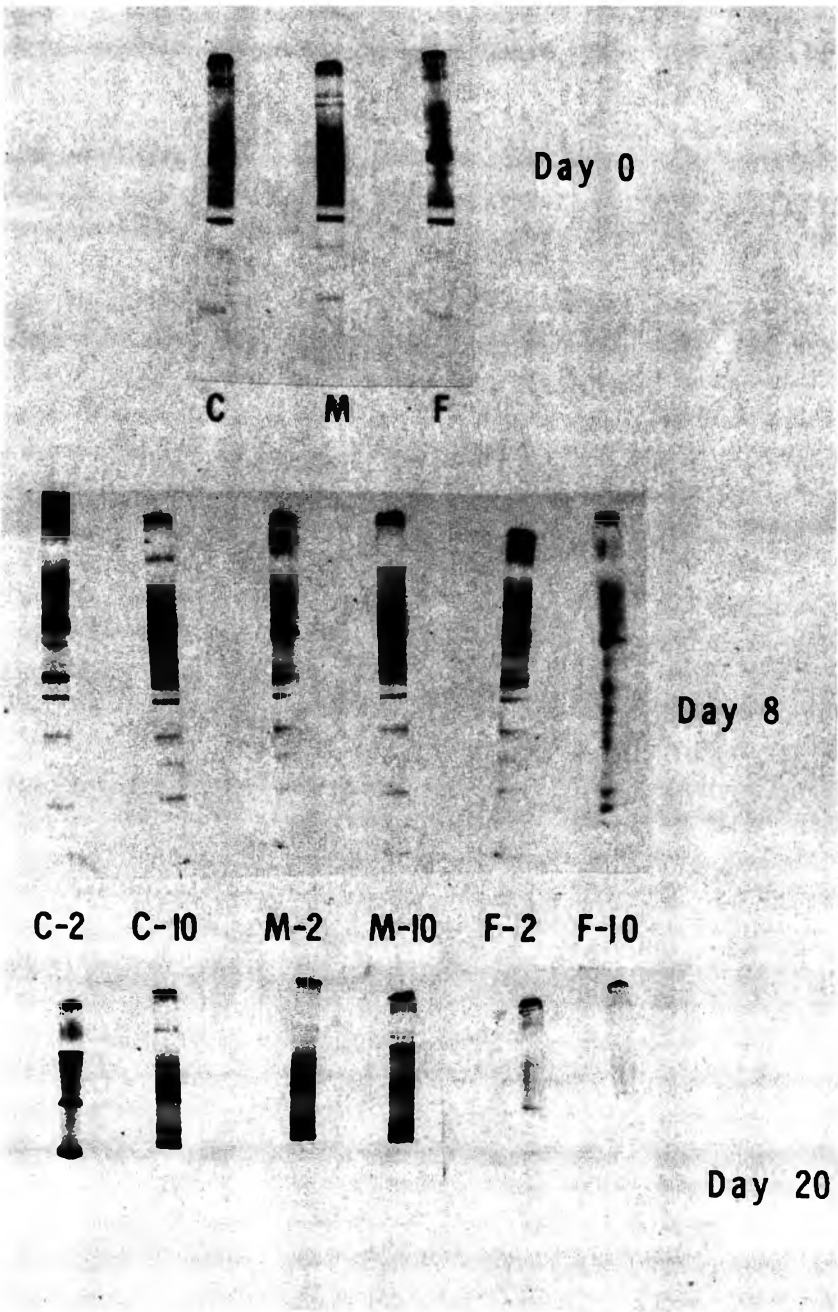

Fig. 4—Disc-urea gel electropherograms of 0.6 M KCl extracts of control porcine muscle samples (C) and samples inoculated with Micrococcus luteus (M) and Pseudomonas fragi (F) stored 0, 8 and 20 days at 2 and 10°C.

The results of disc-urea gel electrophoresis of 0.6 M KCl extracts of control samples and samples inoculated with *P. cerevisiae* and *L. mesenteroides* and stored for 8 and 20 days at 2 and 10°C are shown in Figure 3. There were at least 14 bands present at day 0 as compared to the 7 present on the starch-urea gel of the same extract. There was little change in the band patterns at day 8 and day 20, except stain intensities varied slightly.

The disc-urea gel electropherograms of 0.6 M KCl extracts of control porcine samples and those inoculated with *M. luteus* and *P. fragi* stored at 2 and 10°C for 8 and 20 days are shown in Figure 4. As in Figure 3, 14 bands were evident at day 0. At day 8 the band patterns were similar to those of day 0 except they were more distinct. The pattern of the extract of *P. fragi*-inoculated samples stored 8 days at 10°C was different from the others, in that 3 bands appeared, whereas only a single band was found on the other samples. The increased pH of this sample may have caused some alterations of the protein, thereby changing the banding pattern. By day 20 extracts of the samples inoculated with *P. fragi* and stored at 2 and 10°C had lost many bands, whereas the samples inoculated with the other species showed little change. Difficulty was encountered when separating the water wash from the sample tissue, due to the higher water-holding capacity of the sample associated with a higher pH. This difficulty indicated that possibly some of the salt-soluble proteins were present in the supernatant. However, when the supernatant was collected, recentrifuged at a higher speed, the residue extracted with 0.6 M KCl and subjected to disc-urea gel electrophoresis, the same pattern as shown in Figure 4 for the *P. fragi*-inoculated samples was found. This indicated some proteins may have been washed out, but they were the same proteins as were present in the extracts from the inoculated samples (F-2, F-10, Day 20, Fig. 4); therefore, all that was lost was stain intensity of the protein bands. These results indicate that proteolysis of the salt-soluble protein occurred as a result of growth of *P. fragi*. These results support those reported earlier by Borton et al. (1970).

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Ms. received 2/18/70; revised 6/15/70; accepted 7/3/70.

Journal Article 4997, Michigan Agricultural Experiment Station.

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THE INFLUENCE OF LACTIC CULTURES ON GROUND BEEF QUALITY

*SUMMARY—The effects of adding 5 and 10% lactic culture (*Streptococcus lactis *and* Leuconostoc citrovorum*) grown in skim milk; 2.5, 5, 10, and 20% lactic cultures grown in 20% milk solids; 1 and 2% frozen concentrated lactic cultures; lactic acid to pH 5.0 and 4.5; and 2.5, 5, and 10% skim milk to ground beef stored at refrigeration temperature (7°C) were studied in the 6 preliminary trials. Results obtained were used to formulate the principal investigation. The influence of 10% lactic culture and 10% lactic culture plus 450 ppm of ascorbic acid were tested in 5 replicate trials for the principal investigation. Cultures used in the replicate trials were grown in 20% milk solids. CVT (crystal violet tetrazolium) counts for gram-negative bacteria, pH, VNC (volatile nitrogen content), and organoleptic observations were evaluated. Data from the preliminary study indicated a profound inhibitory action of the lactic cultures on the growth of the inherent gram-negative bacteria in ground beef. The addition of 10% lactic culture grown in 20% milk solids was effective in preventing aerobic growth. The addition of pure lactic acid inhibited microbial growth, but caused an undesirable color and aroma. Frozen concentrated cultures required 1% lactose to inhibit the growth. The CVT count in the uncultured meat significantly increased ($P < .01$) as the storage time progressed. Cultured meat did not exhibit a significant increase in CVT count until 7 days of storage. The pH of the cultured meat significantly declined ($P < .01$) whereas the uncultured meat indicated a significant increase in pH ($P < .01$) during storage. VNC in the uncultured meat was significantly higher ($P < .01$) than in the cultured meat. Cultured meat with 450 ppm of ascorbic acid was consistently preferred for flavor, aroma, and when color was compared with the uncultured meat and the meat with culture alone.*

INTRODUCTION

GROUND BEEF keeps well for only a short period at refrigeration temperatures. This is attributed mainly to the deterioration caused by the rapid growth of spoilage microorganisms which cause decomposition of meat. Ample evidence has been established that the majority of the psychrophilic meat-spoilage bacteria (about 90% of the total population) are of the gram-negative type. A study was made by Brown et al. (1958) on selected properties of 189 psychrophilic bacteria isolated from chilled beef and associated sources. Of these, 182 were gram-negative and 7 were gram-positive. In the same study, they also reported that there were 170 pseudomonads out of 182 gram-negative bacteria isolated. Kirsch et al. (1952), Halleck et al. (1958), Ayres (1960), Gardner et al. (1966) and Stringer et al. (1969) are among several other researchers who also concluded that *Pseudomonas-Achromobacter* group constitute the most predominant microflora in fresh beef under refrigerated storage. During refrigerated storage of meat, coliform bacteria, yeasts, molds, and species of *Micrococcus* and *Streptococcus* increased. At all periods these organisms formed only a minor proportion of the total flora (Gardner et al., 1966).

At present very litle research is being conducted on the use of microorganisms as a tool for retention of meat quality. On the other hand, lactic cultures are commonly used to improve the quality and shelf-life of dairy products. Since milk products and meat are similar in regard to nutritive properties and types of microorganisms (gram-negative non-spore forming, rod shaped bacteria—mostly *Pseudomonas-Achromobacter* group) causing spoilage, it was presumed that lactic cultures could be used to retard bacterial deterioration in ground beef by inhibiting undesired bacterial growth, and thus improving the shelf-life.

Lactic cultures most commonly used in the dairy industry consist of a mixture of *Streptococcus lactis* or *Streptococcus cremoris*, which produce lactic acid from lactose, and *Leuconostoc* species or *Streptococcus diacetilactis*, which produce biacetyl and other volatile compounds from citrates in milk. Production of the inhibitory substances and inhibitory action of lactic cultures towards spoilage organisms in dairy products have been studied by Baribo et al. (1951), Collins (1961), Marth et al. (1962) and Mather et al. (1959). They reported that the lactic cultures produce antibiotic-like inhibitory substances which have a profound effect on a variety of gram-negative spoilage type bacteria in dairy products.

This study was undertaken to determine what effect the inoculation of ground beef with lactic cultures had on the rate and extent of gram-negative bacterial growth, meat deterioration and shelf-life. Since no reports were available in this area of work, preliminary trials using different culture concentrations, combinations, and forms were conducted. These studies were designed to learn their effect on inhibiting the gram-negative bacterial growth thus reducing microbial deterioration in ground beef. Results of these preliminary trials were used to formulate the principal study.

MATERIALS & METHODS

6 PRELIMINARY TRIALS and 5 principal trials were conducted to determine the effect of added lactic cultures on the deterioration and inherent gram-negative bacterial growth in ground beef stored at 7°C.

In the preliminary trials, the following test materials were added to study their effects on the gram-negative bacterial growth (CVT count), pH, volatile nitrogen content (VNC) and organoleptic criteria in ground beef.

Trial 1—Lactic culture (5 and 10%) propagated in regular skim milk; and 5 and 10% sterile skim milk added to the meat homogenate.

Trial 2—Lactic culture (2.5, 5 and 10%) and 2.5, 5 and 10% sterile reconstituted milk added to the meat homogenate.

Trial 3—A pure culture of *S. lactis* (10%); 10% pure culture of *L. citrovorum* plus lactic acid to bring the pH of the meat homogenate to 5.0; 10% pure culture of *L. citrovorum* plus lactic acid to bring the pH of the meat homogenate to 4.5; lactic acid to bring the pH of the meat homogenate to 5.0; lactic acid to bring the pH of the meat homogenate to 4.5; and 10% lactic culture added to the meat homogenate.

Trial 4—A pure culture (20%) of *S. lactis* and 20% lactic culture added to the ground beef.

Trial 5—Lactic cultures (5, 10 and 20%) to the ground beef.

Trial 6—Frozen concentrated lactic cultures (1 and 2%) and 1 and 2% frozen concentrated lactic cultures plus 1% lactose added to the ground beef.

In the principal study, 5 replicate trials were conducted to determine the effects of 10% lactic culture and 10% lactic culture plus 450 ppm of ascorbic acid on CVT count, pH, volatile nitrogen content and organoleptic criteria in ground beef.

Cultures and other materials were added on a ground beef weight basis to obtain the desired concentrations. Frozen concentrated cultures used in the preliminary trial were received fresh from Angevine-Funke Co., St. Louis, Mo. and contained about 14 billion organisms of *S. lactis* plus *L. citrovorum* per ml.

Fresh coarse ground beef was obtained from the meat laboratory and from a retail store. Test materials were directly mixed with the meat samples designated to receive treatment. The control sample received no treatment. Treated and control samples were run through the grinder with 3/16" hole plate to obtain regular size ground beef and then placed in polyethylene bags for storage. The bags were not sealed to provide anaerobic conditions. However, the meat homogenate (200g ground beef blended in 800 ml of sterile distilled water) used in the preliminary trials (Trials 1 to 3) was treated directly with the test materials and dis-

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Table 1–Analysis of variance for CVT count, pH, VNC, flavor, aroma and color as influenced by lactic cultures in ground beef.

	Log CVT count		pH	VNC		Flavor		Aroma	Color
Source	df	F	F	df	F	df	F	F	F
Total	59			119		359			
Treatment	2	24.49**	271.92**	2	259.59**	2	2.69	3.50*	4.25*
Day	3	31.20**	61.38**	3	98.63**	3	34.41**	40.97**	22.52**

*P <.1.
**P <.01.

pensed into sterile screw cap bottles for storage. Samples were stored at 7°C for different intervals before studying the treatment effects.

The lactic cultures used consisted of *S. lactis* and *L. citrovorum* propagated as described by Hammer et al. (1957). These cultures were grown in the reconstituted milk with 20% milk solids (20g milk solids in 80 ml of distilled water) unless otherwise specifically stated. Cultures thus grown contained about twice the number of viable organisms of cultures grown in regular skim milk (about 2 billion/ml). *S. lactis*, *L. citrovorum*, or both *S. lactis* and *L. citrovorum* were inoculated into the reconstituted milk to make the desired culture. In the work herein reported, the term "lactic culture" refers to the culture containing both *S. lactis* and *L. citrovorum.*

Inherent gram-negative bacterial counts in the meat were determined by standard bacteriological pour plate methodology (A.P.H.A., 1967) using crystal violet tetrazolium chloride (CVT) agar for plating. CVT agar was prepared as described by Olson (1967), consisting of Standard Plate Count agar with 1 ppm Bacto-crystal violet (Difco) added at the time of heating the medium. Just before pouring the plates, 50 ppm of 2,3,5 triphenyl tetrazolium chloride was added to the medium as a 1% solution in 50% alcohol. Required meat tissue homogenates were directly pipetted from the test samples for making the serial dilutions where meat homogenate was used. When ground meat was used, 11g representing the meat samples were blended with 99 ml of phosphate-buffered saline diluent (Sulzbacher, 1953; Lewis et al., 1964) and required sample aliquots were pipetted directly from this homogenate to make appropriate dilutions. Duplicate platings at two appropriate decimal dilutions were made for each sample. Plates were incubated at 32°C for 2 days before counting.

The pH of the meat homogenate and ground meat was measured with a standardized (pH 5.0 ± 0.01) pH meter by placing the electrode directly into the samples. When ground meat was used, approximately 10g of the appropriate sample was mixed with 5 ml of distilled water for pH determination. All the samples were allowed to equilibrate with room temperature (22°C) before measuring the pH.

Volatile nitrogen determinations, as described by Pearson (1968), were used to assess the protein degradation in the samples. Duplicate determinations were made for each sample tested.

The general method adopted for organoleptic evaluation was that described by Peryam et al. (1957). A 150g sample was prepared for flavor evaluation. The sample was filled into a glass petri dish cover, placed about 7.5 cm from the flame and broiled in a gas oven. Both meat surfaces were exposed to the flame. All the samples were broiled for approximately the same time and temperature. The cooked sample was divided into 6 approximately equal portions for panel evaluation. About 500g of the uncooked sample was displayed against a white background for color and aroma evaluations. Light intensities and other environmental conditions were the same throughout the study. The panel of 6 members consisted of staff, graduate students and secretaries. The panel was not trained primarily for this study; however, the members were considered competent to evaluate organoleptic criteria of meat. Panel members were asked to score the cooked samples for flavor and the uncooked samples for color and aroma. A 9 point hedonic scale with a neutral point was used for scoring the samples. A score of 9 was the highest rating and 1 the lowest.

Experimental data for the preliminary trials are not reported; however, results of these trials are discussed in brief. Data obtained in the 5 replicate trials of the principle study were used for statistical analysis and reported in the tables.

RESULTS & DISCUSSION

Preliminary trials

CVT count. The addition of milk did not inhibit the gram-negative bacterial growth as indicated by CVT counts in the ground beef. Reduction of the meat pH to 5.0 and 4.5 by adding lactic acid definitely reduced the development of gram-negative bacteria; however, at the same pH level, the sample with added *L. citrovorum* had a considerably lower CVT count, indicating the additional inhibitory action of *L. citrovorum*. Addition of lactic acid to bring the sample pH to 5.0 or 4.5 severely affected the color and aroma of the samples. However, when the pH of the cultured samples was brought to 5.0 or 4.5 by the culture organisms, the color and aroma were not affected to the extent observed in the samples with lactic acid. This suggested the impracticality of the addition of lactic acid to meat to inhibit the inherent gram-negative bacterial growth. The results indicated that the lactic cultures had a pronounced inhibitory effect on the gram-negative bacteria in ground beef. The effect became greater as the amount of culture used increased up to the 10% level (10 ml of lactic culture grown in 20% milk solids exerted greater inhibition than the cultures grown in the regular skim milk, when the same amounts of the cultures were added to the meat). Lactic cultures did not exhibit any pronounced advantage over pure culture of *S. lactis* in suppressing the microbial growth; however, the former had an advantage for flavor and aroma when added to the meat. The addition of 10% lactic culture (grown in 20% milk solids) was found to be completely effective in preventing inherent gram-negative bacterial growth. When 10% lactic culture was added to the meat, CVT counts remained constant during the 7 days of storage and were practically the same as those on the initial day. However, in the control samples, counts increased by 3 to 4 log numbers during storage. Frozen concentrated cultures were effective only when 1% lactose was added. The inhibitory effect of these cultures was not as pronounced as it was with the cultures grown in the 20% milk solids.

pH. Frozen concentrated cultures without lactose, skim milk, and pure culture of *L. citrovorum* did not influence the pH of the meat samples during the storage. However, addition of lactic cultures resulted in a decline in pH of the samples. The pH values of the cultured meat samples decreased during the first 2 days of storage, then held fairly constant during the remainder of the storage.

Volatile nitrogen content. Consistently less VNC was detected in the cultured meat samples and increases in concentration of added cultures resulted in decreased volatile nitrogen production in the samples. Frozen concentrated cultures when added with 1% lactose resulted in slightly lower VNC than in the control meat samples during the storage.

Organoleptic. Taste panel results for flavor and aroma indicated a slight preference for cultured meat samples; however, color scores were generally much lower for cultured samples throughout the study. Hence, in an effort to improve the color of cultured meat, food color and ascorbic acid were tested. A concentration of 450 ppm of ascorbic acid (460 ppm of ascorbic acid is legally permitted in the meat products to be processed) was considered adequate to retain and/or improve the color in the cultured meat samples.

With the above results of the preliminary trials, it was decided to add 10% lactic culture (grown in 20% milk solids) and 10% lactic culture plus 450 ppm of ascorbic acid to further test their effects

Table 2–Log CVT counts, pH and volatile nitrogen as influenced by lactic cultures in ground beef (Trials 7–11).

Treatment	Log CVT Count[a] per gram sample				pH[a]				Volatile Nitrogen mg/100g sample			
Storage (Day)	0	3	5	7	0	3	5	7	0	3	5	7
Meat with no culture (control)	5.65	7.60	8.56	8.70	5.65	5.61	5.70	5.75	25.15	29.64	35.49	40.18
Meat with 10% culture	5.50	5.73	5.78	6.24	5.37	4.84	4.82	4.75	23.80	25.27	26.60	27.93
Meat with 10% culture + Asc. acid 450 ppm	5.54	5.73	5.84	6.26	5.37	4.84	4.83	4.78	23.91	25.97	26.25	27.65

Least Significant Differences (LSD)

		Day	Log CVT Count	pH	Volatile Nitrogen[a] mg/100 g sample
Treatment Effect		0	Not Significant	$5.65 > 5.37$ ($P < .01$)	Not Significant
		3	$7.60 > 5.73$ ($P < .05$)	$5.61 > 4.84$ ($P < .01$)	$29.64 > 25.97$ ($P < .01$)
		5	$8.56 > 5.84$ ($P < .01$)	$5.70 > 4.83$ ($P < .01$)	$35.49 > 26.60$ ($P < .01$)
		7	$8.70 > 6.26$ ($P < .01$)	$5.75 > 4.78$ ($P < .01$)	$40.18 > 27.93$ ($P < .01$)
Day Effect	Treatment				
	Control		$8.70 > 7.60 > 5.65$ ($P < .01$)	$5.75 > 5.61$ ($P < .01$)	$40.18 > 35.49 >$ $29.64 > 25.15$ ($P < .01$)
	10% Cul.		$6.24 > 5.5$ ($P < .05$)	$5.37 > 4.84$ ($P < .01$)	$27.93 > 25.27$ $26.60 > 23.80$ ($P < .01$)
	10% Cul. + Asc. Acid		$6.26 > 5.54$ ($P < .05$)	$5.37 > 4.84$ ($P < .01$)	$27.65 > 25.97 > 23.91$ ($P < .05$); $27.65 > 23.91$, $26.25 > 23.91$ ($P < .01$)

[a]Mean values for five trials.

on the gram-negative bacterial growth, deterioration as measured by VNC and organoleptic criteria in ground beef.

Principal study

CVT count. A significant treatment effect ($P < .01$) indicated the profound influence of the cultures in altering the log CVT counts in the samples (Table 1) A steady increase in CVT count was noted in the uncultured meat sample throughout the storage period (Table 2). The counts on the cultured sample and on the cultured sample with ascorbic acid added were practically identical throughout the storage period and were much lower (at least 2 log count difference) than those of the control sample. These data indicated a definite inhibitory effect of lactic cultures on the growth of the inherent gram-negative bacteria in ground beef. Further analysis, using least significant difference (LSD) test, indicated no significant difference in log CVT counts for the initial day sample (Table 2). This was expected because the cultures had no time to grow and exert their inhibitory effect in the initial day treated samples. However, at the later part of the storage, the counts in the cultured samples were significantly lower than the control sample ($P < .05$, $P < .01$, and $P < .01$ respectively) at 3, 5 and 7 days storage (LSD test, Table 2), sustantiating the inhibitory effect of the cultures.

These results are in general agreement with Baribo et al. (1951); Collins (1961); Marth et al. (1962); and Mather et al. (1959) who reported the inhibitory effect of the lactic cultures towards gram-negative spoilage type organisms in dairy products.

A non-significant difference in log CVT counts was found (LSD test, Table 2) between meat with only the culture and culture with ascorbic acid. This suggested that 450 ppm of ascorbic acid had no inhibitory effect on CVT count in ground meat.

A significant day effect ($P < .01$), suggested that the log CVT count at different storage intervals was different for the same treatment. Analysis by LSD test indicated a significant increase ($P < .01$) in the CVT count in 3 and 7 day stored control samples when compared with the same treated samples at 0 and 3 days storage respectively. On the other hand, cultured samples did not exhibit a significant increase in the CVT count until 7 days of storage.

pH. A significant difference in pH ($P < .01$) between cultured and uncultured meat samples existed due to the treatment effect (Table 1). The pH in both cultured meat samples was essentially the same but lower ($P < .01$) than the uncultured samples. The LSD test, (Table 2) indicated a significantly lower pH ($P < .01$) in the cultured meat samples throughout the study. A significant decline in pH for the initial day cultured samples was due to the acidity (pH 4.5 to 4.6) of the cultured medium.

A significant day effect ($P < .01$) on pH change was substantiated by the analysis of variance (Table 1). The control sample at 7 days storage had a significantly higher pH ($P < .01$) than the sample at 3 days storage (LSD test, Table 2). These results are in agreement with Bodwell et al. (1965); Jay (1964); and Pearson (1968a) who reported a pH rise in meat during storage at refrigeration temperature. During the first 3 days of storage, the cultured samples exhibited a highly significant decline in pH ($P < .01$) which was held constant until 7 days of storage (LSD test, Table 2).

Volatile nitrogen content. Treatment effect was significant ($P < .01$) and superseded the day effect (Table 1). VNC continuously increased ($P < .01$) in the control sample (Table 2) and was always greater ($P < .01$) than that in the cultured samples. These findings are in general agreement with Pearson (1968) who reported an increase in VNC of meat as the days of storage increased. Samples with culture and culture plus ascorbic acid did not significantly differ from each other at any tested storage time.

VNC was not significantly different (LSD test, Table 2) in the control and treated samples at 0-day analysis. However, a significant increase ($P < .01$)

Table 3–Flavor, aroma, and color as influenced by lactic cultures in ground beef (Trials 7–11).

Treatment	Flavor Score[a]				Aroma Score[a]				Color Score[a]			
Storage (Day)	0	3	5	7	0	3	5	7	0	3	5	7
Meat with no culture (control)	6.77	4.77	4.03	3.50	5.97	3.63	3.70	3.13	6.30	4.17	4.17	4.73
Meat with 10% culture	6.67	5.00	4.53	4.83	5.83	4.20	3.93	3.50	6.63	3.63	2.97	2.67
Meat with 10% culture + Asc. acid 450 ppm	6.80	5.80	4.63	4.90	5.60	4.63	4.10	3.93	6.37	5.77	5.10	4.43

Least Significant Differences (LSD)

		Day	Flavor Score	Aroma Score	Color Score
Treatment Effect		0 3 5 7	Not Tested (Treatment effect was not significant by AVO)	Not Tested (Treatment effect was not highly significant by AVO)	Not Tested (Treatment effect was not highly significant by AVO)
Treatment					
Day Effect	Control		6.77 > 4.77 > 3.50 (P < .01)	5.97 > 3.70 > 3.13 (P < .01) 3.63 > 3.13 (P < .01)	6.30 > 4.73 (P < .05) 6.30 > 4.17 (P < .01)
Day Effect	10% Cul.		6.67 > 5.00 (P < .01)	5.83 > 4.20 > 3.13 (P < .01) 3.63 > 3.13 (P < .05) 3.93 > 3.50 (P < .01)	6.63 > 3.63 (P < .01)
Day Effect	10% Cul. + Asc. Acid		6.80 > 5.80 (P < .05) 6.80 > 4.90 (P < .01) 5.80 > 4.63 (P < .05)	5.60 > 4.63 > 4.10 (P < .01) 4.63 > 3.93 (P < .01)	6.37 > 5.10 (P < .05) 6.37 > 4.43 (P < .01) 5.77 > 4.43 (P < .05)

[a]Mean values for 5 trials.

occurred in the 3-, 5- and 7-day control sample. Significantly lower VNC in the cultured samples assured a definite inhibitory action of the lactic cultures on the inherent gram-negative spoilage type organisms in the ground beef.

The analysis of variance (Table 1) also showed a highly significant day effect ($P < .01$) which was supported by further analysis (LSD test, Table 2). The latter indicated a highly significant increase in VNC ($P < .01$) of control samples at 3-, 5- and 7-day storage. Significantly increased VNC ($P < .01$ and $P < .05$) was observed in both the cultured samples as the storage time increased. However, this increase was significantly lower ($P < .01$) than the control (LSD test, Table 2).

Flavor. The analysis of variance revealed a non-significant treatment effect for flavor scores (Table 1). However, mean values (Table 3) clearly showed panel preference for cultured meat samples over the control throughout the storage time. Of the two cultured samples, the one with ascorbic acid was scored higher at any given storage time. Considerably large within sample mean square value (2.74) was noticed indicating a large variation in the panel score for any given sample due to varied preference of the panel members for the cultured meat samples.

The analysis of variance (Table 1) suggested a highly significant day effect ($P < .01$). Further analysis by LSD test (Table 3) indicated significant decreases in flavor scores ($P < .01$ and $P < .05$) in both the treated and untreated samples due to the storage time. The decline in the flavor score during storage was greatest in the control sample and was least in the cultured sample with ascorbic acid. The reason for a non-significant increase in the flavor scores of cultured samples after 5 days storage was not clearly understood.

Aroma. Treatment influenced the aroma score ($P < .1$) and the cultured samples were generally rated higher than the control sample (Table 3 and 1). Cultured samples with ascorbic acid was preferred over the sample with culture alone. Within sample mean square was considerably large (2.30) and indicated a selective preference of the panel members for the treated samples.

Analysis of variance (Table 1) showed a significant day effect ($P < .01$). Scores were significantly decreased ($P < .01$) in the control and treated samples after 3 days and subsequent storage time (LSD test, Table 3). As the storage time increased, a decline in the score was greatest in the control sample and least in the cultured sample with ascorbic acid.

Color. Panel scores for the color preference were affected by treatment ($P < .1$). However, the significance of the treatment effect was not high (Table 1). Mean values (Table 3) indicated a higher rating for color in the cultured meat sample with ascorbic acid than for the control and for the sample with culture alone. The control sample was rated higher than the sample with culture alone except for the initial day. A drop in the panel scores for color was greatest for the sample with culture alone and least for the cultured sample with ascorbic acid. These data suggested that ascorbic acid aided greatly in maintaining the color in the cultured meat samples.

Analysis of variance indicated a highly significant influence ($P < .01$) of storage time, which was substantiated by LSD test (Table 3). The color score for the control and the sample with culture alone significantly decreased ($P < .01$) on 3 days of storage. However, no significant drop in color score in the cultured meat with ascorbic acid was observed until 5 days of storage. Color score for the control sample was improved after 5 days of storage. These findings are in agreement with those of Kontou et al. (1966) and Jay et al. (1964) who reported a rise in ground beef color scores after 3 to 4 days of storage at refrigeration temperature.

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Ms. received 11/5/69; revised 2/16/70; accepted 2/22/70.

Journal Series paper No. 1924 of the Oklahoma Agricultural Experiment Station.

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SUSCEPTIBILITY TO AMYLOLYSIS OF GAMMA-IRRADIATED WHEAT

SUMMARY—Initial reducing sugars and diastatic activity, expressed as "maltose value," are increased in irradiated wheat and are functions of dose levels in the range 20—200 Krad. Both alpha- and beta-amylases retain their activities in irradiated wheat, but the sensitivities of starch to amylolysis are increased with radiation dose levels. Latent beta-amylase present in resting seeds, is also radioresistant. Susceptibility of starch to radiation treatment is more pronounced with moist than dry grain.

INTRODUCTION

RESULTS OF multi-generation feeding trials with rats have demonstrated conclusively the wholesomeness of wheat gamma-irradiated at insect sterilization (20 Krads) or even at ten times the dose levels (200 Krads) (Aravindakshan et al., 1970). However, there is a possibility that ionizing radiations may alter, to subtle degrees, the physicochemical properties of macronutrients in wheat, either by direct (Long and Lirot, 1957; Kertesz et al., 1959) or indirect action (Khenokh, 1955; Oreshko et al., 1962). Several studies on the effect of ionizing radiation on starch in resting seeds (Lai et al., 1959; Milner and Finney, 1959) and in barley endosperm (Faust and Massey, 1966) have been reported.

Quality of wheat for bread making is determined, among other attributes, by its amylases and, to a lesser extent, proteases. Gamma-irradiation at moderate dose levels improves the baking quality of wheat in terms of loaf volume and crumb structure (Milner and Yen, 1956; Nicholas et al., 1958). Therefore, it is of interest to ascertain whether radiation treatment of wheat induces any change in amylase activities or in their response to substrates. In the present paper, the effects of irradiation on initial reducing sugars and on diastatic activity in wheat is reported. Alpha- and beta-amylase activity in irradiated resting seeds, and radiosensitivity of these enzymes in wheat stabilized at higher moisture levels have also been determined.

MATERIALS & METHODS

SAMPLES OF hard red winter variety wheat, *Triticum aestivum*, (obtained from Food Corporation, India) were made into 50g packets, and exposed at room temperature to a Cobalt 60 source of gamma radiation (Gamma cell 220 – Atomic Energy of Canada Ltd) having a flux of 25,000 rads/min at dose levels varying from 20—200 Krads. Absorption of ionizing radiation was checked with ferrous sulfate and ceric sulfate dosimetry (Jarret, 1965). Samples were stored for 3 wk and ground to flour (60 mesh). Soluble starch of analytical grade was purchased from British Drug Houses, Ltd., London.

Reducing sugars

Total reducing sugars were determined using maltose as a reference standard according to A.O.A.C. methods (1960). Maltose was purified by paper chromatography, since various maltodextrins with lower RF values were present as impurities in commercial samples.

Diastatic activity

10g wheat flour was suspended in 46 ml of 0.1M acetate buffer pH 4.8 and incubated under toluene at 30°C for 1 hr with frequent shaking (A.O.A.C., 1960). The reaction was stopped by addition of 2.0 ml 12% sodium tungstate and 2.0 ml 2N sulfuric acid. The mixture was centrifuged and reducing sugars determined in the supernatant. "Maltose value" was expressed as mg maltose liberated/10g flour/hr at 30°C. Necessary blanks were carried out to correct for reducing sugars originally present in the flour samples.

Preparation of enzymes extract

1g wheat flour was suspended in 100 ml distilled water at 20°C for 2 hr under toluene, with occasional shaking. The suspension was centrifuged and the clear supernatant used as the source of alpha- and beta-amylases.

Selective inactivation of alpha- and beta-amylases

Activities of alpha- and beta-amylases, present in enzyme extracts, were measured separately after their differential inactivation. Alpha-amylase was inactivated by acid treatment (pH 3.4) at 0°C for 30 min and beta-amylase, by thermal treatment (70°C for 10 min) at pH 6.5 (Kneen et al., 1943). The enzyme preparation was then assayed for alpha- and beta-amylase activity as the case may be.

Determination of alpha-amylase activity

A modified procedure of Sandstedt et al. (1939) was followed to measure alpha-amylase activity. 20.0 ml 2% soluble starch, 5.0 ml 0.5% sodium chloride, 2 ml 0.2 M acetate buffer (pH 5.5) and 10 ml enzyme extract were incubated at 20°C. 1.0 ml reaction mixture was withdrawn at intervals and added to 5.0 ml iodine solution (0.002%); color was measured in a Klett-Summerson photocolorimeter using #66 filter. Dextrinogenic activity was expressed in terms of the inversely related galvanometer deflections.

Determination of beta-amylase activity

To determine beta-amylase activity, 20 ml 2% soluble starch, 2.0 ml 0.2M acetate buffer pH 4.8 and 2.0 ml enzyme extract were incubated at 20°C. At intervals, aliquots were removed and the enzyme inactivated with 0.5 ml of 0.5N sodium hydroxide. Reducing sugars were estimated as described above. Enzyme activity was expressed either as mg maltose liberated in 1 hr by beta-amylase present in 1g wheat, or as enzyme units, one unit being 1 mg maltose liberated in 3 min at 20°C.

Release of latent beta-amylase

5% wheat flour slurry in 0.1M acetate buffer pH 4.8 was incubated at 37°C for 2 hr with 50 mg papain activated with 5.0 mg glutathione under toluene, with occasional shaking. Beta-amylase activity was determined in the supernatant.

Stabilization of moisture level in wheat

Wheat samples (11% moisture) were stabilized at 20 and 30% moisture levels; it was ascertained in trials that this could be achieved by soaking the seeds in distilled water for 3 and 5 hours, respectively. The samples were packed in aluminum foil and irradiated immediately. Diastatic activity was determined in dried (at 45°C under vacuum) and powdered samples as described above.

RESULTS

Initial reducing sugars and diastatic activity in irradiated wheat

Results of the effect of radiation treatment on reducing sugars and diastatic activities in irradiated wheat are presented in Table 1. Initial water soluble reducing sugars in control seeds (0.9%) increased by 5 to 28% upon irradiation at dose levels from 20—200 Krad. Diastatic activity expressed as "Maltose Value," representing the rate of hydrolysis of starch mainly by the beta-amylase present in wheat, was calculated taking into consideration the initial reducing sugars in corresponding samples. Concentration of maltose liberated (150 mg/10g wheat flour) after 1 hr incubation was increased by 28—43% as a result of irradiation at 20—200 Krad; the effect was more or less the same above the 40 Krad dose level.

This observed increase in "maltose value" in irradiated wheat may be attributed to either of two possibilities: (1) amylases present in wheat are activated by ionizing radiation resulting in the rapid breakdown of long chain polysaccharides into smaller maltodextrin units; and (2) radiation treatment degrades the starch to oligosaccharide units which are more susceptible to enzyme action, with more reducing sugar thereby being liberated. Both these possibilities were tested in the following experiments.

Effect of radiation on alpha- and beta-amylases

No differences in maltose liberation in 30 min were observed when aqueous

Table 1–Reducing sugars and diastatic activities in irradiated wheat.[a] (Values are averages of 3 experiments.)

Dose level (Krads)	Initial reducing sugar[b]	Maltose Value[c]	% increase in maltose value
0	90	150	–
20	95	172	28
40	105	190	41
60	110	196	43
200	125	211	43

[a]Aqueous extracts of wheat flour were analyzed for reducing sugar values. 10g wheat flour (60 mesh) was suspended in 46 ml 0.1M acetate buffer pH 4.8 resulting in 50 ml total volume and incubated at 30°C for 1 hr to measure diastatic activity.

[b]mg maltose/10g wheat flour.

[c]mg maltose liberated/10g wheat flour at 30°C for 1 hr.

Table 2–Susceptibility of irradiated wheat flour to beta-amylolysis.[a] (Values are averages of 3 experiments.)

Dose level (Krads)	Initial reducing sugar[b]	Maltose value[c]
0	11.6	193
20	12.0	207
40	12.0	222
60	11.6	241
200	12.2	268

[a]Gelatinized wheat flour was suspended in 20 ml 0.1M acetate buffer pH 4.8 and 2.0 ml 1% wheat extract (beta-amylase source). Reaction was stopped after 1 hr with 2.0 ml 12% sodium tungstate and 2.0 ml 2N H_2SO_4. Maltose was estimated in the supernatant after centrifugation.

[b]mg maltose/g wheat flour.

[c]mg maltose liberated/g wheat flour at 30°C for 1 hr.

extracts from unirradiated or irradiated samples were used as beta-amylase source; therefore, the first possibility, i.e., activation of amylases by gamma-radiation was ruled out. Beta-amylase activity expressed as enzyme units, 1 unit being 1 mg maltose liberated in 3 min at 20°C, ranged from 163–167 units/g wheat in the irradiated samples and was close to the control value of 165. Similarly, alpha-amylase activity determined by the change in starch-iodine complex caused by the enzyme acting on 2% substrate at 20°C, was also more or less unchanged. After 90 min incubation, galvanometer deflection changes varied from 60–68 in the irradiated samples as compared with 71 from the unirradiated control.

Some experiments were carried out with 1% aqueous amylase extracts of wheat flour, irradiated from 20–200 Krad at 20°C and then used as the enzyme source. No change in maltose liberation in 30 min was observed in the irradiated samples compared with the control: values varied from 156–167 units/g, as compared with 163 for the unirradiated control. Results indicate that amylases are quite radioresistant in the dose ranges employed, either in bound form or even in dilute solutions where the effect is more indirect.

Susceptibility of irradiated wheat flour to beta-amylolysis

The observed differences in initial reducing sugars or in diastatic activity of irradiated wheat may therefore arise from the second possibility, viz. increased susceptibility of starch to enzyme action. Amylases in flour from wheat irradiated at different doses were selectively inactivated by methods described earlier. The residual gelatinized wheat flour was subjected to amylolysis with enzyme preparations from unirradiated wheat flour. It was observed that irradiated wheat flour was a better substrate for beta-amylase action than the unirradiated sample (Table 2). After 1 hr incubation at 30°C, there was increasingly more maltose liberation with samples irradiated at higher doses. There was higher saccharification as compared with values obtained in Table 1, because gelatinized wheat flour was used as substrate. Initial reducing sugar values were also low and more or less the same in all samples, since most of the soluble sugars were removed in the supernatant during enzyme inactivation.

Latent beta-amylase activity of irradiated wheat

Latent beta-amylase in wheat grain bound to glutenin (Rowsell and Goad, 1962) was released and total activity assayed. The initial amylase (free amylase) activity varied between 163–169 units/g wheat in irradiated and unirradiated wheat. The total beta-amylase units ranged from 436–440 in all the samples. Latent activity was calculated as the difference between total and free activity and expressed as % of total. It was observed that about 62% of the total beta-amylase activity in resting seed was in dormant form and remained almost the same in samples irradiated at different dose levels. This suggests that the enzyme was neither inactivated nor released by radiation treatment.

Effect of radiation on wheat stabilized at higher moisture levels

Results of the diastatic activity of irradiated wheat stabilized at higher moisture levels are presented in Table 3. Changes in "maltose value" due to radiation treatment at 20% and 30% moisture levels, as compared with 10% moisture level, reveal greater depolymerization of starch, as evident from appreciable increase at 20–200 Krad.

DISCUSSION

EFFECT OF irradiation on wheat starch in situ has been evaluated by measuring the changes in the initial water soluble reducing sugars and "maltose value" (Table 1). Increases in initial reducing sugars in irradiated samples suggest that starch has been degraded by random cleavage of glycosidic bonds. This modification is a function of dose level in the range studied. This is further evident by the observed increase in maltose liberated dur-

Table 3–Effect of radiation on diastatic activity of wheat stabilized at higher moisture levels.[a] (Results are averages of 2 experiments.)

Dose level (Krads)	"Maltose value" mg maltose liberated/10 gm/1 hr at 30°C					
	10% moisture	% increase	20% moisture	% increase over control (10%)	30% moisture	% increase over control (10%)
0	152	–	197	29	232	52
20	172	13	207	36	236	55
40	190	25	211	39	356	134
60	196	28	247	62	368	142
200	211	38	261	72	368	142

[a]Moisture levels were stabilized at 20 and 30% by soaking seeds in distilled water for 3 and 5 hr respectively. Seeds were irradiated, dried under vacuum, powdered, and diastatic activity was determined as described previously.

ing autolysis of irradiated wheat flour. Such an increase in "maltose value" in irradiated samples can be attributed either to depolymerization of starch or to the activation of amylases. The first possibility seems to be more plausible, since radiation treatment exhibited no effect on alpha- and beta-amylases present either in free or in bound form in wheat. It would imply that the susceptibility of starch to enzyme action was increased due to its degradation. Lee (1959) also noted no activation of beta-amylase due to radiation treatment. However, at high dose levels, beta-amylase in very dilute solution is inactivated (Scoppa and Tafuri, 1964).

It was observed in this study that in resting seeds about 62% of total beta-amylase was in bound form, contrary to 80% as reported by Rowsell and Goad, (1962). This may be due to strain differences.

In the present study, wheat starch degradation was observed even at dose levels as low as 20 Krad even though Kertesz et al. (1959) reported that this takes place beyond 60 Krad.

Radiosensitivity of soaked seeds was more pronounced, since significantly higher "maltose values" were obtained in seeds stabilized at higher moisture levels before irradiation (Table 3). This also corresponds to the water content of seeds. Osborne et al. (1963) and Wallace (1959) found that higher humidities are more damaging. Kamra and Kesavan (1969) reported that microwave restoration of post-irradiation injury is maximum in dry barley seeds (3% moisture) and negligible in moist seeds (11%). Relatively higher metabolic activity of the wet seeds may contribute to their increased vulnerability to radiation damage (Biebl and Mostafa, 1965).

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Ms. received 9/18/69; revised 4/20/70; accepted 5/16/70.

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EFFECT OF GAMMA RADIATION ON WHEAT STARCH AND ITS COMPONENTS

SUMMARY—Studies on the susceptibility of irradiated wheat starch, amylose and amylopectin to alpha- and beta-amylolysis reveal that they are more susceptible to enzyme actions, compared to their unirradiated controls; however, irradiated amylose seems to be comparatively more vulnerable. From irradiated starch, series of oligosaccharides of the maltose series are discernible, while glucose appears only above 200 Krad dose level. Quantitative analysis of the radiolytic breakdown products of starch reveal that at high dose levels (1 Mrad) maltose, maltotriose and maltotetrose are the main products. Results on the separation of radiolytic breakdown products suggest they resemble those produced by alpha-amylolysis of starch.

INTRODUCTION

IN WHEAT ENDOSPERM, due to radiation treatment, starch is degraded into oligosaccharides which are more susceptible to enzymatic hydrolysis (Ananthaswamy et al., 1970). This is shown to be a function of the moisture content of starch (Osborne et al., 1963). Depolymerization of starch seems to be a direct effect of radiation, since anaerobic or aerobic conditions during irradiation exert no differences (Sosedov and Vakar, 1961). The ultimate products of starch breakdown by irradiation have been characterized by several workers (Bourne et al., 1956; Ulmann and Ritcher, 1959) and these include low molecular weight dextrin and deoxycompounds (Scherz, 1968). However, it is to be ascertained whether straight chain amylose or branched-chain amylopectin is responsible for the observed changes in the irradiated starch.

In the present studies, the susceptibility of irradiated starch and its components to alpha- and beta-amylolysis were studied. Initial reducing sugars formed are an index of susceptibility to radiation. Radiolytic breakdown products of starch were characterized, and action patterns of alpha- and beta-amylase hydrolyses studied to elucidate their nature in relation to enzymatic hydrolysis.

MATERIALS & METHODS

FLOUR (60 mesh) from hard red winter variety wheat *Triticum aestivum* (obtained from Food Corporation, India) was kneaded into dough and the starch isolated by expelling it from gluten under running water. The suspension was centrifuged at 3,000 × g for 15 min and the upper pigmented layer scraped out. The sediment was washed with methanol, dried at 50°C in vacuum, and analyzed for moisture (15%) and protein (not detectable).

Amylose, amylopectin, crystalline bacterial alpha-amylase from *Bacillus subtilis* and beta-amylase from barley were Sigma products; taka-diatase was a commercial preparation from Parke Davis & Co., Bombay, India; and Sephadex G-75, medium grade, was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden.

Susceptibility of isolated starch and its components

Aqueous solutions of starch, amylopectin (1%) and amylose (0.5%) were prepared with the addition of a few drops of 1N NaOH. For alpha-amylolysis, pH of the substrate was adjusted to 6.5 (Sandstedt et al., 1939) and 0.1 mg alpha-amylase/ml was added as an enzyme source. For beta-amylolysis, pH was adjusted to 4.8 and 0.1 mg beta-amylase/ml was added.

Fractionation of radiolytic breakdown products of starch

To characterize the radiolytic breakdown products, aqueous extracts of starch irradiated at different dose levels were lyophilized to smaller volumes. Aliquots were spotted on Whatman No. 1 paper and chromatographed (Hough and Jones, 1962) with maltose and glucose as standards. The RF value of glucose was taken as 1 and comparative RF values of other sugars were determined. For quantitative analysis, spots corresponding to different reducing sugars were cut from the undeveloped chromatogram using the developed one as a marker. Sugars were eluted with distilled water and estimated colorimetrically (Umbreit et al., 1959).

Isolation of the products formed by alpha-amylolysis of starch

100 ml of 1% starch in 0.01M acetate buffer pH 5.5, was incubated with 20 mg taka-diastase at 20°C with frequent shaking. At different time intervals, a 5 ml aliquot was withdrawn, heated in boiling water for 3 min to inactivate the enzyme, and stored over a few drops of chloroform to prevent retrogradation and microbial growth, until analyzed.

For further characterization of hydrolytic products, a 4 ml sample of starch hydrolysate was applied on Sephadex G-75 column (1.5 × 50 cms) and eluted with distilled water. 6 ml fractions were collected and analyzed for total carbohydrate content by the colorimetric method described by Umbreit et al. (1959). Unhydrolyzed starch having higher molecular weight was eluted in the first peak (Nordin, 1962). Hydrolyzed products from the second peak were pooled, lyophilized, and chromatographed for the separation of individual maltodextrins.

Procedure for beta-amylolysis

100 ml of 1% starch in 0.01M acetate buffer pH 4.8, was incubated with 10 mg beta-amylase at 20° with frequent shaking. 5 ml aliquots were removed periodically from the incubation mixture; the enzyme was inactivated by the addition of 0.5 ml 2N NaOH and stored at 20°C. The hydrolytic products were characterized similarly as described for alpha-amylolysis.

RESULTS

Alpha-amylase action on irradiated wheat starch and its components

Results on the susceptibility of irradiated starch and its components to alpha-amylase are presented in Table 1. Differences in the galvanometric deflections at 90 min varied from 112–102 (Klett readings) in irradiated starch samples compared with 118 in the unirradiated control. The rate of alpha-amylolysis of irradiated amylose was higher than that of amylopectin. In a typical instance at 200 Krad, calculated differences in galvanometric deflections at the end of 90 min were 37 with amylose and 7 with amylopectin, taking into consideration the initial and final values of their respective controls. This infers that amylose is more susceptible to radiation damage than amylopectin, and is further confirmed by data in Table 2 which show beta-amylol-

Table 1—Alpha-amylase action on irradiated starch and its components. (Values are averages of 3 experiments.)

Dose level (Krads)	Starch[a]		Amylose[a]		Amylopectin[a]	
	Dextrinogenic activity in terms of deflection in the galvanometer					
	0 min	90 min	0 min	90 min	0 min	90 min
0	186	68	145	76	250	33
20	180	68	139	68	246	31
40	175	66	115	61	242	24
60	169	65	101	59	235	22
200	165	63	90	58	230	20

[a]A mixture of 20 ml 1% starch or amylopectin or 0.5% amylose solution (pH adjusted to 6.5), 2 ml 0.5% NaCl, 2 ml phosphate buffer pH 6.5, and 1 ml enzyme (0.1 mg/ml) was incubated at 20°C. At different time intervals, a 0.25 ml aliquot was removed from the incubation mixture and added to 5 ml iodine solution. The volume was adjusted to 10 ml and the color read in a Klett photocolorimeter at 660 mμ.

Table 2–Action of beta-amylase on irradiated starch and its components.[a] (Values are averages of 3 experiments.)

Dose level (Krads)	Starch		Amylose		Amylopectin	
	Maltose mg/gm					
	Initial	15 min	Initial	15 min	Initial	15 min
0	0.14	77.0	11.1	62.9	2.24	119.5
20	0.21	79.5	12.3	65.3	2.36	120.7
40	0.24	84.2	12.5	67.7	2.60	130.4
60	0.46	91.5	12.9	74.9	2.92	140.0
200	0.70	108.0	13.4	96.4	4.10	144.5

[a]Reaction mixture consisted of 20 ml substrate, 2 ml acetate buffer pH 4.8 and 1 ml enzyme (0.1 mg/ml). Incubation was carried out at 20°C for 15 min. The reaction was stopped by the addition of 1 ml 1N NaOH and maltose was estimated as total reducing sugars, using ferric-iron reagent. Color was read against a reagent blank at 520 mμ.

ysis of irradiated amylose resulted in higher maltose liberation. At a 200 Krad dose level, 35% more maltose was liberated from amylose in 15 min incubation, compared with 20.4% from amylopectin with respect to their unirradiated controls.

Characterization of radiolytic breakdown products of starch

Besides glucose and maltose as degradation products of irradiated starch, a series of oligosaccharides with low RF values were discernible and designated as G_1 for glucose; G_2 for maltose; G_3 for maltotriose, etc. (Fig. 1). The degree of polymerization of these maltodextrins was measured after acid hydrolysis (Johnston, 1965), based on the reducing power relative to that of glucose. Results were quite in agreement, even though this method is considered less sensitive for measuring the chain length of starch. Only enzymatic hydrolysis of starch with pullulanase and beta-amylase is claimed to result in complete hydrolysis (Abdullah et al., 1965). All products were obtained at very low dose levels; however, at higher dose levels, oligosaccharides with higher molecular weights were observed up to the point of application and they were not amenable to separation by paper chromatography.

The quantitative analysis of the radiolytic breakdown products of starch revealed that total reducing sugars increased progressively with dose levels (Table 3). Glucose appeared only above 200 Krad dose levels. Though at 400 Krad, G_5 content was high, at higher dose levels (1 Mrad), maltose, maltotriose and maltotetrose were the chief products. No detectable reducing sugars of smaller molecular weights were present in unirradiated samples.

Characterization of products of enzymatic hydrolysis of starch

The first stage in the alpha-amylolysis of isolated wheat starch was characterized by a rapid decrease in the molecular size as shown by rapid loss of iodine staining ability (Fig. 2). A sharp decrease in galvanometric reading, indicated a rapid hydrolysis in the first hour. Achroic point with iodine was reached in 3 hr under present experimental conditions. No appreciable change in the galvanometric reading was observed thereafter up to 48 hr. This indicates that maltodextrins higher than G_8 were not present after 3 hr hydrolysis.

Resolution of products of alpha amylolysis of wheat starch on a Sephadex G-75 column is presented in Figure 3. The compounds estimated as total carbohydrates were resolved into two sharp peaks: one appearing between fractions 4 and 8, and other between 9 and 13. Unhydrolyzed starch, representing the first peak, stained deep blue with iodine and gave no reducing sugar test. Oligosaccharides obtained from the second peak stained purple to red with iodine and 99% of the total reducing sugars present in the sample was collected in these fractions.

The progress of hydrolysis of wheat starch, shown chromatographically in Figure 4, revealed that besides glucose and maltose, a series of maltodextrins with low RF values were discernible. The oligosaccharides were characterized by their RF values. The RF value of glucose was taken as 1 and those of the other sugars were calculated. The RF values of different sugars were G_2 = 0.65; G_3 = 0.43; G_4 = 0.29; G_5 = 0.19; G_6 = 0.15; and G_7 = 0.11. These are in agreement with reported values (Hough and Jones, 1962). Further, the oligosaccharides were rapidly hydrolyzed by crystalline beta-amylase to maltose (French and Wild, 1953), besides small amounts of glucose and maltotriose, as revealed by paper chromatography.

The action pattern of alpha-amylase was the same when isolated starch or soluble or gelatinized starch was used as the substrate. This suggests that fraction-

Table 3–Quantitative analysis of the radiolytic breakdown products of isolated wheat starch.[a] (Values are averages of 4 experiments.)

Dose level (Krads)	Reducing sugar value mg/100g wheat starch					
	Total	G_1	G_2	G_3	G_4	G_5
0	14	–	4.6	5.4	3.4	6
20	19	–	5.4	6.0	6.8	6
40	21	–	6.4	6.4	6.8	2
60	46	–	13.8	12.8	10.8	19
200	70	3.6	13.2	17.4	12.0	35
400	110	4.4	18.4	11.7	26.4	45
600	124	5.2	22.8	31.8	21.0	35
800	140	6.0	31.8	34.8	23.4	33
1000	172	7.2	57.0	57.0	40.8	12

[a]Spots corresponding to each sugar were cut from the undeveloped chromatogram, eluted with distilled water and estimated for reducing sugars.

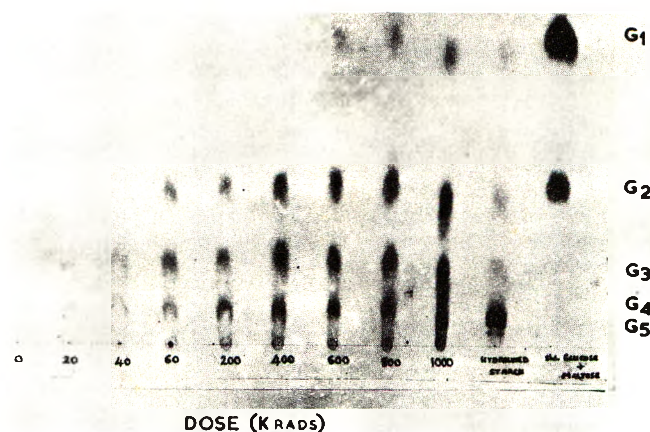

Fig. 1–Radiolytic breakdown products of starch. 1 gram starch either unirradiated or irradiated (0.02–1 Mrad) was extracted with 5 ml distilled water and centrifuged. Supernatant was lyophilized to 1 ml and aliquots (0.05 ml) were applied on Whatman No. 1 filter paper. Descending chromatography using ethylacetate-pyridine-water (8:4:3, v/v) as the solvent system was carried out.

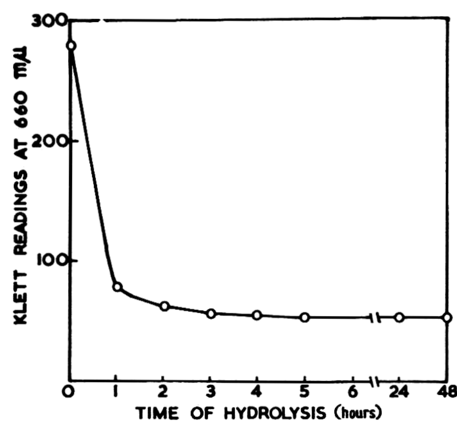

Fig. 2–Changes in iodine coloration during alpha-amylolysis of starch. 0.25 ml of incubation mixture was withdrawn at different time intervals and added to 5 ml iodine solution (0.002%). Final volume was adjusted to 10 ml and color intensity measured at 660 mμ in a Klett-Summerson photocolorimeter.

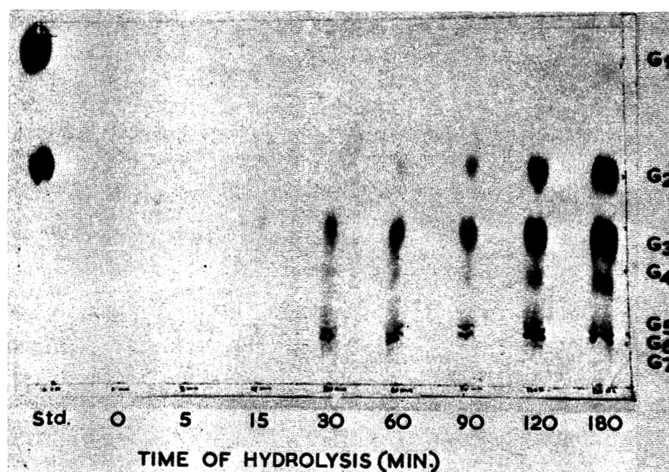

Fig. 4–Action pattern of alpha-amylase on isolated wheat starch. Effluents corresponding to second peak (Fig. 3) were pooled, lyophilized, and separated by descending paper chromatography: G_1-glucose, G_2-maltose, G_3-maltotriose, etc.

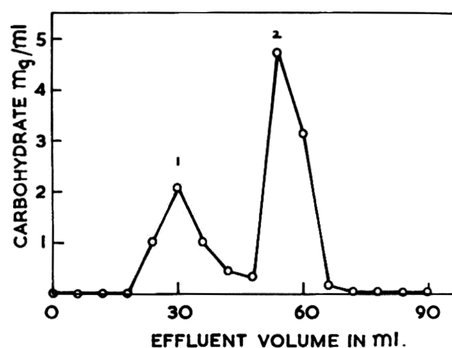

Fig. 3–Fractionation of hydrolyzed wheat starch on Sephadex G-75. 4 ml starch-hydrolysate was applied on a column (1.5 × 50 cm) and eluted with distilled water. Each 6 ml fraction was tested for total carbohydrate content.

Table 4–Quantitative yield of maltodextrins produced by alpha-amylolysis of wheat starch.[a] (Values are averages of 2 experiments.)

Time of hydrolysis in hours	Reducing sugar value mg/g wheat starch							
	Total	G_1	G_2	G_3	G_4	G_5	G_6	G_7
1	144	–	24.1	15.4	19.2	18.2	19.7	27.8
2	190	–	27.8	21.2	35.0	22.6	36.0	29.3
3	204	–	37.0	25.9	44.6	28.2	27.4	19.7
6	504	36.5	110.0	65.3	105.6	74.9	61.4	–
24	624	68.2	235.2	81.6	109.4	91.2	–	–
48	696	139.2	264.0	85.4	96.0	76.8	–	–

[a]Spots corresponding to each sugar were cut from the undeveloped chromatogram, eluted with distilled water and estimated for reducing sugars.

ation of wheat starch to oligosaccharides is a characteristic property of this enzyme and is not substrate specific.

Quantitative analyses of various maltodextrins produced by alpha-amylolysis of wheat starch at different incubation times are presented in Table 4. At the early stage of hydrolysis, higher maltodextrins appeared although small quantities of lower sugars were also present. Glucose appeared only after 6 hr of hydrolysis. As the hydrolysis proceeded, all the maltodextrins from G_1-G_6 increased in amount. After 3 hr hydrolysis the higher ones began to decrease; and at 24 hr, G_6 and G_7 disappeared completely with glucose and maltose being the main hydrolytic products (57% of total reducing sugars).

With crystalline beta-amylase as the enzyme source, maltose was the only detectable sugar in the starch hydrolysate.

DISCUSSION

THE OBSERVED INCREASE in susceptibility of isolated starch and its components to amylases, on radiation treatment, could be attributed to their degradation as revealed by (1) an initial decrease in iodine staining capacity (Table 1); and (2) an increase in reducing sugars (Table 2) in irradiated samples. Deschreider (1959, 1966) attributed the decrease in gelatinization viscosity of starch and the increase in amylopectin solubility in water to shortening of polysaccharide chains, depending upon the radiation dose. Decrease in amylograph units on irradiation of starch (Deschreider, 1959) and the shift in the iodine complex towards shorter wave lengths with irradiated amylose (Bourne et al., 1956) also support this view.

The ultimate products of radiolytic breakdown of starch have been characterized as homologues of the maltose series (Fig. 1). This suggests the hydrolytic splitting in starch due to radiation, and not that of pyranose structure, as suggested by Ulmann and Ritcher (1959), although oxidative degradation (Finney et al., 1960) and a direct effect of radiation on the starch molecule (Sosedov and Vakar, 1961) are also considered responsible for the depolymerization of starch.

For further elucidation of the nature of the products of radiolytic breakdown, a comparison was made with enzymatic hydrolysis of starch. It is known that alpha-amylase degrades the starch at alpha-1:4 linkages by random or nonrandom action (Deschreider, 1960). That the characteristic property of this enzyme is not substrate-dependent is evident since the action pattern of wheat alpha-amylase resembles that of barley (Bird and Hopkin, 1954), sorghum, saliva, and *Bacillus*

subtilis (Dube and Nordin, 1962). According to Samec (1960), radiation treatment also causes degradation in a similar way, and at 2 Mrad, 33% of the products released have a molecular weight lower than 5,000. The formation of maltotriose, etc., at 0.02–1 Mrad also supports this view, although after 6 hr of enzymatic hydrolysis (Table 4) glucose appears in the hydrolysate. This may be due to maltase impurities or the slow hydrolysis of maltose by amylase as suggested by Nitta et al. (1968).

Detection of only maltose as the end product of beta-amylolysis of starch could be explained, since, this enzyme degrades the linear starch chain of alpha-1:4-linked glucose residues from the non-reducing end with stepwise formation of maltose (Mishina and Nikuni, 1959).

Thus, these experiments suggest that radiolytic breakdown resembles the process of alpha-amylolysis. Degradation takes place by the random cleavage of starch into maltodextrins of different molecular size and not by systematic cleavage from the non-reducing end, as in the case of beta-amylolysis, where maltose is the only final detectable product.

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Ms. received 9/18/69; revised 4/20/70; accepted 5/16/70.

APPLIED SCIENCE and ENGINEERING

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COMPUTER-AIDED PREDICTIONS OF EXTENT OF BROWNING IN DEHYDRATED CABBAGE

SUMMARY—The feasibility and accuracy of prediction of extent of browning was studied on freeze-dried cabbage stored in packages made of materials with different water-vapor permeabilities. Kinetic data on the browning reaction were obtained for samples maintained at various constant-moisture contents. In each case, browning increased linearly with time. An equation relating rate of browning to moisture content was chosen on the basis of minimum variance. The kinetic data were combined with mass transfer characteristics of the packages using assumptions discussed previously. The validity of the assumptions was tested and the magnitudes of needed corrections evaluated. Increase of moisture content and changes of the sorption capacity of the cabbage, caused by browning, proved to be the major factors requiring corrections. An iteration technique for the prediction of the browning, using a computer and a method for estimation of the variance of the predicted values, was devised. The standard deviation ranged from 2–10% for the low and high extent of browning, respectively. Most of the actual data obtained from package storage tests at 37°C and 61% RH were within 1 standard deviation of prediction.

INTRODUCTION

PREDICTION of storage life in a given food-package combination and the related problem of prediction of packaging protection required for a given food to be stored for a specified time are of obvious importance in food technology. Traditionally, storage tests on the packaged food, either at the expected storage conditions or under "accelerated test" conditions, have been used for this purpose. Attempts to predict storage life or packaging protection requirements, or both, on the basis of properties of the food and the package have been limited in scope and have made use of greatly simplified assumptions (Oswin, 1945; Felt et al., 1945; Charie et al., 1963 and Paine, 1963).

Karel (1967) reviewed the scope of the problem and suggested some mathematical procedures which would simplify the task of prediction of packaging requirements for foods deteriorating through reactions sensitive to infiltration of atmospheric gases, such as water vapor and oxygen. Karel and his coworkers (Karel and Labuza, 1969; Simon, 1969; and Labuza et al., 1969) applied these procedures to the evaluation of package requirements of space rations. They developed prediction of storage stability as a function of package properties for 6 spacefood items; 5 were deteriorating through moisture-sensitive reactions and 1 through an oxidative reaction. The predictions were tested in storage tests.

Results of their work showed the mathematical methods promising, but further improvement on the accuracy of the prediction was considered necessary. 1 of the areas requiring further exploration was the evaluation of validity of various assumptions made in predicting storage stability. Another area requiring further work was the evaluation of variances arising from various experimental and curve-fitting errors. Neither of the above was adequately evaluated in previous studies, which were exploratory and involved a limited number of samples.

The present study is aimed, therefore, at these 2 areas. It presents the evaluation of validity of assumptions made in a mathematical model for prediction of storage stability of a product deteriorating through nonenzymatic browning; this reaction is a strong function of moisture content and it changes as water vapor infiltrates through a semipermeable package. It also presents evaluation of variance due to the experimental and curve-fitting errors in the above case.

The food product chosen was freeze-dehydrated cabbage. The mathematical model was based on combination of kinetic data for the browning reaction, the sorption properties of the cabbage and the permeability characteristics of the packages. The general procedure for combining such data was described by Karel (1967) and Karel and Labuza (1969).

EXPERIMENTAL

Preparation of samples

Savoy cabbage was cored, washed, shredded and blanched in steam for 2 min. It was then freeze dried, ground in a Model D Fitz mill with a screen having 0.030-in. openings, then redried to remove moisture picked up during grinding. The cabbage powder was blended to give homogeneous samples.

Humidification

Samples of 2.50 ± 0.01 g were spread in a thin layer on a side wall of 2-oz square bottles. They were humidified in evacuated desiccators

Table 1—Equations correlating rate of nonenzymatic browning with moisture content.

Equation No.	Equation	Remarks
2	$R = k_1 \{1 + \sin(-\frac{\Pi}{2} + \frac{m\Pi}{m_x})]^n$	m_x = Moisture content at which browning rate is a maximum. (In the present case m_x = 18.)
3	$R = k_2 a^s$	
4	$R = c_1 + c_2 m + c_3 m + \ldots$	Four terms are adequate for the desired precision.

a = Water activity. m = moisture content (g water/100 g solids). R = rate of browning (Klett units/day). $k_1, k_2, c_1, c_2, c_3, n, s$ = constants in correlation equations.

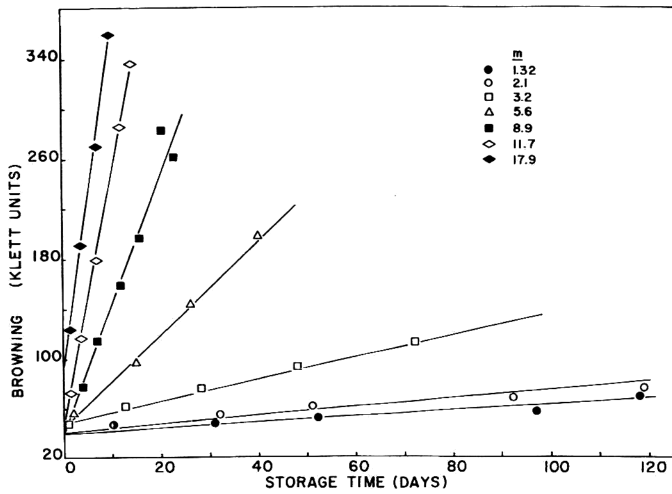

Fig. 1–Nonenzymatic browning of dehydrated cabbage as a function of storage time at specified moisture contents (m = moisture content; g water/100 g solids).

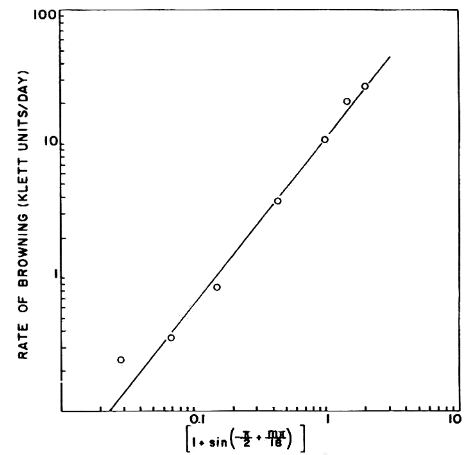

Fig. 2–Correlation between rate of browning and moisture content; based on Equation [1].

over saturated salt solutions maintaining a constant humidity for 48 hr, and the resultant change in moisture content was determined by weighing the bottles. The initial moisture content was determined by placing similar samples in an evacuated desiccator containing Drierite (anhydrous calcium sulfate) for 30 days, during which time periodic weighings were made to determine when the samples came to equilibrium with the desiccant.

Determination of browning rates

Samples were adjusted to different moisture contents by humidification over different saturated salt solutions. 8 samples at each moisture content were placed in stoppered bottles in an incubator maintained at 37 ± 0.5°C. At suitable intervals samples were removed from the incubator and kept in a freezer at −20°F until they could be analyzed for extent of browning. Extent of nonenzymatic browning was determined using a modification of a method reported by Hendel et al. (1950).

The 2.5-g sample was extracted in a 250-ml Erlenmeyer flask with 100 cc of water for 1 hr. Extract was filtered through S&S #595 filter paper. 40 ml of the filtrate were placed in a centrifuge tube and clarified by centrifugation on a refrigerated centrifuge (International Model B-20) for 30 min at 23,500 g. The clear supernatant was acidified with 0.5 ml of 40% acetic acid and its light absorbance measured in a Klett-Summerson colorimeter using filter No. 42. The extent of browning was expressed in Klett units at the specified conditions involving an initial concentration of 2.5 g of sample per 100 ml of water. When measured in the above unit, browning was found to be directly related to optical density at 420 mμ, as it was when measured on a Beckman B spectrophotometer, by the following relationship: optical density at 420 mμ is equivalent to 1.3×10^{-3} Klett units. For experimental convenience the Klett-Summerson instrument was used throughout the study.

Storage tests on samples in permeable packages

To compare predictions of browning with storage tests in which moisture content of samples was changing due to infiltration of moisture through the packaging materials, samples were packaged in flexible films. Polyethylene (2.5 mil) and Scotchpak 48 (Minnesota Mining and Manufacturing Co.) were used. Both films are hydrophobic and the rate of water-vapor transfer through them is proportional to partial pressure difference (Karel et al., 1959).

A 15-g sample of cabbage was placed into 10- by 13-cm pouches of polyethylene and a

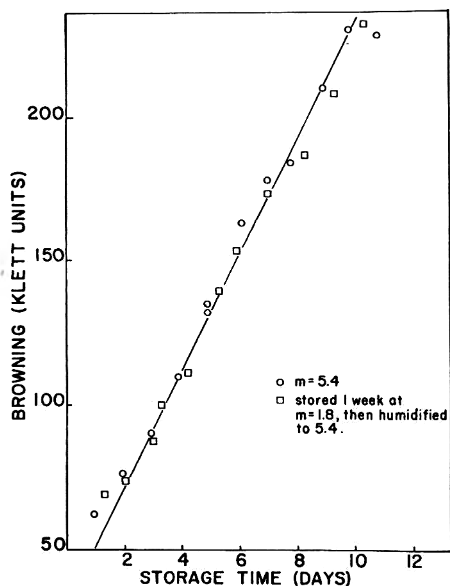

Fig. 3–Effect of sample history on rate of browning.

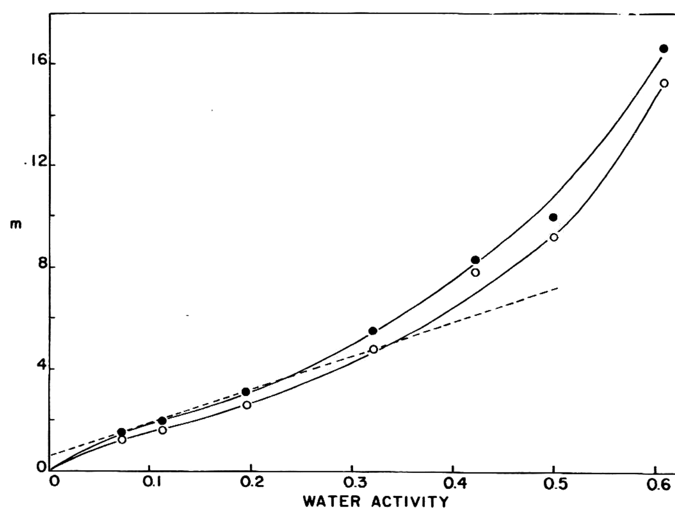

Fig. 4–Effect of browning on sorption isotherms of dehydrated cabbage. —●—●—●— Sample prior to storage. Extent of browning is 58 Klett units. —o—o—o— Sample after storage. Extent of browning is 410 Klett units.

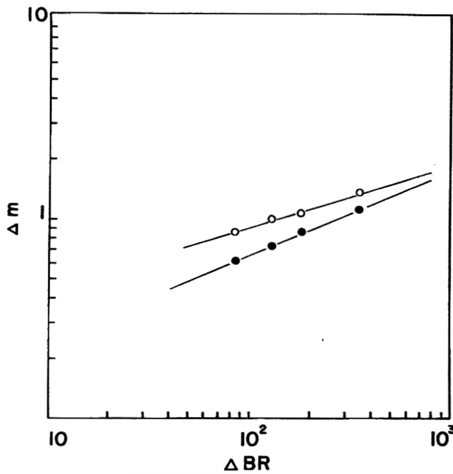

Fig. 5–Generation of water by the browning reaction. —●—●—●— Increase in moisture content due to browning. —o—o—o— Increase in moisture content due to browning, corrected for change in sorption isotherm.

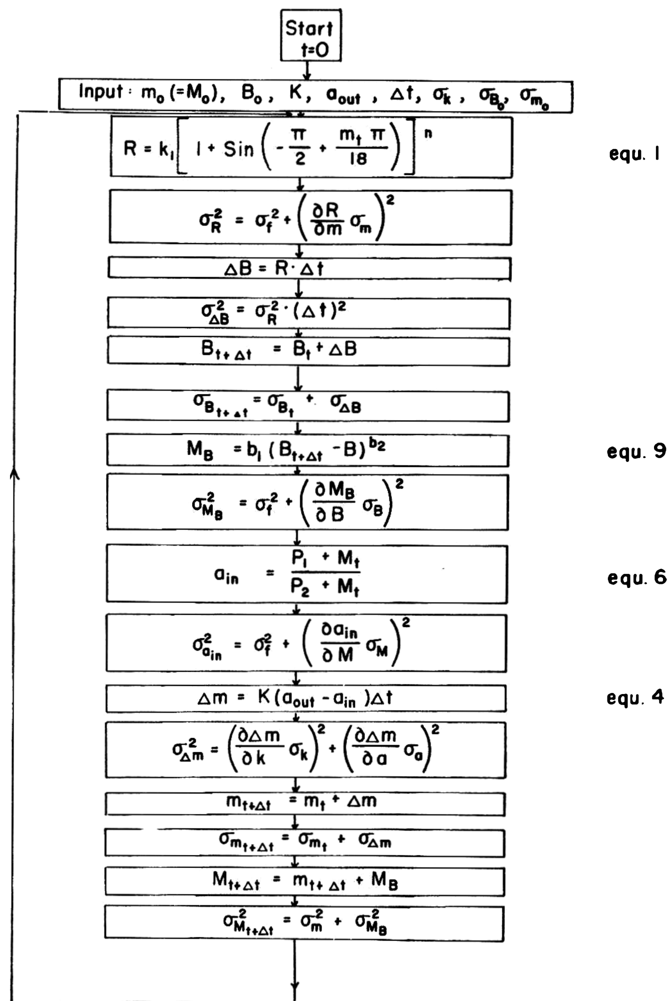

Fig. 6–Flow sheet for computer-aided calculation of increase in moisture content, increase in browning and variances of functions.

20-g sample into those of Scotchpak 48. The pouches were sealed in air and then stored in desiccators containing solutions maintaining a relative humidity of 61% at 37°C. Periodically, samples were removed for weighing and several were analyzed for extent of browning according to a predetermined schedule.

Function fitting and estimation of the function variance

Function fitting and estimation of the variance of the function (σ_f^2) were done according to the procedures described by Wolberg (1967) using least-squares analysis. Essentially, for any function $f(x;\ p_1 \ldots p_n)$; $p_1 \ldots p_n$ are the parameters, whose magnitudes and variances are determined by least squares. The variance of the calculated dependent variable is given by:

$$\sigma_f^2 = \left(\frac{\delta f}{\delta p_1}\right)^2 \sigma_{p_1}^2 + \ldots + \left(\frac{\delta f}{\delta p_n}\right)^2 \sigma_{p_n}^2 + 2\left(\frac{\delta f}{\delta p_1}\right)\left(\frac{\delta f}{\delta p_2}\right) \zeta_{1,2}\, \sigma_{p_1} \sigma_{p_2} + \text{other cross-product terms} \quad [1]$$

When several alternative equations were available, 1 was chosen on the basis of minimizing the variance of the prediction, which usually meant minimum σ_f.

All the calculations in this study were carried out using an IBM Systems 360 Model 65 Computer at the M.I.T. Computation Center.

RESULTS & DISCUSSION

THE PREDICTION of extent of browning in packaged cabbage stored at 37°C required the prior development of the following information: 1. A function relating extent of browning to time of storage and to moisture content. 2. A function relating moisture content within the food to partial pressure of water. 3. Function(s) relating change of moisture content in the samples to properties of the package, the food and the environment.

The first task was to determine how browning increased with time at various constant moisture contents. Figure 1 indicates that at each moisture content browning increased linearly with time. Rates of browning, as given by the slopes of the straight lines in Figure 1, were then correlated with moisture content. It was determined that several functions could be used for this purpose; 3 such functions are listed in Table 1 (Equations 2, 3 and 4). Of these Equation [2] was chosen because it gave the lowest variance. The correlation curve between rate of browning and the function of moisture content is shown in Figure 2. This curve was obtained by regression analysis using a statistical weight (Wolberg, 1967) of 0.06 R, calculated from the variance of browning rate at each moisture content.

It was considered necessary to determine whether moisture dependence of browning rate derived from experiments at constant-moisture content could be applied to the dynamic situation in which moisture content increases during storage. A test of this assumption was conducted at 45°C. Browning of a sample humidified at 11% RH, stored for 1 week and then rehumidified to 32% RH was compared to that of a sample humidified directly to 32% RH. Results are shown in Figure 3, and they, as well as other checks carried out at 37°C, showed that under the conditions involved in the present study, relationships developed in static storage tests could be applied to the dynamic situation.

Prediction of browning requires calculation of increase in moisture content due to permeation of water through the package material. The following rate equation was used:

$$\frac{dm}{dt} = K\,(a_{out} - a_{in}) \quad [5]$$

where:

m = moisture content (g water/100 g solids)
t = time (days)
a = water activity

"out" and "in" refer to conditions outside and inside the package, respectively.

K is defined by Equation [6]:

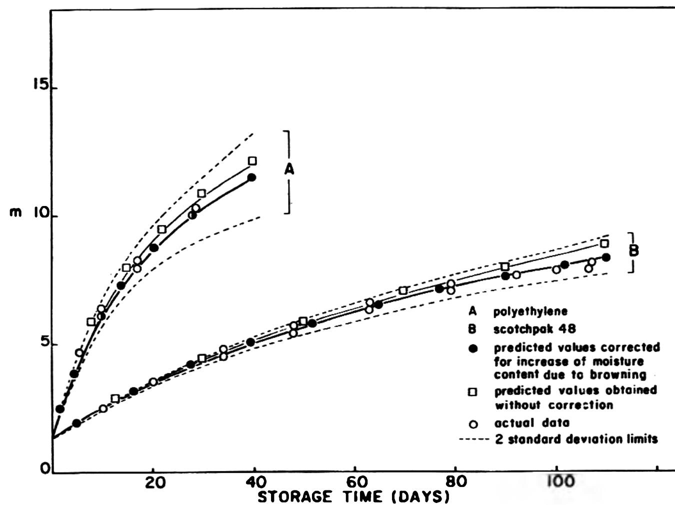

Fig. 7–Comparison of predicted increases in moisture content of samples stored in 2 types of packages, and experimental results.

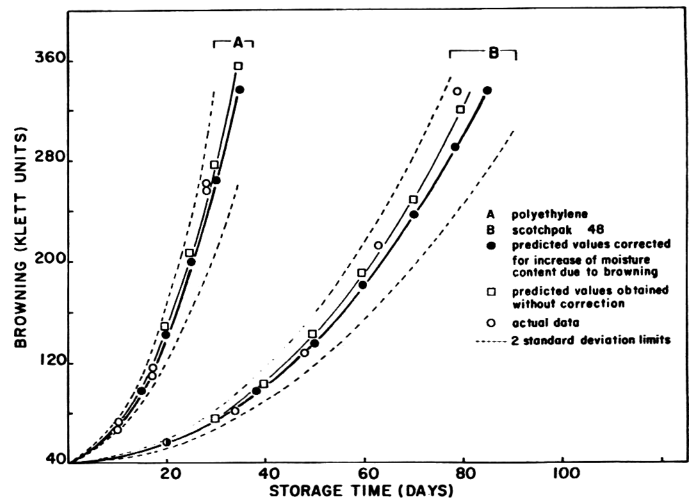

Fig. 8–Comparison of predicted increases in browning of samples stored in 2 types of packages, and experimental results.

$$K = 100\left[\frac{(k_{H_2O})(p_0)(A)}{(W_S)(X)}\right] \qquad [6]$$

where:

k_{h_2o} = permeability to water vapor $\left(\frac{g \cdot mil}{torr \cdot m^2 \cdot day}\right)$

p_0 = vapor pressure of water (torr)

A = package area (m^2)

X = packaging material thickness (mil)

W_s = weight of solids per package (g)

The water activity a_{in} can be assumed to be in equilibrium with food moisture content m only if there are no significant moisture gradients in the food, i.e., resistance of the food to water transport is negligible compared to the resistance of the package.

A test was conducted in which a 2-cm-thick layer of the cabbage was placed in a can that had a Scotchpak 48 membrane sealed to its top. In this case, moisture could enter the cabbage only from the top. Checks on extent of browning in the uppermost and the lowest layers of this sample showed a maximum difference in extent of browning of less than 5%. Thus, it appears that the assumption of equilibrium is justified, since mean thicknesses in the test packages were much lower than 2 cm.

The relationship between moisture content and water activity (sorption isotherm) was determined for samples immediately after drying, as well as on samples which had undergone extensive browning. Typical results are shown in Figure 4. It is evident that browning results in some reduction of sorptive capacity of the dried cabbage. In addition, the browning reaction results in an increase in moisture content. The extent of this increase is shown in curve A in Figure 5.

It was found that the most efficient way (minimum contribution to the over-all variance) to correct for both effects (change in isotherm and moisture production) was to transform the sorption isotherms to coincide with the fresh sample isotherm by adding correction factors to the moisture content values. The magnitudes of the correction factors were determined by minimizing the differences between the sorption isotherm of the fresh sample and the browned samples using least-squares analysis. The correction factors were added to the values of moisture content increase, caused by the browning, to give the "effective moisture content increase" presented in curve B of Figure 5.

Different functions were tried in fitting the observed sorption isotherm and it was found that for the range of values of interest, the best fit was given by Equation [7]:

$$a = \frac{r_1 + M}{r_2 + M} \qquad [7]$$

where:

m = moisture content including correction for moisture generated by browning

r_1, r_2 = equation parameters

The statistical weight (Wolberg, 1967) used in this case was 0.028 M. It was estimated from the variance of M on 10 samples after humidification at each water activity.

The constant K was determined for the 2 types of packages used in the storage study. 2 different methods were used to compute this constant. By the first method, permeability tests on the packaging materials were conducted using the water-vapor permeability test on desiccant-filled packages (Davis et al., 1960). The water-vapor permeability constant was calculated and from it the over-all constant K was obtained by Equation [6].

The second method allowed the estimation of constant K on the packages as they were actually used in the storage study. A comparison of values from the 2 methods, therefore, allowed a test of various assumptions on which Equations [5] and [6] are based, including lack of significant mass-transfer resistances within the packages and lack of significant imperfections in each package as constructed and sealed.

This second method was conducted as follows: It was assumed that the sorption isotherm for the cabbage could be approximated by a straight line for the portion of the isotherm of significance in the study. The line is shown as a broken line in Figure 4 and its equation is given by:

$$m = \alpha a + \beta \qquad [8]$$

where:

α, β = constants

By substituting into Equation [5] and integrating, an expression is obtained which relates increase in moisture content to the constants K and time:

$$\ln\left[\frac{m_e - m_0}{m_e - m}\right] = \frac{K}{\alpha} t \qquad [9]$$

where:

m_e = moisture content in equilibrium with a_{out}

m_0 = initial moisture content

Since m_0 and m_e were known, and m readily determined by periodic weighing of the packages, K could be calculated.

The results were: For 2.5 polyethylene packages described previously, the values of K were 1.37 using the first method and 1.37 using the second method (standard deviation of 0.05 for 12 samples). For Scotchpak 48, the first method gave a value of 0.248 and the second, 0.244 (standard deviation of 0.0008 for 12 samples). Thus, the assumptions used in Equations [5] and [6] are well justified.

The information from the above tests was combined in a mathematical model for prediction of extent of browning. An iteration procedure over time intervals of 1/10 of a day was used with the aid of an IBM 360 digital computer. The iteration procedure is shown as a flow sheet in Figure 6 and is briefly summarized below. In the following summary, subscripts "t" and "t + Δt" refer to values at the beginning and end of interval "Δt", respectively.

1. Rate of browning at the beginning of the interval "Δt" is determined using Equation [2]. Increase in browning (ΔB) is added to B_t to obtain $B_{t+\Delta t}$. (B is extent of browning in Klett units.)

2. Moisture content increase due to browning is then calculated using Equation [10] derived on the basis of Figure 5.

$$M_B = b_1 (B_t - B_0)^{b_2} \qquad [10]$$

where:

M_B = total moisture generated by browning reaction
B_0 = extent of browning at beginning of storage
b_1, b_2 = equation parameters

3. Water activity in the package a_{in} is calculated from Equation [7].

4. Moisture increase due to water-vapor permeation is calculated using Equation [5], and this amount is added to m_t to obtain $m_{t+\Delta t}$. (The symbol m refers to moisture content uncorrected for water generated by the browning reaction. The symbol M is used for moisture content including the water due to browning.)

5. Corrected moisture content $M_{t+\Delta t}$ is calculated by adding M_B to $m_{t+\Delta t}$. The process is then repeated to calculate changes occurring in the next time interval.

Predicted values of the increase of moisture content due to permeation and the predicted extent of browning for samples packaged in polyethylene and in Scotchpak 48, obtained by the mathematical model described above, are presented in Figures 7 and 8. The estimated uncertainties of the calculations are expressed as 2 times the standard deviation and are shown in Figures 7 and 8 as broken lines. The actual increases in moisture content, obtained by weighing the samples during storage, are scattered around the predicted values with maximum deviation less than 3% (Fig. 7). In the case of the extent of browning (Fig. 8), the deviations between the actual and the predicted values are less than 10%.

The effect of the correction for the increase of moisture content due to browning can be evaluated by comparing the predicted values obtained with and without this correction (Fig. 7 and 8). In the particular case studied here, the correction for moisture generated in browning improves the prediction, but the error due to ignoring this correction is not large. However, it can be shown that the error due to omission of this correction will increase when storage of packages is conducted at low humidities, and there may be other conditions in which disregard of it may have greater significance.

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Ms. received 2/17/70; revised 4/3/70; accepted 4/16/70.

This research was supported by U.S.P.H.S. Grant No. FD-00140 (formerly UI-00385).

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FEASIBILITY OF ACCELERATED TESTS FOR BROWNING IN DEHYDRATED CABBAGE

SUMMARY—Feasibility of using accelerated tests for deterioration of dehydrated vegetables through nonenzymatic browning was evaluated. Studies were made on unsulfited freeze-dehydrated cabbage. Two procedures for shortening the time required to predict shelf life were used: (1) Extrapolation from high to low moisture contents; and (2) Extrapolation from high to low temperatures. A combination of the two procedures was also evaluated. It was determined that activation energy for browning was dependent on moisture content, and an equation expressing this dependence was developed. Equations relating rates of browning to moisture content at a given temperature were also obtained. Extrapolations based on either procedure, or on a combination of both, gave satisfactory results. The variance of the extrapolation-based estimates of browning rates was dependent on the number of points used for the extrapolation. The procedures used reduced the time required to obtain browning-rate data at low moisture contents from over a year to only 10 days.

INTRODUCTION

WE HAVE RECENTLY reported studies designed to predict storage life of dehydrated foods packaged in semipermeable containers and subject to various types of chemical deterioration (Karel and Labuza, 1969; Simon, 1969; Mizrahi et al., 1970). In a study concerned with nonenzymatic browning of dehydrated, unsulfited cabbage packaged in pouches of known permeance and stored at 37°C, we determined browning rates for samples maintained at various moisture contents (Mizrahi et al., 1970). Browning in these samples increased linearly with time.

Several equations were found suitable for relating the rate of browning to moisture content, and one was chosen on the basis of minimum variance.

Using assumptions which were discussed previously by Karel and Labuza (1969), we combined the kinetic data with mass transfer characteristics of the packages. The validity of the assumptions was tested and the magnitudes of needed corrections were evaluated. Increase of moisture content and changes, caused by the browning, in the sorption capacity of the cabbage proved to be the major factors requiring corrections.

An iteration technique using a computer for the prediction of the browning, and a method for estimating the variance of the predicted values were devised. The standard deviation ranged from 2–10%. Most of the actual data obtained from package storage tests at 37°C and 61% RH were within one standard deviation of prediction.

Having developed a method for predicting storage life of materials that undergo chemical reactions dependent on moisture content and that are under conditions in which moisture content is changing, we considered the practical aspects of such predictions, and, in particular, methods by which time required for accumulation of needed kinetic data could be shortened.

Acquisition of kinetic data under conditions of constant temperature and moisture content requires a very long experimental period. Materials stored at constant moisture, such as dehydrated vegetables stored in hermetically sealed cans, have long been recognized as needing an accelerated test. For a narrow range of moisture contents, such a test was studied by Gooding and Duckworth (1957) who accelerated deterioration of dehydrated vegetables by using elevated temperatures.

The use of high temperatures for accelerated tests for systems with changing moisture contents is complicated by the possible variation in temperature dependence with changing moisture content. In a study using a range of moisture contents, Legault et al. (1947) and Legault et al. (1951) found that the apparent energy of activation for nonenzymatic browning depended on moisture content. Legault et al. (1951) were able to show that an increase in moisture content of dehydrated cabbage from 2.1–7.1% (moisture-free basis) caused a decrease in apparent energy of activation from 43–35 kcal/mole. The same trend was found for other dehydrated vegetables, but the correlation between the apparent energy of activation and moisture content was not established. Legault et al. (1951) suggested that the high temperature and moisture content coefficient might be used for devising an accelerated test.

Table 1—Estimated minimum time (days) necessary to obtain kinetic data.

Storage temp (°C)	Moisture content (g H_2O/100g solid)						
	1.4	2.1	3.2	5.6	8.9	11.7	17.9
52	47	10	4	2	1	<1	<1
45	220	28	16	4	2	1	<1
37	300	210	100	22	9	4	1
30	>300	>300	250	60	26	13	3.5

EXPERIMENTAL

Preparation of samples

Savoy cabbage was cored, washed, shredded, and blanched in steam for 2 min. It was freeze dried, ground in a model D Fitz mill with a screen having 0.030-in. openings, and then redried to remove moisture picked up during grinding. The cabbage powder was blended to give homogeneous samples.

Humidification

Samples of 2.50 ± 0.01g were spread in a thin layer on a side wall of 2-oz square bottles. They were humidified in evacuated desiccators over saturated salt solutions maintaining a constant humidity at 30°C for 48 hr; the resultant change in moisture content was determined by weighing the bottles. The initial moisture content was determined by placing similar samples in an evacuated desiccator containing anhydrous calcium sulfate for a period of 30 days, during which the samples were periodically weighed to determine when they came to equilibrium with the desiccant.

Determination of browning rates

Samples were adjusted to different moisture contents by humidification over saturated salt solutions. Eight samples at each moisture content were placed in stoppered bottles in incubators maintained at 30, 37, 45 and 52°C (±0.5°C). At suitable intervals, samples were removed from the incubator and kept in a freezer at −31°C until they could be analyzed for extent of browning. Extent of nonenzymatic browning was determined with a modification of a method reported by Hendel et al. (1950). The 2.5g sample was placed in a 250 ml Erlenmeyer flask with 100 cc of water for 1 hr. Extract was filtered through S & S No. 595 filter paper. 40 ml of the filtrate were placed in a centrifuge tube and clarified on a refrigerated centrifuge (International Model B-20) for 30 min at 23,000 × g. The clear supernatant was acidified with 0.5 ml of 40% acetic acid and its light absorbance was measured in a Klett and Summerson colorimeter using filter No. 42. The extent of browning was expressed in Klett units under the specified conditions involving an ini-

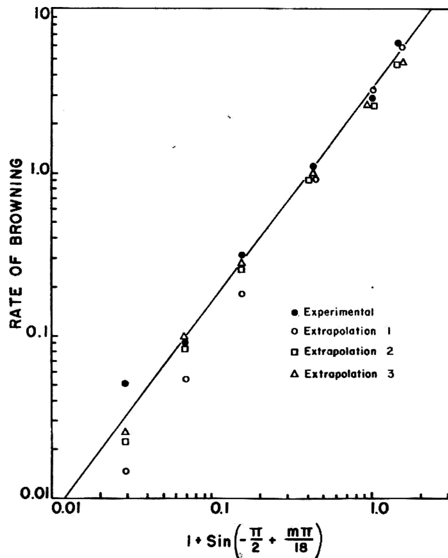

Fig. 1–Correlation between rate of browning and moisture content. Results of studies at 30°C and of extrapolations from high moisture contents and from other temperatures. Extrapolation no. 1 is based on the 4 high moisture points and Eq. 4. Extrapolation no. 2 is based on activation energy calculated from 7 moisture contents at 4 temperatures and on results at 52°C. Extrapolation no. 3 is based on activation energy calculated from the 3 highest moisture contents at 4 temperatures and on results at 52°C using 4 moisture content points.

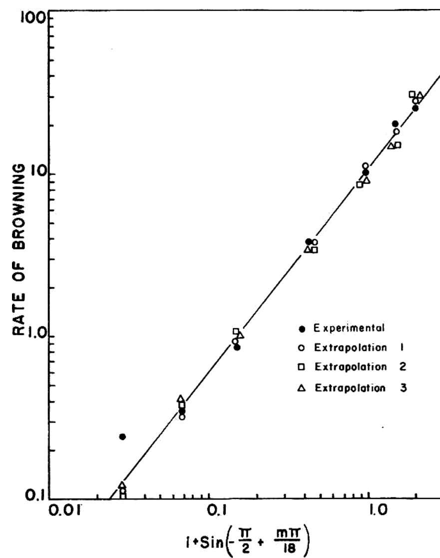

Fig. 2–Correlation between rate of browning and moisture content. Results of studies at 37°C and of extrapolation from high moisture contents and from other temperatures. Extrapolation no. 1 is based on the 4 high moisture points and Eq. 4. Extrapolation no. 2 is based on activation energy calculated from 7 moisture contents at 4 temperatures and on results at 52°C. Extrapolation no. 3 is based on activation energy calculated from the 3 highest moisture contents at 4 temperatures and on results at 52°C using 4 moisture content points.

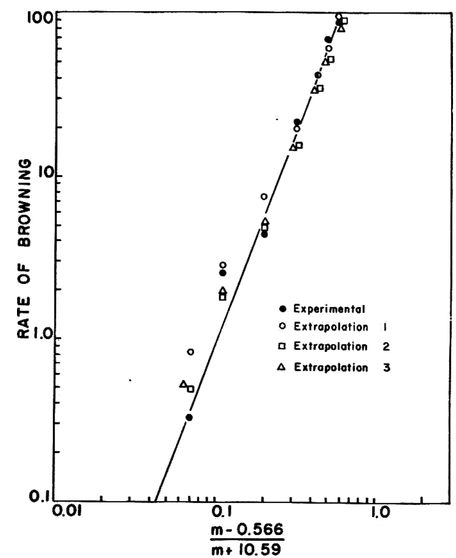

Fig. 3–Correlation between rate of browning and moisture content. Results of experimental studies at 45°C and of extrapolation from high moisture contents and from other temperatures. Extrapolation no. 1 is based on the 4 high moisture points and Eq. 5. Extrapolation no. 2 is based on activation energy calculated from 7 moisture contents at 4 temperatures and on results at 52°C. Extrapolation no. 3 is based on activation energy calculated from the 3 highest moisture contents at 4 temperatures and on results at 52°C using 4 moisture content points.

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Function fitting and estimation of the function variance

Function fitting and estimation of the variance of the function (σ_f^2) were according to the procedures described by Wolberg (1967) using least squares analysis. Essentially, for any function f (x; $p_1 \ldots p_n$); $p_1 \ldots p_n$ are the parameters, whose magnitudes and variances are determined by least squares. The variance of the calculated dependent variable is given by:

$$\sigma_f^2 = \left(\frac{\delta f}{\delta p_1}\right)^2 \sigma_{p_1}^2 + \ldots + \left(\frac{\delta f}{\delta p_n}\right)^2 \sigma_{p_n}^2 + 2\left(\frac{\delta f}{\delta p_1}\right)\left(\frac{\delta f}{\delta p_2}\right)\zeta_{1,2}\ \sigma_{p_1}\sigma_{p_2} + Z \quad \text{(Eq. 1)}$$

Where Z = other cross-product terms.

When several alternative equations were available, the one was chosen that would minimize the variance of the prediction, which usually meant the one with minimum σ_f.

All the calculations in this study were carried out using an IBM Systems 360 Model 65 computer at the M.I.T. Computation Center.

The extrapolation from high to low temperature was carried out according to the following equation:

$$R_2 = R_1 e^{\left[E_{a(m)}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)\right]} \quad \text{(Eq. 2)}$$

and the variance of the extrapolated rate is

$$\sigma_{R_2}^2 = \left(\frac{\delta R_2}{\delta R_1}\sigma_{R_1}\right)^2 + \left(\frac{\delta R_2}{\delta R_{a(m)}}\sigma_{E_a}\right)^2 \quad \text{(Eq. 3)}$$

where R_2 is rate of browning at absolute temperature T_2;
R_1 is rate of browning at absolute temperature T_1;
$E_{a(m)}$ is activation energy at moisture content (m) determined in the usual manner from the slope of the plot $\log_e$ vs 1/T.

RESULTS & DISCUSSION

RATES OF BROWNING in dehydrated unsulfited cabbage were studied at four different temperatures and at seven different moisture contents at each temperature. The extent of browning increased linearly with time, except in the case of low-moisture samples at the lowest temperature studied (30°C) when the constant-rate period was preceded by a brief induction period. The rates of browning were calculated from the slopes of linear browning vs time plots.

Rates of browning were then correlated with moisture content. Legault et al. (1947), working with nonsulfited dehydrated vegetables including cabbage at a narrow range of moisture contents, found an exponential relationship between rate of browning and moisture content. One of our previously reported studies demonstrated that several equations could be used to correlate rates of browning with moisture content (Mizrahi et al., 1970). The two equations providing the best fit for the correlation of data in the present study were Equations 4 and 5.

$$R = k_1\ [1 + \sin(-\frac{\pi}{2} + \frac{m\pi}{m_x})]^n \quad \text{(Eq. 4)}$$

$$R = k_2\left(\frac{r_1 + m}{r_2 + m}\right)^s \quad \text{(Eq. 5)}$$

where m is the moisture content (g water/100 g solids);
R is the rate of browning (Klett units/day);
m_x is the moisture content at

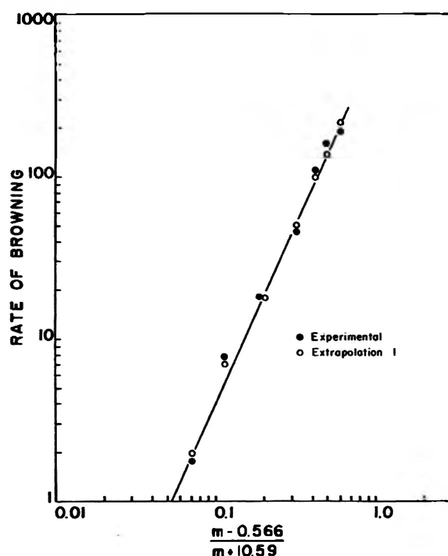

Fig. 4—Correlation between rate of browning and moisture content. Results of studies at 52°C and of extrapolation from high moisture contents and from other temperatures. Extrapolation no. 1 is based on the 4 high moisture points and Eq. 5.

which browning rate is maximum. (In this case $m_x = 18.0$);

r_1, r_2, k_1, k_2, n, s are constants;

π is 3.1415 . . .

It was found that Equation 4 gives slightly lower variance when used for relating rate of browning to moisture content at 30 and 37°C than Equation 5, but Equation 5 served that purpose better at 45°C and 52°C.

The approximate time necessary to obtain the kinetic data at the different constant temperatures and moisture contents is presented in Table 1. The data show that either elevating the temperature or increasing the moisture content shortens the experimental period. One method for shortening time required to determine browning rates is based on the accelerating effect of high moisture contents. If the form of correlation between rate of browning and moisture content is known, it is possible to extrapolate from high to low moisture content.

We performed this extrapolation using the best equation for each temperature and the four moisture-content points for 30, 37, 45 and 52°C. The calculated data are shown in Figures 1 to 4 respectively, together with the points obtained by actual experiments. The results show that this method can be considered as feasible and reliable.

At relatively low temperatures, however, even the acquisition of data for samples with high moisture content is a time-consuming process. The time required to obtain the four high-moisture points (m = 17.9, 11.7, 8.9, 5.6) at 37°C and 30°C was 22 and 60 days respectively (Table 1).

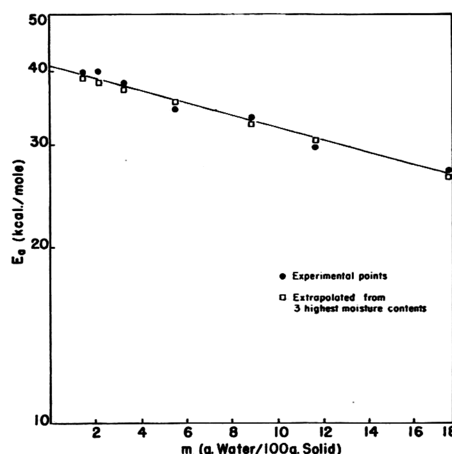

Fig. 5—Dependence of apparent activation energy for browning on moisture content.

The second and most widely used method of accelerating storage tests is to extrapolate from high temperatures to lower temperatures. In order to determine the relationship between rates of browning and temperature, the apparent activation energy (E_a) for browning at each moisture content was determined by plotting the data according to the Arrhenius equation. Data presented in Figure 5 indicate that the apparent energy of activation depends on moisture content and that this dependence is given by

$$E_a = c_1 e^{-c_2 m} \qquad \text{(Eq. 6)}$$

where E_a is the activation energy (kcal/-mole);

c_1, c_2 are constants.

The values of the constants in this case were $c_1 = 41.1$ and $c_2 = 0.025$. For moisture contents near zero, E_a is 41 kcal/mole, a value close to the one obtained by Legault et al. (1951). At higher moisture contents, E_a decreases and tends to level off at around 26 kcal/mole when moisture reaches 18g/100g solids. This value is similar to that obtained by Stadtman et al. (1946) for apricots at a moisture content of 23%.

In a study on nonenzymatic browning of potatoes, Hendel et al. (1955) found moisture content to have a similar effect on activation energy. At a moisture content of 4.9%, activation energy was 37 kcal/mole; this value dropped to 28 kcal/mole when moisture content reached 15%, and leveled off at about 25 kcal/-mole as moisture was brought to high levels. It is interesting that activation energies for nonenzymatic browning show such marked similarities for different dehydrated vegetables. If the dependence of activation energy on moisture reported here could be established as generally valid for dehydrated vegetables, it would facilitate greatly further development of accelerated tests.

Having established that Equation 6 correlates the apparent energy of activation and moisture content, we used it together with the data obtained at 52°C to calculate rates of browning at 30, 37 and 45°C. These data are presented in Figures 1, 2 and 3 respectively. Though the calculated data are a good estimation, this is still a time-consuming method, mainly because of the long experimental period necessary to obtain energy of

Table 2—Comparison of actual and extrapolated rates of browning of cabbage at 37°C and 2.14 g H_2O/100 g solids.

Method of extrapolation	Rate of browning[a] (Klett units/day)	Time required to obtain data (days)
Experimentally determined rate	0.35 ± 0.02	200
Extrapolated from 5 high moisture points at 37°C	0.29 ± 0.03	100
Extrapolated from 4 high moisture points at 37°C	0.32 ± 0.08	22
Extrapolated from 3 high moisture points at 37°C	0.32 ± 0.26	9
Extrapolated from data at 52°C (7 moisture points) and (E_a)[b]	0.39 ± 0.14	40
Extrapolated from data at 52°C (5 moisture points) and (E_a)[b]	0.41 ± 0.16	10
Extrapolated from data at 52°C (4 moisture points) and (E_a)[b]	0.41 ± 0.20	10

[a]Rate ± standard deviation.

[b]E_a was estimated from 3 highest moisture points at 4 temperatures using Equation 6.

activation data at the low moisture contents. This obstacle can be overcome by an extrapolation procedure using Equation 6 and the values of apparent energy of activation obtained for high moisture content. The results of such an extrapolation, using data obtained for moisture contents of 8.9, 11.7, and 17.9%, are shown in Figure 5. The calculated data did not differ significantly from the actual ones, and it was found that using them to extrapolate the rate of browning from high to low temperature gave valid results.

Assuming that more experiments may be carried out within the range of 37°C to 52°C and within moisture range of 9–18%, the estimated minimum time necessary to obtain the energy of activation data is 10 days, compared to about a year required when these determinations are made directly at the low moisture contents.

We attempted to reduce the time required at low temperatures by combining the two methods, namely, extrapolation from high to low moisture content at constant temperature and extrapolation from high to low temperature. The results of such an extrapolation procedure, using energy of activation obtained from the three highest moisture points at four temperatures (using Eq. 6) and rates of browning at four moisture contents at 52°C (using Eq. 5), are shown for 30, 37 and 45°C in Figures 1, 2 and 3 respecexperimental points decreased. A comparison of different methods requiring the same time (10 days) shows that extrapolation at 37°C from the three upper points gave standard deviation of 0.26 whereas combination of 5 points at 52°C and three points for energy of activation gave a standard deviation of 0.16. Preference for any method depends on the dispersion of the data along the curve used, the number of points available, and the magnitude of the error arising from the combination of two equations each of which has its own variance.

The success of the accelerated test depends mainly on previous knowledge of the form of correlation between rate of browning and moisture content and between apparent energy of activation and moisture content. More experiments are needed in order to establish whether the equations we used can be applied generally for other dehydrated foodstuffs as well as for different batches of dehydrated cabbage.
tively. It appears that this extrapolation yields satisfactory results and decreases the required time to about 10 days, as compared to over a year required for direct determination of rates.

Decreasing the number of experimental points used for extrapolation inevitably increases the uncertainty of the calculated values. Standard deviations of the values determined by extrapolation were calculated from different combinations of data and are shown in Table 2 for the case of extrapolated browning rates at 37°C and 2.14% moisture. Generally, the standard deviation increased as available

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Ms. received 4/2/70; revised 6/5/70; accepted 6/17/70.

Contribution no. 1618 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology.

This research was supported by United States Public Health Service Grant no. FD-00140 (formerly UI-00358).

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THERMAL BROWNING OF TOMATO SOLIDS AS AFFECTED BY CONCENTRATION AND INHIBITORS

SUMMARY—The amount of brown color developed was always determined by reconstituting the sample to 5.6% total solids and measuring optical density (O.D.) of an acetone extract at 420 mμ. Tomato solids at concentrations of 5.6 to 98% were heated for 15 min at 95°C. The O.D. increased slowly with increasing concentration to about 50% solids. Thereafter, the O.D. increased rapidly to a peak at 95% and decreased to half this value at 98%. The effect of heating time at 55, 75 and 95°C and at 5.6 and 45% solids was studied. The browning rate was slight at the lower 2 temperatures relative to that at 95°C, and was much more rapid at the higher concentration than at single strength. Under each temperature and solids condition, the rate of browning was highest at the earlier times. The inhibition effects of pH and SO_2 were studied. Browning was at a minimum at pH 2.5. Browning could be further reduced at each pH by increasing levels of SO_2. The protective effect of acidification and SO_2 addition increased with increasing solids concentration.

INTRODUCTION

UNDESIRABLE quality changes occur during processing and storage of commercial tomato products. One of the most serious of these is development of browning caused by the Maillard reaction (Wong et al., 1956; Luh et al. 1964). The naturally occurring constituents responsible for browning of tomato products are mainly reducing sugars and amino acids.

El-Miladi et al. (1969) reported the effect of heat processing on tomato juice constituents. A loss of 17.2% total fructose and 19.1% total glucose was observed after a 20-min treatment at 220°F. Loss in total sugar was 19.4%. In spite of the hydrolysis of starch during processing, the sugar content did not increase. This was probably due to the reaction of sugar with amino acids. Sugars can also undergo caramelization, a reaction requiring no amine. Processing resulted in increased concentrations of all 19 amino acids present, probably due to partial hydrolysis.

Reynolds (1969), in her review on browning, reported that browning decreased as the pH decreased, that sulfur dioxide and bisulfites can be used to inhibit browning in dried fruits and vegetables and that the inhibition of browning by bisulfite is due to the formation of a bisulfite addition compound.

The objective of this investigation was to study the effect of total solids concentration and of time and temperature of heat treatment on the browning of tomato solids. The solids concentration range studied included concentrations of single-strength juice, paste and powder. The inhibition of the browning reaction by control of pH and addition of sodium bisulfite also was investigated.

EXPERIMENTAL

THE TOMATOES were Campbell 18 variety grown at Urbana, Illinois. After washing, juice was extracted with a 180°F hot break and a Langsenkamp finisher (Model 185S, Indianapolis, Indiana), having a screen opening of .045 in. Part of the juice was filled in 2.5-gal cans frozen at −40°F and stored at 0°F until needed for single-strength juice experiments. The remainder was freeze dried at room temperature and under 1 Torr pressure to a moisture content of 1.9% and stored at 0°F until needed for preparing samples of different total solids concentration.

A portion of this same juice was centrifuged (Gyro test, DeLaval Corp., Poughkeepsie, New York) and serum collected. One lot of serum remained untreated, a second was adjusted to pH 2.5 and a third adjusted to pH 2.5 and 300 ppm SO_2 as $NaHSO_3$ was added. These batches were freeze dried as described above.

A commercial spray-dried tomato powder with a moisture content of 1.9% was also used for the first experiment.

Samples were prepared from powders by adding the calculated amount of distilled water and holding at 34°F for 24 hr to reach equilibrium.

For heat treatment, samples of the desired solids concentration were added to 20- by 150-mm screw-capped tubes placed in a constant-temperature water bath shaker (Eberbach Co., Ann Arbor, Michigan) at 95, 75 or 55°C. The amount placed in the tube varied with concentration: 25 g in case of single-strength (5.6%) juice, 10 g for 15−35%, 5 g for 45−65% and 3 g for 75−98% total solids. Heating time was 15 min unless otherwise specified.

The pH, measured with a Beckman research pH meter (Model 1019), was adjusted before heat treatment to cover the range of 1.5−4.5 with concentrated HCL

Sulfite was added to tomato juice in the form of a sodium bisulfite stock solution containing 10,000 ppm SO_2 before heat treatment. To 0.5, 1.0 or 2.0 ml of this bisulfite solution in a 100-ml volumetric flask single-strength juice was added to the mark so that final concentrations were 50, 100 and 200 ppm SO_2. This was followed by adjustment of pH to the desired level as described above.

The determination of brown color was a modification of that reported by Luh (1960). The samples that had been heat treated at the different concentrations were all reconstituted to 5.6% total solids before analysis. A 25-ml aliquot of single-strength concentration was centrifuged for 10 min at 2500 rpm in an International Centrifuge Size 2 Model V. 10 ml tomato juice serum were pipetted into a tube and 10 ml acetone added. The mixture was centrifuged for 10 min at 2500 rpm and the supernatant filtered through Whatman No. 1 paper. Optical density (O.D.) of the acetone extract from this single-strength concentration was measured with a Beckman DB-G Grating spectrophotometer at 420 mμ against a blank, as described below.

Within a given set of samples made from the same powder, a 1:1 distilled water-acetone blank was used. However, the amount of brown color already present in a tomato product will vary with origin, time-temperature history of processing, etc. Therefore, when comparing browning in samples made from different powders, the blank was obtained from an unheated, single-strength juice made from the same pow-

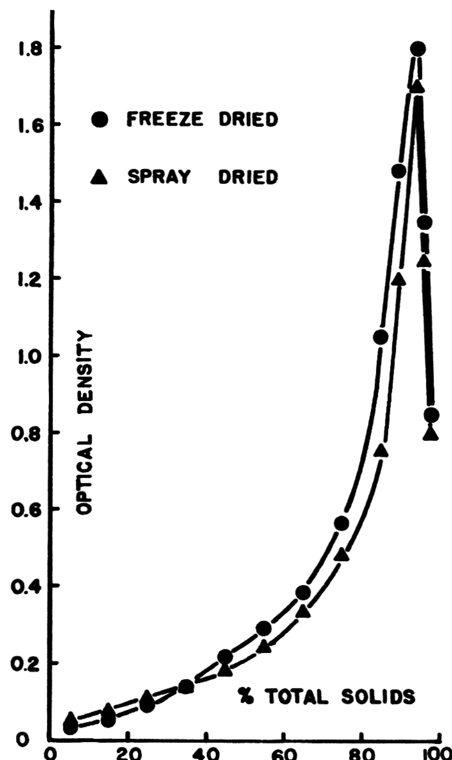

Fig. 1—Effect of tomato solids concentration on browning development in juice from 2 sources during a 15-min heat treatment at 95°C, as measured against an unheated blank after reconstitution to 5.6%. Optical density of unheated juice (5.6%) against water-acetone blank: freeze dried—0.182 and spray dried—0.324.

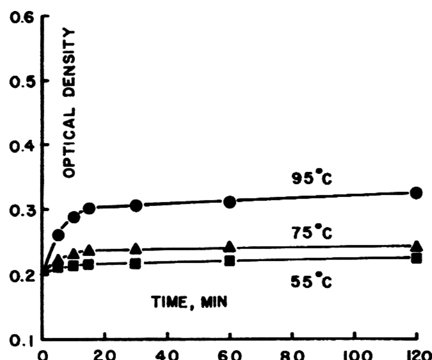

Fig. 2–Effect of time of heat treatment, at 3 temperatures, of single-strength tomato juice on browning development.

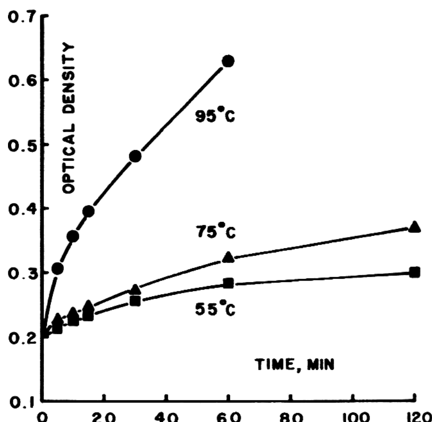

Fig. 3–Effect of time of heat treatment, at 3 temperatures, of 45% solids tomato paste prepared from freeze-dried powder on browning development.

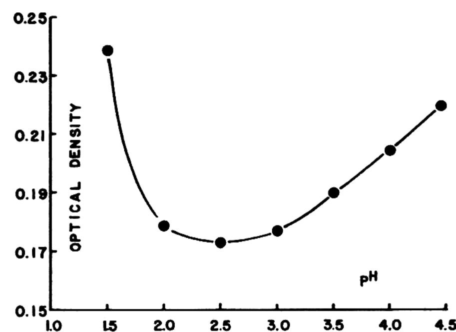

Fig. 4–Effect of pH of single-strength tomato juice on browning development during a 15-min heat treatment at 95°C.

der. The O.D. of this blank was measured against the water-acetone blank to indicate initial browning color content in each powder.

All experiments were carried out in duplicate and figures reported here are averages. The coefficient of variation of the O.D. determination (Steel and Torrie, 1960) was calculated to be 2.4%. Effect of 3 variables, temperature, concentration and time of heating, on O.D. was determined in a split-split factorial experiment. An analysis of variance of the data from the 15- and 60-min heat treatments was performed (Steel and Torrie, 1960). Similarly, the effect of 3 variables, SO_2 concentration, pH and delay of heating, was studied in another split-split factorial experiment and similarly analyzed. Only significance at the 1% level was considered.

RESULTS & DISCUSSION

Solids concentration

The effect of total solids concentration on browning of tomato products caused by heat treatment was investigated with tomato solids from 2 sources. 1 was a laboratory-prepared freeze-dried tomato powder and the other a commercial (Tri-Valley Growers, San Francisco, California) spray-dried tomato powder. The optical density (O.D.) readings were 0.182 and 0.324, respectively, using the water-acetone blank. The higher figure for the spray-dried product indicates some previous heat effect. These powders were rehydrated to the desired solids concentration from 5.6–98% for these studies.

The effect of tomato solids concentration on browning is shown for the 2 products in Figure 1. Almost identical results were obtained with the 2 sets of samples; the minor difference between the 2 curves was assumed to be due to variations in constituents such as sugar, acid or metal contamination and, in case of low moisture samples, to nonhomogeneous moisture distribution. The important finding was a coincidence in concentration of maximum browning, occurring at 95% total solids.

Also of interest was the finding that browning increased slowly with increasing concentration between 5 and about 50% solids, as compared to the increase at higher concentrations (Fig. 1). This indicates successful production of highly concentrated tomato pastes in the 40–50% solids range.

The O.D. decreased sharply at concentrations above 95% solids, so that at 98% it was down to about one-half that at 95%. The implication is that the moisture content and storage temperature of tomato powder must be reduced as low as economically possible to achieve storage stability of color. This elucidates results of storage stability studies with powders of varying moisture content by Miers et al. (1958) and Wong et al. (1956), who recommended use of in-package desiccation of tomato powder.

Time and temperature of heating

Single-strength juice was heated at 55, 75 and 95°C for various times to 2 hr. The O.D. readings shown in Figure 2 increased very little at both 55 and 75°C. A 0.12 unit increase in O.D. from 0.20 was noted at 95°C; 80% of the total increase during 2 hr occurred during the first 15 min and thereafter the rate of increase was very small.

Freeze-dried powder rehydrated to 45% solids was given the same treatments with different results (Fig. 3). At 55 and 75°C browning continued to increase after 15 min to an appreciable level by the end of 2 hr. At 95°C browning took place quite rapidly; by the end of 1 hr the

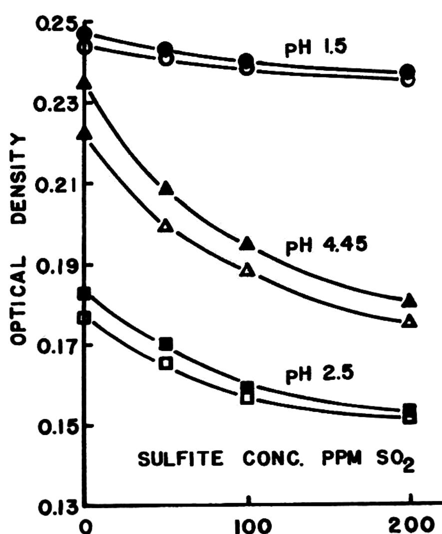

Fig. 5–Effect of sulfite concentration and pH in single-strength tomato juice on browning development during a 15-min heat treatment at 95°C. Closed points–heated immediately after adjustment and open points–delayed the heat treatment for 30 min after adjustment.

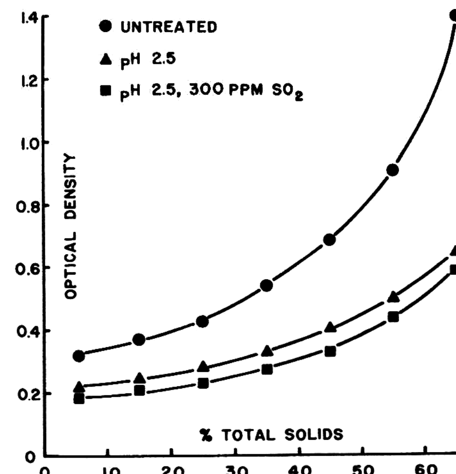

Fig. 6–Effect of sulfite concentration and pH, in tomato serum of various solids concentrations, on browning development during a 30-min heat treatment at 95°C, as measured after reconstitution to 5.5%.

browning was so severe that the O.D. rose to 0.63 as compared to about 0.3 at 2 hr for the lower temperatures.

It was concluded that increasing the freeze-dried tomato solids concentration from 5.6 to 45% and the temperature from 75 to 95°C greatly increased the rate of browning. The data were statistically analyzed. All the main factors (temperature, concentration and heating time) had a significant effect on browning as measured by optical density. All the 2-factor interactions were significant. Thus, there was an increased browning due to the combined effect as the level of each factor was increased.

pH

The effect of pH of single-strength juice on browning was studied over the range from pH 4.5, the normal level, down to 1.5. The O.D. values obtained are shown in Figure 4.

A decrease in O.D. was observed while reducing the pH of tomato juice to pH 2.5. A further reduction in pH caused an increase in O.D. (Fig. 4.), reversing the original trend. A change of one-half unit on either side of pH 2.5 caused only a small increase in browning. This pH for minimum browning coincided with the pH suggested by Wagner et al. (1968) for an acidified hot break to obtain maximum consistency of tomato products. Since the degree of acidity affects flavor, the acidified juice should be neutralized to its natural pH range after heating. The net effect of using NaOH to neutralize the acidified product is equivalent to the addition of about 0.5% NaCl (less than the usual concentration of salt in tomato products).

Sulfur dioxide

SO_2 was added at concentrations of 0, 50, 100 and 200 ppm in the juice. One portion remained at pH 4.45, another was adjusted to pH 2.5, which was previously found to be optimum for inhibition of browning and the third adjusted to pH 1.5. The data are shown as closed points in Figure 5. The O.D. decreased with increasing concentration of SO_2 at each pH. This decrease was slight at pH 1.5, where browning was at a maximum, and was most marked at the normal juice pH. Adjusting the pH to 2.5 with no SO_2 resulted in the same browning as addition of 200 ppm SO_2 at the normal pH of 4.45. At a concentration of 200 ppm SO_2, decrease of pH from 4.45 to 2.5 gave a further decrease in browning.

These results are in agreement with the theory that the bisulfite ion is the inhibitory form of SO_2. Although HSO_3 does not combine with fructose, it combines with the active aldehyde groups of its decomposition products as well as with glucose (Burton et al., 1963). The decreased inhibition obtained at pH levels below 2.5 is explained by the accelerated shift in equilibrium from HSO_3 to H_2SO_3 (inactive) caused by the pH decrease (Joslyn and Braverman, 1954). Kaufman et al. (1955) decreased heat damage by addition of sodium bisulfite to tomato paste before vacuum drying.

Delayed heating.

In a similar series of experiments the standard heat treatment was delayed for 30 min after addition of SO_2 and subsequent adjustment of pH, because it was noted that such a delay decreased browning. It was reasoned that this delay might allow greater development of the protective reaction. The data are shown as open points in Figure 5. It was found that the time effect was slight at pH 4.45 and became even less as pH was decreased and SO_2 was increased.

The data were statistically analyzed. All the main factors (SO_2 concentration, pH and delayed heating) had a significant effect on browning as measured by optical density. The interactions of pH with SO_2 and pH with delay in heat treatment were significant. Thus, the maximum benefit from addition of 200 ppm SO_2 was obtained at pH 4.45 and this benefit became less valuable as pH was reduced. Similarly, the delay became less valuable as the pH was decreased.

Combined effects

The effect of pH and SO_2 adjustment at different solids concentrations was studied in another experiment using serum separated from juice. The concentration varied from 5.5 to 65% at pH 4.45 and 2.5, the latter with and without 300 ppm SO_2. As shown in Figure 6, the inhibitory effect of pH and SO_2, found previously for single-strength juice, held true over the concentration range studied. Advantageously, the protective effects of pH 2.5 and 300 ppm SO_2 increased with increasing solids concentration.

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Ms. received 2/2/70; revised 4/6/70; accepted 4/9/70.

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EXPLORING AIRBORNE SOUND IN A NONVACUUM FREEZE-DRYING PROCESS

SUMMARY—A study was made to determine if sound energy could be used to enhance freeze-drying rates of foods and to analyze the kinetics in such a system. A prototype pilot freeze-dryer with airborne sound was designed and built to freeze-dry liquid foods, e.g., tea and coffee extracts. The sound source was a stem-jet whistle producing 10.8–12.2 kcps with a maximum sound intensity level of 149 db. Desiccated compressed air at 70° to 90°F served as the driving force of the whistle as well as the carrier of the sublimed moisture. Experimental results showed this to be a feasible process. Sample temperature, inlet air pressure, the freeze-dryer tube size, and the sound source, all had significant effects on the freeze-drying rates. Freeze-drying rates were 11–100% higher in the stem-jet whistle runs than in the dummy whistle runs; 7% of this improvement was attributed to the thermal effect on the air resulting from friction and adiabatic compression. The remaining increase was due to the sound pressure energy, the increase in heat and mass transfer coefficients, and the "reduced-pressure" effect in the sweeping air stream during the rarefaction cycle of the sonic vibration. The flaking action of the sound waves on near-dried tea and coffee was considered a processing advantage because of the ease in product recovery and because a new surface was continuously being exposed for sublimation.

INTRODUCTION

NONVACUUM freeze-drying is a process based on the phase characteristics of water. This concept is evidenced by clothes being freeze-dried outdoors on a cold, dry, and windy winter day. Applied to food, it requires that the food be kept frozen throughout the freeze-drying cycle; that energy be available to sublime the ice; and that there be a sufficiently large vapor pressure gradient between the frozen food and the surroundings for mass transfer. Liquid foods such as coffee and tea extracts containing varying amounts of soluble solids could conceivably be dried with this process. The obvious advantage is the elimination of high-vacuum equipment and strong-wall drying chamber. However, when desiccated cold air is used in a nonvacuum freeze-drying process, the drying rate is low. Instead of relying only on regulating the temperature and humidity of the air to control the freeze-drying rate, sonic energy could be added to the sweeping air stream to enhance the drying rate.

This study is believed to be the first attempt to use sonic energy in freeze-drying, although in air-drying, sonic energy has been found effective in drying-rate improvement (Boucher, 1959b). Both sonic and ultrasonic energy are mechanical energies, capable of doing work. Ultrasound has the arbitrarily defined frequency range of 20 kilocycles per second (kcps) and above, whereas the sonic range is 20 kcps and below. Both sound and ultrasound are transmitted in the form of compressional waves through fluids or solids at a velocity characteristic of the condition of the transmitting medium.

In a fluid stream with sonic pulsation, a building-up of pressure is followed by an expansion. This alternating compression and rarefaction takes place thousands of times a second depending upon the frequency of the sound source. Boucher (1959b) pointed out that when a high intensity sound field is produced above a liquid-coated material, moisture is continuously released, either as a liquid or as a vapor, because the effect of expansion always predominates over that of compression. Thus in a system where a source of airborne sound with a frequency of, for example, 10,000 cps, passes over a frozen material, a vapor pressure gradient will develop every ten-thousandth of a second during which sublimation can take place. The sublimed moisture is then carried away by the sweeping air stream.

Several problems arise in applying sonic energy to a nonvacuum freeze-drying process. These are: (1) keeping the material frozen at all times; (2) providing a large vapor pressure gradient for mass transfer; and (3) utilizing the sonic energy efficiently. The objective of this study is to determine whether or not sonic energy can be used to enhance the freeze-drying rates, and to analyze the kinetics of such a process.

LITERATURE REVIEW

MERYMAN (1959) discussed the feasibility of freeze-drying without vacuum, and obtained a patent for his apparatus for freeze-drying at atmospheric pressure (Meryman, 1963). Monkey kidney, in 2-mm cubes, was freeze-dried at −22°F in 8 hr. Lewin and Mateles (1962), who studied the feasibility of dehydrating frozen peas, carrots, and chicken meat in a flow of desiccated air, reported that the drying time was long. For example, white chicken meat of unspecified size at 29°F required 26 hr to dry to 7.0% moisture. Woodward (1963) studied freeze-dehydration without vacuum by passing a stream of dry gas through a bed of frozen vegetables. He called this the Carrier Gas Sublimation Process. Gas temperature, sample temperature and surface area were significantly related to drying rates, but a three-fold increase in gas velocity had practically no effect on drying time.

Drying by sound or ultrasound is not a new idea. Stephanoff obtained a patent in 1938 for an ultrasonic drying method (Greguss, 1961). Vang (1942) proposed to create sonic or ultrasonic vibrations inside a dryer by using the high-frequency induction heating technique for producing artificial turbulence with sound waves. Wenk (1951) reported that the fog-producing effect of ultrasonic waves could be used for drying paper and textiles.

Boucher (1959a) pointed out that sonic and ultrasonic waves developed alternating intervals of pressure and expansion, known as compression and rarefaction. Repeated thousands of times a second, it would create a surface cavitation that would break the boundary layer and allow liquid to evaporate under partial vacuum.

Borisov and Gynkina (1961) used a dynamic siren to dry thin capillary porous materials. They suggested that, in addition to sound pressure, intensive circulation flows be induced on the drying surface to enhance drying.

Researchers in recent years have tried to improve convective heat and mass transfer with various sound sources, but as Lemlich (1961) pointed out, results were inconclusive. Improvement depends upon the transfer system, sonic frequency, flow velocity, fluid properties, and the Reynolds number.

EXPERIMENTAL

Sound source

The sound source used was a stem-jet whistle (Lelandais, 1960) developed and manufactured by Demister AB in Sweden. It is an acoustic air-jet whistle which transforms compressed-air energy into acoustic energy. The main frequency range of the stem-jet whistle is 10.8–12.2 kcp at which about 60 watt are radiated. Figure 1 shows the Demister AB stem-jet whistle.

To determine the effect of airborne sound

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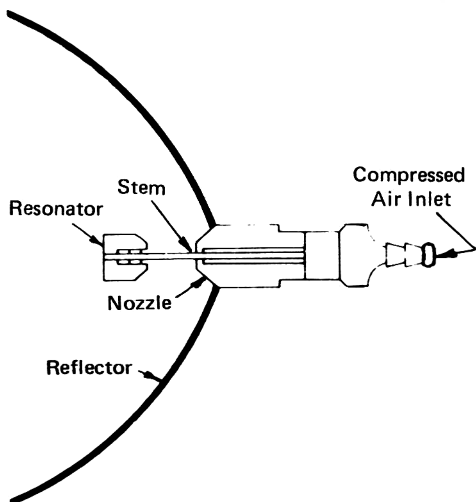

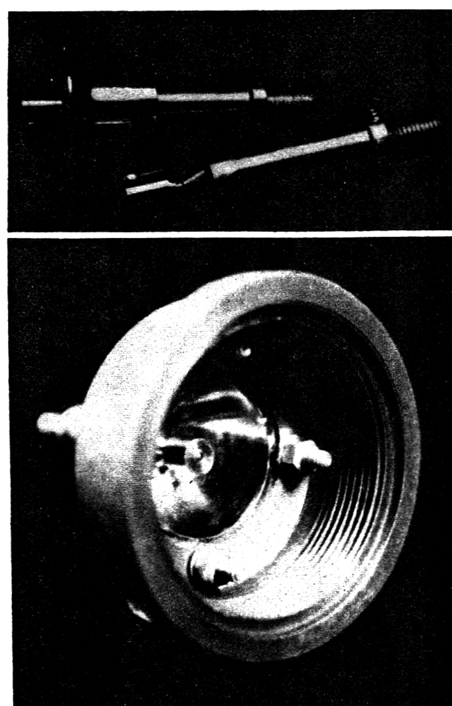

Fig. 1–Demister AB stem-jet whistle: (top) cross-sectional view; (center) as assembled; (bottom) assembled with 2 in. parabolic reflector and cover plate.

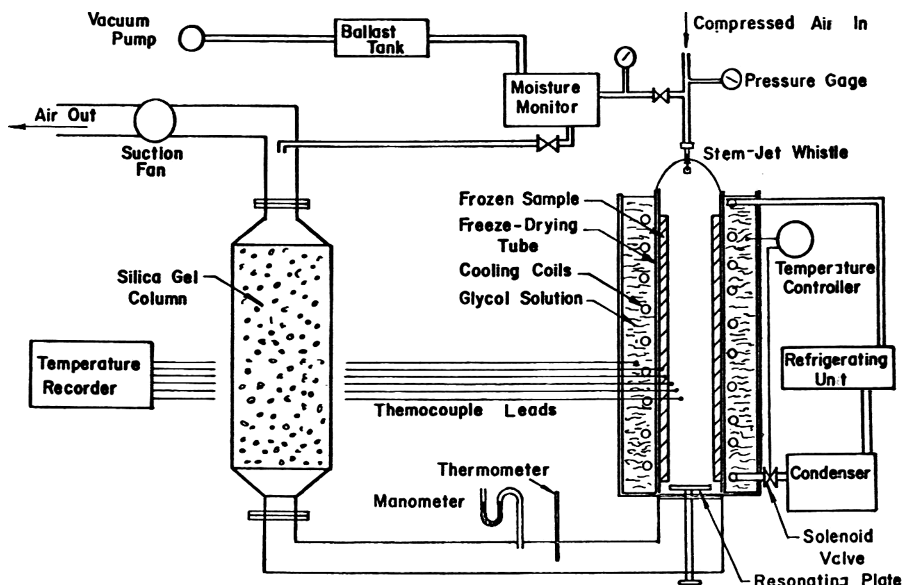

Fig. 2–Schematic diagram of airborne sonic freeze-drying system.

on freeze-drying rates, both sonic and soundless runs were made under various sets of identical operating conditions. Any gain or loss in freeze-drying rates from the sonic run was then attributed to the effects of sound. For soundless runs, a dummy whistle was used. This dummy whistle was identical to the stem-jet whistle except that the resonating chamber was replaced by a solid cylinder. Without the resonating chamber, the dummy whistle had a very low sound intensity level of 130 decibels (db) as compared with 145–149 db for the stem-jet. On the other hand, measurements of air velocities with an anemometer showed that the dummy whistle gave the same air velocity profile in the freeze-drying tube as the stem-jet whistle.

Design considerations

To activate the whistle and transmit sound energy onto the frozen sample, desiccated compressed air was used. In addition to being readily available and continuous, the compressed air also served as the moisture-carrier in the freeze-drying system. A compressed inert gas would have been desirable for foods from the standpoint of minimizing oxidation and being very dry, if it were an economical and continuous source of gas supply.

In a nonvacuum freeze-drying system, the sample temperature is affected by the temperature, pressure, and humidity of the air. Without external cooling, the sample is kept frozen by evaporative cooling, or by sublimative cooling. Regardless of the sublimation rates, it would be desirable to keep the sample frozen at a fixed temperature so that the effect of sample temperature on the freeze-drying rate could be determined. As a result, a 2-ton −40°F refrigeration unit was built to serve a dual purpose: to freeze the sample on the inside wall of the freeze-drying tube and to maintain the sample temperature at a set point.

Measuring the freeze-drying rate of a sample frozen on the inner wall of a tube posed a problem. Of several techniques considered, the one chosen was that by which a material balance on the moisture in the air streams around the system was taken. The air flow rates were measured with a Schutte & Koerting rotameter in CFM, and then converted to lb/hr. The humidities in the inlet and outlet air streams were monitored with an electrolytic moisture meter in ppm by volume (Consolidated Electrodynamic Corp. Model 26-303 MA). The bulk of the subliming vapor from the sample was adsorbed in a silica-gel column which was part of the system. Figure 2 shows the schematic diagram of the freeze-drying system using airborne sound. There are four moisture streams around the system. If we denote:

r_i = water vapor in inlet air, lb/hr (Inlet 1)

r_d = vapor sublimed from the frozen sample, lb/hr (Inlet 2)

r_c = moisture adsorbed in silica-gel column, lb/hr (Outlet 1)

r_o = water vapor in outlet air, lb/hr (Outlet 2)

and equate the inlet streams with the outlet streams:

$$r_i + r_d = r_c + r_o \quad [1]$$

we will obtain the freeze-drying rate as:

$$r_d = (r_c + r_o) - r_i. \quad [2]$$

Further modifications will yield drying rate in weight of water removed per unit weight of bone-dry solid per hr, or weight of water removed per hr per unit surface area.

The inlet air humidity was brought to below 100 ppm by an elaborate desiccation system consisting of an aftercooler, three air dryers and two air filters. The temperature of the compressed air entering the whistle was between 70–90°F. The air pressure was regulated downstream of all the air dryers and before the whistle.

Since sound intensity is a major contributing factor in effective acoustic air drying (Boucher, 1959b), it was desirable to maximize the sound intensity of the stem-jet whistle for freeze-drying. A resonating plate was therefore set up at the discharge end of the freeze-drying tube. The position of the plate was determined with a microphone connected to a sound intensity meter. The point of maximum sound intensity at a given nozzle pressure was also the point of resonance.

The freeze-drying system

A view of the constructed system is shown in Figure 3. The freeze-drying tubes were 20 in. long × 2 in. I.D. and 20 in. long × 3 in. I.D., surrounded by a refrigerating coil containing

Table 1—Freeze-drying rate increase in the stem-jet whistle runs over the dummy whistle runs.

Inlet air pressure psig	Sample temp. °F	Avg rates, lb/hr/ft² Stem-jet	Avg rates, lb/hr/ft² Dummy	% Increase = (Steam-jet—Dummy)/Dummy	Avg % Increase
			In a 2-in. tube		
22	− 15	.0635	.0531	19.4	
	− 5	.0846	.0697	21.4	15.3
	5	.1003	.0955	5.0	
25	− 15	.0860	.0582	47.8	
	5	.0972	.0942	3.2	24.7
	− 5	.1216	.0987	23.2	
30	− 15	.0774	.0900	-14.0	
	− 5	.1024	.0855	19.8	10.6
	5	.1210	.0960	26.0	
			In a 3-in. tube		
22	− 15	.0466	.0354	31.6	
	− 5	.0482	.0361	33.5	32.6
	5	.0484	.0364	33.0	
25	− 15	.0534	.0425	25.6	
	− 5	.0580	.0520	11.5	17.8
	5	.0684	.0588	16.3	
30	− 15	.0481	.0310	55.2	
	− 5	.0537	.0510	5.3	31.8
	5	.0870	.0644	35.1	

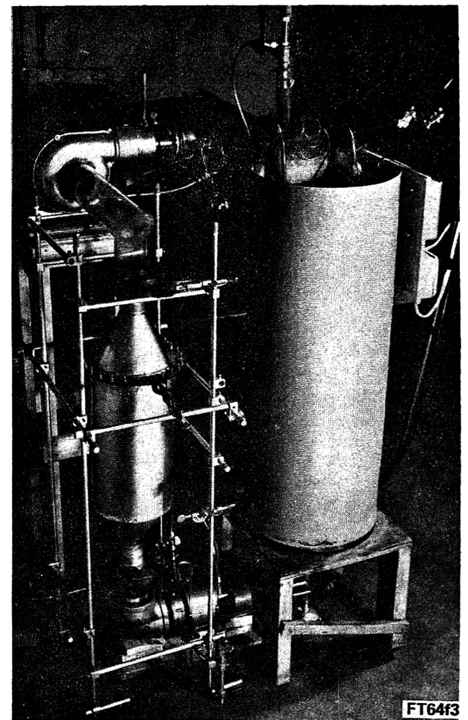

Fig. 3—Airborne sonic freeze-drying pilot unit designed and built for this study.

Freon 502 (Dupont trade-marked refrigerant). A tank, 36 in. high and 15 in. in dia, accommodating both the coil and the tube, was filled with 65% ethylene glycol bath to maintain the tube at −30° to 0°F.

When the tube was cooled to the desired temperature, a liquid sample at 40°F was introduced into the tube. After a frozen layer had developed on the inner wall, the excess sample was drained off and the drain valve removed. A series of 10 thermocouples, spaced 0.1 in. apart radially from the inside wall, measured the sample and air temperatures.

The freeze-drying cycle began when desiccated, compressed air was admitted through the whistle and into the tube. The subliming vapor from the frozen sample was carried away by the sweeping air stream. The bulk of the vapor was then adsorbed in the silica-gel column. The residual moisture in the outlet air was measured by the moisture monitor. The short tubing between the freeze-drying tube and the silica-gel column housed a manometer and a thermometer. An appreciable pressure drop occurred through the silica-gel column which slowed down air flow before the column. Since this would give a false picture of the freeze-drying rate, the pressure drop was cancelled by creating a suction with a variable-speed suction fan on the downstream of the column.

Variables studied

A 4-factor randomized complete block of 3 x 3 x 2 x 2 experiment was carried out. With and without the whistle, the air pressure was varied at 22, 25, and 30 psig; sample temperature was varied at −15°, −5° and 5°F; and tube sizes were 2 in. and 3 in. nominal I.D. by 20 in. long. Duplicate freeze-drying runs were made on both the stem-jet and the dummy whistles, with distilled water and tea and coffee solutions as samples. 10–25% tea and coffee solutions were prepared by dissolving soluble tea and coffee in distilled water.

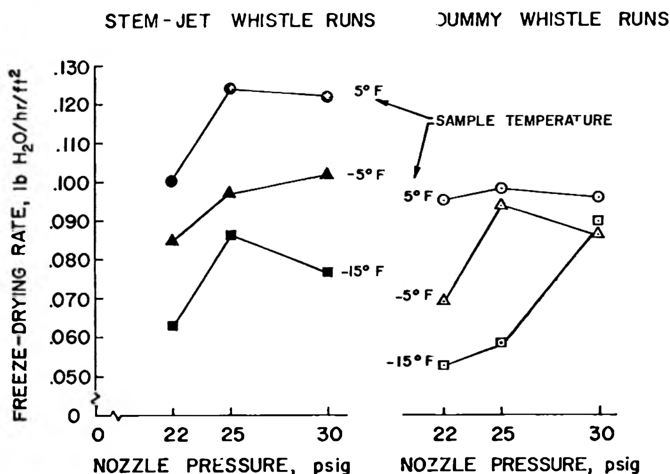

Fig. 4—Effects of processing conditions on the freeze-drying rates of ice in a 2-in. tube with and without sound.

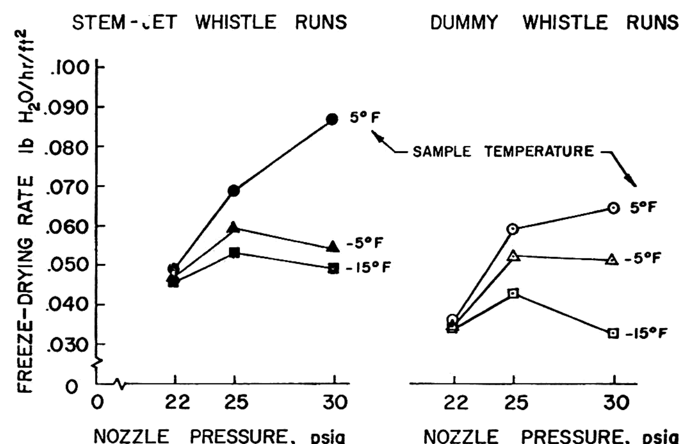

Fig. 5—Effects of processing conditions on the freeze-drying rates of ice in a 3-in. tube with and without sound.

Table 2–Freeze-drying rates of coffee and tea extracts in a 2-in. freeze-drying tube with a stem-jet whistle and a dummy whistle.

Inlet air pressure psig	Sample temp. °F	% Sol solids, (wet basis)	Freeze-drying rates, lb H_2O/hr/ft^2 Stem-jet whistle runs	Dummy whistle runs
19	– 5	20% coffee	.0519	.0207
22	– 5	20% coffee	.0461	.0393
25	– 5	10% coffee	.0675	.0320
22	– 5	25% Tea[a]	.0272	–
22	0	25% Tea[a]	.0404	.0168
22	– 5	10% Tea[a]	.0532	.0280
25	– 5	10% Tea[a]	.0610	.0453

[a]The solids content: 50–50 tea and malto-dextrin.

Table 3–Analysis of variance of the airborne sonic freeze-drying rate data.[a]

Source of variation[b]	Degrees of freedom	Sums of squares	Mean square	F ratio
Total	35	219.5755	–	–
T	2	33.9405	16.9702	353.10**
P	2	20.9282	10.4641	217.73**
J	1	10.0594	10.0594	209.30**
D	1	137.0850	137.0850	2852.33**
T × P × D	4	6.2972	1.5743	3.28*

*Significant at p = 0.05.
**Significant at p = 0.01.
[a]All other effects due to two-factor interactions such as T × P, and three-factor interactions, such as T × P × J, not shown here, were found to be insignificant.
[b]Notation: T = Sampling temp. P = Pressure. J = Stem-jet whistle D = Tube diameter

RESULTS

Freeze-drying system performance

The prototype freeze-drying pilot unit designed and built for this experimental study with airborne sound has performed satisfactorily. A few minor problems arose, such as fluctuations of temperature and humidity of the incoming air, temperature of cooling water and conditions of various desiccants. These sometimes caused fluctuation of the humidity in the compressed air, but the humidity was below the limit for nonvacuum freeze-drying.

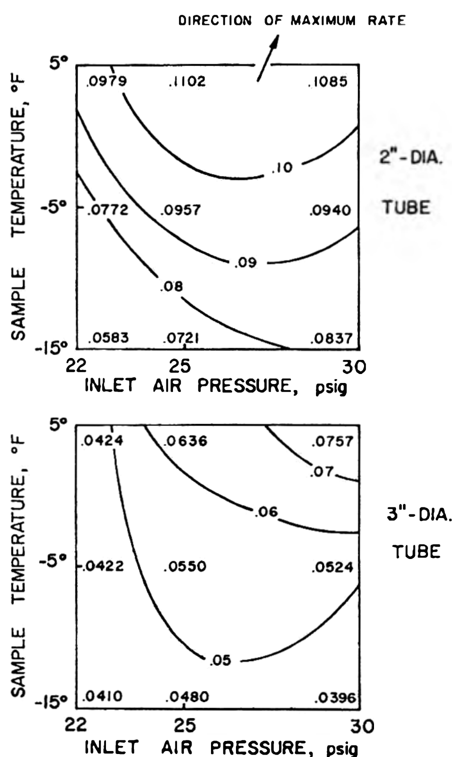

Fig. 6–Response surface of the airborne sonic freeze-drying system. All numbers in the squares are average freeze-drying rates in lb/hr/ft² from the stem-jet and the dummy whistle runs.

Freeze-drying rate studies

The effects of sound and three other variables on the freeze-drying rates of ice were first studied. The average rates were measured hourly over an 8–10 hr period. As shown in Table 1, the drying rates from the stem-jet whistle runs were higher than those from the dummy whistle runs except in one instance. The rates were measured in lb of water removed per hr per sq ft of sample surface area. On this same basis, the drying rates were about 50–100% higher in the 2-in. tube than in the 3-in. tube. On the other hand, the effect of inlet air pressure did not show a definite trend on the drying rates as shown in Figures 4 and 5.

Based on results obtained with ice as a sample, a number of runs on coffee and tea extracts at 10–25% soluble solids were made. The dual purpose was to test the feasibility of freeze-drying liquid foods in such a system and to see if the sonic effect was significant. Table 2 shows that significant increases in drying rate were obtained in the sonic runs which demonstrated that frozen tea or coffee extracts could be freeze-dried readily with airborne sound. Also, the sound waves caused some flaking at the interface of the near-dried layer. In the soundless runs, on the other hand, the frozen extract did not flake and had a much lower drying rate.

Analysis of data

Analysis of variance on the freeze-drying data showed significant effects at the 1% level from the four variables being studied, namely, sample temperature, inlet air pressure, tube size, and the stem-jet whistle, as shown in Table 3. In addition, there was an interaction among the first three variables significant at the 5% level. It was also found that a difference, significant at the 5% level, existed in the freeze-drying rates between the sonic runs and the soundless runs.

The variances were homogeneous among all the drying rate data. The best estimate of the standard deviation from all the replicate runs was 0.00504 lb/hr/ft² with 46 degrees of freedom.

Optimizing the process

To see if the drying rate in this process can be optimized, a response surface as a function of the sample temperature and inlet air pressure were plotted using all the drying rate data from the stem-jet and dummy whistle runs on ice. Figure 6 shows the response surface for the 2 in. and 3 in. tubes. Because of the ranges of variables chosen for the experiment, the maximum did not appear on the response surface. The maximum rate was, however, in the direction of a higher sample temperature and an inlet air pressure above 25 psig.

Energy distribution and system efficiency

The energy input to the airborne sonic freeze-drying system came from three sources: (1) enthalpy in the air; (2) mechanical energy produced from the sound pressure; and (3) additional enthalpy in the air due to the thermal effect produced by the stem-jet whistle. In a typical case where air entered the whistle at 82°F and 22 psig and was discharged at 62°F and 15 psia, an energy balance around the system can be set up as shown in Table 4. It can be seen that the energy contribution from the sound source was 27%. However, it was not easy to estimate the efficiency of the whistle as used here. The usual mechanical efficiency of many sonic devices, such as an air-jet whistle, is in the neighborhood of 5–10%. As Table 4 also shows, the

Table 4—An energy balance in the airborne sonic freeze-drying system.[a]

	Input			Output	
	BTU/hr	%		BTU/hr	%
Net enthalpy from the inlet air 82–62°F[b]	435	56.0	Heat of sublimation[e] for 0.05 lb of ice	61	7.9
Energy due to sound pressure[c]	210	27.0	Sensible heat[f] for .05 lb of ice	1	0.1
Additional enthalpy of inlet air due to adiabatic compression[d]	132	17.0	Heat loss to the system by radiation and convection[g] (By difference)	715	92.0
Total	777			777	

[a]Basis: Air at 32°F and atmospheric pressure.

Methods of calculations for the three input terms

[b]Calculated from experimental data:

Inlet air pressure: 22.0 psig; Inlet water vapor: 4.72×10^{-5} lb/hr;
Air flow rate (w): 92.0 lb/hr ; Outlet water vapor: 0.552 lb/hr.

Enthalpy in inlet air $= (w)_{air} \int_{t_o}^{t_2} (C_p)_{air}\, dt = (92.0) \int_{32^\circ}^{82^\circ} (0.2404) dt$

$= 1105.8$ Btu/hr (enthalpy of inlet water vapor is negligible)

Enthalpy in outlet air $= (w)_{air} \int_{t_o}^{t_1} (C_p)_{air}\, dt + (w)_{H_2O} \int_{t_o}^{t_1} (C_p)_{H_2O}\, dt$

$= (92.0) \int_{32^\circ}^{62^\circ} (0.2404) dt + (0.552) \int_{32^\circ}^{62^\circ} (0.444)\, dt$

$= 663.5$ Btu/hr + 7.4 Btu/hr

$= 679.9$ Btu/hr

Available enthalpy in the air stream = 1105.8 – 670.9 = 434.9 Btu/hr

[c]Sound pressure energy E_p (Beranek, 1954):

$E_p = P \int (v)\, dt = (\rho c)(v) \int (v)\, dt = (\rho c) \int (v^2)\, dt$

where
P = Sound pressure
v = Air velocity = 6.0 ft/sec = 1.83 m/sec (measured experimentally)
ρc = (air density) × (sound velocity) = Characteristic impedance
= 407 Newton-sec/m³ at 72°F (Beranek, 1954)

$E_p = (\rho c) \int (v^2)\, dt = (\rho c)\, v^2 \Big|_0^1$

$= (407)$ Newton-sec/m³) × (1.83 m/sec)²

$= 0.648$ ft-lb/sec-in²

$= 432$ Btu/hr/ft²

E_p in 2" tube $= E_p$ (Surface area) = (432 Btu/hr/ft²) × (0.50 ft²)

$= 216$ Btu/hr

From the rated output of the stem-jet whistle of 60 watt:

$E_p = (60\ w) \times (3.415$ Btu/hr/w)

$= 204.9$ Btu/hr

Average sound pressure energy from the two calculations:

$E_p = 210.4$ Btu/hr

[d]Additional enthalpy in the inlet air stream due to friction and adiabatic compression in the resonating chamber of the stem-jet whistle, which caused a temperature rise of 6°F by direct measurements:

$E = (w)_{air} (C_p)_{air} (\Delta t)$

= (92.0 lb/hr) (0.2404 Btu/lb/°F) (6°F)

= 132.5 Btu/hr

Methods of calculations for the three output terms

[e]Heat of sublimation for 0.05 lb of ice:

Latent heat = 1220.2 Btu/lb (for ice at 15°F) (from Charm, 1963)

Energy used = (0.05 lb/hr) (1220.2 Btu/lb)
= 61.0 Btu/hr

[f]Sensible heat for 0.05 lb of ice:

Average specific heat of ice = 0.505 (from Henderson and Perry, 1955)

Energy used = (mass) (specific heat) (Δ t)
= (0.05 lb) (0.505 Btu/lb-°F)* (30°F)
= 0.76 Btu/hr
≅ 1.0 Btu/hr

*Strictly speaking, specific heat has the same units as heat capacity, but need not have the units shown, as in the case of specific gravity. However, this has not been universally agreed upon by various authors in various disciplines.

[g]Heat loss to the system by radiation and convection:

Total energy input – total energy used = heat loss

777 Btu/hr – (61 + 1)Btu/hr = 715 Btu/hr

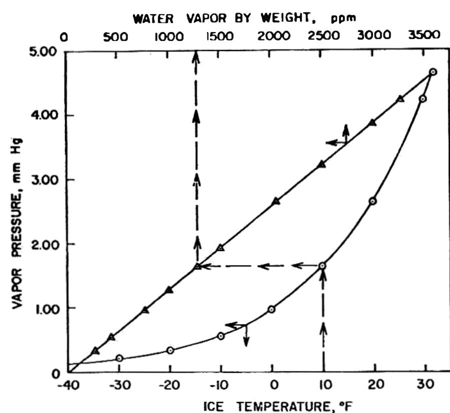

Fig. 7–Vapor pressure limiting curve for nonvacuum freeze-drying.

thermal efficiency of the system in terms of energy utilization for sublimation was only about 8%.

DISCUSSION

The freeze-drying system

As far as is known, there have been no previous attempts to utilize sound or ultrasound in freeze-drying. These experiments, therefore, were essentially exploratory, although basic in determining whether or not sound could be used to enhance freeze-drying rates and the kinetics involved in such a system. A few problems were encountered in the operations of the system. The inlet air humidity was not well controlled, but was below the limits for nonvacuum freeze-drying as determined from Figure 7, the vapor pressure limiting curve adapted from established vapor pressure-temperature data on ice. The temperature of the inlet air should be controlled over a wide range and more precisely than had been possible. The moisture readings were not as consistent as expected.

Isolating the sonic effect

Using a dummy whistle to distinguish the sonic effect from the stem-jet whistle on the freeze-drying rate was satisfactory. Another way of studying the sonic effect would be to separate entirely the effect of air flow from the freeze-drying tube by using a membrane for sound transmission. However, any moisture sublimed from the frozen sample would have to be carried away in order to determine the freeze-drying rate. For further discussion of this problem refer to Moy (1965).

Sonic effect on freeze-drying rates

The highest freeze-drying rate of ice in this experiment was obtained when the sample temperature was 5°F and the inlet air pressure 25–30 psig. This pressure range corresponds to a linear velocity of 5.0–5.2 ft/sec in the 2 in. tube. The average drying rate obtained was 0.120 lb/hr/ft^2. Lewin and Mateles (1962) obtained drying rates of 0.0615 lb/hr/ft^2 in one apparatus and 0.178 lb/hr/ft^2 in a modified apparatus where both ice samples were at 20–23°F with an air velocity of 3.5 ft/sec.

Drying rates on 10% tea and coffee extracts at –5°F and 5.0 ft/sec in this study were 0.0610 and 0.0675 lb/hr/ft^2 respectively. Higher sample temperature could conceivably result in higher drying rates within the limits of the eutectic temperature.

Kinetic theory of freeze-drying with airborne sound

In this study, the concept of heat energy having the same dimension as mechanical energy was explored. Both heat energy and mechanical energy were supplied to the freeze-drying systems as energy for sublimation, the mechanical energy being the sound energy.

To carry the subliming moisture away, a flow system is needed. The system used in this study has the feature of forced convective heat and mass transfer with turbulent flow. The Reynolds numbers vary from about 11,000–22,000. Generally, one thinks of the phenomenon of turbulence as being random motion all around. In a tube such as the one set up for freeze-drying in this study, the motion is not entirely random throughout. Around the center of the tube, the velocity fluctuations are almost completely random. By the wall, fluctuations in the axial direction are greater than those in the radial direction, and all fluctuations approach zero at the wall itself. Three zones are customarily designated within a tube in turbulent flow: the laminar sublayer, which is the layer immediately adjacent to the wall; the buffer zone, in which the laminar and turbulent effects are both important; and the region of fully developed turbulence.

For convective flow in a tube, the transport mechanism is convection in the turbulent core but conduction in the laminar sublayer. Resistance of the laminar sublayer is proportional to its thickness. Any reduction of the thickness will result in a comparable increase in the heat transfer rate between the wall and the fluid. Between the 2 in. and 3 in. tubes used in this experiment, the smaller tube had a higher Reynolds number and a thinner laminar sublayer. Hence the heat transfer is more effective in the 2 in. tube, which agrees with the experimental findings that freeze-drying rates were higher in the 2 in. tube than in the 3 in. tube.

With the use of a whistle in the freeze-drying system, the sound might be considered as a turbulence promotor. The vibration and pulsation would tend to increase the turbulence of the flowing stream. The effect might be helpful in reducing the thickness of the laminar sublayer and increasing the heat transfer coefficient and hence mass transfer coefficient between the air stream and the frozen layer.

At the same time, the pressure pulses from the sound waves create alternating cycles of compression and rarefaction. During the compression cycle, the sound pressure energy is transmitted as kinetic energy to the air molecules, also increasing heat conduction between molecules due to the compressive effect. During the rarefaction cycle, the effect of sound on drying rate can be viewed in two ways:

(1) Pioneers in acoustic drying such as Boucher (1959b) have suggested that the significance of this rarefaction cycle is the partial vacuum effect. He stated that even though the change in pressure was about 6% of the total pressure, it became appreciable when repeated many times a sec. If this theory is accepted, then the increase in airborne sonic freeze-drying rate would be due to this partial vacuum effect.

(2) Another theory suggested from the results of this experiment is the reduced vapor pressure effect in the ambient air stream. The change in pressure in the tube due to the presence of sound pressure was calculated to be 0.112 psig which is only about 0.75% of the total pressure in the system. According to Dalton's Law, the partial pressure of the water vapor in the air is reduced during the expansion cycle, which predominates over that of compression (Boucher, 1959b). The vapor pressure difference between the air stream and the frozen layer is accordingly increased, enhancing the sublimation rate. This is especially true when this phenomenon is repeated 12,000 times a sec.

Further experimental work to determine the magnitude of this "partial vacuum" or "reduced pressure" would be worthwhile.

In addition to promoting turbulence for a higher heat transfer rate, and providing a larger vapor pressure difference for a high mass transfer rate, the freeze-drying rates were increased by the following sonic effects.

(1) Increased eddy diffusion induced by the high-frequency vibration increased the mass transfer rate.

(2) An additional amount of thermal energy was obtained from the whistle due to friction and adiabatic compression of the air as it enters the resonating chamber of the whistle. Measurements of air temperatures before and after the whistle showed a rise of 5° to 8°F in all the stem-jet freeze-drying runs. By using convective heat and mass transfer calculations, the increase in freeze-drying rate due to this effect was estimated to be 7%. This effect may not be an advantage, since the same thermal effect is readily obtained in other ways.

(3) A hystersis effect caused the moisture from the frozen layer to desorb more readily than to re-adsorb. This effect is based on Boucher's observation and theory that the rarefaction is larger in magnitude than the compression in a sound pressure system. The experimental procedure was not set up to measure this effect. Further work in determining the sound pressure variation in a tube, especially with a turbulent air stream passing over a layer of ice, would be of great interest and would contribute to further understanding of the effects of sound on freeze-drying.

CONCLUSIONS

THE FOLLOWING conclusions were drawn from the study of nonvacuum freeze-drying with airborne sound:

(1) With the pilot unit designed and built for this study, freeze-drying with airborne sound was feasible for drying liquid foods such as tea and coffee extracts.

(2) Freeze-drying rates in the sonic runs were significantly higher than those in the soundless runs. The four processing variables, sample temperature, inlet air pressure, tube size, and sound source, had significant effects on freeze-drying rates.

(3) Increased freeze-drying rate in the sonic runs was probably due to (a) the increase in heat and mass transfer coefficient; (b) the effect of reduced vapor pressure in the air stream during the rarefaction cycle; (c) the thermal effect on the air resulting from friction and adiabatic compression; and (d) the sound pressure energy as a source of work energy.

(4) Flaking action of the sound waves on the near-dried surface of the frozen material was also a probable significant factor in increasing the freeze-drying rates in the sonic runs, and could be considered a processing advantage.

(5) The process optimum was not achieved in this study due to lack of precise control of the inlet air humidity and temperature, and also to the limited range selected for the sample temperature and air flow rate.

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THE NITRATE DETINNING REACTION IN MODEL SYSTEMS

SUMMARY—As part of a coordinated research program on internal can corrosion, the action of nitrates on tinplate was studied in model systems simulating foods of varying acidities. Detinning rates were measured in citrate buffered nitrate solutions ranging in pH from 3.0–7.0. At pH values less than about 5.2–5.5 the detinning is extremely rapid, with slower rates at higher pH values. Nitrate concentrations of 125 ppm in pH 4 buffers completely detinned cans in about 2 months at ambient temperatures. Ammonia is the principal nitrate reduction product in the pH range of acid products. Nitrous oxide is also produced and acts as an effective detinner at pH 4 and 5. Nitrogen may also be produced at pH 5.

INTRODUCTION

ORGANIZATION of the canning industry research project on rapid detinning and the general plan of the research have been described (Farrow, 1970). In addition to the study and analysis of experimental packs grown and canned under controlled conditions at cooperating universities, the project included studies of the nitrate detinning reaction carried out on well defined model systems. The objectives of this work were to determine the conditions under which nitrate detinning might be important, and to identify the nitrate reduction products resulting from the reaction.

Most of the literature relating to the chemistry of the reaction between tin and nitrates is concerned with the action of relatively concentrated acid on the metal, usually in the presence of air. An early review by Bancroft (1924) outlines the reduction products of nitric acid, but the discussion has only limited application to interactions between nitrates in food products and tinplate containers. Kasbekar and Normand (1933) published data on the gaseous products of the reaction of nitric acid on tin. The reduction products they list include NO, N_2O, N_2 and NH_3.

A detailed study of the action of nitrates in food products on tinplate containers has not been published. Culpepper and Moon (1928) suggested that nitrate could be responsible for rapid detinning in pumpkin and beets. Strodtz and Henry (1954) presented information on the effect of pH on the reaction and studied nitrate detinning in green beans and spinach.

In the experimental work described here, citrate buffered nitrate solutions were used to obtain detailed data on the effect of pH on nitrate detinning. The decrease in the nitrate concentration and the increase in the concentration of ammonia were followed in the canned buffered nitrate solutions throughout suitable storage periods. Buffered nitrate solutions canned under helium were used to study gaseous reaction products.

EXPERIMENTAL

ALL MODEL BUFFER solutions used in this study were canned in the containers specially prepared for this project (Farrow, 1970). Three levels of nitrate—50, 125, and 250 ppm—were studied. These were prepared in 0.15% citric acid adjusted with sodium hydroxide solution to final pH values of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, and 7.0. Some experiments were prepared in 0.8% citric acid and these have been identified.

Solutions were canned using a process suitable for an acid product. They were heated to 205°F in a water bath, filled into 303 × 406 cans, closed and processed in water at 210°F for 5 min. Cans were cooled immediately in cold water and kept at room temperature throughout their storage period.

At suitable intervals samples of each variable were taken from storage and analyzed for dissolved tin, nitrate, ammonia, and various additional constituents as indicated.

To detect the gaseous nitrate reduction products, an experimental procedure was required that would take account of the possible production of nitrogen. For this portion of the

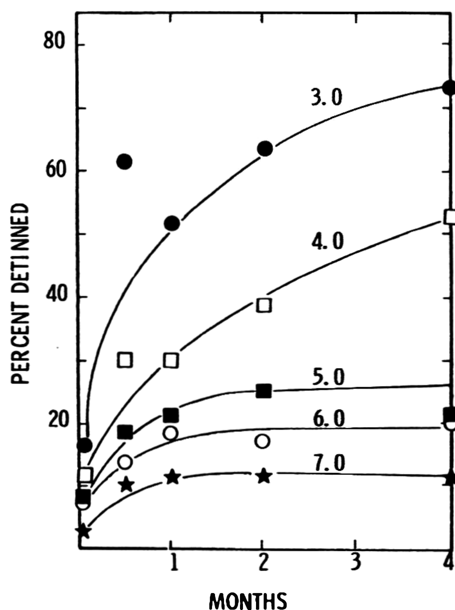

Fig. 1—Detinning due to 50 ppm of nitrate in citrate solutions buffered to pH 3.0, 4.0, 5.0, 6.0, and 7.0.

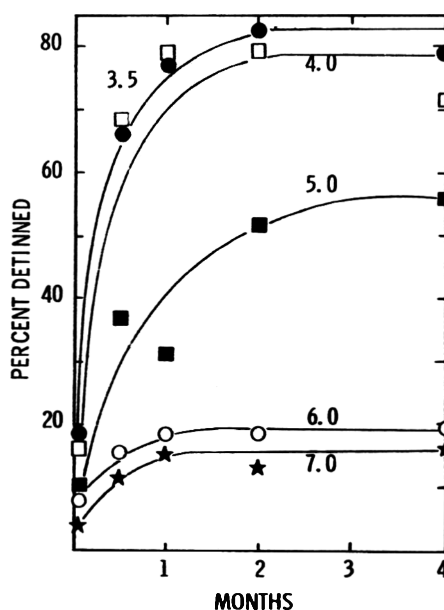

Fig. 2—Detinning due to 125 ppm nitrate in citrate solutions buffered at 3.5, 4.0, 5.0, 6.0 and 7.0.

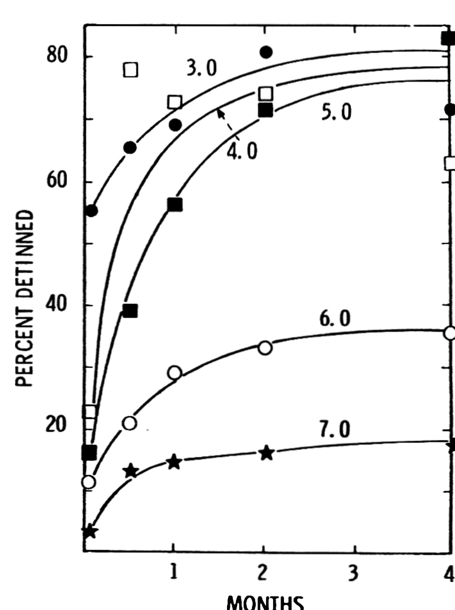

Fig. 3—Detinning due to 250 ppm of nitrate in citrate buffers from pH 3.0–pH 7.0.

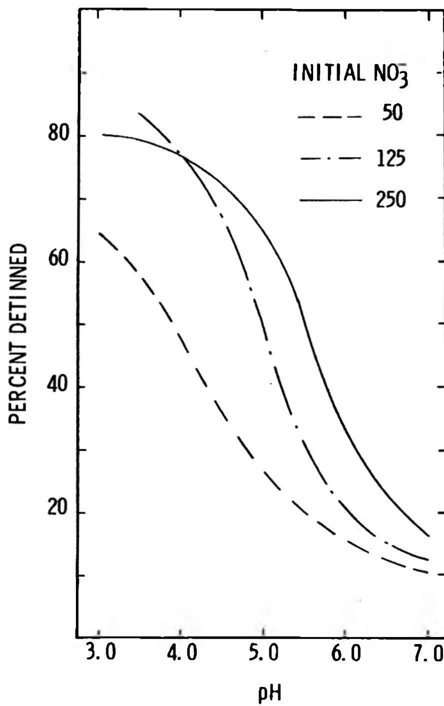

Fig. 4—Influence of pH on detinning of citrate buffered nitrate solutions stored 2 months at ambient temperature.

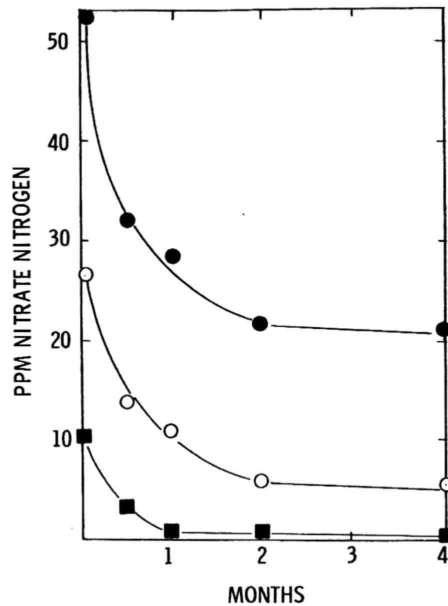

Fig. 5—Decrease in nitrate, expressed as ppm of nitrate nitrogen, during detinning of pH 3.5 solutions having initial nitrate concentrations of 50, 125, and 250 ppm (11, 27, and 55 ppm as nitrate nitrogen).

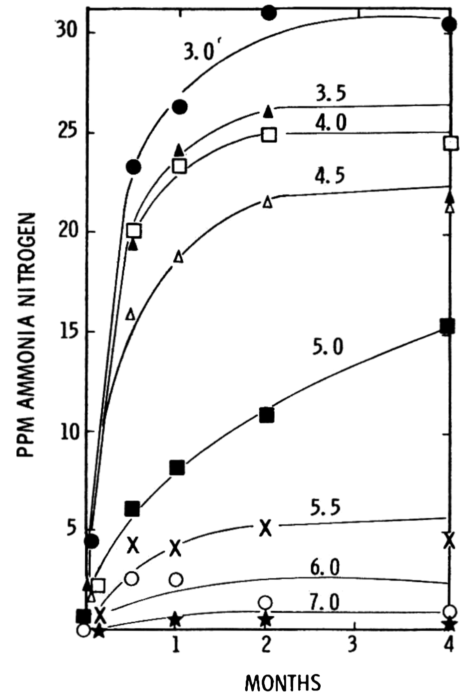

Fig. 6—Formation of ammonia, expressed as ppm of ammonia nitrogen, during detinning of citrate buffer solutions containing 250 ppm of nitrate (55 ppm as nitrate nitrogen).

Tin was determined in a nitric-sulfuric acid digest using a polarographic procedure of Condliffe and Skrimshire (1961). The "percent tin removed" was calculated from analytical data determined on the contents of the can, not by analysis of tin remaining on the container.

Ammonia was determined by distillation from a strongly alkaline solution into standard 0.1N sulfuric acid and back titration of the excess acid.

Headspace gas analyses were carried out by gas chromatography by a method utilizing two Fisher-Hamilton Gas Partitioners. The two-column system provided a clean separation of N_2, O_2, CO_2, CO, and N_2O. The details of the method were described by Elkins et al. (1969).

Tin dissolution results are expressed in terms of the percent tin removed from the container. All of the cans used throughout the project were produced from the same lot of tin plate. The total amount of tin on the inside of the 303 cans was determined by analysis to be 297 mg. The "percent tin removed" is the product of the net contents of the container and the tin concentration divided by 297 and expressed as a percent.

work citrate buffered nitrate solutions were canned under helium to minimize the oxygen and nitrogen content of the headspace gas. Only one nitrate level, 250 ppm, was used in two citrate buffers having pH values of 4.0 and 5.0. These were prepared from 0.8% citric acid and contained a final 1% NaCl concentration Control packs containing no added nitrate were also prepared. The solutions were sterilized by means of a Millipore filter. Containers were also sterilized before filling to prevent formation of mold growth during the experiment.

A Pacific Semi-automatic Vacuum Closing Machine was modified to permit a flow of helium directly across the headspace of the filled can while the can end was held away from the can body. The filled unsealed cans were placed in the vacuum chamber under a vacuum of about 25 in. for 5 sec. The helium was then introduced using a vigorous flow rate for about 30 sec. The helium was turned off, pressure in the vacuum chamber reduced, and the can sealed. These cans had no heat treatment.

Analytical methods

Analytical methods utilized in this project remained essentially unchanged throughout the four years of the study. In addition to the buffer solutions described in this paper, these methods have been used for the analysis of more than 8,000 cans of tomatoes, carrots, green beans, and spinach.

Nitrate was determined by a procedure adapted from the method described by AOAC (1965). The procedure depends upon the nitration of m-xylenol in a strongly acid solution and distillation of the nitrated xylenol into sodium hydroxide solution whose optical density is determined promptly at a wave length of 450 mμ.

RESULTS

Effect of pH on nitrate detinning

Data on the detinning rate of buffered nitrate solutions at five different pH values and three nitrate concentrations were obtained on a total of 250 samples over a storage interval of 4 months (Fig. 1 through 3). The initial analyses represent the extent of tin removal during the first 24 hr. The remaining cans were stored at ambient temperatures and analyzed at the indicated intervals.

The most corrosive of the variables studied removed a maximum of about 80% of the tin from the container. In fact, these cans were completely detinned over all surfaces in contact with the solutions. About 12–15% of the tin was in the headspace area of the can not in contact with the liquid.

The data in Figures 1 to 3 show that in the pH interval between 6 and 7, nitrates have not appreciably accelerated tin removal, except at high concentrations. At pH 5, nitrate detinning assumes increasing importance and at values less than 5, nitrate detinning in citrate solutions is extremely rapid. These relationships are illustrated in Figure 4, in which the percent tin removal after 2 months' storage is plotted as a function of pH. After 2 months' storage the 125 and 250 ppm nitrate solutions having a pH of 4 or less were, for practical purposes, completely detinned.

The nitrate content of all cans was also determined at each examination and these data exhibit the expected decrease at the lower pH values. The data presented in Figure 5 for the pH 3.5 buffers are typical. Nitrate concentrations are shown as nitrate nitrogen. At the lowest concentration studied, the nitrate was exhausted at this pH in about 1 month. At the higher concentrations there was an excess

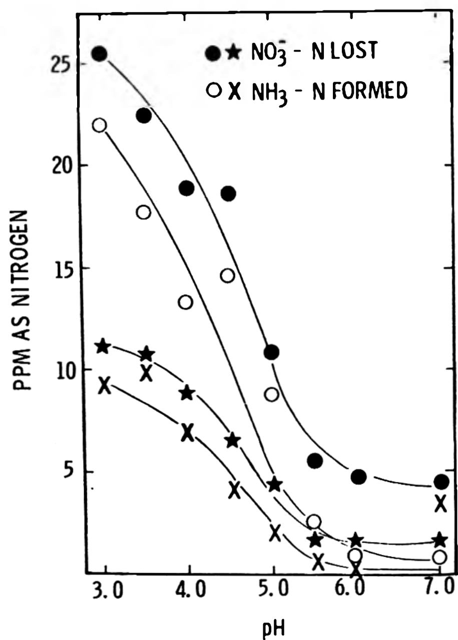

Fig. 7–Ppm nitrate nitrogen lost and ppm of ammonia nitrogen formed during 2 months detinning in citrate buffer solutions containing 50 ppm (lower curves) and 125 ppm (upper curves) of nitrate (11 and 27 ppm as nitrate nitrogen).

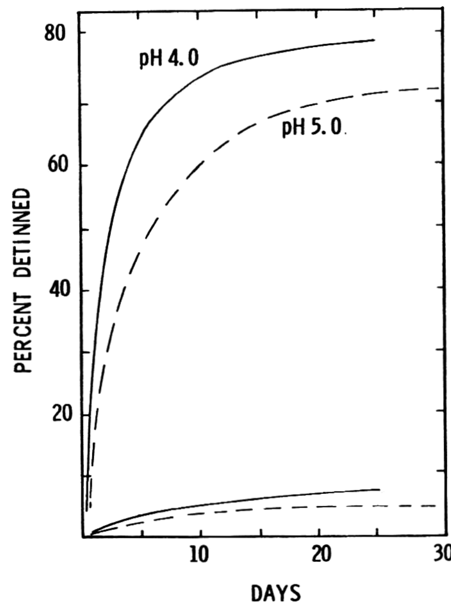

Fig. 8–Detinning in citrate buffered nitrate solutions canned under helium and containing 250 ppm nitrate (55 ppm as nitrate nitrogen). Two lower curves show detinning in nitrate-free controls.

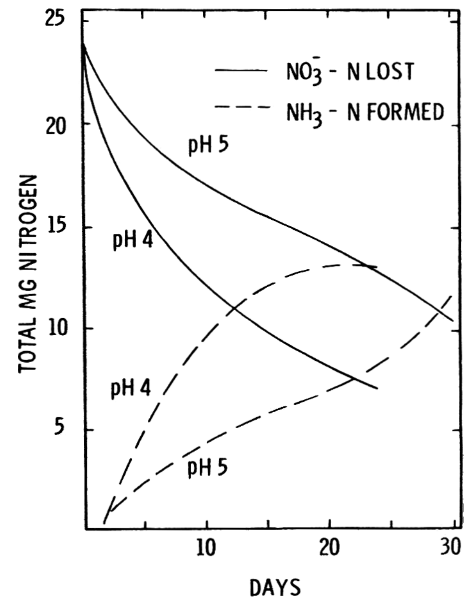

Fig. 9–Total nitrate nitrogen lost and total ammonia nitrogen formed per can during detinning of helium-packed buffered nitrate solutions containing 250 ppm nitrate (55 ppm as nitrogen).

of nitrate; at 125 ppm detinning was almost complete after 2 months and there was no further loss in nitrate. A similar pattern was exhibited at the highest nitrate concentration. The data for the other low pH values would exhibit a similar pattern with respect to loss in nitrate.

At pH values of 5 and higher, nitrates were lost much more slowly and at pH 6 and 7 there was little loss of nitrate throughout the storage period. For example, the 250 ppm pH 7 nitrate buffer had 213 ppm nitrate after 4 months.

Nitrate reduction products

Ammonia was expected to be the principal reduction product in the reaction between nitrate and tinplate. It was determined in each of the cans examined as a part of this study. Figure 6 illustrates the results obtained on the 250 ppm (55 ppm as nitrate nitrogen) buffered nitrate solutions stored for periods up to 4 months and covering the pH range from 3.0–7.0. Results follow the expected pattern: very little detinning is taking place at the higher pH values and little or no ammonia is produced.

In the pH range from 3.0–5.0, the rate of detinning is appreciable and the concentration of ammonia increases accordingly. A comparison of Figure 6 with the detinning rates shown in Figure 3 suggests that after detinning was complete, there was little or no further production of ammonia.

The absolute quantities of ammonia produced are relatively small, about 25–30 ppm of ammonia nitrogen in the most reactive of the solutions studied. If it is assumed that the tin is oxidized to the stannous condition, then 1 mole of nitrate is reduced to ammonia for every 4 moles of tin dissolved. For the fill weights and containers used in this series of experiments, the total amount of tin on the inside of the can should account for somewhat less than 20 ppm of ammonia nitrogen in the completely detinned solution. In this investigation maximum values are in excess of this amount, even though reduction products other than ammonia are produced during detinning.

It is of interest to compare the proportion of reacted nitrate nitrogen converted to ammonia at different pH values. Figure 7 presents such a comparison for the 125 and 50 ppm buffered nitrate solutions after detinning had progressed for 2 months. The reacted nitrate nitrogen is taken as the difference between the initial nitrate nitrogen content and that determined by analysis at the end of the indicated storage interval.

Results presented in Figure 7 are similar to those obtained at other concentrations or storage periods. As the detinning reaction nears completion, roughly 80% of the reacted nitrate nitrogen has been converted to ammonia nitrogen at pH values of about 5 or less. In the higher pH buffers some nitrate has been reduced but the proportion reduced completely to ammonia is smaller. At the higher pH values, other reduction products at higher oxidation states may assume a greater importance. Data in Table 1, obtained from the 250 ppm nitrate solutions, show that at this higher concentration, the proportion of nitrate reduced to ammonia is roughly similar to that obtained in the lower concentration nitrate buffers displayed in Figure 7.

The titration procedure used for the ammonia determination was operating near its lower limit of sensitivity. Also considerable can-to-can variation in detinning was encountered due to variations in headspace detinning and other factors. These conditions limit the precision of the above results especially where the loss in nitrate has been small.

To obtain information on gaseous reduction products a second series of buffered nitrate solutions was canned under helium, using methods intended to minimize the concentration of nitrogen and oxygen in the headspace. Two series of packs at pH 4 and 5 were prepared both with a nitrate concentration of 250 ppm. The buffer solutions were 0.8% citric acid, adjusted with NaOH to the indicated pH, and containing 1% NaCl. Nitrate-free control solutions at both pH values were canned under identical conditions. Immediately after canning, the oxygen content in the headspace of these samples was about 3–5% and the nitro-

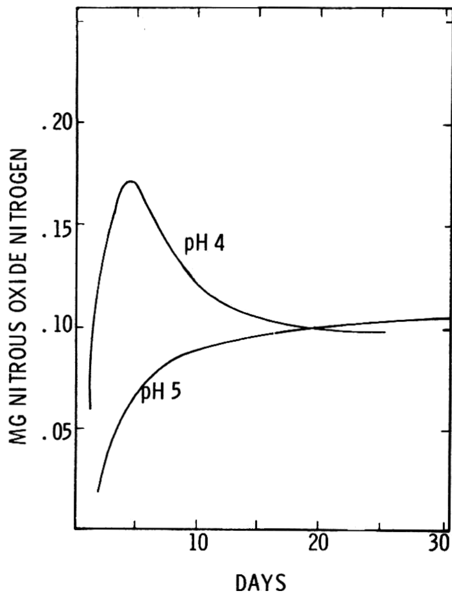

Fig. 10—Total nitrous oxide nitrogen in the headspace of helium-packed buffered nitrate solutions having an initial nitrate concentration of 250 ppm (55 ppm as nitrogen).

gen content about 10–20%. The solutions were stored at 30°C for 24 and 30 days. This temperature is somewhat higher than the ambient temperatures at which the previously reported experiments were stored.

The corrosion histories of these two packs and their controls are presented in Figure 8 in terms of the percent of the tin removed from the can. The nitrate-free controls showed little detinning. The solutions that contained 250 ppm of nitrate detinned according to the expected pattern.

The loss of nitrate nitrogen and the formation of ammonia nitrogen for the two series of buffers is shown in Figure 9.

One of the objectives of this series of experimental packs was to produce cans having very low oxygen and nitrogen content in the headspace in order to determine if nitrogen was produced as a result of nitrate reduction. However, the initial nitrogen content was appreciable and subject to some can-to-can variation.

The results of the pH 5 solutions are presented in Table 2. The nitrogen content of the headspace gas in the control cans can be compared with that of the buffered nitrate solution. The variation is unfortunate but control of the initial headspace gas composition was not as good as would be desired. The figures presented in the last column of Table 2 have been corrected for some of the experimental variation. The mean nitrogen content of the headspace in the control cans has been subtracted, and the total amount of nitrogen in the headspace of the buffered nitrate solutions has been calculated from headspace volume and can vacuum data. The resulting corrected nitrogen figures suggest that nitrogen gas is produced as a result of detinning at pH 5.0, and that nitrogen production increases after the detinning nears completion. The nitrogen data for the pH 4.0 samples were inconclusive and when subjected to similar corrections provided no suggestion of an increase in nitrogen as a result of the detinning reaction at the lower pH.

A second objective of the helium purged packs was to examine the possibility that nitrous oxide or nitric oxide might be formed as nitrate reduction products. No gas chromatographic evidence for the presence of nitric oxide was obtained. Nitrous oxide, however, was found in easily detectable quantities. The results for the two buffer solutions are shown in Figure 10, in terms of total nitrous oxide nitrogen in the headspace of the cans as calculated from vacuum and headspace volume data.

The absolute amounts of nitrous oxide nitrogen produced by the reaction are relatively small. Also nitrous oxide has a considerable solubility in the liquid phase and the nitrous oxide in solution was not determined. The total amount in solution was very probably considerably larger than that in the headspace gas.

The concentration of nitrous oxide in the headspace passes through a maximum in the pH 4.0 buffer solutions after the detinning reaction has proceeded for about 4 days. Thereafter, the amount of nitrous oxide nitrogen in the headspace decreases, gradually leveling off at an amount similar to the maximum value obtained in the pH 5.0 solutions.

These results suggested that the nitrous oxide was itself taking part in the detinning reaction at pH values of about 4.0. A separate series of experiments was designed to establish this.

Other reduction products which might result from the nitrate detinning reaction include nitrite and hydroxylamine. Nitrite is an extremely reactive detinner. Its reactivity would not permit it to exist for any appreciable length of time in solution in a plain can in the pH range ordinarily encountered in canned foods. This has been confirmed by a number of analyses in this laboratory over a period of years. Analytical methods capable of detecting nitrite have consistently failed to demonstrate its presence in more than trace amounts in food products in unenameled tinplate containers.

Hydroxylamine might also be considered as a possible reduction product in the detinning reaction. A number of cans from the first series of buffered nitrate solutions were examined for hydroxylamine after detinning had proceeded for about 3 months. Only trace quantities, of the order of a few ppm were indicated. The method utilized was that of Yashphe et al. (1960). Some differences in response of the developed color was encountered possibly as the result of interference by tin or nitrate ions. The indicated level of hydroxylamine was so low however, that it was not considered worthwhile to make the procedure strictly quantitative.

Nitrous oxide detinning

A third series of experimental packs were prepared to determine the extent to which nitrous oxide might act as a detinning agent. Citrate buffer solutions adjusted to pH values of 4.0 and 5.0 and containing no nitrate were saturated with nitrous oxide for more than an hour at room temperature. These were then sealed in the same 303 cans used throughout this project, and stored at ambient temperature. Control cans not saturated with nitrous oxide were closed under similar

Table 1—Proportion of reacted nitrate reduced to ammonia in 250 ppm nitrate solutions.

pH	Percent Converted 2 months	4 months
3.0	78	77
3.5	76	63
4.0	82	74
4.5	85	64
5.0	56	55
5.5	56	31
6.0	15	7
7.0	11	7

Table 2—Nitrogen content of helium purged 250 ppm nitrate buffers after detinning.

	Percent N_2		
Days Storage	Control Cans	250 ppm nitrate	mg N after corrections
1/6	20	20	0.65
1-1/6	17	18	0.72
2-1/6	16	18	0.07
3	19	19	0.28
4	–	20	0.52
5	18	20	0.15
7	23	24	0.76
8	–	24	0.79
9	18	22	0.81
12	17	19	0.29
13	–	19	0.36
14	19	20	0.54
15	19	21	0.47
16	19	21	0.40
19	19	21	0.59
20	19	21	0.69
21	19	22	0.78
22	19	23	0.70
23	19	21	1.04
27	19	20	1.25
29	24	23	1.78

Table 3–Detinning in pH 4 and 5 citrate buffers saturated with nitrous oxide.

Days	pH 4.0 Buffer		pH 5.0 Buffer	
Storage	Control	N_2O Sat.	Control	N_2O Sat.
1/6	5	4	4	4
7	24	37	21	41
12	27	45	24	53
19	24	64	20	67
26	28	64	25	82
62	31	–	24	–
95	–	75	–	79

conditions. The buffer was 0.8% citric acid and contained 1% sodium chloride. No attempt was made to remove oxygen from the headspace of the control cans or to deaerate the control buffer solutions.

Tin determinations were carried out at intervals with the results collected in Table 3. In the control cans, oxygen detinning near the headspace area removed up to 31% of the tin in the pH 4.0 buffer and 24% in the 5.0 pH buffer.

The solution saturated with nitrous oxide detinned rapidly and to a much greater extent than the control cans. The pH 5.0 buffer removed somewhat more tin from the container than the pH 4.0 solution. Since nitrous oxide is a weak acid, its solubility should be somewhat greater at the higher pH and therefore this difference in the extent of the detinning is probably accounted for by the fact that a larger quantity of nitrous oxide was in solution.

DISCUSSION

INSOFAR AS the results obtained with these buffer solutions are representative of detinning in food products, they indicate that in tomatoes and other acid products ranging in pH from about 4–5, relatively low concentrations of nitrates could result in extensive detinning. At pH 4, concentrations as low as 50 ppm expressed as nitrate, removed 50% of the tin from the containers in this study. The buffer solutions containing 125 ppm and having a pH value of 4.0 were completely detinned in about 2 months.

At the somewhat higher pH values of vegetable products such as green beans, carrots and spinach, it appears that substantially higher concentrations of nitrate could be tolerated. The indications from this work are that the critical pH for nitrate detinning lies in the neighborhood of about 5.2–5.5. Nitrate detinning rates could be relatively slow at higher pH values, but at lower pH values the rates increase very rapidly.

In the pH range below 5, ammonia is the principal nitrate reduction product; however, the total amount of ammonia resulting from detinning is not large. Completely detinned cans showed a gain in ammonia nitrogen on the order of 30 ppm This quantity is too small to be of use in diagnosing the cause of internal can corrosion in samples of canned food products that are already detinned. The ammonia nitrogen content of most canned products varies by an amount considerably greater than this. Differences in ammonia concentrations due to nitrate detinning could not be distinguished from normal product variation.

Nitrous oxide is also produced. It is an excellent oxidizing reagent and hence it reacts with tin under acid conditions to result in further tin dissolution. As a result, nitrous oxide may be found only temporarily in the headspace of acid products undergoing nitrate detinning. This has been confirmed by subsequent experience in the examination of nitrate containing acid products. Nitrous oxide is rarely found after several weeks of storage, and only in trace amounts. Some, or all of the nitrous oxide could result from the reduction of nitrate by stannous ions.

Under less acid conditions, in the pH range above 5, nitrous oxide may still be an effective detinner but at a somewhat reduced rate. In low acid products it can accumulate in the headspace gas, and this has also been confirmed in work on carrots, green beans and spinach that will be reported separately.

Nitric oxide was not seen in any of the chromatograms resulting from the headspace gas analyses of these buffer solutions. The analytical system used provides a good separation of NO. It reacts rapidly with oxygen to form NO_2, which has a strong affinity with water and which would have been absorbed on the chromatographic columns. The detection of trace quantities of nitric oxide would depend upon oxygen-free conditions inside the can and during sampling.

It is likely that nitrogen is also a reduction product of the nitrate detinning reaction at higher pH values.

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FABRICATION AND CORROSION PERFORMANCE OF TIN-MILL PRODUCTS MADE FROM CONTINUOUSLY CAST STEEL

SUMMARY—During the past year, large tonnages of tin-mill products made from continuously cast steel were supplied to the can-making industry for almost every type of container and closure. Most of the product was used for the manufacture of beer-can bodies. Without exception, fabrication performance was as good or better than that attained with ingot-cast steel. Results of pack tests of both heavy- and light-coated electrolytic tinplate, lead to the conclusion that continuously cast steel containing 0.03–0.08% silicon has corrosion resistance equal to that of ingot-cast steels containing a maximum of 0.010% silicon. Continuously cast steel is an addition to the family of steels now available to the container industry, and its use may open new vistas for improved container concepts.

INTRODUCTION

THE STEEL SUBSTRATE of tinplate used in the manufacture of food containers contains about 0.6% of elements other than iron. Two of these elements (carbon and manganese) are required to obtain desired metallurgical and mechanical properties; the others are present in small amounts as impurities and do not make a significant contribution to the properties of the steel. However, different steel compositions have been developed for various applications and through careful control, steel producers have been able to produce these steels with accuracy and dispatch.

Concurrently, steel producers conduct research on methods to produce better and more economic steels. U.S. Steel is a leader in this field of process metallurgy and has recently developed a proprietary method of continuously casting steel that represents a large step toward the achievement of superior steels. Conventionally, molten steel is poured from a ladle into ingot molds. After solidifying in the molds, the ingots are heated in soaking pits and then hot-rolled into slabs. In contrast, this new continuous-casting process involves a continuous operation from melting to slabs. The process results in a more homogeneous steel and in improved yields, inasmuch as losses associated with cropping of slab ends following rolling of the ingots are eliminated.

This continuous casting process has been described by Wiebel (1969), Kalin et al. (1969) and by Laubscher and Lesney (1970).

This casting concept—characterized by high casting speed—requires a steel essentially free of dissolved oxygen. This steel can be cast at higher speeds and results in improved slab-surface quality, decreased reheating time for subsequent hot reduction, and improved characteristics of the cast steel. The optimum steel for continuous casting is one that is deoxidized mainly with silicon. A smaller quantity of aluminum deoxidizer is also used. Although most of the silicon combines with the oxygen in the steel and is removed prior to actual casting, the silicon content of the solidified continuously cast steel (0.03–0.08%) is slightly higher than that of ingot-cast steels (0.010% maximum).

The two major classifications of ingot-cast steels for container applications are Type L and Type MR, chemical specification for which are presented in Table 1. Type MR differs from Type L principally in that copper is less restrictive, the allowable maximum being 0.20%. These restrictions on phosphorus, silicon, and copper in steels for tinplate are based on food-pack corrosion considerations. Chemical specifications for the new continuously cast steel, USS RIBAND 1, are also shown in Table 1. The 0.010% restriction on silicon in ingot-cast steels was imposed as a result of corrosion failures obtained with hot-dipped tinplate produced from hot-pack-rolled steel containing up to 0.09% silicon (Hartwell, 1951). Hot-pack rolling is no longer used, having been superseded by the cold-reduction process in the 1930's (Meneilly, 1951).

Because the development of the new continuously cast steel involved changes in both processing conditions and chemical composition, the determination of its adaptability to the fabricating and corrosion requirements imposed by modern can-manufacturing and canning technology was required. This paper describes the information gained in fabricating trials in manufacturing plants and the results of the corrosion pack tests made by our laboratory.

MATERIALS & METHODS

Characteristics of continuously cast steel

Uniformity of chemical composition. In comparison with ingot-cast steel, one metallurgical characteristic of continuously cast steel is its uniformity of chemical composition. This uniformity yields the excellent flatness and formability required in the fabrication of containers. A continuously cast slab solidifies in only a few minutes, whereas an ingot cools so slowly that it may take hours to solidify. As a result of rapid solidification, the chemical segregation typical of a rimmed or capped ingot is almost completely absent in a continuously cast slab.

Cleanliness. The cleanliness of steel can be rated by measuring the area of inclusions such as oxides, sulfides, and silicates with quantitative television microscopy. Results of measurements (Table 2) show that continuously cast steel is both markedly and consistently cleaner than the ingot-cast steel. For 49 samples of continuously cast steel, the average area occupied by inclusions was 0.046% with a range of 0.014–0.170% compared with an average area of 0.239% and a range of 0.083–0.481% for 23 samples of ingot-cast rimmed steel. Representative micrographs of continuously cast steel and ingot-cast rimmed steel illustrate the magnitude of this difference in cleanliness (Fig. 1). There is a general trend toward improved fabrication performance with cleaner steels.

Table 1—Chemical specifications of different types of steel.

Type	Composition, maximum %					
	Carbon	Manganese	Phosphorus	Sulfur	Silicon	Copper
Continuously cast (RIBAND 1)	0.08	0.60	0.015	0.020	0.080	0.06
Type L	0.13	0.60	0.015	0.050	0.010	0.06
Type MR	0.13	0.60	0.020	0.050	0.010	0.20

Table 2—Inclusion area of tin-mill products measured with a quantitative television microscope.

Type of steel	No. of samples	Area occupied by inclusions, percent[a]	
		Avg	Range
Continuously cast	49	0.046	0.014–0.170
Ingot-cast	23	0.239	0.083–0.481

[a]25 fields per sample.

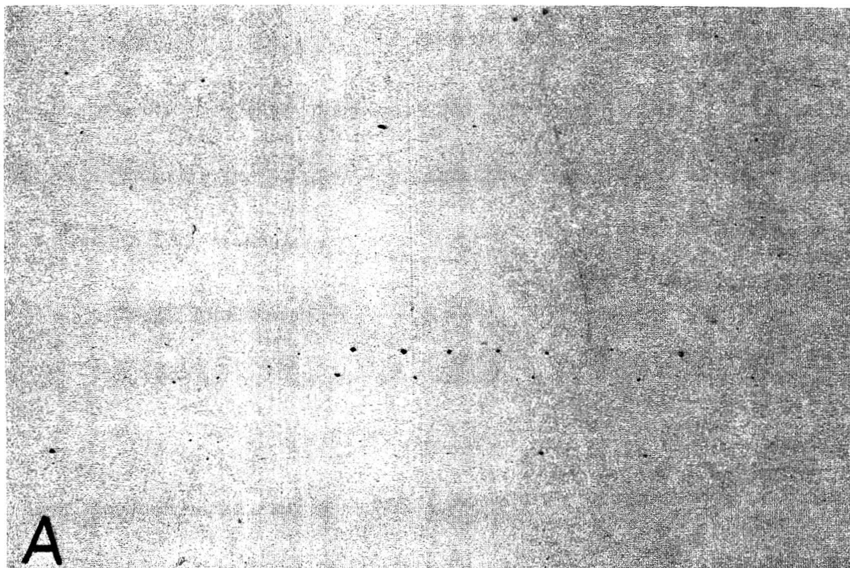
Fabrication performance

To evaluate the fabricability of tinplate made from continuously cast steel, continuously cast slabs were processed to different types of tinplate for manufacturing into various container components.

Beer-can bodies. During 1969, over 35 million beer-can bodies (211 × 413) were made from double-cold-reduced tinplate and tin-free steel made from continuously cast steel. No fabricating problems were encountered and no adjustments were required on the can-making lines. Can body flanging performance was about equal to that of similar products processed from ingot-cast steel.

Table 3–Special properties–Heavy-coated tinplate produced from continuously cast steel.

Special property	Average	Range	Type K limits
Alloy-tin-couple test (ATC) (μamp/cm²)	0.051	0.02–0.21	0.12 max
Iron solution test (ISV) (μg of iron)	2	1–6	20 max
Pickle lag test (time in seconds)	1.4	1–4	10 max
Tin-crystal size (ASTM nonferrous grain size)	7.0	6–9	9 min



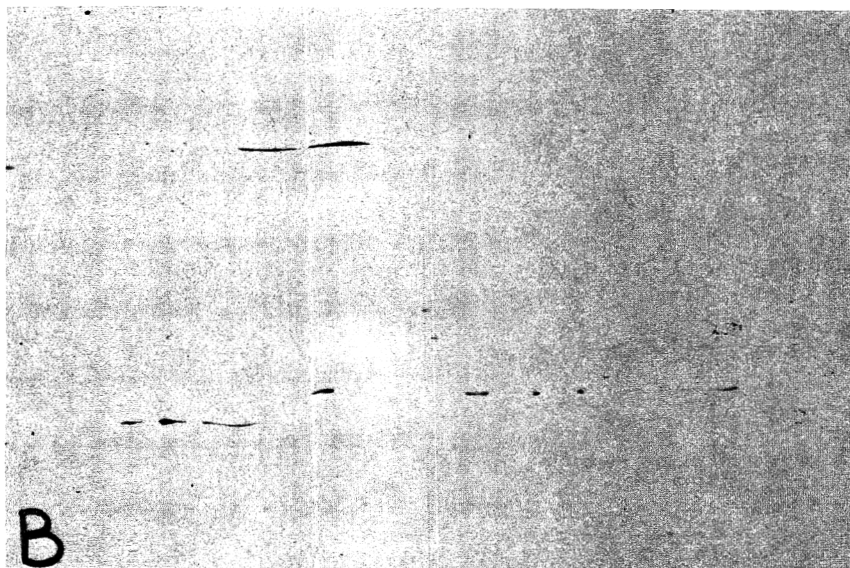


Fig. 1–Micrographs showing typical inclusions in tinplate made from: A–continuously cast steel; B–rimmed steel. (Magnification: ×500)

Many cans are fabricated from thin double-cold-reduced (2CR) steels, which are produced by cold reducing after annealing. Normally, for cans processed from these severely cold-reduced steels in the form of tinplate or the recently developed tin-free steel, it is mandatory that the rolling direction be perpendicular to the side seam to prevent the development of cracked flanges. However, when using an experimental flanging technique, continuously cast steel performed very well when formed with the rolling direction parallel to the side seam; this factor will permit greater flexibility in can-manufacturing procedures.

Beaded can bodies for pet foods. Several manufacturers and packers of pet food cans have produced beaded can bodies from 2CR tinplate made from continuously cast steel (Fig. 2). While some cracking of the beads has been experienced with ingot-cast steel, continuously cast steel has performed satisfactorily.

1-gal paint can bodies with clinched ears. The manufacture of 1-gal paint can bodies with clinched ears necessitates a localized draw to permit the clinching operation for the ears (Fig. 3). With ingot-cast steels, 2 to 3 fractures per thousand cans are commonly obtained. However, with continuously cast steel no fractures have been reported in commercial production to date.

Tapered 5-gal can bodies. Tapered 5-gal can bodies are fabricated initially with a straight wall and a welded side seam. The taper is developed by expansion of the top end of the bodies to 11-¼ in. dia. as compared with the 10-¾ in. dia. at the opposite end. The body is then flanged at both ends to permit double seaming of the cover and bottom end. With ingot-cast steel it has always been necessary to use the favorable grain direction to keep fractures at a minimum. However in trials with continuously cast steel, using the reverse grain direction, no fractures were encountered.

Domed tops for aerosol cans. Domed tops for 211-dia. aerosol cans require a special steel with excellent drawing properties. The domed tops shown in Figure 4 were drawn at normal press speed from 0.013- and 0.014-in.-thick tinplate made from continuously cast steel with excellent performance.

Drawn covers for aerosol cans. The drawn protective covers for tops of aerosol cans also require steel with excellent ductility. Those shown in Figure 5 were produced satisfactorily from continuously cast steel at commercial press speeds.

Corrosion resistance

Various grades of tinplate having a wide range of tin coating weights are produced to meet the requirements of the packaging industry.

Heavy-coated electrolytic tinplate. For the past six years U.S. Steel has produced heavy-coated electrolytic tinplate having certain performance characteristics related to corrosion resistance. These characteristics, denoted as Type K special properties, are desired for tinplates used in packing corrosive food products, Table 3. For example, Type K plate has been useful for packing such food products as citrus fruits in plain cans.

During the past 5 yr, 40 lots of heavy-coated electrolytic tinplates were produced from continuously cast steel and, with the exception of a

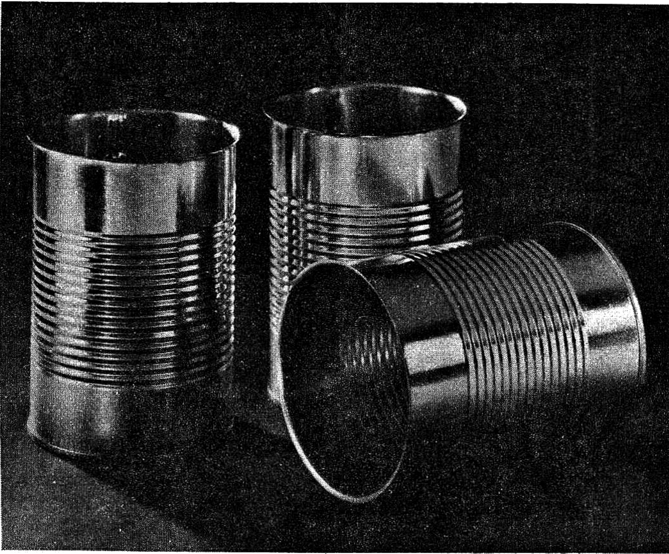


Fig. 2–Beaded can bodies for pet foods.

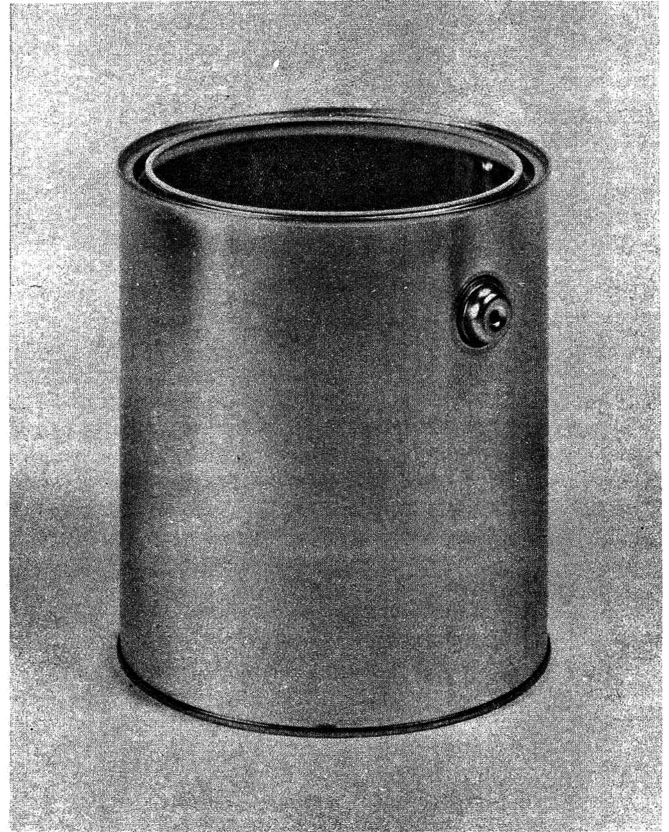


Fig. 3–1 gal paint can bodies with clinched ears.

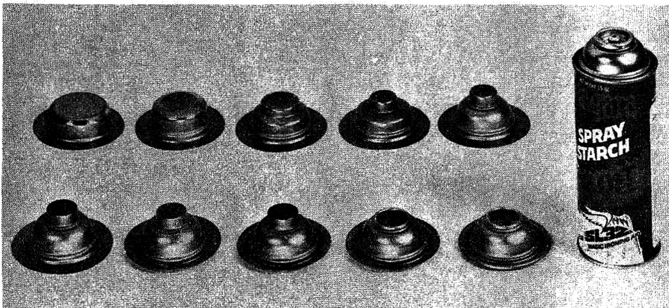


Fig. 4–Domed tops for aerosol cans.

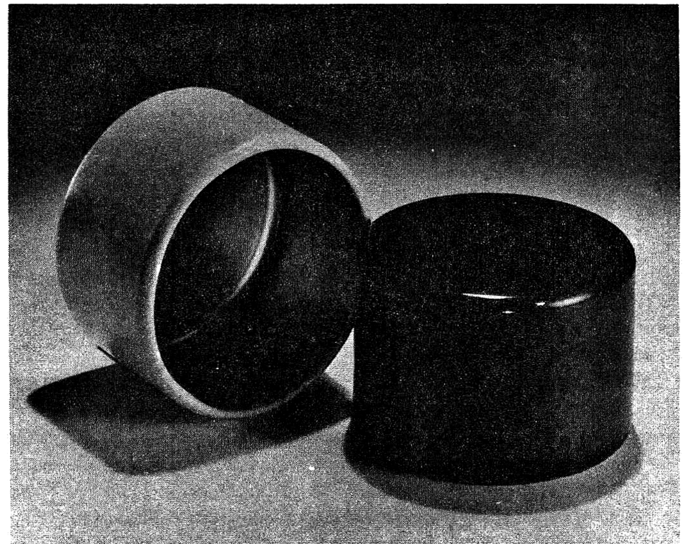


Fig. 5–Drawn covers for aerosol cans.

few alloy-tin-couple (ATC) tests, all the special properties were within the Type K limits. Of particular interest is the performance observed with the Iron-Solution Test (ISV), which is greatly affected by steel base cleanliness and soundness. The overall ISV average for continuously cast steels was 2, with a max value of 6. This is considered excellent performance and good indication of the superior surface quality of the continuously cast steel compared with that of rimmed and capped ingot-cast steels.

To determine the corrosion performance of heavy-coated electrolytic tinplate produced from continuously cast steels, our laboratory observed test packs of cans for the past five years. All the elements in the steels were within the Type L specification with the exception of silicon which covered a range of 0.008–0.20%. The major portion of the continuously cast steel in test had silicon contents in the range specified for RIBAND 1: 0.03–0.08%.

To evaluate the corrosion performance of tinplate produced from continuously cast steel for products packed in cans with plain (unlacquered) bodies and lacquered ends, test packs were originally made with grapefruit juice, orange juice, fruit cocktail, pears, prunes, sauerkraut, and green asparagus. The corrosion resistance of the continuously cast steel, based on average time for development of springers, was equal to that of the ingot-cast steels. (See Table 4 and Fig. 6 and 7.) Continuously cast steel containing high silicon (0.16 to 0.20%) had performance equal to that of the companion lots containing less than 0.020% silicon.

Table 4—Corrosion resistance of heavy-coated tinplate plain (unlacquered) can bodies with lacquered ends.

Product packed	Can size	Cold reduction	Storage temp. °F	Continuously cast steel: Time in wk to 10% Springers	Continuously cast steel: Range in avg pack life	Ingot-cast steel: Time in wk to 10% springers	Ingot-cast steel: Range in avg Life
Single-strength grapefruit juice	Beaded 307 x 409	Single	100	87/79	102/109	79/113	89/135
Single-strength grapefruit juice	Beaded 307 x 409	Single	100	79/87	89/102	83/86	94/104
Single-strength grapefruit juice	202 x 314	Double	100	101/107	113/122	106	107.1
Single-strength orange juice	202 x 314	Double	100	91/105	99/113	102/112	113/114
Green asparagus	300 x 407	Single	100	112	129	45/102	93/119
			77	No failures after 122 wk		5% Springers after 112 wk	
Fruit cocktail	Beaded 303 x 406	Single	100	69	75.2	74	77.7
Pears	Beaded 303 x 406	Single	100	63	66.0	63	67
Dried prunes in water	Beaded 303 x 406	Single	70	97/154	133/190	82/156	100/182
		Double		89/120	111/153	97/130	125/160
Sauerkraut	Beaded 303 x 406	Single	100	14/33	23/42	19/27	25/35
			77	59/121	75/168	65/84	80/114
		Double	100	19/33	35/44	21/28	33/34
			77	79/127	105/158	78	100/142

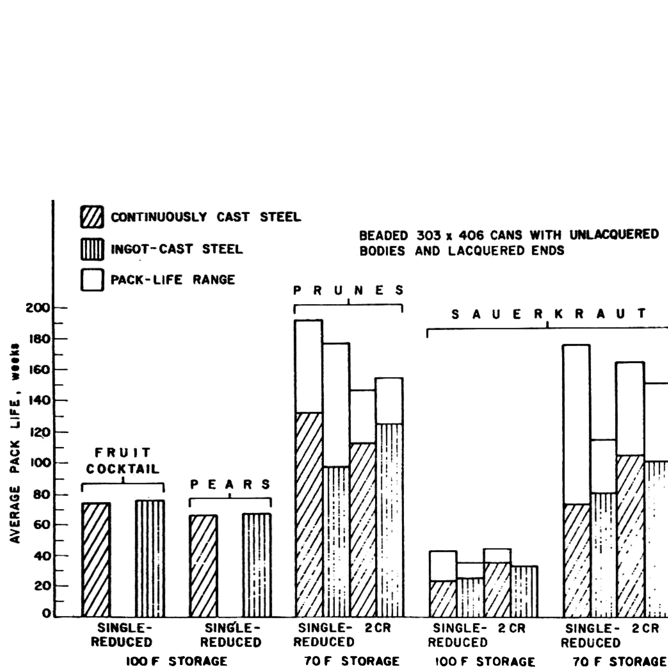


Fig. 7—Corrosion resistance of heavy-coated tinplate.

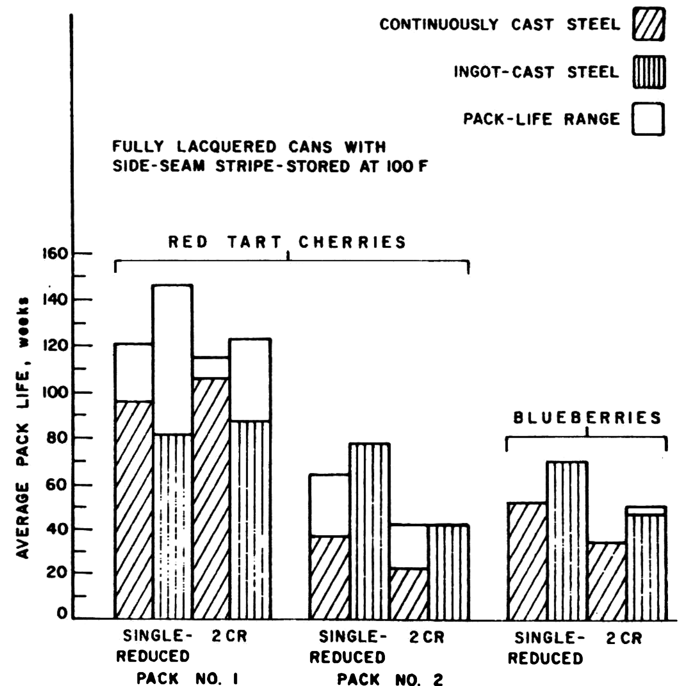


Fig. 8—Corrosion resistance of heavy-coated tinplate.

Packs with plain bodies and lacquered ends have also been made with grapefruit sections, cling peaches, green beans, pears, acidified red peppers, and diced carrots. In all cases, after at least 1 yr storage at 100°F, performance of the continuously cast steel is equal to that of the ingot-cast steels.

The good performance of the cold reduced high silicon content continuously cast steel is attributed to the complete absence of surface ruptures or cracks that caused the corrosion failures previously mentioned for tinplate produced from high silicon steel. This difference is due to the use of the cold reduction process rather than the hot pack rolled process which was in use in the 1930's.

To evaluate the corrosion performance of tinplate produced from continuously cast steel for products customarily packed in fully lacquered cans, test packs were made with red tart cherries and blueberries. In one pack of red tart

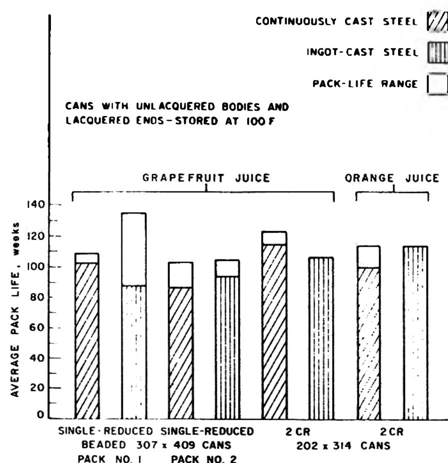


Fig. 6–Corrosion resistance of heavy-coated tinplate.

Table 5–Corrosion resistance of heavy-coated tinplate fully lacquered cans with side-seam stripe.

Product packed	Can size	Cold reduction	Storage temp. °F	Continuously cast steel: Time to 10% perforations (in wk)	Continuously cast steel: Range in avg pack life (wk)	Ingot-cast steel: Time to 10% perforations (in wk)	Ingot-cast steel: Range in avg pack life (wk)
Red tart cherries	Beaded 303 × 406	Single	100	76/104	96/121	69/128	81/146
		Double	100	80/93	106/115	70/101	85/122
Red tart cherries	Beaded 303 × 406	Single	100	30/49	36/64	52	78.4
		Double	100	23/31	24/41	26	41.5
Blueberries	Beaded 303 × 406	Single	100	43	52	50/61	69/72
		Double	100	26	35	34/42	48/54

cherries in fully lacquered cans with striped side-seams, the continuously cast steel showed pack life equal to that of tinplate from ingot-cast steels. As with plain cans, the high-silicon (0.16–0.20%) continuously cast steel lots had pack life equivalent to the lots with low silicon (0.008–0.018%). A second red-tart-cherry pack and a blueberry pack showed that the continuously cast steel had somewhat shorter pack life than the ingot-cast steels, Table 5 and Figure 8. This difference between the two types of steel in the latter two packs and the greater difference in overall level of pack life between the two cherry packs are due to factors other than steel composition. These packs were stored at 100°F, a temperature that accelerates the corrosion rate. With 70°F storage, which is more nearly representative of actual conditions in commercial handling, the pack life would be several times longer.

Light-coated electrolytic tinplate. 12 oz soft drink cans (211 × 413) were fabricated with bodies made of 2CR electrolytic tinplate-25 produced from continuously cast steel. These cans had the standard two lacquer coats on the interior, the protective system normally used for beer and carbonated beverage containers. The cans were packed in a commercial plant with orange soda and a cola-type drink and stored at 77°F. For comparison, cans produced from the ingot-cast steel were packed with orange soda by the same commercial plant. The time to 2% perforations for the two lots of cans was 11 mo. The cola-type drink was much less corrosive for both types of steel than was the orange soda.

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Ms. received 1/16/70; revised 7/1/70; accepted 7/3/70.

Presented at the 30th Annual Meeting of the Institute of Food Technologists, in San Francisco.

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THE HELICAL PUMP: USE AS A MULTIZONE PROCESSING SYSTEM

SUMMARY—A hydrostatic processing system consisting of a compression zone, a holding zone, and a decompression zone has been built from a single coil of pipe. Theoretical maximum operating pressure is the product of the number of turns in the compression zone and the diameter of the coil. Problems of air compression and disruptive air expansion, noted in a previous model, have been overcome by an innovative air feedback system. Crosstubes, another innovation, are used to maintain a constant pressure in the holding zone of the coil. Experimental results on a model operating at 9 psig show that hydrostatic pressures in the holding zone under continuous conditions are about 90% of theoretical. Packages moving through the unit were found to have a pressure history similar to conventional hydrostatic processing systems that use single tall vertical legs for compression and decompression.

INTRODUCTION

HYDROSTATIC COOKERS represent a significant development in thermal processing technology (Conley, 1965). The hydrostatic leg provides a smooth, valveless method for introducing packages into a sealed chamber.

Present hydrostatic systems use tall vertical legs and in common with the new horizontal units (Lawler, 1967) require long chains to convey packages. Munz (1956) developed a method for pumping cans through a continuous sterilizer; however, mechanical valves are required and only rigid packages can be handled.

The helical pump (Farkas et al., 1969; Farkas and Lazar, 1970) has been proposed as a valveless, compact, mechanically simple, continuous, hydrostatic, processing system capable of handling foods in cans or plastic pouches. This design eliminates the need for tall hydrostatic legs, long chains, and mechanical valves. The hydrostatic leg is replaced by a series of short additive liquid legs in coil form. Packages are augered through in the manner of a screw conveyor by rotation of the coil.

In our previous paper, we proposed a continuous helical pump processing system with a pair of coils rotating side by side. This paper describes a completely new form built from a single length of coiled pipe.

EXPERIMENTAL

THE TEST APPARATUS (Fig. 1) consisted of a 22-turn helix of 2 in. I.D. transparent vinyl tubing wound around a 2-1/2 ft diameter by 5 ft long sheet metal drum.

The helix was divided into three zones, a compression zone of 9 turns, a holding zone of 3 turns, and a decompression zone of 10 turns. The three zones were delineated by a pair of crosstubes joining diametrically opposite points of turns #10 and #12. The crosstube in effect shorted out the pressure differential.

Equal volumes of air and water leave the constant pressure holding zone at turn #12 to re-form the necessary heads on the descending side of the coil for decompression (Fig. 2). Small diameter "feedback" tubes joined each compression turn to a decompression turn of similar pressure.

Water and packages (small bottles) were fed into the rotating helix at one end and discharged at the opposite end. At the same point in each turn of the helix, packages were quickly introduced through a horn-shaped feed scoop, along with a volume of water to fill a half turn of the tubing. Feeding was synchronized with helix rotation by a cam-operated microswitch. At the discharge end, water and packages flowed out of the helix through an axial outlet. The feed water flow rate was adjusted to give the desired volume in a 2 sec "pulse." The period of the "pulse" was set at 10% of one revolution.

Studies of system stability and package movement, and mechanical and hydraulic observations were made using a speed of 3 rpm. Pressures in each turn were determined by measuring the height of water in the ascending and descending halves of the coil. Pressures relative to coil position during rotation were measured after stopping the coil periodically. The pressure history of a package traveling through the unit was measured with a small conventional pressure gauge fitted with a valve and pre-pressurized to 11 psig. The gauge traveled along the bottom of the coil turns, oriented so that its dial could be read through the transparent plastic tubing. Therefore $P = 11 - P_{gauge}$ was the true pressure value at any point the gauge could be read.

RESULTS & DISCUSSION

APPLICATION OF THE helical pump to processing posed several problems, including (a) loss of effective head due to the reduced volume of the leg of compressed air in the compression section of the helix; and (b) instability in operation caused by disruptive air expansion, or "unloading." Unloading was uncontrolled expansion of compressed air between the columns of liquid in the decompression turns of the coil. The expanding air normally bubbled up through the column of water to reach the next air column. At coil pressures below about 75% of theoretical maximum, excess air volume could

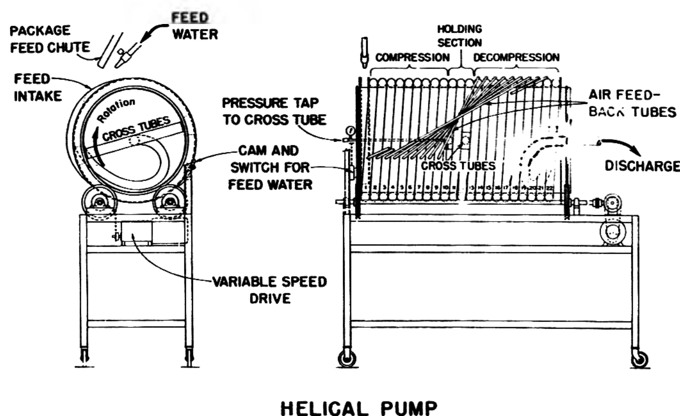

Fig. 1—Experimental three-zone hydrostatic system. Feedback tube connections in the decompression zone are 180° from those in the compression zone.

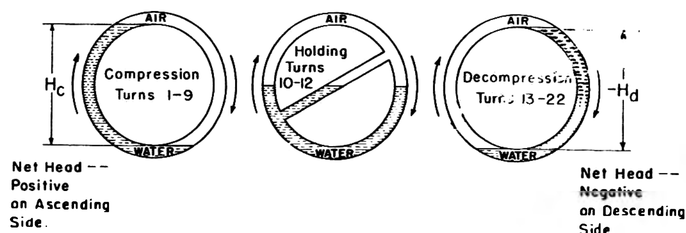

Fig. 2—Cross section of helix in each zone showing the position of the water legs. Coil rotation is clockwise looking at feed end.

Table 1—Description of two successful feedback tube configurations.

Arrangement A:	With feed scoop in ascending position, feedback tube ends are in a horizontal line on both the descending side of the compression section and on the ascending side of the decompression section 180° apart. Feedback tube #1 starts one-half turn from the feed scoop and connects turns #1 and #21. The tube bundle passes over the top of the coil, as shown in Figure 1.
Arrangement B:	Same as A except tube bundle passes under the bottom of the coil.

be smoothly dissipated in this fashion. However, at higher operating pressures, the expansion effect was cumulative so that the volume of bubbles built up in violent, chain-reaction fashion. At the discharge end, the "piston" of air drove the last columns of water ahead of it out through the coil discharge.

Instability was corrected by feeding back excess air from the decompression zone to the compression zone. This was done by connecting small "feedback" tubes to join each turn of the helix in the decompression zone to a turn in the compression zone operating at the same pressure. Tubes were sized to act as check valves against the unwanted backward flow of water. Since water has about 50 times the viscosity of air, feedback tubes with 2% or less of the coil tube cross sectional area proved satisfactory. Feedback stabilizes the operation because the excess air that is decompressed in one zone is smoothly returned to augment the air volume in the compression zone. Table 1 describes two arrangements of feedback tubes.

Table 2 shows the distribution of hydrostatic heads for several operating conditions. Without feedback tubes the maximum stable operating pressure was 7.5 psig. With feedback tubes the maximum stable operating pressure, ΣH, was found to approach the theoretical value for this unit; namely, the product of the number of compression turns, N_C, and the diameter of the compression coil, D, or 22.5 ft of water (9.7 psig).

The modified unit was capable of self-pressurization by a mechanism depicted in Figure 3. During self-pressurization the pressure in the holding zone was found to increase at the rate of 0.25 ft of water per revolution. A hydrostatic head (ΣH) of about 20 ft of water (8.7 psig)

Table 2—Distribution of hydrostatic heads by turns for the three-zone helical pump (values in inches of water).[a]

Experimental condition		Turn number 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
		Compression									Holding			Decompression									
No feedback																							
External pressurization required	(H)[b]	27	20	15	16	21	26	25	29	28	0	-1	0	-6	-10	-20	-25	-27	-24	-18	-26	-23	-27
	(ΣH)[c]		47	62	78	99	125	150	179	207	207	206	206	200	190	170	145	118	94	76	50	27	0
Feedback tubes in Arrangement A																							
Heads measured before feeding	(H)	30	30	30	29	30	29	30	28	21	7	-7	-5	-10	-28	-30	-29	-26	-26	-29	-29	-23	-6
	(ΣH)		60	90	119	149	178	208	236	257	264	257	252	242	214	184	155	129	103	74	45	22	16[d]
Heads measured after feeding	(H)	30	30	30	28	28	30	29	22	16	4	-12	-4	-22	-29	-28	-27	-27	-28	-30	-28	-11	-1
	(ΣH)		60	90	118	146	176	205	227	243	247	235	231	209	180	152	125	98	70	40	12	1	0
With coil rotated 1/3 revolution from feed position	(H)	27	29	28	28	29	29	29	26	20	17	-11	-6	-15	-29	-29	-28	-28	-27	-30	-29	-20	-9
	(ΣH)		56	84	112	141	170	199	225	245	262	251	245	230	201	172	144	116	89	59	30	10	1
With coil rotated 2/3 revolution from feed position	(H)	29	30	29	28	28	30	29	27	21	5	-4	-8	-9	-26	-28	-28	-27	-26	-28	-27	-21	-11
	(ΣH)		59	88	116	144	174	203	230	251	256	252	244	235	209	181	153	126	100	72	45	24	13[d]

[a]Positive values are for net heads on the ascending side of the coil; negative values are for net heads on the descending side of the coil (Fig. 2).

[b]top rows are individual turns.

[c]bottom rows are summed values.

[d]net head in discharge spiral.

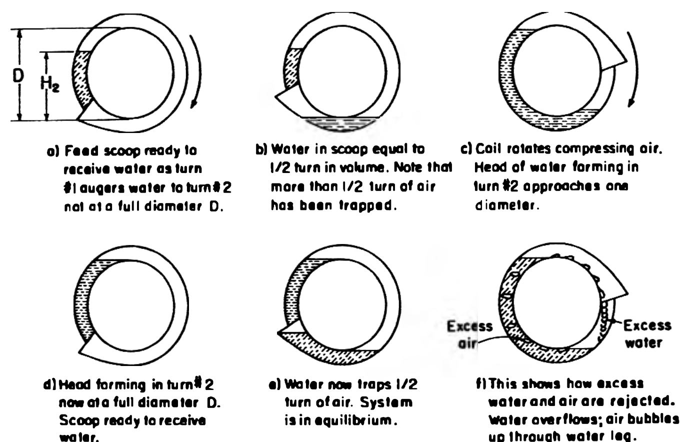

Fig. 3–Self-pressurization mechanism. Steps (a) to (c) are repeated until the water in turn #2 forms a head, H_2, equal to one diameter, D. The rate of self-pressurization approaches zero as maximum pressure is approached.

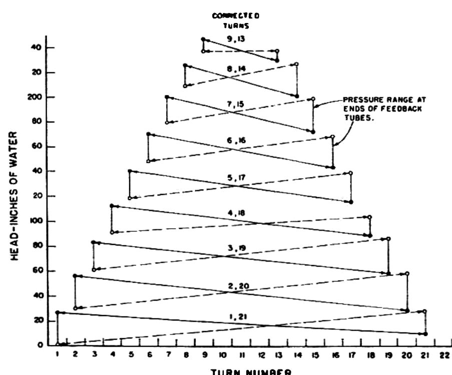

Fig. 4–Pressure at the ends of the feedback tubes in Arrangement A (Table 1), for two positions during coil rotation. Holding section at 250 in. of water. ● No feedback taking place, ends entering water; ○ Feedback taking place, ends entering air.

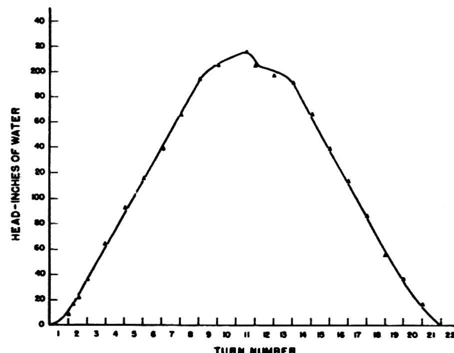

Fig. 5–Pressure history of a package passing through the unit operating at 215 in. of water.

was attained after 80 revolutions. A self-limiting pressure ceiling contributed to the stability of the system.

The maximum pressure attained in the unit during self-pressurization was influenced by the location of the feedback tubes. Operating pressures of 6.2–9.0 psig were attained, depending upon the feedback tube arrangement.

Figure 4 shows the pressure at the ends of the feedback tubes for Arrangement A (Table 1) during one revolution. Arrangement A resulted in undesirably large pressure changes across the feedback tubes during one revolution. This was due in part to their inverted manometric action as they passed over the top of the coil during feedback.

Feedback took place smoothly with Arrangement B (Table 1). As the tubes passed under the coil during feedback they acted as normal manometers, thus providing a small balancing pressure between the decompression and compression turns. The "B" arrangement is the preferred feedback tube configuration.

Tests were made on the effect of a 10% change in feed water volume (from the 0.7 gal needed to fill a one-half turn of tubing). Excess water volume had no effect. A maximum operating pressure of 8.5 psig was obtained (feedback tubes in Arrangement A) with the surplus water spilling out of the feed scoop (Fig. 3, f). A 10% shortage of feed water resulted in a slightly reduced maximum operating pressure (8.2 psig). Excess air was rejected by bubbling.

Figure 5 shows the pressure profile indicated by the measuring gauge as it traveled through the unit. Operating pressure was 7.8 psig (215 in. H_2O). The pressure profile is similar to conventional hydrostatic systems (Conley, 1965), except for some variation due to the action of the feedback tubes.

CONCLUSIONS

SUPERIOR OPERATING characteristics of this multizone helical processing system over previously proposed arrangements have been demonstrated. The pressure profile of a conventional continuous hydrostatic cooker requiring tall vertical legs can be duplicated in this new compact continuous system.

Stable, truly hydrostatic operation has been obtained, independent of package load and rate of coil rotation. Stable hydrostatic characteristics have been obtained by means of an innovative feedback mechanism.

The improvement of this unit over earlier models and the widespread interest shown in this new helical pumping and processing concept for a wide range of potential industrial applications have encouraged construction and study of prototype three-zone systems.

Studies are underway on an all-metal unit simulating commercial operating conditions for thermal processing at about 250°F and 15 psig pressures. Operational problems in package handling and heat transfer will be studied.

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Ms. received 3/6/70; revised 6/15/70; accepted 7/3/70.

The authors express their appreciation to Mr. Edison Lowe of this laboratory for his contributions in the preparation of this manuscript.

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DEVELOPMENT OF A NEW PRODUCT FOR THE CIVIL DEFENSE PROGRAM

SUMMARY–Development of a new cereal-based product–proposed as a replacement for existing rations now used in the Civil Defense Ration Program–is described. Major emphasis was placed on developing a product containing adequate amounts of protein (46 g/day vs 14 g/day for existing rations) and at a competitive cost. Selection of raw materials, nutritive value and processing conditions were developed to meet these and other criteria of the Office of Civil Defense. Major ingredients were whole wheat flour, casein and lard. The finished product cost was 20c/lb vs 23c/lb for the existing rations. Application of these efforts to other civilian areas is discussed.

INTRODUCTION

CONSIDERABLE emphasis is currently being placed on the nutritional adequacy of foods. Since the selection of such foods is important in the economics of feeding hungry people, it is surprising that little attention is being directed to the efforts of Civil Defense Rations Programs. More than 10 years ago, the Office of Civil Defense (OCD) became concerned with the need for food supplies to be stored in shelters for use by occupants during times of disaster (Hundley, 1952; Stone 1965). A concerted research effort by numerous individuals and companies resulted in the development of three products: biscuit, cracker and wafer, which had specifications established through guidelines offered by the National Academy of Sciences (1962), in association with OCD. Of the many factors concerned with ration development, those most important to the research described herein are: storage stability of 7–10 yr, maintenance nutritive value (i.e., minimal protein to minimize increased water demand); provision of 2,000 cal/person/day at 900 cal/lb; and a cost not to exceed 23¢/person/day, including packaging and shipping.

These three products were subsequently subjected to numerous tests and feeding trials, including shelter habitability studies (Browe, 1964; Chow, 1964; Hammes and Osborne, 1964; Longnecker and Sarett, 1963; Morris, 1963). Of particular interest were the nutritional experiments carried out in shelters. People who consumed these rations for periods of up to 14 days exhibited a negative nitrogen balance and a concomitant weight loss. In those tests, the food and water consumed were considerably less than expected, despite the availability of an excess. The food consumption was 400–900 cal/person/day, from more than 2,000 calories available. The water intake was 0.8–1.4 qt/person/day, with an available supply of 1.4 qt. Since increasing the protein level did not reverse the nitrogen imbalance, Chow (1964) concluded that the protein quality was inadequate. Of course, raw material availability was more limited at that time, and the cost of some specific ingredients (e.g., the L-amino acids) was high. Since people would consume these products for a limited time, sufficient protein of good quality was not considered important. Provided subsequent food consumption was adequate, a 10- to 15-day period of negative nitrogen balance was not considered serious. Alternatively, improving the nutritive value of these products without substantially increasing the price would be advantageous. Nutritional stress during confinement in a shelter would certainly add to the overall stress on all occupants, particularly on pregnant and lactating women, babies, the injured, and the sick.

Another consideration was the possibility that the water requirement would increase above the total available of 1.4 qt/person, if the amount of protein in the ration was increased. Existing rations contain 8.5% protein, whereas the proposed product contains 18.5% protein. However, Chow (1964) reported that with subjects consuming 40g protein/day, coupled with 1.4 l water/day, there were no significant weight losses. The subjects did not consume all the available water.

With the advent of new, better quality, and lower cost raw materials, it seemed prudent to consider the development of an improved product–one that would definitely be better than existing products from the standpoints of nutrition, cost and, hopefully, acceptability.

EXPERIMENTAL

IN DESIGNING a replacement product it was decided to retain the existing cracker shape and form, to develop a process that would be applicable to industrial baking equipment, and to use the nutritional guidelines as proposed by the NAS-NRC Recommended Dietary Allowances (1968).

Raw materials

Various combinations of grains and protein supplements were ranked according to their nutritive value, usage in baked goods, acceptability, and large-volume costs. Some of the products considered were whole wheat flour, spray-dried casein, whole corn flour, bakers dried yeast, lactalbumin, whole eggs, soy flours (containing 50, 66 and 90% protein), alfalfa meal, wheat gluten, rolled oats and wheat bran. The final selection of the ingredients was influenced by the quality of the finished product as well as protein level, amino acid composition, cost, and possible flavor value.

The quantities of the raw ingredients were adjusted to give an acceptable balance of the essential amino acids, as well as the correct amount of protein and calories. Certain vitamins and minerals were also added.

The two most promising formulations were presented to a 59 member flavor panel for preference testing. One product was based on wheat as the major component, and the other formulation contained wheat and corn as the cereal ingredients. Two trials for each panel member were run, where the order of sampling was reversed in one of the trials. Out of the 118 responses, 56 indicated a preference for the wheat formulation, and 62 favored the wheat-corn sample, therefore indicating no significant preference. Since the wheat-based formulation was slightly superior from a nutritional viewpoint, it was selected as the prototype product.

The formulation and ingredient costs for this product are presented in Table 1.

Product formulation

Formulation of the product involved a series of relatively simple unit operations. The yeast and micronutrients were carefully blended with water at 95°F. The remaining dry ingredients and fat were blended separately for 10 min and

Table 1–Product composition and cost per 900-calorie portion.

Ingredients	Grams	Approximate West Coast Cost in Cents[a]
Whole wheat flour	197.0	2.896
Casein	19.0	1.174
Baking powder (Calumet)	2.1	0.067
Sodium chloride	5.4	0.014
Sucrose	4.1	0.090
Lard	13.7	0.363
Yeast	8.2	0.723
Water	140.0	–
Calcium citrate	2.93	0.428
Ferrous gluconate	0.06210	0.013
Thiamine hydrochloride	0.00325	0.005
Riboflavin	0.00323	0.010
Niacinamide	0.02900	0.012
Total	392.53	5.795

[a]Cost of ingredients only.

then slowly added to the aqueous mixture. Agitation was accomplished in a Hobart mixer. The dough was rolled to a thickness of about 1/8 inches, cut to shape, and allowed to ferment at 95°F for 30 min. The product was then removed from the proofing cabinet, baked at 360°F for approximately 7 min, and dried at 212°F for 2 hr. The baking time was influenced by the thickness of the cracker and the initial amount of moisture present.

Discussions with a major biscuit and cracker manufacturer supported our conclusion that the process could be adapted to commercial operations.

Once formulation of a prototype product was completed, samples were analyzed to ascertain the extent of nutritive losses due to baking. Table 2 shows that heating reduced the levels of amino acids.

It can be seen that the new prototype meets the requirements for all the essential amino acids. Although it contains somewhat smaller amounts of tryptophan, methionine and cystine, phenylalanine, and histidine than the recommended levels, this was considered not to be an impediment since the recommended levels are double the daily requirement.

Tables 2 and 3 show similar data for protein and selected vitamins and minerals. Although levels of thiamine, niacin, and riboflavin were also reduced they were still above the required amounts, and in most instances equal to or above the recommended levels. The only deficiencies are the vitamins A and C. Since both are destroyed during processing, they were not added to the present formulation. The absence of these vitamins in the finished product was of no great concern, since the recommendation of the NAS-NRC was to have vitamins in capsule form available in the medical kit.

Nutritional evaluation

The nutritive value of the prototype product was investigated by means of a rat-feeding study. 21-day-old, Sprague-Dawley rats were divided into three groups of 15 each, based on weight, and housed in individual, suspended cages. Diets were prepared in advance and frozen until needed. The test diets are described in Table 4. Following a 3-day holding period, the animals were fed the test diets for 20 days.

The diets fed to Groups 1 and 3 were isocaloric (39.66 cal/day), based on calculations from the recorded food intake. Since water intake was not monitored in the present experiment, it was not possible to estimate the effect, if any, of the increased protein on the water requirement (see Chow, 1964).

Some of the results are summarized in Table 4 which shows the average daily weight gain, weight of solids consumed, and the protein efficiency ratio (PER) values. The PER values are in excellent agreement with the previously reported results of Longnecker and Sarett (1963): their lysine-supplement group had a PER of 1.39 vs 1.37 in this study; their control had a PER of 3.06 vs 2.92 in this study. The PER for the prototype ration was 2.12, which is consistent with PER values for diets consisting mainly of cereal grains. It is interesting to note that the animals in the lysine-supplement group exhibited poor growth even though they received food and water *ad libitum*. This is believed to be due to a marginal amount of certain amino acids, as mentioned earlier. Although the PER values were based on a 20 day feeding trial vs a recommended 28 days, we believe the results are still meaningful. More definitive nutritional experimentation is warranted once production volumes are achieved.

The initial objective of this research was realized in the development of a prototype product that possessed an improved nutritive value according to analysis and an initial rat-feeding trial. Obviously additional feeding experiments are required, preferably in shelter environments, before the product can be properly compared with existing shelter rations.

Cost analysis

The question of cost also received attention. With the cost of the raw materials known, an

Table 2–Effect of processing on the amino acid composition of the prototype product (in grams).

	Essential								Partially Essential	
	Tryptophane	Threonine	Isoleucine	Leucine	Lysine	Methionine + cystine	Phenylalanine	Valine	Arginine	Histidine
Calculated amount	0.306	1.778	2.684	4.000	2.497	1.784	2.615	2.763	2.013	1.228
Amt remaining after processing[a]	0.306	1.574	1.710	3.332	1.750	1.170	2.194	2.133	1.715	1.030
Required per day[b,c]	0.25	0.50	0.70	1.10	0.80	1.10	1.10	0.80	–	–
Recommended per day[b]	0.50	1.00	1.40	2.20	1.60	2.20	2.20	1.60	1.5	2.0

[a]Data based on analysis of sample rations.
[b]Burton, 1965.
[c](Blanck, 1955).

Table 3–Effect of processing on the protein, vitamin, and mineral composition of formula 6300-060-19.

	Protein (g)	Calcium (mg)	Phosphorus (mg)	Iron (mg)	Sodium (mg)	Potassium (mg)	Vitamin A (I.U.)	Thiamine (mg)	Riboflavin (mg)	Niacin (mg)	Vitamin C (mg)
Theoretical total in raw ingredients	46.7	840	883	15.0	2333	893	0	4.15	3.91	40.5	0
Expected total remaining after processing[a]	–	802	883	13.4	2333	768	0	3.50	3.64	39.8	0
Required/day[b]	45.0	800	–	10.0	–	–	5000	1.20	1.70	19.0	70.0
Recommended/day[b]	–	–	–		2123		5000	2.00	–	20.0	100.0

[a]Data based on analysis of sample rations. This value for protein is expected to be only slightly less than the theoretical total.
[b]National Academy of Sciences, 1962.

Table 4—Weight gain, food intake, and protein efficiency ratio (PER) for the three test groups.[a]

Group		Avg Daily Weight Gain (g)	Avg Daily Intake of Food Solids (g)	PER
1	Casein control[b]	6.71	10.16	2.92
2	Survival biscuit supplemented with 1.22% L-lysine HCl[c]	0.73	5.65	1.37
3	Prototype product[d]	4.93	10.98	2.12

[a]Each diet was fed moist to minimize spillage; however, reported values are on a dry-weight basis.

[b]Percentage by weight of ingredients is as follows: casein, 18.1; sucrose, 55.3; shortening, 7.7; vitamin fortification mixture (without fat soluble vitamins), 2.2; alphacel, 7.6; water, 9.1. Fat soluble vitamins were fed by dropper twice weekly.

[c]Percentage by weight of ingredients is as follows: wheat flour, 63.0; corn flour, 6.3; soy flour, 3.8; sucrose, 12.5; corn sugar or corn syrup solids or a combination of both, 6.3; shortening, 7.0; salt, 1.1; water, as needed. For this study, lysine was added at 1.22% of the total weight.

[d]Percentage by weight of ingredients is as follows: whole wheat flour, 50.2; casein, 4.8; baking powder, 0.5; sodium chloride, 1.4; sucrose, 1.0; lard, 3.5; yeast, 2.1; water, 35.7; calcium citrate, 0.7; ferrous gluconate, .0022; thiamine hydrochloride, .00086; riboflavin, .0017; niacinamide, .0021.

Table 5—Product logistics comparison.

	OCD Present	Proposed Prototype Product
Calories/lb	2,000	1,720
Calories/day	720	900
Calories, total ration	10,000	12,600
Percent increase; calories/day	–	25
lb/person (14 days)	5	7.3
lb/day	0.36	0.52
Percent protein	8.5	18.5
Protein/day, g	14	46
Mineral supplement	none	yes
Percent increase; weight, lb/person	–	45
Cost, cents/day	8.3	10.6
Cost, dollars/lb	0.23	0.20
Percent increase; cost	–	28

estimated selling price of the proposed ration was computed from the ratio of raw materials cost to gross sales for a major cracker and biscuit company for the years 1963–67. It was found that the raw materials and supplies comprised 54.7% of the selling price during those 5 years. Therefore, the total estimated cost of the proposed ration would be 10.594¢/900-cal portion (5.795¢ for the raw materials and 4.799¢ for operating costs and profit). Table 5 presents a comparison of the proposed product and the existing rations in terms of cost, weight, calories and protein. Thus, one could manufacture an improved product without materially altering its shape, form, process and cost to the government. Certainly the improved nutritive value is well worth the very slight cost increase (8.3 vs 10.6¢/day). The new product should have more widespread appeal, although such tests have not yet been made. Various supplements (e.g., spreads) could be used to further enhance product appeal (Stone et al., 1968). At a time when much attention is focused on nutrition and health, a product of this type could find wide application in certain areas.

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Ms. received 3/23/70; revised 7/3/70; accepted 7/17/70.

The authors ackowledge the cooperation of Mr. James Halsey of Stanford Research Institute; Dr. Norman Desrosier of National Biscuit Company who kindly reviewed the process and provided helpful comments on commercial baking operations; and Dr. Peyton Davis of Stanford Research Institute who kindly reviewed the manuscript.

This research was supported by the Civil Defense Technical Office under Contract OCD-DAHC20-67-C-0136. As a CDTO research paper, it does not reflect the policy of the Office of Civil Defense or the Department of Defense.

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INTEGRAL TECHNIQUES APPLIED TO SUBLIMATION DRYING WITH RADIATION BOUNDARY CONDITION

SUMMARY—An analytical solution is presented for the temperature distribution in a semi-infinite slab with a radiation boundary condition and sublimating phase front. 2 cases are considered. First, a solution is presented for the transient surface temperature holding the heater temperature constant. Secondly, a solution is presented for the heater temperature variation required to hold the surface at the same prescribed value reached in the first case. The application of this technique to sublimation drying is considered, along with calculations to demonstrate the effect of various parameters on the drying time. For example, a steak ½-in. thick with a porosity of 0.8 can be dried 35% faster if the initial heater temperature is 200 rather than 150°F. In contrast, for the same sample, the drying time is increased by only 15.5% if the initial heater temperature is 250 instead of 200°F. Graphs are included to show how optimum drying rates may be obtained.

INTRODUCTION

IN SUBLIMATION drying, the latent heat of sublimation is usually supplied by energy radiated from a heated platen to the exposed surface of the product and then conducted through the dried layer to the interface between the frozen and dried regions. Several authors have published results for the drying time and interface position, assuming the exposed surface instantaneously reaches a temperature slightly under the scorch temperature and remains at that value during the entire drying process.

A comprehensive study was conducted by Harper and Tappel (1957) concerning the role of heat and mass transfer during the sublimation drying of food. They used a 1-dimensional model with heat transfer by thermal radiation to the surface of the sample, where it was conducted through the dried porous layer to the sublimation front. The surface temperature was assumed constant and the temperature profile through the dried layer was assumed to be linear. They also suggested that the vapor flow from the interface to the chamber was either hydrodynamic due to the total pressure gradient across the dried layer or diffusional due to the partial pressure gradient of the water vapor.

Hatcher (1964) used the attenuation of gamma rays to measure the moisture distribution during sublimation drying. He showed that no more than 3% of the drying process occurred on either side of the interface and that the sublimation front was less than 5 mm thick for freeze drying slabs of beef. He also obtained data for the transient temperature distribution during freeze drying.

Dyer and Sunderland (1967) presented an exact solution for the transient temperature distribution in the dried layer as well as the interface position as a function of time. Mass transfer was not included in the analysis and the temperatures at the exposed surface and the interface were assumed constant. Heat transfer in the frozen region was assumed to be negligible. An example was presented and compared to the data obtained by Hatcher. Differences in results were attributed to the possibility of some backface heating during the actual drying process as investigated by Hatcher.

Hardin (1965) and Dyer and Sunderland (1968a) presented improved solutions for the drying rates and times, considering the internal flow to be in the transition regime. Hill (1967) calculated approximate drying times in all molecular flow regimes. Dyer and Sunderland (1968b) included backface heating, i.e., heating through the frozen layer, in calculating drying times. It was also concluded that under normal conditions, the role of convection is small compared to conduction in the internal problem.

Massey (1968) carried out an extensive analytical investigation to determine the heat and mass transfer mechanisms of a binary mixture of gases flowing in a parallel plate channel where mass ejection occurred at 1 wall. The results were applied to sublimation drying. He showed that thermal radiation is the predominant mode of heat transfer to the surface of the product. Although Massey included the variation of surface temperature with time, the temperature distribution was used as an independent boundary condition instead of the nonlinear heat flux, due to radiation to the surface.

These papers have approached freeze drying from varying considerations and with different applications. Freeze drying is so complicated that a simple general solution does not seem to be obtainable. Therefore, different solutions apply to the different applications encountered. The purpose of this paper is 2-fold. First, a method is presented to calculate interface position, surface temperature and drying time for slabs during the period in which the product surface temperature varies. The heater temperature is assumed constant for this analysis. Secondly, a method is presented that gives a means of calculating how the heater temperature must be varied to keep the surface temperature at a constant value just under the scorch temperature. Calculations are presented to show the effect of heater temperature and the porosity (ratio of void to total volume) on drying time.

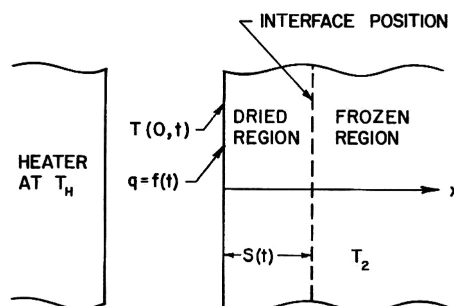

Fig. 1—Drying model.

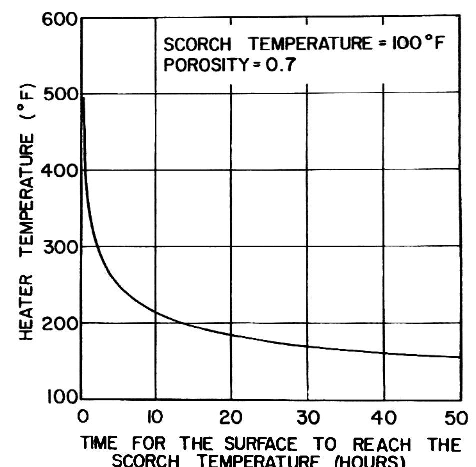

Fig. 2—The influence of heater temperature on the time to reach scorch temperature.

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ANALYTICAL INVESTIGATION

Case 1: Varying surface temperature, constant heater temperature

The 1-dimensional model used in this analysis is shown in Figure 1. Initially, the slab is entirely frozen and its temperature is equal to the sublimation temperature corresponding to the water vapor pressure in the vacuum chamber. Variations of the initial temperature from this value will have only a minor influence on the drying rate. As drying proceeds, a dried layer is formed and is distinctly separated from the frozen layer by a thin interface. In view of the measurements made by Hatcher (1964), this interface will be assumed to be planar. The surface temperature of the slab rises due to radiation heat transfer from the heater to the surface of the product. The heater is assumed to radiate as a black body at a constant temperature, T_H. Due to the small gap between the heater and slab, the view factor for radiation is approximately 1. Convection heat transfer between the heater and the exposed surface of the slab is neglected (see Massey, 1968). The energy radiated to the surface of the slab contributes to raising the temperature of the dried region and to supplying the latent heat of sublimation. The energy transfer due to mass transfer of the vapor from the interface through the porous dried layer is neglected (see Dyer and Sunderland, 1968b) and all properties except temperature are assumed to remain constant. The following analysis is used until the temperature of the surface reaches the scorch temperature.

The transient temperature distribution satisfies the following differential equation:

$$\frac{\partial^2 T}{\partial x^2} = \frac{1}{\alpha}\frac{\partial T}{\partial t} \text{ for } 0 \leqslant x \leqslant s(t). \quad [1]$$

Since initially there is no dried region, the initial condition is satisfied by

$$s = 0 \text{ for } t = 0 \quad [2]$$

The following boundary conditions involve the temperature of the interface (which is constant) and an energy balance at the exposed surface:

$$T = T_2 \text{ at } x = s(t) \quad [3]$$

and

$$-k\frac{\partial T}{\partial x} = f(t) \text{ at } x = 0 \text{ and } 0 \leqslant t \leqslant t_0. \quad [4]$$

An energy balance at the interface will provide 1 additional relation needed to determine the interface position. Thus

$$-k\frac{\partial T}{\partial x} = \lambda\rho L\frac{ds}{dt} \text{ at } x = s(t). \quad [5]$$

To determine Equation [5] it is assumed that any heat transfer through the frozen region to the interface is negligible compared to the heat transfer through the dried region. Following the analysis of Goodman (1964), Equation [1] is integrated over the region from x = 0 to x = s(t) to obtain

$$\int_0^{s(t)} \frac{\partial^2 T}{\partial x^2} dx = \frac{1}{\alpha}\int_0^{s(t)} \frac{\partial T}{\partial t} dx.$$

This equation can be rearranged by using Leibniz's rule,

$$\frac{d}{dt}\int_0^{s(t)} T\, dx = T_2\frac{ds}{dt} + \int_0^{s(t)} \frac{\partial T}{\partial t} dx$$

to obtain

$$\int_0^{s(t)} \frac{\partial^2 T}{\partial x^2} dx = \frac{1}{\alpha}\left[\frac{d}{dt}\int_0^{s(t)} T\, dx - T_2\frac{ds}{dt}\right].$$

Carrying out the indicated operations on the left-hand side and making use of Equations [4] and [5],

$$\frac{d}{dt}\left[\int_0^{s(t)} T\, dx - T_2 s\right] = \alpha\left[\frac{f(t)}{k} - \frac{\lambda\rho L}{k}\frac{ds}{dt}\right]$$

or

$$\frac{d}{dt}\left[\theta - T_2 s + \alpha A s\right] = \frac{\alpha f(t)}{k} \quad [6]$$

where

$$A = \frac{\lambda\rho L}{k}$$

and

$$\theta = \int_0^{s(t)} T\, dx.$$

The rate of heat transfer between the heater and the product surface is given by

$$f(t) = \epsilon\sigma\left[T_H^4 - T^4(0,t)\right]. \quad [7]$$

To calculate θ, the temperature distribution is approximated by the following quadratic equation

$$T = a + b(x - s) + c(x - s)^2. \quad [8]$$

The parameters a, b and c are functions of time and can be determined from Equations [3] and [4] and the following equation

$$\left(\frac{\partial T}{\partial x}\right)^2 = A\alpha\frac{\partial^2 T}{\partial x^2} \text{ at } x = s(t) \quad [9]$$

which is an auxiliary condition derived by Goodman so that Equation [6] remains first order in t.

Using Equations [3], [4] and [9] to find a, b and c, the temperature profile is found to be

$$T = T_2 + \frac{1}{2}\frac{A\alpha}{s}\left[1 - (1 + 4\mu)^{1/2}\right][x - s] + \frac{A\alpha}{4s^2}\left[1 - (1 + 4\mu)^{1/2} + 2\mu\right][x - s]^2 \quad [10]$$

where

$$\mu = \frac{f(t)\, s}{A\alpha k}. \quad [11]$$

Substituting Equation [10] into the expression for θ and carrying out the indicated operations, yields

$$\theta = T_2 s + \frac{1}{6}A\alpha s\left[\mu + (1 + 4\mu)^{1/2} - 1\right]. \quad [12]$$

Substitution of Equation [12] into Equation [6] yields

$$\frac{d}{dt}\left\{s\left[5 + (1 + 4\mu)^{1/2} + \mu\right]\right\} = \frac{6f(t)}{Ak}. \quad [13]$$

Evaluating Equation [10] at x = 0 gives the following expression for the surface temperature as a function of time:

$$T(0,t) = T_2 + A\alpha\left[\frac{\mu}{2} + \frac{1}{4}(1 + 4\mu)^{1/2} - \frac{1}{4}\right]. \quad [14]$$

At this point it is useful to define the following dimensionless quantities

$$f^* = \frac{f(t)}{f(0)} \quad [15]$$

$$s^* = \frac{s f(0)}{\alpha A k} \quad [16]$$

$$t^* = \frac{t f^2(0)}{\alpha A^2 k^2} \quad [17]$$

and

$$T^* = \frac{T}{A\alpha} \quad [18]$$

where

$$f(0) = \epsilon\sigma\left(T_H^4 - T_2^4\right). \quad [19]$$

With these definitions, Equations [7], [13] and [14] become respectively

$$f^* = \frac{T_H^{*4} - T^{*4}(0,t^*)}{T_H^{*4} - T_2^{*4}} \quad [20]$$

$$\frac{d}{dt^*}\left\{s^*\left[5 + (1 + 4\mu)^{1/2} + \mu\right]\right\} = 6f^* \quad [21]$$

and

$$T^*(0,t^*) = T_2^* + \left[\frac{\mu}{2} + \frac{1}{4}(1 + 4\mu)^{1/2} - \frac{1}{4}\right] \quad [22]$$

where

$$\mu = f^* s^*. \quad [23]$$

Since Equation [21] is a differential equation with 2 dependent variables, s^* and μ as functions of t^*, it is convenient to eliminate s^*. Differentiating the left-hand side of Equation [21] and making use of Equation [23] to eliminate $\frac{ds^*}{dt^*}$ in terms of $\frac{df^*}{dt^*}$ and $\frac{d\mu}{dt^*}$ yields

$$\left[5 + 2\mu + 2\mu(1 + 4\mu)^{-1/2} + (1 + 4\mu)^{1/2}\right]\frac{d\mu}{dt^*} - \frac{\mu}{f^*}\left[5 + (1 + 4\mu)^{1/2} + \mu\right]\frac{df^*}{dt^*} = 6f^{*2}. \quad [24]$$

Next, substitute Equation [22] into Equation [20] and differentiate the resulting expression with respect to t^*.

Solving the resulting equation for $\frac{df^*}{dt^*}$ and substituting the resulting expression into Equation [24] yields

$$\frac{dt^*}{d\mu} = F(\mu) \quad [25]$$

where

$$F(\mu) = \frac{z(\mu)}{6f^{*2}} \quad [26]$$

$$z(\mu) = \left[5 + 2\mu + 2\mu(1 + 4\mu)^{-1/2} + (1 + 4\mu)^{1/2} - \mu\left(5 + (1 + 4\mu)^{1/2} + \mu\right)\left[\frac{T^*(0,t^*)]^3\, h(\mu)}{f^*}\right] \quad [27]$$

where f^* and $T^*(0,t^*)$ are given by Equations [20] and [22], respectively, and

$$h(\mu) = \frac{-2\,(1 + (1 + 4\mu)^{-1/2})}{T_H^{*4} - T_2^{*4}}. \quad [28]$$

Evaluating $F(\mu)$ at $t^* = 0$ (i.e., $\mu = 0$) one has the following initial condition

$$\frac{d\mu}{dt^*} = 1.$$

Equation [25] is solved by numerical integration. Simpson's Rule is the quadrature method used in this paper. As discussed in Scarborough (1966), this method should provide sufficient accuracy for problems of the type given by Equation [25] provided the initial slope is not large. In the case considered here, the initial slope is unity and thus Simpson's Rule is applicable. For s, t, T(0,t) and f to be found in the appropriate dimensional variables, the following information must be given: Scorch temperature–Density of the frozen material–Porosity of the dried region–Thermal conductivity–Diffusivity–Latent heat of sublimation–Emissivity–Temperature of the interface and Heater temperature. The scorch temperature must be given so that when T(0,t) becomes equal to the scorch temperature, Case 2 is initiated.

Case 2: Constant surface temperature, varying heater temperature

The same general assumptions are made in Case 2 as in Case 1. The equations of Case 1 are used until the surface temperature of the product reaches the scorch temperature. After that time, the surface temperature remains constant and thus the boundary condition given by Equation [4] no longer holds. The final temperature distribution calculated from the Case 1 analysis becomes the initial condition for Case 2. Thus, the heater temperature is varied for the remainder of drying so that the surface temperature of the product remains at a constant value just under the scorch temperature.

If t_0 denotes the time at which the surface temperature first reaches the scorch temperature, T_s, then for $t > t_0$, $T(0,t) = T_s$ and from Equation [22], μ is a constant which we will define as μ_0. Under these conditions, Equation [21] becomes

$$\frac{ds^*}{dt^*} = \frac{6f^*}{5 + (1 + 4\mu_0)^{1/2} + \mu_0} \quad [29]$$

From Equation [23]

$$f^* = \frac{\mu_0}{s^*}. \quad [30]$$

Hence

$$s^*\,ds^* = \frac{6\mu_0\,dt^*}{5 + (1 + 4\mu_0)^{1/2} + \mu_0}. \quad [31]$$

Integrating Equation [31] yields

$$s^{*2} = \frac{12\mu_0\,t^*}{5 + (1 + 4\mu_0)^{1/2} + \mu_0} + C \quad [32]$$

at $t^* = t_0^*$, $s^* = s_0^*$, hence

$$s^{*2} - s_0^{*2} = \frac{12\mu_0}{5 + (1 + 4\mu_0)^{1/2}\,t\mu_0}(t^* - t_0^*). \quad [33]$$

Since s_0^*, μ_0 and t_0^* are known from the first case considered, Equation [33] gives s^* as a function of t^*. Equation [30] then gives f^* as a function of t^*.

Let $T_H^*(t^*)$ represent the heater temperature at any time $t^* > t_0^*$. From the definition of f^* given by Equation [20] and noting that $T_H^*(0,t^*) = T_s^* =$ constant, it can be shown that

$$T_H^*(t^*) = \left[\frac{T_s^{*4} - T_2^{*4}\,f^*}{1 - f^*}\right]^{1/4}. \quad [34]$$

RESULTS OF SAMPLE CALCULATIONS

TO ILLUSTRATE the use of the relations derived in the previous section, the following example is presented: Beef steak is chosen as the product and is sufficiently thick to satisfy the assumptions discussed earlier. For a chamber pressure of 1 torr, the necessary input variables are:

ρ = 57.4 ft^3/lbm (Dickerson, 1968)

k = 0.042 B/hr ft°R (Massey and Sunderland, 1967)

a = .06 ft^2/hr (Dyer and Sunderland, 1967)

L = 1488 B/lbm (Dyer et al., 1966)

ϵ = .74 (Sevcik and Sunderland, 1962)

T_2 = 5.5°F (Hatcher, 1964).

Using the results of Case 1 and assuming the scorch temperature to be 100°F, a plot of heater temperature versus time for the surface to reach the scorch temperature is presented in Figure 2. The porosity of the dried meat is 0.7. Notice how much longer it takes for the surface to reach the scorch temperature when the heater temperature is 150 instead of 175°F. Table 1 shows the effect of porosity on the time to reach the scorch temperature for 4 typical values of the heater temperature.

In Figure 3 the heater temperature is plotted as a function of time for porosities of 0.6 and 0.8. As before, the scorch temperature is 100°F. During the initial drying phase, the heater temperature is constant and the equations for Case 1 are used. As drying proceeds and the scorch temperature is reached, the heater temperature is modulated to hold the surface at 100°F. Practically, this modulation could be accomplished by a simple temperature controller coupled with a silicon control rectifier for proportional control. Figures 4–6 give the surface temperature as a function of time for 3 values of porosity. The maximum scorch temperature has been taken as 140°F but for scorch temperatures less than 140°F, the plots should be discontinued at the actual scorch temperature.

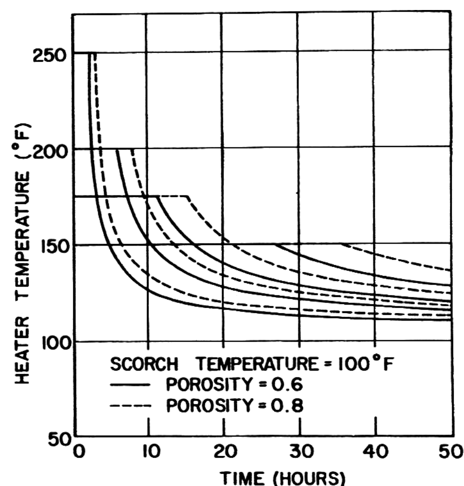

Fig. 3—Heater temperature as a function of time with porosities of 0.6 and 0.8.

Figures 7–9 show the interface position as a function of time for various heater temperatures and porosities. Experimental data obtained by Hatcher (1964) are also included. His data were obtained under thermal conditions that would correspond approximately to our highest heater temperature. His data showed faster drying rates than our predictions. This may be explained by considering the heat transfer to the interface by way of the back face and edges of the sample.

Figure 10 shows the effect of heater temperature and porosity on the time to dry a sample 0.5-in. thick. As expected, increasing the heater temperature decreases drying time and decreasing the porosity decreases the drying time.

These results are presented to give the reader insight into the types of calcula-

Table 1—Time required for surface to reach scorch temperature for various porosities and heater temperatures.

Scorch temp = 100°F	Time to reach the scorch temperature (hr)		
Heater temp (°F)	Porosity .6	Porosity .7	Porosity .8
150	53.7	62.7	71.6
175	22.7	26.5	30.3
200	11.9	13.9	15.8
250	4.4	5.1	5.8

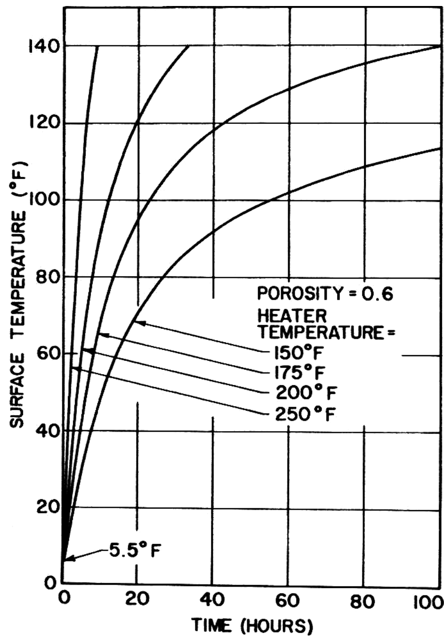

Fig. 4—Surface temperature as a function of time and heater temperature with porosity of 0.6.

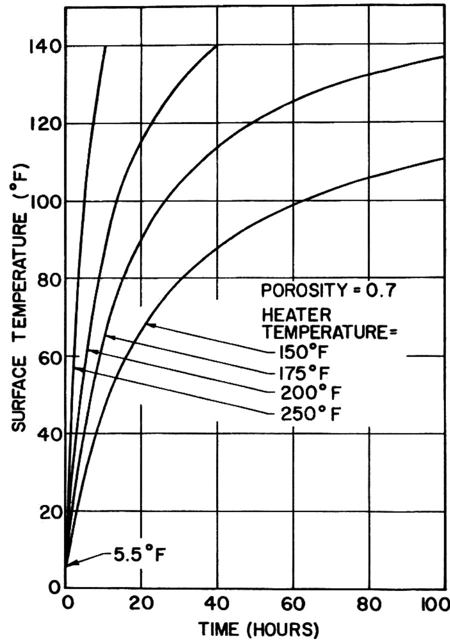

Fig. 5—Surface temperature as a function of time and heater temperature with porosity of 0.7.

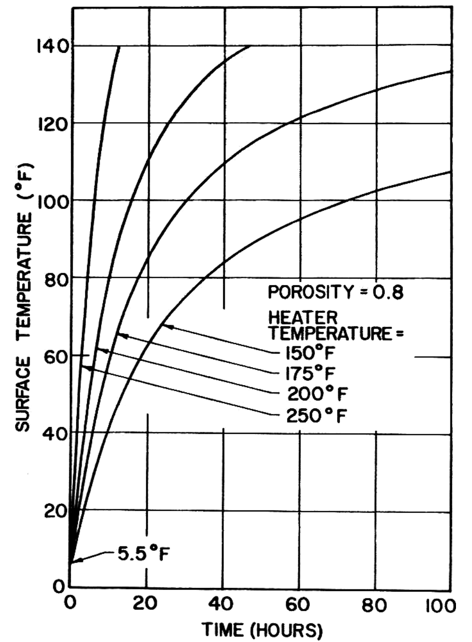

Fig. 6—Surface temperature as a function of time and heater temperature with porosity of 0.8.

tions and data that can be determined from the analysis. Although the calculations were obtained for freeze drying beef, the actual numbers presented may also be close to values that could be expected for other lean meats.

CONCLUSIONS

THE ANALYSES presented are useful for predicting the interface position as a function of time for freeze drying, where conventional heated platens are used to provide the energy needed for the sublimation process. It is assumed that the platen temperature remains constant until the product surface reaches the scorch temperature. The platen temperature is then controlled to maintain a constant product surface temperature. The interface position can be determined from the numerical solution of Equation [25] for μ as a function of t* and the subsequent use of Equations [20], [22] and [23] during the time when the platen temperature is held constant. After the surface reaches the scorch temperature, Equation [33] applies.

The transient surface temperature is easily calculated from Equation [22] after μ has been determined on a function

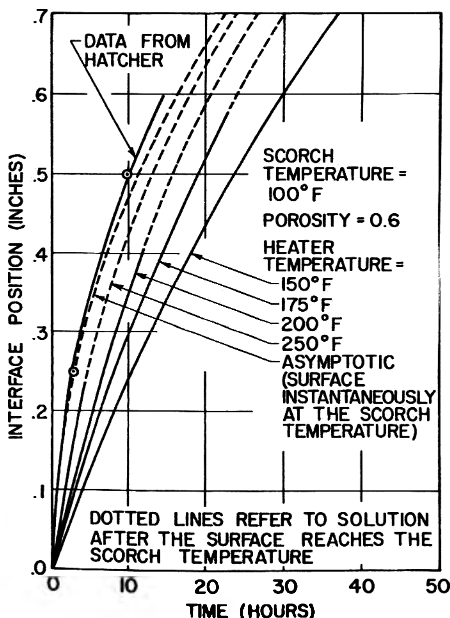

Fig. 7—Interface position as a function of time and heater temperature with porosity of 0.6.

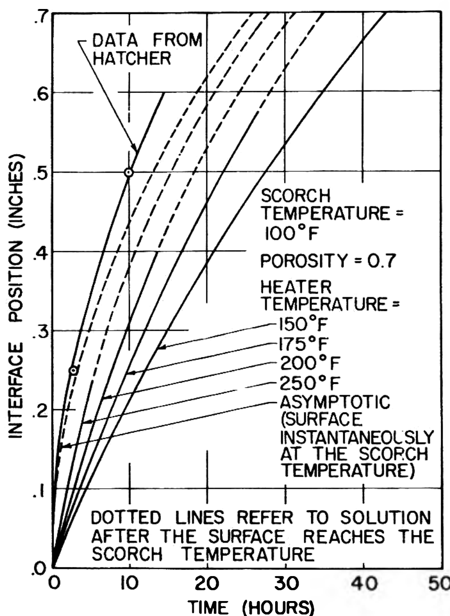

Fig. 8—Interface position as a function of time and heater temperature with porosity of 0.7.

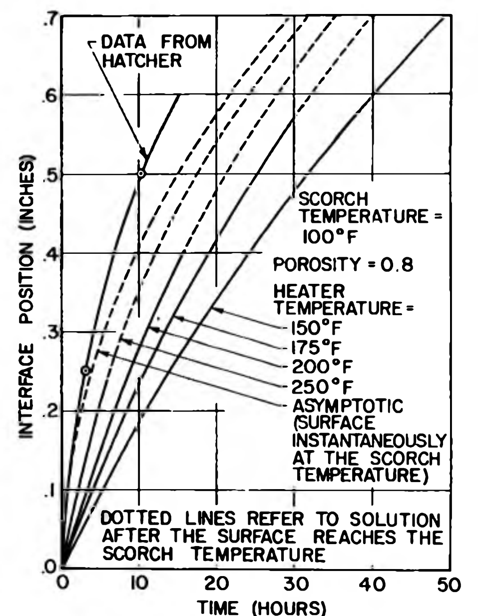

Fig. 9—Interface position as a function of time and heater temperature with porosity of 0.8.

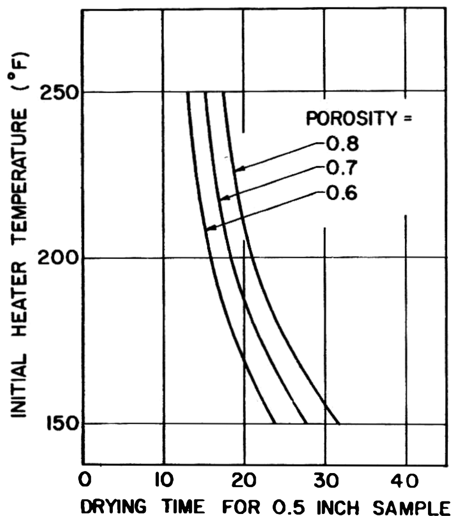

Fig. 10–The influence of initial heater temperature and porosity on drying time for a sample 0.5-in. thick.

t^*. The temperature distribution can be calculated from Equation [10]. The rate of heat transfer to the surface can be calculated from Equation [20] when the platen temperature is constant, and from Equation [30] when the surface temperature is held constant. In the latter case, Equation [34] can be used to calculate the transient platen temperature.

The approximate integral techniques presented for 1 set of boundary conditions can serve as a guide for the solution of problems with other restraints. Although these techniques have certain inherent inaccuracies, the accuracy of the final results obtained will be limited primarily by the specification of the boundary condition; the influence of 2-dimensional effects caused by heat transfer through the sides of the product and nonuniformities of the product structure and the selection of the property values used.

NOTATION

A	$\lambda\rho L/k$, hr °R/sq ft
a,b,c	constants defined by Equation [8]
f(t)	radiation heat flux at the surface, B/sq ft hr
f^*	dimensionless variable defined by Equation [15]
$F(\mu)$	variable defined by Equation [26]
$h(\mu)$	dimensionless variable defined by Equation [28]
k	thermal conductivity of the dried region, B/hr ft °R
L	latent heat of sublimation, B/lbm
s	interface position, ft
s^*	dimensionless variable defined by Equation [16]
t	time, hr
t_0	time at which surface temperature reaches T_S, hr
t^*	dimensionless variable defined by Equation [17]
T	temperature of the dried region, °R
T_2	temperature of the interface and the frozen region, °R
T_H	heater temperature, °R
T_S	scorch temperature, °R
T^*	dimensionless temperature defined by Equation [18]
x	distance from exposed surface, ft
$z(\mu)$	dimensionless variable defined by Equation [27]
α	diffusivity of dried region, sq ft/hr
ϵ	emissivity of the product surface, dimensionless
θ	$\int_0^{s(t)} T\,dx$, °R ft
λ	porosity, ratio of void volume to total volume, dimensionless
μ	dimensionless variable defined by Equation [11]
ρ	density of ice at given pressure, lbm/cu ft
σ	Stefan-Boltzmann constant B/sq ft hr °R^4

The subscript $_0$ denotes the time when $T = T_S$ at the surface. The superscript * denotes the dimensionless nature of the variable.

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Ms. received 7/10/69; revised 9/30/69; accepted 5/1/70.

This research was supported by Public Health Service Research Grant FD-00156-02 from the Food and Drug Administration.

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AN EXPERIMENTAL DRY CAUSTIC PEELER FOR CLING PEACHES AND OTHER FRUITS

SUMMARY—An experimental cling peach peeler was developed for continuous removal of alkaline peel as a solid rather than the dilute slurry of common industrial practice. The peeler consists of rows of soft rubber disks rotated in such a manner that peel material is gently wiped from the surface of fruit and flung into collectors. Cling peach halves were successfully peeled in a number of tests, with peeling losses comparable to those normally obtained commercially. Both mature and immature Bartlett pears were also successfully peeled. Dewaxing of immature pears with isopropyl alcohol was necessary to obtain adequate peeling. In single tests, whole apricots and freestone peaches were also successfully peeled.

INTRODUCTION

ALTHOUGH AIR and water pollution was once a minor problem, present day urbanization and industrialization have advanced to the point where pollution affects everyone. Water from wells and other sources is now limited; consequently dilution is no longer a solution to water pollution. Waste waters must be treated to reduce or destroy the pollutants. To do this economically, waste waters must be concentrated through reuse, or water pollutants must be kept at a minimum by reducing waste material entering plant effluents.

Many canneries once located on the outskirts of cities are now surrounded by city growth. These plants usually have to dispose of their effluents through municipal sewage treatment plants, paying a charge for the volume of effluent, the BOD, and sediment loads. In many cases they have to install systems to continually correct the pH of their effluent to avoid damaging sewage equipment or upsetting operation of the sewage disposal system.

One approach to this problem is to remove cannery peeling wastes in a concentrated form so that they do not enter plant effluents. An example of this is the "Dry Caustic Peeling" process developed at the Western Regional Research Laboratory. In this process white potatoes receive an infrared heat treatment after the usual lye treatment (Graham et al., 1969a, 1969b). The loosened, alkaline peel material is then removed by spinning rubber rolls covered with 1/2-in. rubber projections. As the rolls spin, peel material is flung off and collected as a wet solid.

This process has performed satisfactorily on white potatoes in a large scale pilot plant (Anon., 1970) and is currently being tested in full scale plant operation. Operation on a 5,000–6,000 lb/hr pilot plant parallelling a standard caustic peeling line kept all of the peel material and caustic out of the plant effluent, reduced by at least 50% the amount of caustic used, and made a substantial reduction in peeling loss. Results indicated a 40% reduction in strength of plant effluent could be achieved.

Although waste material from fruit canneries is not as enormous as that from white potato processing, it is still substantial. In 1968, fruit peeled prior to preservation was about 1,540,000 tons. Each ton of fruit processed produced about 12 lb of BOD and 9 lb of suspended solids in rinse waters used after conventional lye peeling. If only 40% reduction of BOD in fruit processing effluent were achieved by dry caustic peeling, some 7.4 million lb of BOD and 5.5 million lb of suspended solids would be removed from treatment plant loadings each year.

Cling peaches comprise the largest tonnage of tree fruit normally lye peeled. The fruit is firm and clings tightly to the pit; because of this difficulty, peaches are pitted prior to lye peeling. The usual peeling procedure after pitting is to place peach halves cup down, cascade hot dilute lye over them for 5 to 10 sec, hold in a warm atmosphere for 15 to 20 sec, and then wash with high pressure sprays of cold water. Another tree fruit similarly peeled is Bartlett pear. Mature pears are dipped in lye, drained, then water washed to remove the peel. Immature pears receive a similar lye treatment but, due to the greater difficulty of peeling, receive an added treatment consisting of a short stay in high pressure steam followed by a rapid release of pressure. The peel is then washed from the pear.

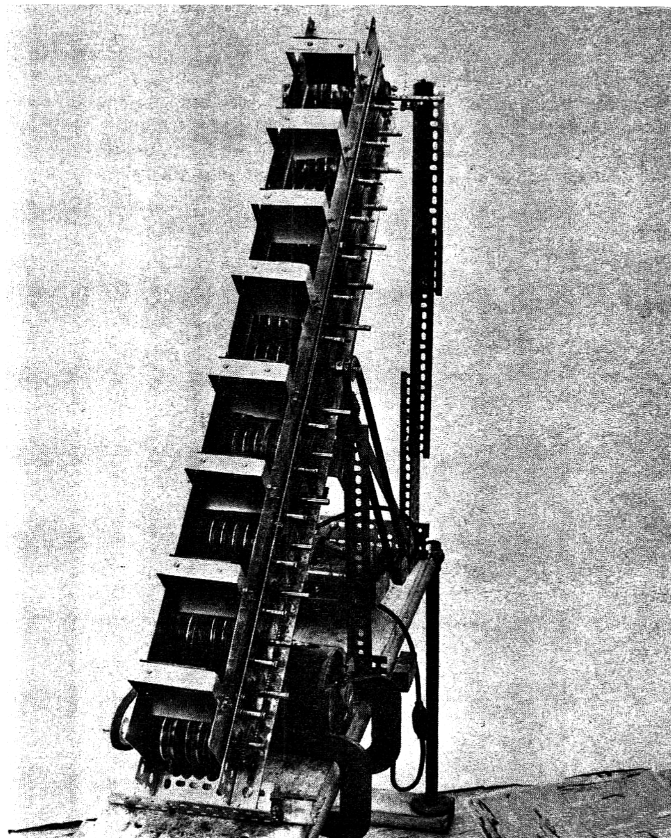

Fig. 1—Experimental peeler for dry removal of alkali treated peel from fruit.

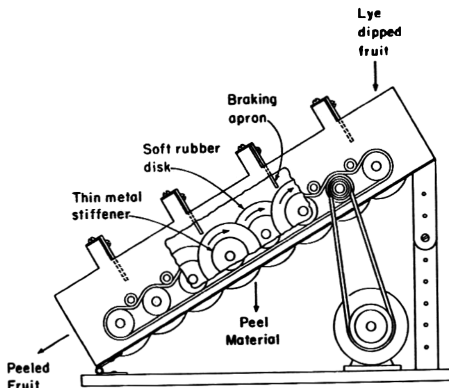

Fig. 2–Diagram of experimental peeler.

During washing, partially disintegrated peel and flesh along with any remaining caustic enter the plant effluent. In this dilute condition these wastes must eventually be removed by biological treatment.

EXPERIMENTAL

Equipment

To apply the dry caustic peeling process to cling peach halves, it was necessary to determine what equipment and process changes had to be made.

Normally when a cling peach half is exposed to caustic, the skin partially disintegrates. Consequently no abrasive action is needed and simple washing or wiping is sufficient to remove the alkaline tissue. However, the combined use of caustic and infrared heat on potatoes was not successful on cling peaches as the heat tended to make the peel adhere more firmly.

The shape of peach halves also poses a problem for mechanical removal of the alkaline peel material. There is an indentation at the stem end of the peach and also a slight crease corresponding to the edge of the pit. In addition to removing peel from these difficult areas, the peeler must be capable of manipulating the peach halves.

The peeler described by Graham et al. (1969b) for dry caustic peeling of potatoes is capable of removing peel from all surfaces but is too abrasive for peaches and cannot manipulate fruit halves. Tests using steam or air to blow loosened peel off the peach halves were successful, but the cost of this method is prohibitive. A peeler with two differential speed belts handled the peach halves well but did not satisfactorily remove peel from the creases and indentations.

The successful peeler developed (Fig. 1) consists of banks of soft rubber disks. In operation, peach halves are dropped into the upper end of the peeler. The disks are spun, gently wiping the fruit clean of peel material as the halves tumble down the incline. In this experimental model, peel material, after being flung from the disks, was merely collected in pans underneath the disks. Provision for continuous peel removal could be incorporated in production equipment. Upon exit from the peeler, peach halves were rinsed to remove any residual lye or loose material.

The diagram of the peeler (Fig. 2) shows disks made from 1/32-in. thick rubber sheet cut into 4-1/2-in. dia circles. On either side of each disk is a thin metal stiffener, 2-3/4 in. dia and 1/16-in. thick. Disks are spaced 3/4 in. apart and mounted so the disks on one shaft will extend between the disks on the next shaft. Shafts are mounted 3 in. apart. Rotation of the disks is clockwise and counter to the downward flow of the fruit. Rotational speed of the disks and incline of the peeler are adjustable. Spaced over every other shaft is a flexible rubber apron which acts as a brake to prevent extra large or heavy fruit from spurting through the peeler without sufficient wiping. Overall the peeler is 4-in. wide and 4-ft long.

In Figure 3 the braking aprons are removed to present an unobstructed picture of the disks. As fruit impinges on the rubber disks, the rubber above the stiffeners flexes and deforms to wipe all peel surfaces. In order to minimize abrasion and obtain enough flexibility in the disks, a smooth rubber was chosen with a Shore A hardness durometer reading of 50. The disks are kept self-cleaning by rotating them at a speed sufficient to throw off peel.

The stiffeners, mounted on both sides of each disk, are important because they keep the disks aligned and support the fruit as it rides down the peeler. Without the stiffeners the peach halves would simply push the rubber disks aside and fall through the peeler.

Spacing between disks and between rows is dictated by the size of fruit to be peeled; no gap may be large enough to allow any fruit to fall through. The disks overlap each other to prevent a deep valley between each row of disks where small fruit might hang up and to provide ample traction in conveying fruit onto the next valley.

Residence time in the peeler can be controlled by a combination of disk speed and peeler incline. Usual operation of the peeler was at a disk speed of 60 rpm and an incline of 45° to achieve an average residence time of approximately 20 sec. However, if desired, the peeler could be operated horizontally, combining peeling and conveying in a single step.

Fruit selection

In order to achieve representative samples in tests of the peeler, cannery fruit stocks were used. An effort was also made to simulate the usual cannery peeling procedure.

Cling peaches. Peaches were washed, halved, and pitted. Sample weights were noted for determination of peeling loss. Halves were placed face down on a stainless steel mesh belt and 3% boiling lye solution poured over the halves for specified times between 5 and 10 sec. Although normally used in dry caustic peeling at this point, infrared heating was not used on peaches. After a short draining period the halves were placed in the disk peeler. Weights were noted following exit of the peaches from the peeler and again after subsequent washing.

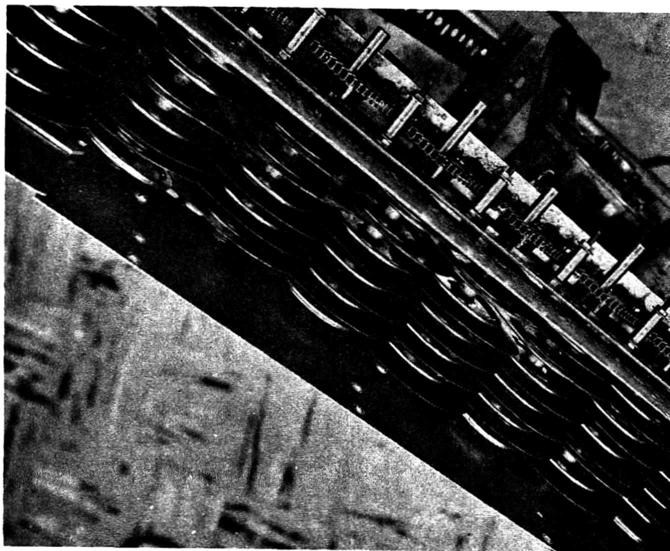

Fig. 3–Disk arrangement in experimental peeler with braking aprons removed.

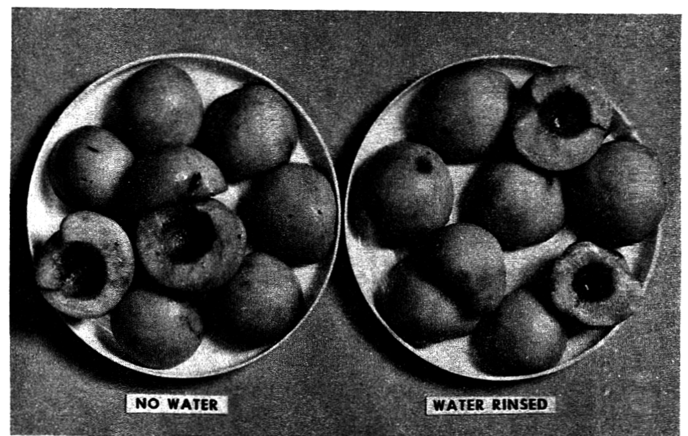

Fig. 4–Samples of cling peach halves peeled by experimental peeler, before and after water rinsing.

For comparison purposes the same conditions were repeated, substituting a water wash for the disk peeler.

Bartlett pears. Mature pears were dipped in a 20% lye solution maintained at 210° to 215°F, for 20–45 sec and drained. The pears were then exposed to a brief infrared heat treatment or placed directly in the peeler. Weights were noted for calculating peeling losses.

Since usual steam treatment of immature pears could not be simulated in the laboratory, dewaxing was tried instead. Harrington and Hills (1964) extensively investigaged dewaxing of various fruits, especially apples, prior to lye peeling and suggested that dewaxing would be beneficial for any fruit possessing a waxy skin.

Two alternate methods of dewaxing were used. In one, whole pears, including stems, were immersed for 30–60 sec in a bath of isopropyl alcohol maintained at 160°F. In the other, vapors of isopropyl alcohol were condensed on pears for 2–4 min over a bath of boiling isopropyl alcohol. Immediately after dewaxing, each pear was drained briefly and then immersed in a 20% lye solution maintained at 210° to 215°F After specified times the pears were removed, drained, and placed in the disk peeler. A comparison was made with pears receiving no alcohol dip prior to the lye dip.

RESULTS & DISCUSSION

RESULTS INDICATE the peeler will completely peel cling peach halves with losses comparable to those obtained by water washing. Peeling loss will vary with lye treatment and maturity of the peach. However, by cascading lye for 5 sec over peach halves before placing them in the peeler, losses as low as 4 to 5% were obtained with complete peel removal. Peaches receiving the same lye treatment followed by a water wash sustained similar peeling losses.

As shown by Figure 4, the peeler gently peels the halves without damage or marks and leaves little residue after they pass through the peeler. Any remaining residue consists of peel material already wiped loose from the surface but not flung off by the disks and is easily removed by a brief water rinse. Difference in peeling loss before and after rinsing varied from 0 to 0.1%.

While the peeler was designed primarily for cling peaches a number of tests were performed on whole Bartlett pears. In tests on both mature and immature pears, the infrared heat treatment partially cooked the pears, yet failed to aid in peeling and was thus of no value. Pears not treated with infrared exhibited less abrasion of the soft flesh underneath the skin.

With a lye-dip time of 20 sec, peeling losses for mature pears varied from 14–20%, depending upon the degree of maturity of the pear. Pears were completely peeled except for a small area at the base which was removed during coring.

In mature pears some scoring of the soft flesh was done by the edge of the disks. This was eliminated by attaching small rubber tubing to the perimeter of the disks.

Peeling of immature pears was easily accomplished after dewaxing. Both immersion and vapor condensation methods of wax removal worked equally well. Treatment times of 1 min immersion or 3 min vapor condensation followed by a lye dip of 25 sec gave good results. Peeling losses varied from 9–13% under these conditions. To obtain comparable results, untreated pears had to be immersed in a lye bath for 75 sec, resulting in greater heat softening of the pear. Because of the firmness of these immature pears no problem of surface scoring occurred.

Whole freestone peaches and apricots were also successfully peeled in single exploratory tests.

During the 1970 fruit canning season extensive tests will be made on a larger peeler measuring 12-in. wide by 10-ft long. This peeler will be set up in the Richmond cannery of California Canners and Growers for a direct comparison with existing processing and to obtain pilot scale data on peeler capacity. The primary investigation will be on cling peach peeling. However, fruits such as apricots, pears, and other available crops will be tried. Several design variations will also be evaluated.

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Ms. received 5/5/70; revised 5/21/70; accepted 6/16/70.

Presented at the 30th Annual Meeting of the Institute of Food Technologists in San Francisco.

Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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BACTERIOLOGICAL ANALYSIS OF FROZEN SHRIMPS. 1. Total Plate Count—Coliforms and Enterococci in Precooked Frozen Chilean Shrimp

SUMMARY—392 samples of precooked frozen shrimps from 2 Chilean industries (A and B) were analyzed for total bacterial count, coliforms and enterococci throughout a period of 8 months. 1-lb samples of breaded shrimp were received directly from the manufacturers after a freezing period of 10 days at −18°C. Total bacterial count ranged from 10^4 to 10^5 organisms per g. Coliforms were absent in 65% of the samples from A, and in 40% of those from B; 89.6% of the samples from A and 50.1% of those from B were bacteriologically acceptable considering a limit of not more than 50 coliforms per g. 98% of the frozen shrimp samples belonging to A contained enterococci, as did 66% of the samples from B. Smaller percentages (17% for A and 54% for B) of acceptable samples are obtained from both industries when 100 enterococci per g is considered as the limit. During the period of observation some sanitary measures were adopted and subsequent coliform counts improved. In plant A working conditions are better and the understanding of bacteriological grounds for the proper handling of food materials has led to the elaboration of a product of consistently better quality. Enterococci counts are in contradiction with coliform counts, since the low-level coliform samples are rejected on the basis of their enterococcal content. In plant B there is a better correlation between coliform and enterococcal counts. Though not investigated, this may be related to the precooking system employed: steaming in an enclosed conveyor in A versus immersion in boiling sea-water in B.

INTRODUCTION

THE FROZEN shrimp industry of Chile has grown in recent years and shrimp now represents an important export item. The 3 common species processed are: yellow shrimp *(Cervimnunida johni)*, carrot shrimp *(Pleurocondes monodon)* and nylon shrimp *(Heterocarpus reedi)* (Hancock and Henríquez, 1968). They are processed and exported as peeled, breaded, precooked and frozen.

Establishment of international bacteriological specifications for this product requires controlled processing under sanitary conditions (Tressler and Clifford, 1957). To control the process and the quality of the final product, our department agreed to assist 2 industries, Exporters A and B, to improve the sanitary aspects of the processing line and to perform periodical bacteriological analyses.

It must be emphasized that the peeling, cleaning and packaging of shrimp tails is performed entirely by hand, since available automatic equipment cannot handle Chilean shrimp because of their size. Consequently, the risk of contamination is greater than in fully mechanized processing plants. Results of this bacteriological control and the changes observed after sanitary recommendations were put into practice are reported.

MATERIALS & METHODS

100 g FROZEN shrimp samples thawed at room temperature were homogenized in 200 ml of peptonized saline solution at pH 7 (Mossel and Quevedo, 1967) in a sterile food blender. Dilutions were made to inoculate the different media (Hartman and Huntberger, 1961).

Total plate counts were obtained after 72 hr of incubation at 35°C in Difco glucose peptonized skim milk agar (Hartman and Huntberger, 1961; Mossel and Quevedo, 1967).

Coliforms were counted after 24 hr of incubation at 37°C in Difco desoxycholate lactose agar (Raj and Liston, 1961a; Silverman et al., 1964). Representative suspected colonies equivalent to $\sqrt{n}$, where n is the number of colonies (not less than 3) were confirmed by making the IMViC test. At the same time transfers to brilliant green lactose broth and to a tryptonized broth for the MacKenzie, Taylor and Gilbert test were made, incubating them at 44°C for 48 hr (Mossel and Quevedo, 1967).

Enterococci plate counts on 176 samples were obtained by inoculating Packer medium (Packer, 1943). The violet-colored colonies were counted after 72 hr of incubation at 37°C. The typical colonies were then identified by inoculating them in these broths: 4% bile, 1% sorbitol, 6.5% NaCl and pH 9.6. The catalase test and the thermal test, heating for 10 min at 60°C, also were performed (Raj et al., 1961b).

RESULTS

TOTAL plate counts of the samples from both manufacturers (shown in Table 1) gave less than 10^5 organisms per g, and the highest distribution frequency was between 10^3 and 10^4 organisms per g. Coliform counts are shown in Table 2.

3 months after the work was started, enterococci counts were done on all the samples from both manufacturers. Results of this counting are shown in Table 3.

DISCUSSION

BACTERIOLOGICAL specifications for imported precooked frozen shrimp from different countries are not uniform; even in the same country, several different

Table 1—Total plate count distribution of 392 precooked frozen shrimp samples.

Number of organisms per g	Manufacturer A		Manufacturer B	
	No. of samples	%	No. of samples	%
1–100	3	1.2	12	8.5
101–1000	60	23.9	27	19.2
1001–10.000	162	64.5	89	63.1
10.001–100.000	26	10.4	13	9.2
Totals	251	100	141	100

Table 2—Coliform count distribution of 392 precooked frozen shrimp samples.

Number of organisms per g	Manufacturer A		Manufacturer B	
	No. of samples	%	No. of samples	%
0	163	65	40	28.4
1–10	25	10	12	8.5
11–50	37	14.5	19	13.5
51–100	5	2	6	4.2
>100[1]	21	8.4	64	45.4
Totals	251	100	141	100

[1] None of the samples reached counts of over 2×10^4 organisms per g.

Manufacturer A–Identification of the colonies gave: 11.4% *Escherichia coli*, the rest being other coliforms.

Manufacturer B–35.6% Escherichia coli, the rest being other coliforms.

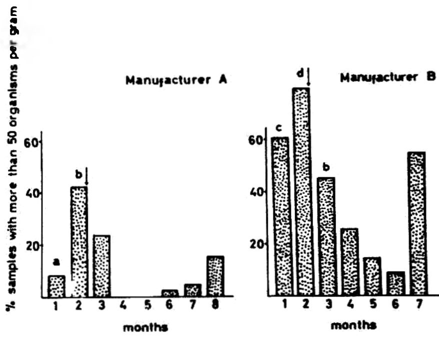

Fig. 1–Relation between enforced sanitary measures and coliform counts in Industries A and B. a) 30 ppm Cl_2 in potable water used normally before starting study; b) 50 ppm Cl_2 in potable water and other sanitary measures (see text); c) sea water 500 m off-shore used normally before starting study; d) chloramine T, 20 g/liter.

Table 3–Enterococci count distribution of 176 precooked frozen shrimp samples.

Number of organisms per gram	Manufacturer A No. of samples	%	Manufacturer B No. of samples	%
0	2	1.6	18	34.0
1–100	19	15.5	11	20.7
101–1000	85	69.1	24	45.3
> 1000[1]	17	13.8	0	0
Totals	123	100	53	100

[1] None of the samples contained more than 5×10^3 organisms per g.

Manufacturer A–Further identification of the colonies gave: 62% *Streptococcus faecalis*, 21.4% *S. faecium* and 16.6% *S. durans*.

Manufacturer B–62.9% *Streptococcus faecalis*, 28.6% *S. faecium* and 8.5% *S. durans*.

Table 4–Bacteriological specifications and standards for precooked frozen foods.

Source	Total plate counts No. per g	Coliforms per g	*E. coli* per g	Enterococci per g	*S. aureus* coagulase per g	+ Salmonella
International Committee on Microbiological Specifications for Foods (I.C.M.S.F.)	10^5	20	–	–	100	–
United States FDA 1967	$<10^6$	<50	<3.6 MPN	–	–	–
England, London. 1963	10^5	100	–	–	100	–
Medical Officer of Health England, Liverpool	$<10^6$	–	<100	–	0	0 in 50 g
Canada	2.5×10^5	–	20/100 g	–	–	–
India	2×10^5	–	0	100	–	–
Codex Alimentario FAO/OMS	2.5×10^5	–	<250 MPN	–	<100	–

standards or specifications may exist. Table 4 shows some of these standards.

The specifications refer to total plate counts, the acceptable limit being 10^5 to 10^6 organisms per g (Thatcher, 1963). All the samples tested were within these limits, most of them containing less than 10^4 per g, and none of them over 10^5 per g. However, when coliform counts were made nearly 90% of the samples from A and only 50% of those from B met FDA specifications. If the London standard is considered, these percentages become slightly higher. *Escherichia coli* constituted 11.4% of the total coliforms from A, and 35.6% of those from B; these figures show a fairly wide variation in the *E. coli* content of the coliform populations from different sources. In these industries higher coliform counts are coincident with higher relative proportions of *E. coli*, but there is not always a direct correlation between the number of *E. coli* and that of coliforms. If this were the case, *E. coli* counts would be more representative of true fecal pollution (Raj and Liston, 1961a).

It is clear that the samples from Manufacturer B showed higher coliform counts than those from A (Table 2). When these results were related to the sanitary conditions of the manufacturing processes in both industries, the following differences were observed: Manufacturer A used chlorinated (30 ppm Cl_2) potable water for all its requirements, whereas sea water brought from 500 m offshore without adding any chlorine was used by Manufacturer B, and the sanitary conditions required of the food handlers were less stringent. Chlorinated water with 50 ppm of Cl_2 was recommended for sanitizing (Tanner, 1950) all counters, trays, dishes and utensils, and also for disinfecting the hands of the food handlers before starting their work and after any interruption. In addition, use of sanitized clothes and surgeon's masks was compulsory.

Figure 1 shows a striking decrease in the coliform counts of A when these suggestions were put into practice at the end of the second month of testing. From that data on, counts began to descend until reaching negligible values that were then maintained for 4 months. Chloramine T, which does not have a strong chlorine flavor, was preferred by Manufacturer B, but the concentration of 20 g per liter was not sufficient to provide satisfactory sanitation, as shown in Figure 1. When B added 50 ppm of chlorine to water supplies, contamination decreased, but never reached low enough levels.

It may be concluded that the use of chlorine alone by Manufacturer B does not justify disregarding other sanitary rules for handling the materials, and that satisfactory results are obtained only when bacteriological considerations are taken into account. Neglecting this point accounts for the poor results and for the rise in contamination at the end of the period, when supervision was over.

Manufacturer A also showed some increase in contamination towards the end of the study period, but this increase was of restricted value. This probably represents the standard level of contamination acceptable in this industry, since their products are accepted abroad without difficulty.

Another index for fecal contamination, recently introduced by the Indian specifications, consists in the determination of enterococci, and allows a maximum of 100 organisms per g (Table 4). If this standard is taken into account, 54.1% of the samples from B, but only 17.1% of those from A, would fall within its limits (Table 3). This means that samples from B have similar acceptability from the point of view of their contamination with coliforms or enterococci, but those from A, showing a far better coliform count, would be unacceptable according to their enterococcal index.

Several authors (Larkin et al., 1956; Zaborowski et al., 1958; Buttiaux, 1959; Raj et al., 1961b; Silverman et al., 1964) have emphasized the value of enterococci as an index of fecal contamination. They

have found that these microorganisms are generally present in frozen foods and that they persist much longer than coliforms under prolonged storage at freezer temperatures. This, however, is not a valid explanation for samples from A since, though maintained at −18°C, the period of freezing did not exceed 2 weeks. Since all of the samples contained over 100 enterococci per g, it is clear that despite the absence of coliforms, these microorganisms are nearly always present. Preliminary experiments on fresh unprocessed shrimps kept apart from the raw shrimps on board showed that they did not contain enterococci, proving that enterococci come from environmental contamination. Since the sanitary conditions in the processing line were maintained, we concluded that the origin of enterococci was on the fishing ship and that they were less affected by the sanitary measures employed during the industrial process (Tressler and Clifford, 1957). This was probably due to their resistance to any unfavorable conditions, even chlorination (Buttiaux and Mossel, 1961).

Comparing the acceptable counts of coliforms and enterococci in plant B, most of the samples are coincident; only about 12% showed a clear difference; of these, about 3 times as many showed higher enterococcal counts than coliform counts. In this case enterococci as well as coliforms are recontaminants of the products during the subsequent manufacturing process after the thermal treatment (Larkin et al., 1956; Niven, 1963). This conclusion is reached because the precooking procedure is different in both industries. While Manufacturer A employs a continuous steaming system, plant B dips the product in a boiling sea-water bath. If steaming is not effective in killing the whole population of enterococci (Deibel, 1964), this may be the explanation for the discrepancy in the results from both plants.

Enterococcal count is a useful fecal contamination index in precooked frozen foods when employed in conjunction with the coliform index. The Indian standard of 100 microorganisms per g is probably too low for such an ubiquitous bacterium with any clear relationship to food poisoning (Deibel, 1964), and the establishment of a higher acceptable numerical limit should be considered if enterococci are to be used as an index for precooked frozen shrimp.

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Ms. received 2/17/69; revised 11/12/69; accepted 6/18/70.

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BACTERIOLOGICAL ANALYSES OF FROZEN SHRIMP.
2. Staphylococci in Precooked Frozen Chilean Shrimp

SUMMARY—392 samples of precooked frozen shrimp from two Chilean manufacturers, A and B, were quantitatively examined for the presence of Staphylococcus aureus *by direct plating on Difco mannitol salt agar (MSA, 10% NaCl). 140 samples (35.7%) were found to contain Staphylococci but only half of these had counts of over 100, 82.4% remaining within the acceptable limit. Frozen shrimps are prone to contamination by Staphylococcus during processing, especially if hand-processed, but these results show that it is possible to obtain a good-quality product when stringent sanitary measures are observed. MSA was compared with Baird Parker's egg yolk medium (BPM) by plating simultaneously on it 141 samples from manufacturer B; BPM detected* S. aureus *in a smaller number of samples (7.8%), and gave rise to fewer colonies than MSA. BPM seemed to be inhibitory even to some Staphylococcus strains, i.e., it is unsuitable for use in these frozen foods. From 2 other manufacturers, C and D, 80 samples of frozen shrimp, together with 60 nasal swab samples from food handlers were plated to investigate some cultural characteristics of* S. aureus. *57 strains of this organism were obtained, 41 belonging to shrimp samples and 16 to nasal carriers from both manufacturers. The strains were isolated and, when tested by anaerobic fermentation of mannitol, deep growth in cysteine agar, catalase and coagulase reactions, all gave positive tests. Phosphatase and DNase reactions were less constant. All the strains with 1 exception were sensitive to 8 antibiotics tested. Thus, the general properties ascribed to* S. aureus *species appear unaltered in frozen shrimps. 28 strains (49.1%) could be typified with the set of 21 international phages, most of them belonging to group III; only in manufacturer D strains coming from food and food handlers were phage type related. In manufacturer C most strains were untypable.*

INTRODUCTION

IF LARGE NUMBERS of coagulase-positive Staphylococci are allowed to grow in a food, they constitute a frequent cause of food intoxication (Nikodemusz, 1967; Thatcher and Clark, 1968). Public Health Services have emphasized the importance of this microorganism in relation to human intoxication and, in the case of frozen foods, have set precise bacteriological specifications that accept a limited number of these cocci per gram (Thatcher, 1963). As Staphylococcus is a widely distributed microorganism that may infect foods in a variety of easily conceivable ways, food industries must be aware of its role as an agent of spoilage and must take care to avoid those factors involved in the processing, handling and storage of foods that lead to contamination (Tanner and Tanner, 1953).

In our country, the process of deveining and peeling shrimps is done by hand. There are no available machines that fit the size of Chilean shrimps. This fact badly handicaps the industry, if it is considered that contamination is generally related to human contact and that there is a high incidence of nasal carriers among food handlers (Elliot, 1963; Williams, 1963). These facts made necessary an enhanced adherence to work conditions that would minimize the possibility of bacterial contamination. An agreement was reached with 2 factories to advise them on some sanitary measures, which were then put into practice under the supervision of this department. The sanitary measures and the samples obtained were the same as described previously (Virgilio et al., 1969).

This paper deals with the counting of *Staphylococcus aureus* in precooked frozen shrimps in these 2 factories during an 8-month period of supervision. In 2 other factories the study was directed to the physiological properties of *S. aureus*, so the strains isolated from the food samples, together with those obtained from the respective food handlers, were subjected to several biological tests.

MATERIALS & METHODS

Samples

392 frozen shrimp samples from 2 industries, A and B, were subjected to Staphylococcus countings. 80 frozen shrimp samples of industries C and D were plated to investigate *S. aureus*; 60 nasal swabs taken from the food handlers at factories C and D were soaked in 2 ml of brain heart infusion (Difco) and carried to the laboratory, where they were plated. All the isolated strains from industries C and D were tested for several biological properties.

Counting methods

Samples. 100 g of frozen shrimp samples thawed at room temperature were homogenized in 200 ml of peptonized saline solution at pH 7 in a sterile food blender. The tests were made inoculating 0.2 ml of the homogenate on Difco mannitol salt agar modified to contain 10% NaCl, and the same quantity on Baird-Parker's (1962) medium, but the latter was used only in 141 samples of manufacturer B. The colonies with the characteristic appearance of *S. aureus* obtained in both media were counted after 48 hr of incubation at 37°C. A representative number of the typical colonies equivalent to $\sqrt{n}$, where n is the number of colonies, but in no case less than 3, were further identified by the coagulase reaction.

Biochemical reactions

Coagulase-positive strains obtained from C and D samples were plated again on nutrient agar; the purified colonies were checked by a gram stain, and stored in agar slants at 4°C until the following tests were performed:

Coagulase reaction. To 0.5 ml of Difco brain heart infusion culture incubated 18–24 hr at 37°C is added 0.5 ml of Difco reconstituted plasma and the mixture incubated for 24 hr at 37°C. The reaction was observed intermittently during and at the end of the incubation period. As a control test, a tube with the same plasma solution and broth without bacteria was incubated at the same time.

Anaerobic fermentation of mannitol. This reaction was carried out in a solid medium (Mossel, 1962) with 1% mannitol and bromocresol purple as indicator. A reaction was considered positive when a deep growth and a yellow color were observed throughout the medium after 72 hr of incubation at 37°C.

Respiratory type. The strains were inoculated deeply in 0.05% of cysteine agar (Mossel and Quevedo, 1967). The tubes were observed after 24–48 hr of incubation at 37°C, a reaction being considered anaerobic when deep growth was clearly evident.

Phosphatase reaction. This was carried out in brain heart agar (Difco) with 0.01% of phenolphthalein diphosphate (Barber and Kuper, 1951). A streak of a young culture (12–18 hr) was inoculated on the medium's surface and, after incubation for 18 hr at 37°C, the plate was exposed to ammonia vapors. Phosphatase-positive colonies turned brilliant red in color.

DNase reaction. This was performed by inoculating a streak of 12–18-hr culture on DNase agar (Difco). After 18 hr of incubation at 37°C the reaction was developed with the following reagents:

N/1 HCl. The DNase-positive colonies depolymerize DNA. When N/1 HCl is added clear zones appear around the colonies.

Toluidine aqueous solution. A positive reaction changes the color of the toluidine blue solution in contact with the colonies to violet pink after a few seconds (Buttiaux et al., 1966).

Catalase reaction. A bit of a colony of *S. aureus* taken with the tip of a Pasteur pipet was added to a small amount of 3% hydrogen peroxide. Gas bubbles evolved by the culture indicated the presence of catalase.

Antibiotic sensitivity test. Commercially available 7-mm-diameter disks of the following antibiotics were employed: Penicillin G (10 μg), ampicillin (25 μg), methicillin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), cephalloridin (15 μg), novobiocin (30 μg) and ryfamycin (30 μg). 0.5 ml of 1/200 dilution of an 18–24 hr broth culture of Staphylococci was added to 20 ml of molten nutrient agar at

Table 1–Staphylococcus aureus count distribution of 392 precooked frozen shrimp samples.

No. of organisms per gram	Manufacturer A MSA[1] No. of samples	%	Manufacturer B MSA No. of samples	%	BPM[2] No. of samples	%
0	167	66.5	85	60.3	96	68.1
1 – 100	45	18	26	18.4	36	25.5
> 100[3]	39	15.5	30	21.3	9	6.4
Totals	251		141		141	

[1] = Mannitol salt agar.
[2] = Baird-Parker's medium.
[3] = None of the samples reached counts over 3000 organisms per gram.

Table 2–Origin and biochemical reactions of 57 strains of Staphylococcus aureus isolated from frozen shrimps and food handlers.

Origin	No. of strains	Anaerobic f. of mannitol	Cysteine medium	Catalase	Coagulase	Phosphatase	D-nase
Manufacturer C							
Shrimps	10	+	+	+	+	+	+
	1	+	+	+	+	–	+
	6	+	+	+	+	–	–
	1	+	+	+	+[1]	+	+
	6	+	+	+	+[1]	–	–
Food handlers	8	+	+	+	+	+	+
Manufacturer D							
Shrimps	17	+	+	+	+	+	+
Food handlers	7	+	+	+	+	+	+
	1	+	+	+	+	–	+

[1] = Slow positive reaction after 24 hr of incubation at 37°C.

45°C, thoroughly mixed and poured into sterile petri dishes. After the medium had solidified the disks were carefully put in place and incubated for 24 hr at 37°C. The diameter of the growth inhibition "halos" was measured in millimeters.

Phage typing. The following 21 international phages and their corresponding propagating strains, obtained from the Central Public Health Laboratory, Collindale, London, were used:

Group	Phage types
I	52,52A,79,80
II	3A,3B,3C,55,71
III	6,7,42E,47,53,54,73,75,77
IV	42D
Miscellaneous	81,187

The propagation phase and the titration test were done according to the description of Blair and Williams, 1961. Bacto nutrient broth (Difco) and trypticase soy agar (BBL) were chosen as liquid and solid media, respectively.

The routine test dilution values (RTD) were determined for each phage, with their corresponding propagating strains. The phage titers varied from 10^3 to 10^6 lytic doses per ml. All the strains were inoculated on nutrient agar slants and incubated for 18–24 hr at 37°C. A transfer of this culture to a nutrient broth (Difco) was made and incubated for 12–18 hr at 37°C. A transfer was then made from this latter culture to a new nutrient broth, and after 2–3 hr of incubation at 37°C this served to inoculate the surface of trypticase soy agar plates. The plates to be used in the test were prepared 48 hr earlier to allow them to dry well at 30°C and to prove their sterility. They were then inoculated with a sterile cotton swab dipped in the broth cultures and allowed to dry 1 hr at 30°C. A small drop of each phage corresponding to a RTD was added on each of the squares marked on the plates. Addition of the RTD of the phages was done with a fine-tipped capillary pipet from a distance of 1 cm without touching the agar surface and, after leaving them undisturbed for 1 hr until they were dry, the plates were incubated for 24 hr at 30°C. Only a few strains showed lysis with 1 RTD. Therefore, the same procedure was carried out again, using the phages at 1000 RTD.

RESULTS

S. AUREUS counts in 251 shrimp samples from manufacturer A and 141 from B, these latter in 2 selective media, are shown in Table 1.

57 strains of *S. aureus* were obtained from manufacturers C and D, 41 belonging to shrimp samples and 16 to nasal carriers. After isolation, all these strains were tested by catalase, coagulase, phosphatase and DNase reactions, by anaerobic fermentation of mannitol and respiratory type in cysteine agar. Furthermore, all the strains were subjected to an antibiotic sensitivity test and to phage typing. Results of the biochemical tests are shown in Table 2.

Nearly 100% of the strains were sensitive to the eight antibiotics tested. Only 1 strain was resistant to tetracycline, 2 others were little sensitive to ampicillin or chloramphenicol and 18 little sensitive to penicillin. Results are shown in Table 3.

From the 57 strains of *S. aureus* subjected to phage typing only 28 (49%) were sensitive to 1 or more phages, the remaining 29 being classified as "untypable." When the test was done with 1 RTD, only 4 strains from shrimp samples of manufacturer D could be typed. Therefore, all the strains were tested again with 1000 RTD. Phages 55, 71, 47 and 54 did not lyse any one of the strains. The phage typing results are shown in Table 4.

DISCUSSION

BACTERIOLOGICAL specifications for precooked frozen shrimp accept a limited number of *S. aureus* (100 microorganisms/g) (Thatcher, 1963), probably considering that low counts of this microorganism in frozen foods are not dangerous and that its total exclusion is rather difficult to achieve (Elliot, 1963).

The implantation of some simple sanitary measures (Virgilio et al., 1969) seemed to be successful in keeping the number of Staphylococci low, and is reflected in the high percentages (82.4%) of samples within the acceptable limit (Table 1).

In plant A, with a better understanding of bacteriological problems, an attempt was made to lower the probability of contamination by carriers that in a previous study (unpublished results) had been shown to attain an incidence of 42%. A topical nasal application of neomycin drops was given to all the personnel (about 250 workers) during 1 week and a medical checkup of the food handlers was performed to remove or treat those presenting signs of upper respiratory illness. For nearly 2 months after this, none of the samples reached the critical level. A rise was subsequently observed, but remained below the original count (Fig. 1).

A reduction in the number of *S. aureus* obtained from the nose of nasal carriers after local application of penicillin was reported by Moss et al., 1948. We assume that a similar effect through the action of neomycin would explain the lower counts obtained after this treatment.

When samples from manufacturer B were plated simultaneously on MSA and BPM the number of colonies in the latter was markedly reduced. The number of

Table 3—Antibiotic sensitivity test of 57 strains of Staphylococcus aureus isolated from frozen shrimps and food handlers.

No. of strains	Pe	Am	Me	Te	Chl.	Ce	No.	Ry
36	S	S	S	S	S	S	S	S
18	LS	S	S	S	S	S	S	S
1	S	LS	S	S	S	S	S	S
1	S	S	S	S	LS	S	S	S
1	S	S	S	R	S	S	S	S

Pe = penicillin G, Me = methicillin, CHl = chloramphenicol, Am = ampicillin, Te = tetracycline, Ce = cephalloridin, Ry = Ryfamicin, S = sensitive (over 18 mm "halo"), LS = little sensitive (less than 18 mm "halo"), R = resistant.

Table 4—Phage typing test with 1000 RTD performed on 57 strains of Staphylococcus aureus isolated from frozen shrimps and food handlers.

Origin	No. of strains Typed	Untyped	Phage scheme*	Group
Manufacturer C				
Shrimps	6		75/6	III
	1		75	III
	1		52+/42E+/42D+	I,III,IV
		16	Untypable	
Food handlers	1		52A/52/79/80/81	I,M
		7	Untypable	
Manufacturer D			79/42E/42D+	I,III,IV
Shrimps	3		77/6+	III
	2		77	III
	1		77/75/42E	III
	1			
	1		77/79	I,III
	1		79/75/73+	I,III
	1		6/79+/75+	III,I
	1		3A/3C/3B/7+	II, III
	1		3A/3C/3B	II
	1		187	M
		4	Untypable	
Food handlers	2		75/53/77	III
	1		77	III
	1		79/42E/77	I, III
	1		3A/52+	II, I
	1		187	M
		2	Untypable	
Totals	28	29		

*Lysis was considered positive when confluent, semiconfluent lysis or more than 50 lysis plaques were observed. Weak reactions, 20–50 lysis plaques are marked with +. Phages are listed in decreasing order of reaction intensity or groups I, II, III, IV, Miscellaneous.

samples with high Staphylococcal counts dropped off at the same time that an increase was observed in the samples with lower counts. In this way the samples with no detectable Staphylococcus were 7.8% higher, and the over-all count with less than 100 organisms per gram increased from 78.7% in MSA to 93.6% in BPM.

It seems that BPM is a very selective medium but too inhibiting to be used with frozen shrimps. If the results are influenced by the medium, it is obvious that microbiological standards or international specifications should be specified in detail, not only as to the number of bacteria per gram but also as to the methods and the media employed (Elliot, 1963).

The biochemical properties studied on the strains from manufacturers C and D were practically constant (catalase, coagulase, DNase, phosphatase reaction, deep growth in cysteine agar and anaerobic fermentation of mannitol). Almost all the strains were sensitive to the antibiotics tested; a single strain resistant to tetracycline was isolated from a food handler and this was the only one showing the 80/81 phage scheme commonly found in Staphylococcus isolated from pathological conditions (Baird-Parker, 1962). Thus, the entire set of strains from both food and food handlers retain their biological characteristics, constituting a very homogeneous group. It may be concluded that these properties of the species are not affected by the conditions met with in the new ecological system to which they are transferred.

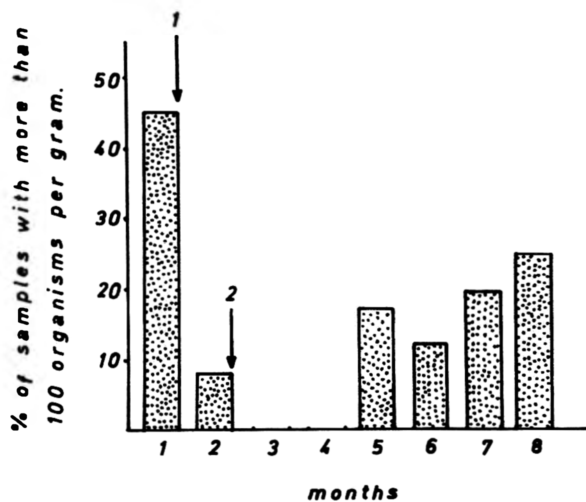

Fig. 1—Relation between sanitary measures and Staphylococcus aureus counts in manufacturer A. 1) Sanitization 2 sanitized mask changes every day. 2) Neomycin applications.

Although the number of typable strains is low, a salient feature is the difference between the 2 manufacturers; in C, the single typable strain from food handlers was lysed by phages 80/81, a sensitivity not present in the strains coming from food; in shrimps, only 8 strains out of 24 were typable, and 7 of these were lysed by group III phages 75 and 6. Thus, 2/3 of the food strains and nearly all of the food handlers' strains were untypable and no definite conclusions can be drawn relative to the source of Staphylococcal contamination in this plant.

In manufacturer D, 13 out of 17 shrimp strains, and 6 out of 8 from food handlers' strains was sensitive to phages in a very similar pattern, group III being predominant; i.e., strains coming from plant D showed an intimate relationship between food and food handlers' strains. On the other hand, in manufacturer C this relationship is lacking unless the

condition of most of the strains being untypable could account for some kind of relation; this would be demonstrated if new phages able to lyse these strains were available (Munch-Petersen, 1963).

Silverman et al. (1961) found differences in the percentages of typable strains coming from different producers. In our case, 1/3 of the strains from manufacturer C were typable, similar to the 35% over-all rate of Silverman et al. (1961). Typable strains from manufacturer D, in contrast, reached about 75%.

Manufacturers C and D are geographically separated, D being a new company located about 600 km south of C, and its workers are recruited from among a healthy community of country people. This probably explains the difference in the strain's susceptibility to this set of phages. In manufacturer C one might speculate about the possibility of certain types of Staphylococcus being spread within the factory personnel and firmly established in the carrier state, precluding, or interfering with, the acquisition of other types, as has been described by Eichenwald et al. (1965).

Since about 82% of the strains typed belong to group III, the group shown to include most of the Staphylococcal strains responsible for food intoxication, this study's results are in agreement with other authors (Silverman et al., 1961; Elliot, 1963; Munch-Petersen, 1963; Wentworth, 1963).

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Ms. received 9/22/69; revised 12/16/69; accepted 4/9/70.

The authors gratefully acknowledge the technical assistance of Dr. Luis Courtin in the phage typing and of Miss Victoria Rodriguez in the isolation of the strains. This work was done with the collaboration of the Instituto de Investigaciones y Ensayos Farmacológicos, Universidad de Chile.

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PREMORTEM STRESS AND POSTMORTEM BIOCHEMICAL CHANGES IN SKIPJACK TUNA AND THEIR RELATION TO QUALITY OF THE CANNED PRODUCT

SUMMARY—Live skipjack were caught, brought to shoreside and held in tanks 24–48 hr. At this time some were exercised to simulate stress during commercial capture, sacrificed, held at 74 or 32°F for 6 hr, sampled for chemical analyses, then canned. Unstressed (rested) skipjack were treated similarly. Some autolytic degradation products post-mortem were measured and the differences noted. Organoleptic evaluation was made on canned fish from the various treatment groups. While there were differences in various organoleptic parameters among the groups subjected to different treatments, there was no overwhelming evidence connecting stress or temperature of holding to quality in the canned product.

INTRODUCTION

PRACTICAL experience in the tuna industry has shown that the quality characteristics of seine-caught tuna are, in general, more variable than those caught by bait boats (i.e., using live bait and poles). In fact, the quality of seine-caught fish is often somewhat lower than that of the latter. It has been observed that the color and texture of the seine-caught fish usually are not rated as highly as similar characteristics of bait-caught fish. One of the reasons for the lower quality of the seine-caught fish has been thought to be related to or dependent on the conditions of glycolysis both pre- and post-mortem. For example, it is known that the water-binding capacity of muscle protein is an important consideration in processing technology and influences the texture of fish. The water-binding capacity, in turn, has been shown to be largely dependent on muscle pH. It also appears that color formation during processing is in part related to the oxidative state of myoglobin and hemoglobin (Barrett et al., 1965) and to the sugar (both free and phosporylated) content of the tissue.

Although a good deal of variation in these factors can be found among fish prior to processing, it has been fairly well established by investigators working with other species of fish that this variation is largely dependent on the physiological state of fish before death and on environmental factors after death (Dyer and Dingle, 1961; Connell, 1964).

In an effort to control product quality, a considerable amount of research has been done in the salmon and cod industry on studies involving development and extension of rigor, and on the effect of postmortem glycolysis and glycolytic intermediates on quality of processed products. Attempts to control product variability in the above industries have been quite successful by using the simple expedient of preconditioning fish before processing.

In this paper, and others to follow, studies on the species skipjack will be reported, since the orderly expansion of the tuna industry requires heavier reliance upon catches of skipjack. It is acknowledged that skipjack more readily undergoes undesirable quality changes than other tunas, but good-quality skipjack can be produced if good handling practices are followed before processing. However, few objective data have been gathered. Little regard is given to the fact that skipjack is a relatively small fish; consequently, when it is put into wells of boats with larger tunas, physical damage can occur leaving the muscle exposed to the brine, thus allowing the possible intrusion of bacteria. This situation can add to oxidative and enzymatic degradation if the temperature in the well remains relatively high for a prolonged period.

Most of the domestic skipjack is purse-seined. When this fish is caught and brought aboard the vessel for chilling and freezing, it is usually dead and in a stressed (highly exercised) condition. Very little is known about rigor formation and the physiological and biochemical changes in skipjack in the stressed or unstressed (rested) condition.

In this paper the following studies were done on skipjack captured alive, then held for 1 or 2 days in tanks at shoreside. Some autolytic degradation products post-mortem were determined and compared, using fish that were either rested or exercised prior to sacrificing, then held in 74 and 32°F seawater for 6 hr. Some fish were also frozen after holding, to note the effect of freezing (whether rigor had formed was observed). All fish were canned after samples of muscle were taken for biochemical evaluation. The organoleptic quality of the canned product was also determined.

MATERIALS & METHODS

Materials

The fish used in this experiment were caught by the Bureau of Commercial Fisheries' *M. V. Charles H. Gilbert* off Honolulu, Hawaii, using live bait and barbless hooks. Only those skipjack in excellent condition were retained and returned alive to Kewalo Basin in tanks developed by the BCF Biological Laboratory (Honolulu). The fish were caught on 2 different trips, yielding 27 and 28 skipjack, respectively, and the catches kept in separate tanks.

We utilized the 27 skipjack from the first catch as follows:

1. Sacrificed (blow on head) 7 rested fish, sampled them for chemical analysis and canned them immediately (controls).
2. Sacrificed 10 rested fish and held them 6 hr in 74°F circulating seawater. Sampled and canned 5 of them The others were frozen to 20°F in circulating brine and then blast frozen to −20°F, stored glazed for 2 days, then thawed in running seawater (3–4 hr), sampled and canned.
3. Exercised 10 fish to simulate stress, sacrificed them and disposed of them as in 2 above.

We used the 28 skipjack from the second catch as follows:

1. Sacrificed 6 rested fish and disposed of them as in 1 above (controls).
2. Sacrificed 10 rested fish and disposed of them as in 2 above, except they were held for 6 hr in 32°F sea water.
3. Exercised 10 fish and disposed of them as in 3 above, except they were held for 6 hr in 32°F sea water.

Sampling

The muscle sampling procedure has been described (Crawford and Finch, 1968). Briefly, a 100-g wedge was removed from the dorsal side of the fish.

Chemical analyses

Immediately after taking a muscle wedge, half of it was frozen in liquid N_2 and stored at −90°C for reference. 20 g of the other half was ground in the cold with 50 ml of 0.6 N $HClO_4$. Half of this extract was stored frozen and the other half neutralized to pH 6.8 with 5 N KOH, then frozen and stored. These samples and extracts were shipped to the BCF Laboratory on Terminal Island, California, for analysis.

Fructose and fructose phosphate were determined on the neutralized extracts by the

Table 1–Results[1] of chemical analysis on raw tissue of exercised and rested skipjack tuna held at 74 and 32°F.

Condition	Glucose (μM/g)	Glucose PO4[2] (μM/g)	Fructose PO4[3] (μM/g)	Total reducing sugars (μM/g)	Pyruvate (μM/g)	Lactate (μM/g)	NAD (mg/g)
			Held in 74°F seawater for 6 hr				
Unfrozen and exercised	7.6	2.8	1.2	11.5	0.60	159	0.17
Frozen and exercised	5.8	1.6	0.8	10.6	0.75	181	0.00
Unfrozen and rested	9.1	2.6	1.2	11.6	0.50	150	0.11
Frozen and rested	6.2	2.9	1.6	19.8	0.60	139	0.00
Controls	7.9	6.9	2.2	24.0	0.65	67	0.26
			Held in 32°F seawater for 6 hr				
Unfrozen and exercised	9.1	3.3	1.1	10.9	0.45	145	0.13
Frozen and exercised	7.2	3.3	0.9	9.5	0.55	151	0.00
Unfrozen and rested	12.5	7.0	2.4	20.6	0.55	153	0.17
Frozen and rested	6.6	6.7	2.5	26.1	0.48	130	0.00
Controls	7.3	6.7	1.5	52.9	0.45	80	0.43

[1] All results are the averages of analyses run in duplicate on 5 individual fish.
[2] Total reducing sugars and glucose PO_4 were calculated as glucose.
[3] Fructose PO_4 was calculated as fructose.

method of Roe (1934). Glucose phosphate was determined with Dreywood's anthrone reagent by the method of Morris (1948) after separation of the neutralized extract on a column of Dowex 1 × 8 (chloride) resin (Spinelli et al., 1964). Total reducing sugars were determined with Dreywood's anthrone reagent on the neutralized extract before resin treatment. Glucose, pyruvate and lactate were determined with kits purchased from Calbiochem of Los Angeles, California. NAD was determined by an assay with alcohol dehydrogenase (Ciotti and Kaplan, 1957) on perchloric acid extracts of muscle neutralized just before assay. Fructose phosphates were calculated as fructose; total reducing sugars and glucose phosphate were calculated as glucose.

Organoleptic evaluation

Taste panel methods and procedures were previously described by Crawford and Finch (1968). A brief description follows:

Scorch. 1–Severe scorch in the headspace area to 5–no scorch in the headspace area.

Color. 1–Very dark red or brown to 10–very light, almost white.

Flakiness. 1–Very flaky to 10–very cohesive.

Firmness. 1–Very soft and mushy to 10–very firm and hard.

Fibers. 1–Very tender to 10–very tough.

Off-odor. 1–Very strong off-odor to 5–no off-odor.

Off-flavor. 1–Strong off-flavor to 5–no off-flavor.

RESULTS & DISCUSSION

THE DESIGNATION frozen refers to fish that were sacrificed, held for 6 hr at 74 or 32°F, frozen in brine to 20°F and then blast frozen to −20°F and stored glazed at this temperature for 2 days before thawing and canning. Unfrozen refers to fish sacrificed, held for 6 hr at 74 or 32°F and then canned. Controls are fish sacrificed, then canned immediately.

As would be expected, exercised fish went into strong rigor within half an hour, whereas the rested fish showed only weak signs of rigor from the time of sacrifice to canning.

Chemical changes

There was no determinable free fructose. Free glucose, glucose phosphate, fructose phosphate and total reducing sugars at 74 and 32°F were lower in exercised skipjack than in rested fish (Table 1). These substances were lower than controls in both exercised and rested fish held at 74°F but not in those held at 32°F. Pyruvate showed no change. Lactate content in fish held at both temperatures was higher in the exercised and rested fish than in their controls.

In many instances, the sugars, sugar phosphates and total reducing sugars were higher in the frozen samples, probably due to glycolysis during sampling and preparation. No real significance is attributed to this.

Levels of NAD were appreciably higher in controls than in any treatment group not frozen. Differences among the latter groups do not appear significant. No NAD was found in any sample from any of the groups that had been frozen. It is not clear whether this is related to the freezing process per se or because these samples were held an additional 48 hr

Table 2–Taste panel scores[1] for exercised and rested skipjack tuna held at 74 and 32°F.

Condition	Scorch	Color	Flakiness	Fibers	Odor	Flavor	Firmness
			Held in 74° seawater for 6 hr				
Unfrozen and exercised	1.4	5.8	5.8	4.0	4.8	2.2	6.0
Frozen and exercised	2.8	4.5	3.8	3.4	3.8	2.8	5.2
Unfrozen and rested	2.0	5.6	5.6	4.2	3.8	2.6	6.0
Frozen and rested	3.0	5.5	5.0	7.3	3.3	2.8	5.0
Controls	3.2	4.8	3.5	5.0	4.3	4.5	4.3
			Held in 32°F seawater for 6 hr				
Unfrozen and exercised	1.3	3.3	6.5	5.3	4.8	2.5	7.5
Frozen and exercised	2.8	5.0	4.2	3.4	4.4	4.0	4.8
Unfrozen and rested	1.8	3.4	7.2	4.8	3.4	2.0	7.2
Frozen and rested	3.8	6.2	4.2	6.0	3.8	3.4	3.8
Controls	3.5	5.0	4.5	6.2	4.5	4.2	5
Commercial pack from the same waters and caught in the same fishing season as the above experimental packs.	3.2	2.9	7.4	6.4	2.1	1.0	6.4

[1] Scores are the average results of 5 cans representing 5 individual fish. In general, the higher scores are more favorable organoleptically. See text for details.

before being extracted. However, a recent report (Atkinson et al., 1969) on the disappearance of NAD in lamb muscle samples held at 0° describes losses of NAD of about 50% of the initial values over a 48-hr period, the initial values being similar to those in our controls. It may be that freezing results in sufficient tissue damage to release enzymes involved in the degradation of NAD. Shimizu et al. (1969) found similar levels of NAD plus NADH in the white muscle of bonito, with the total amount being twice as high in the red muscle (mostly as NADH).

The procedures employed in this study did not allow for the determination of NADH, i.e., it would undergo oxidation in the acid extractant medium. Future studies will utilize means of extraction that ensure the stability of NADH, since NADH may help provide a more stable and desirable color by maintaining the reduced form of myoglobin in muscle (Brown and Snyder, 1969).

Organoleptic changes

All results are the average of 5 cans representing 5 individual fish.

Fish held at 32 and 74°F for 6 hr and frozen showed more severe scorch in the canned product, whether they had been exercised or rested. Poorer color was found in the exercised and rested fish held at 32°F and frozen. Fish receiving all other treatments had good color, better or equal to the controls.

The canned frozen exercised skipjack held at 32 and 74°F, and the rested unfrozen fish held at 32°F showed a tendency to be very flaky (i.e., ready separation of myotomes). The controls were also very flaky. In contrast, the myotomes of the frozen fish from all treatments showed no tendency to separate. All fish tended to be firm with the exception of the controls and the unfrozen exercised and rested fish held at 32°F, which were more or less soft.

The canned rested unfrozen fish held at 74°F were fibrous (chewy). There was very little, if any, off-odor in any sample. Some off-flavor was found in fish from all treatment groups with the exception of the controls and the unfrozen rested and exercised fish held at 32°F.

When comparing the chemical changes to organoleptic changes, some trends were noted but no significant relationships were established. In general, there were no dramatic differences between the rested and exercised fish, regardless of treatment. Commercial packs of tuna from the same waters, caught during the same season as the above experimental packs, were included in the taste panel. Results show that the experimental packs are, in general, of better quality. All of the above results were also arrived at by industry experts in a separate taste-panel session.

CONCLUSION

IT IS somewhat surprising that the quality of the fish held at 32°F did not show marked superiority over the quality of fish held at 74°F. Overall, fish in this experiment were judged by industry experts to be of fairly good commercial quality. They also found these fish to be generally better in quality than the average domestic commercial packs of skipjack. This may indicate that more technological abuse occurs in commercial practice than was involved in this experiment, or that there are factors other than those included in this study that are of importance in determining quality.

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Ms. received 5/11/70; accepted 6/18/70.

We gratefully acknowledge the indispensable help of Richard Shamura and Eugene Nakamura, Bureau of Commercial Fisheries Biological Laboratory, Honolulu, Hawaii; Scotti McCloud and Bill Coleman, Hawaiian Tuna Packers, Honolulu, Hawaii; Fritz Jermain, Bumble Bee Seafoods, Astoria, Oregon; Herman Groninger, Bureau of Commercial Fisheries Technological Laboratory, Seattle, Washington, and Suzanne Green, University of California, Berkeley. That portion of the work done in Berkeley was supported by a grant from the Tuna Research Foundation and a contract with the Department of Agriculture, Commonwealth of Puerto Rico.

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STABILITY OF OIL-IN-WATER EMULSIONS. 1. Effects of Surface Tension, Level of Oil, Viscosity and Type of Meat Protein

SUMMARY–Surface tension responses for solutions of salt-soluble protein from cow meat, beef hearts, beef cheek meat, pork trim and pork cheek meat were found to follow the Type III curves assigned to surface-active agents. The stability of emulsions prepared utilizing salt-soluble protein of the meats was significantly affected by concentration of protein and level of oil. As either concentration of protein or of oil was increased, higher and more significant stability of the emulsions was observed. Emulsions prepared from protein of each type of meat had similar responses for stability. High and significant correlation was found between protein surface activity and emulsion stability. Little change in emulsion viscosity was found except at the upper protein and oil levels tested.

INTRODUCTION

THE EMULSIFYING capacity of muscle protein slurries and extracts have been investigated with model systems by numerous workers (Swift et al., 1961; Hegarty et al., 1963; Carpenter and Saffle, 1964; Christian and Saffle, 1967; Borton et al., 1967). The emulsification procedure in all cases has been the mixing or blending of the protein solution with an initial aliquot of oil, followed by addition of oil until the emulsion collapsed. Variation in rate of shear, rate of fat addition, temperature rise during emulsification and type of fat added have been investigated with basically the same method for emulsification.

The dilution of the meat slurry, the effects of pH, concentration of NaCl, and various anions and the effect of rigor mortis on the emulsifying capacity of the water- and salt-soluble proteins have been studied (Swift and Sulzbacher, 1963; Hegarty et al., 1963; Acton and Saffle, 1969). Carpenter and Saffle (1965) investigated additional chemical and physical factors such as limiting viscosity numbers, shape and charges on the protein molecule and ionic strengths on the emulsifying capacity of meat proteins.

There is a large difference between the emulsifying capacity and the emulsion stabilizing ability produced with meat proteins. Emulsion stability is not a measure of maximum oil addition, but rather the ability to remain durable and unchanged for a serviceable lifetime. At the present time, there is a limited amount of research literature pertaining to the stability of emulsions containing meat proteins. Swift et al. (1961), Hegarty et al. (1963), Carpenter and Saffle (1964), and Borton et al. (1967) have examined emulsions that contained from 70–88% oil for stability by heating, centrifugation and aging. In each report, the emulsions were examined at oil levels slightly below that found for the emulsion collapse point. Saffle's (1968) recent review of meat emulsions has pointed to the need for additional studies in the area of basic emulsion science as applied to meat proteins.

The objectives of this study were to determine the effects of surface tension of solutions, level of oil emulsified and emulsion viscosity on the stability of oil-in-water emulsions containing protein of various types of meat.

EXPERIMENTAL

Source of meat

Fresh cow meat, beef hearts, beef cheek meat, pork trim and pork cheek meat were obtained from a local slaughterhouse. All meats were trimmed free of fat, ground three times through a 3 mm plate, thoroughly mixed after each grinding, packaged in 225g packages, frozen and stored at −25°C. Beef hearts were ground only twice in the above procedure. Packages were thawed at 5°C for 12–16 hr before extracting for salt-soluble protein.

Extraction of salt-soluble protein

A procedure similar to the method of Saffle and Galbreath (1964) was used for extraction of salt-soluble protein from the various meats. 75g of meat and 150 ml 3% NaCl (w/v) solution were placed in a 250 ml beaker and the meat was thoroughly dispersed using a spatula. The pH of the sample was adjusted to pH 6.0 with either 0.1N NaOH or 0.1N HCl solutions. The 0.1N NaOH and HCl solutions were prepared using 3% NaCl solution.

The pH-adjusted meat slurry was placed in an Osterizer blender bowl and sufficient 3% NaCl solution added to make a final volume of 300 ml of 3% NaCl solution. The slurry was blended for 2 min, allowed to stand for 2 min, and blended again for 2 min. The blending speed was 5700 rpm and the temperature range during extraction was maintained at 10–14°C.

The slurry was centrifuged at 12,100 × G for 24 min at 0°C and the supernatant filtered twice through Whatman #4 filter paper. The extract was stored at 5°C until used for subsequent analyses. When needed, several extractions were conducted and the extracts combined and used as a single batch.

Preparation of meat protein extract dilutions

The salt-soluble protein extracts were diluted volumetrically to make solutions of desired protein concentration. Dilutions were

Table 1–Surface tension (dynes/cm) of salt-soluble protein of cow meat at 24, 48 and 72 hr after extraction.[1]

Protein conc mg/ml	Time after extraction		
	24 hr	48 hr	72 hr
0.25	60.06[a]	60.17[a]	61.16[a]
0.50	55.22[b]	55.11[b]	55.16[b]
5.00	52.65[c]	52.05[c]	52.75[c]

[1] Any two treatment means having the same letter are not significantly different at the 5% level of probability.

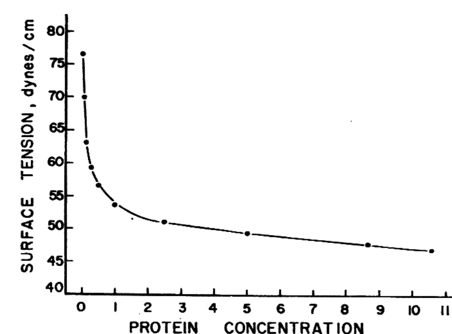

Fig. 1–Surface tension response with solutions of salt-soluble protein from beef cheek meat.

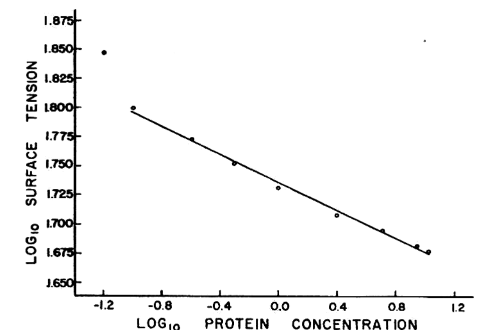

Fig. 2–The linear response from regression analysis of surface tension obtained with salt-soluble protein of beef cheek meat.

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Table 2–Surface tensions (dynes/cm) of salt-soluble protein from extracts of various meats.[1]

Protein conc mg/ml	Type of Meat[2]				
	CM	BH	BC	PT	PC
0.05	70.8[a]	71.1[a]	70.6[a]	73.7[a]	71.9[a]
0.10	61.9[b]	64.5[b]	63.1[b]	70.0[b]	68.3[b]
0.25	60.3[b]	57.6[c]	59.1[c]	62.0[c]	57.5[c]
0.50	54.9[c]	53.5[dc]	56.6[d]	57.2[d]	55.3[d]
1.00	52.8[cd]	54.2[d]	53.9[e]	55.3[e]	53.0[e]
2.50	53.3[cd]	52.1[de]	51.2[f]	51.5[f]	49.0[f]
5.00	52.4[d]	50.9[ef]	49.6[fg]	50.1[fg]	50.2[f]
7.18	–	48.3[f]	–	–	–
8.63	48.0[e]	–	48.1[g]	49.7[g]	–

[1] Any two means within a type of meat having the same letters are not significantly different at the 5% level of probability.

[2] Types of meat coded as follows: CM–cow meat, BH–beef hearts, BC–beef cheek, PT–pork trim, PC–pork cheek.

Table 3–Regression equations and correlation coefficients for surface tension (y, dynes/cm)–protein concentration (x, mg protein/ml) relationships at 0°C.

Type of Meat[1]	Simple Linear Regression Equation	Correlation Coefficient, r
CM	$\log Y = 1.7380 - 0.0505 \log X$	−0.86**
BC	$\log Y = 1.7361 - 0.0606 \log X$	−0.96**
BH	$\log Y = 1.7343 - 0.0561 \log X$	−0.90**
PC	$\log Y = 1.7348 - 0.0691 \log X$	−0.92**
PT	$\log Y = 1.7512 - 0.0734 \log X$	−0.96**

** Highly significant at the 1% level of probability.

[1] See footnote to Table 2 for coding.

prepared within 12–18 hr of extraction using 3% NaCl (w/v) solution and protein extract at a temperature of 5°C. Since the protein concentration (determined from Kjeldahl N) of the diluted extracts showed only slight deviation from the desired concentration, the volumetric error due to temperature was considered negligible.

Total N and nonprotein N were determined following the Kjeldahl method (AOAC, 1965). After subtraction of nonprotein N from the total N, protein concentration was determined using the factor of 6.25.

Surface tension of salt-soluble protein solutions

The capillary rise method (Harkins, 1949; Becher, 1965) was used to determine the surface tension of protein solutions.

Six surface tension measurements were recorded from duplicate protein solutions using three capillaries of 0.0963 ± 0.0003 cm radius. The average of three height recordings from a solution was used with each capillary. The measurements were conducted in an ice-water bath maintained at 0 ± 0.5°C. The concentration of protein in the solutions ranged from approximately 0.05 mg/ml–10 mg/ml.

Density was calculated for each solution from the weight of six 50 ml aliquots in tared volumetric flasks. The solutions were equilibrated at the same temperature used for surface tensions and the volume adjusted at that temperature.

Preparation of oil-in-water solutions

Following extraction of the salt-soluble protein, solutions of 0.10, 0.25, 0.50, 1.00, 2.50, 5.00 and 7.50 mg of protein/ml were prepared. Aliquots of each solution were placed in 100 ml dilution bottles and corn oil at 5°C was added to give a final volume of 50 ml of oil-solution mixture. The amounts of solution and oil were added to attain either 20, 35 or 50% oil by volume.

The mixture was shaken sufficiently to mix the two liquids. This dispersion was immediately placed in a laboratory hand homogenizer and pumped through a 1 mm orifice twice. Microscopic and water-dilution tests showed that with each mixture an oil-in-water emulsion resulted. Homogenization was used for all emulsion samples tested for stability and viscosity.

Emulsion stability test

A stability test which would determine the extent of moisture homogeneity between initial and tested samples of the emulsion was used. The method devised is a modification of that introduced by Titus et al. (1968). The modifications were the measurement of change in moisture rather than oil and the lengthening of the stability testing period from 2 hr to 24 hr.

Two 10 ml samples of each homogenized mixture were placed in 15 × 150 mm test tubes. The two 10 ml samples were held at 5°C for 1–3 hr and then placed in a 37 ± 2°C incubator for 24 hr. Two additional 5 ml aliquots of the emulsion were immediately analyzed for moisture following the AOAC (1965) procedure.

Following the 37°C incubation period, the bottom 5 ml of the tube samples were removed with a 5 ml pipette and analyzed for percent moisture. A stability rating for each sample was determined on the basis of the percent change in moisture. The following equation was used:

$$SR = \frac{100 - M_{test}}{100 - M_{original}} \times 100$$

where SR is the stability rating; M_{test}, the percent moisture of the bottom 5 ml of the sample stored at 37°C for 24 hr; and $M_{original}$ is the initial percent moisture of the sample. Tests for emulsion stability were replicated three times.

Viscometry of oil-in-water emulsions

The viscosity of the emulsions relative to water was determined for single preparations of the oil-in-water emulsions stabilized by salt-soluble protein of cow meat. A Brookfield Synchro-Lectric Viscometer, Model RVF, was used at a shear rate of 20 rpm with the No. 6 spindle. The emulsions were examined immediately after preparation at 4°C.

Statistical analysis

Surface tension results for each meat were analyzed statistically by analysis of variance and the significance of means tested by the least significant difference method following Ostle (1966).

For the emulsions, a randomized complete block design involving a factorial arrangement was selected (Ostle, 1966). Protein concentrations were blocked and oil levels within blocks were random.

RESULTS & DISCUSSION

Surface tension of salt-soluble protein solutions

The surface tension of solutions of salt-soluble protein from cow meat at 24, 48, and 72 hr after extraction are shown in Table 1. No significant changes occurred over this aging period. Thus, it was assumed that equilibrium between surface-denatured molecules and the protein in solution had been attained within the first 24 hr after extraction.

Surface tensions recorded at 0°C for salt-soluble protein of cow meat, beef hearts, beef cheek meat, pork trim and pork cheek meat are given in Table 2. For each type of meat, as the concentration of protein was increased by small increments from 0.05–1.00 mg/ml, the surface tension decreased significantly from that of preceding concentrations except for cow meat and beef hearts. With these two meats, the increase in protein concentration decreased the surface tension value found for the preceding concentration although the change was not always significant. Increasing the protein concentration about 1.00 mg/ml to 7–10 mg/ml showed a slight reduction of surface tension for the solutions.

The large effect of the first and second concentration tested, 0.05 and 0.10 mg protein/ml, is clearly shown by comparing the solvent surface tension to that of 3% NaCl solution. A surface tension of 76.3 dynes/cm at 0°C was found for the 3% NaCl (w/v) solution used as the solvent.

All of the surface tension responses found, irrespective of the type of meat, correspond to the Type III response presented by Becher (1965) for substances classified as surfactants (see Fig. 1). Examination of Figure 1 shows that the surface tension varied with the concentration of protein in a logarithmic response. When the relationship was examined on a log surface tension versus log protein concentration basis, a linear response was evident from the highest

Table 4–Stability ratings of oil-in-water emulsions containing 20% corn oil and stabilized by salt-soluble protein from various meats.[1]

Protein Conc	Type of Meat[2]				
mg/ml	CM	BH	BC	PT	PC
0.10	20.3[a]	13.4[a]	15.1[a]	14.8[a]	14.3[a]
0.25	24.7[ab]	20.6[b]	19.7[ab]	20.7[b]	21.1[bc]
0.50	24.3[ab]	20.9[b]	21.9[bc]	21.5[b]	20.6[b]
1.00	27.1[b]	24.3[bc]	26.1[cd]	25.1[bc]	25.3[cd]
2.50	34.0[c]	27.1[c]	28.0[d]	26.6[c]	29.4[d]
5.00	39.4[d]	33.8[d]	34.1[e]	31.8[d]	36.3[e]
7.50	43.9[d]	37.2[d]	36.8[e]	38.5[e]	35.8[e]

[1] Any two means within a type of meat having one of the same letters are not significantly different at the 5% level of probability.

[2] See footnote to Table 2 for coding.

Table 5–Stability ratings of oil-in-water emulsions containing 35% corn oil and stabilized by salt-soluble protein from various meats.[1]

Protein Conc	Type of Meat[2]				
mg/ml	CM	BH	BC	PT	PC
0.10	11.1[a]	10.3[a]	9.6[a]	9.4[a]	8.9[a]
0.25	21.6[b]	18.2[b]	20.2[b]	22.8[b]	21.7[b]
0.50	29.5[c]	25.5[c]	27.8[c]	24.2[b]	30.3[c]
1.00	46.4[d]	39.5[d]	38.3[d]	34.1[c]	50.7[d]
2.50	70.8[e]	56.5[e]	63.1[e]	61.4[d]	68.2[e]
5.00	76.5[f]	69.7[f]	71.9[f]	68.5[e]	73.2[f]
7.50	82.1[g]	79.9[g]	78.6[g]	80.2[f]	83.9[g]

[1] Any two means within a type of meat having one of the same letters are not significantly different at the 5% level of probability.

[2] See footnote to Table 2 for coding.

protein concentration to 0.10 mg of protein/ml. Figure 2 shows this relationship for beef cheek meat protein. Prediction equations using regression analysis and correlation coefficients are given in Table 3.

Effect of protein concentration on emulsion stability

Stability ratings of the oil-in-water emultions prepared at 20, 35 and 50% levels are given in Tables 4, 5 and 6. In general, there appears to be little difference in the stability responses obtained between the meats.

For each type of protein extract, stability ratings at the 20% level (Table 4) always increased as the protein concentration increased from 0.10 to 5.0 mg/ml. The increase in stability was not always significantly greater between each increment change of protein concentration, but was significantly greater than some of the previous concentrations.

Stability ratings of emulsions containing 35% oil (Table 5) were significantly greater with each increase of the protein concentration from 0.10 to 7.50 mg/ml. At the 50% oil level (Table 6), the emulsion stability also increased significantly with each increment change of protein concentration up to 5.00 mg/ml. In every case, there was no significant difference between stability ratings in 50% oil-emulsions at 5.0 and 7.50 mg/ml protein levels.

Table 6–Stability ratings of oil-in-water emulsions containing 50% corn oil and stabilized by salt-soluble protein from various meats.[1]

Protein Conc	Type of Meat[2]				
mg/ml	CM	BH	BC	PT	PC
0.10	8.4[a]	9.1[a]	9.6[a]	7.4[a]	7.6[a]
0.25	37.3[b]	29.5[b]	32.4[b]	36.5[b]	42.5[b]
0.50	55.3[c]	56.8[c]	55.5[c]	53.0[c]	62.0[c]
1.00	70.6[d]	71.5[d]	73.0[d]	71.4[d]	66.8[d]
2.50	86.4[e]	77.1[e]	84.1[e]	81.5[e]	89.9[e]
5.00	96.1[f]	94.1[f]	91.7[f]	91.3[f]	93.6[ef]
7.50	98.5[f]	97.7[f]	96.1[f]	95.2[f]	97.6[f]

[1] Any two means within a type of meat having one of the same letters are not significantly different at the 5% level of probability.

[2] See footnote to Table 2 for coding.

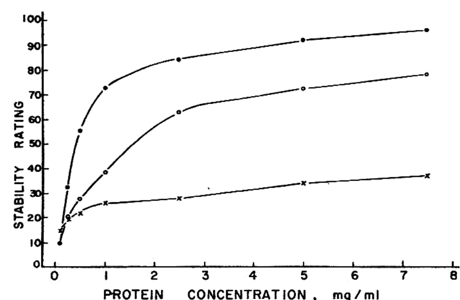

Fig. 3–Stability ratings of emulsions stabilized by salt-soluble protein of beef cheek meat (x x, 20% oil; o o, 35% oil; o o, 50% oil).

Effect of level of oil on emulsion stability

Examination of Figure 3 shows the significance of the level of oil in the emulsion preparation at each protein concentration. For the emulsions prepared with the various meat proteins, stability was usually significantly higher at 20% oil than at 35 or 50% oil at a protein concentration of 0.10 mg/ml. At the protein concentration of 0.25 mg/ml, the emulsions containing 50% oil were significantly more stable than those of either 20 or 35% oil which were not significantly different from each other. Thus, the effect of the level of oil was reversed as stability shifted from being highest at 20% to highest at 50% oil when the protein concentration changed from 0.10 to 0.25 mg/ml.

Increasing the percent oil from 20% to 35% or to 50% within each protein concentration in the range of 0.50–7.50 mg/ml significantly increased the emulsion stability with each type of salt-soluble protein. The stability ratings became significantly higher, reaching their maximum at the 50% oil level, which was the highest oil level studied. The effect of oil level on stability is in agreement with that reported by Titus et al. (1968) for emulsions stabilized by nonionic surfactant mixtures of varying HLB.

Correlation of emulsion stability and protein surface activity

Although both surface tension and emulsion stability were dependent on the concentration of protein in solution, surface tension was used as an independent variable in simple correlation analysis between stability ratings and surface tensions. The correlation coefficients between these two factors in the protein concentration range of 0.10–7.50 mg/ml are listed in Table 7.

For each of the meat proteins in emulsions containing 20, 35 or 50% oil, there were high and significant coefficients between the two variables. It should be pointed out that the level of oil for each correlation did not significantly

Table 7–Correlation coefficients between stability ratings (Y) and surface tension (X) of various meats in protein concentration range of 0.10–7.50 mg/ml.[1]

Type of Meat	% Oil of Stability Rating	Correlation[2] Coefficient, r
Cow Meat	50	−0.95
	35	−0.90
	20	−0.83
Beef Cheek Meat	50	−0.99
	35	−0.98
	20	−0.98
Beef Hearts[3]	50	−0.94
	35	−0.81
	20	−0.89
Pork Trim	50	−0.99
	35	−0.92
	20	−0.91
Pork Cheek Meat	50	−0.98
	35	−0.92
	20	−0.89

[1] Surface tension value at 7.50 mg protein/ml estimated using log surface tension-log protein concentration prediction equations.

[2] All correlation coefficients are significant at the 5% level of probability.

[3] Protein concentration range of 0.10–5.00 mg/ml used for beef hearts.

alter this relationship. Although no interfacial tension determinations were conducted, the surface tension responses were considered to reflect the same type of activity and adsorption that would occur at the oil phase. The ability to reduce surface tension and stabilize the emulsion were two requirements for an emulsifier as listed by Lowe (1966).

Table 8–Relative viscosities[1] *of oil-in-water emulsions stabilized by salt-soluble protein of cow meat at 4°C.*

Protein Conc mg/ml	% Corn Oil 20	35	50
0.10	1.0	1.0	1.0
0.25	1.0	1.0	1.0
0.50	1.0	1.2	2.0
1.00	1.0	1.3	2.0
2.50	1.3	1.5	2.0
5.00	1.5	21.7	41.7
7.50	1.7	33.3	58.3

[1] Relative to the viscosity of water at 4°C.

Emulsion viscosity

Table 8 shows the relative viscosities of the emulsions prepared with salt-soluble protein of cow meat. From observations and handling, the viscosity of emulsions prepared using the protein of the other meats appeared very similar at the same protein and oil level. Within each protein concentration and within each oil level, there is no large difference in emulsion viscosity up to 2.50 mg of protein/ml. However, at 5.00 and 7.50 mg of protein/ml, the emulsion viscosity increased considerably as the level of oil was increased. At 7.50 mg of protein/ml, the emulsion containing 50% oil is approximately 35 times as viscous as the emulsion containing 20% oil. The viscosity increase due to increased volume of the dispersed phase has been shown by Sherman (1963) to retard the coalescence of dispersed phase droplets and has been cited by Becher (1965) as a primary factor in retaining a stable emulsion.

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Ms. received 5/28/70; revised 7/15/70; accepted 7/17/70.

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CALCIUM POLYPHOSPHATE INTERACTION IN CURING PICKLES AND THE EFFECT OF CALCIUM ON CURED HAM YIELDS

SUMMARY—The formation of a calcium polyphosphate precipitate in curing pickles is dependent upon brine strength, brine temperature, tripolyphosphate concentration and calcium concentration. The highest concentration of calcium at which no precipitate is formed under various conditions is referred to as the critical calcium concentration. A series of curves is presented from which the critical calcium concentration can be determined for any practical combination in solution of NaCl, sodium tripolyphosphate and calcium ions. The effect on the critical calcium concentration of temperature, pH and added sucrose was also determined and graphically presented. In some cases, added $CaSO_4$ caused a significant increase in the water holding capacity of cured ground ham, and in no instance did the calcium salt cause a reduction in water-holding capacity. The yield of fully-cooked cured hams was not affected by curing with phosphate pickles containing abnormally high levels of $CaSO_4$.

INTRODUCTION

THE EFFECTS OF alkaline earth metals on water holding capacity and swelling of meat proteins have been rather extensively investigated. Hamm (1956) indicated that polyvalent cations caused a tightening of meat structure which he attributed to cross linking of proteins. Bozler (1955) extracted calcium and magnesium ions from muscle with chelating agents and observed increased swelling of the muscle. Hamm (1958) used a cation exchanger to remove calcium from beef muscle which was followed by increased muscle hydration. Wierbicki et al. (1957) reported that calcium and magnesium chlorides added to meat prior to heating resulted in increased water holding capacity when the meat was heated to 70°C. Hamm (1960) pointed out that this increase in muscle hydration upon the addition of calcium or magnesium chlorides is the result of the predominant hydrating effect of the Cl^- ion.

The major impurity in NaCl is $CaSO_4$. Rock salt, used in brine generating systems of most large packing plants, contains from 0.3 to 1.75% $CaSO_4$. Though $CaSO_4$ is sparingly soluble in water, its solubility is increased in the presence of NaCl, and a brine to be used for curing can easily become saturated with $CaSO_4$.

It has been suggested that high calcium levels in phosphate curing pickles would reduce yields of cured ham. This is based on the hypothesis that high levels of added calcium combine with the polyphosphates in a curing pickle inhibiting their effectiveness in reducing shrink.

A related problem is the occasional occurrence of a dense flocculant precipitate in phosphate pickles which contain high levels of calcium. In order to avoid this problem, it would be helpful to know what specific combinations of factors lead to the formation of this precipitate.

This experiment was designed to resolve two basic questions concerning sodium tripolyphosphate (Na-TPP) and calcium levels in ham curing pickles:

(1) What is the maximum amount of calcium that can be tolerated at any given brine strength and Na-TPP level (this is from the standpoint of precipitate formation in the pickle)? The precipitation point is referred to in terms of a critical calcium concentration at a defined NaCl and Na-TPP concentration.

(2) Do high levels of calcium reduce the effectiveness of Na-TPP in meat curing, and, if so, to what degree?

EXPERIMENTAL

Determination of critical calcium concentration

Curves were constructed for four commonly used brine strengths: 18%, 15%, 12% and 9.5%. Three experimental points (at 1%, 2% and 3% Na-TPP) were determined for each brine strength. All percentages in this work are calculated on a weight/weight basis. Each experimental point for critical calcium concentration was determined as follows: A series of test tubes was set up containing the required amount of Na-TPP and NaCl for each point in question, e.g., consider 18% NaCl and 1% Na-TPP. Calcium content was varied from one tube to the next in regularly increasing increments making calcium the only variable in the system. Each of the three compounds in the system was dissolved separately before mixing as NaCl interferes with the solubilization of Na-TPP. If all additives are dissolved separately, they can then be mixed without difficulty. The highest level of calcium at which no precipitate occurred was designated as the critical calcium concentration for a particular combination of Na-TPP and NaCl. This point was then plotted on a graph with % calcium on the horizontal axis and % Na-TPP on the vertical axis (see Figure 1). All observations were made at room temperature, within a few minutes after mixing the solutions, unless otherwise indicated.

Determination of processing and cooking shrink

A test tube method was used for the shrink tests on cured, ground ham. Fresh defatted ham was ground twice through a 1/8-in. plate. The appropriate amount of curing pickle was thoroughly mixed with 100g of ground ham. The basic curing pickle contained 18% salt and 0.1% $NaNO_2$. 14g of this solution added to 100g of meat gave a final concentration based on meat weight of 2.5% salt and 140 ppm $NaNO_2$. Calcium and Na-TPP were varied as outlined in Tables 1 and 2. This meat-pickle mixture was held overnight at 4°C. Approximately 25g of the cured meat was extruded through a glass tube into a tared test tube (200

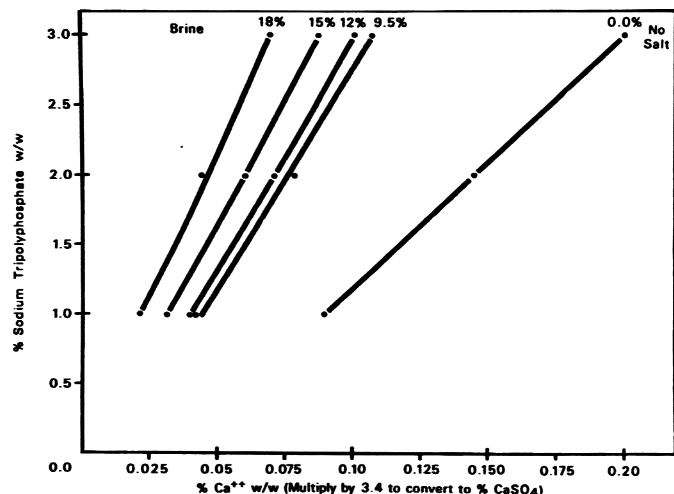

Fig. 1—Critical calcium concentration curves for various NaCl concentrations.

Table 1–Percent moisture loss for cured ground meat. Test 1, with and without calcium, 2% Na-TPP in the pickle and 14% pickle added to ham.

pH	% Calcium in Pickle[a] 0	0.03
5.5	9.6	8.8
5.75	9.0	4.9
5.9	9.1	4.9
6.2	5.8	5.1

[a]Cured meat contained 42 ppm Ca.

Table 2–Percent moisture loss for cured ground meat. Test 2, with and without calcium no Na-TPP and 14% pickle added to ham.

pH	% Calcium in Pickle[a] 0	0.03
5.5	17.7	17.4
5.75	21.8	19.5
5.9	17.9	15.6
6.2	8.8	5.2

[a]Cured meat contained 42 ppm Ca.

mm length × 29 mm O.D.). The plug of meat was deposited about 50 mm from the bottom of the tube and remained rather firmly attached in this position throughout the test. The weight of the raw meat was obtained to the nearest tenth of a gram. The tubes were heated for 40 min in a water bath maintained at 70°C. The samples were allowed to cool to room temperature, and the meat plugs were removed with a spatula and weighed. Triplicate determinations were made on each variable. Percent weight loss (shrink) was calculated on the basis of raw cured meat weight.

Hams from the 12–14 lb range were utilized for the processing experiments. In the first ham shrink test, hams were paired according to pH. Left and right hams from the same carcasses were used for the second test. Hams were randomized so that each variable contained both left and right hams. Hams were artery pumped to 12% with an additional 2% stitch pumped into the ham butt. The pickle was prepared according to a commercial formula. Pumped hams were allowed to drain for 1-1/2 hr before processing. Hams were processed to an internal temperature of 66°C in a conventional smokehouse. Ham weights were obtained at the completion of the process and after 16 hr of storage at 4°C.

After approximately 48 hr of storage at 4°C, a 2-in. center slice was removed from each ham. Each slice was baked in a 180°C oven to an internal temperature of 60°C. Cooking shrink was calculated for each center slice.

Analytical

All pH measurements were made with a Beckman Model G pH meter. Meat pH was taken at room temperature. The pH of intact hams were taken on the exposed area of the gracilis muscle.

Calcium analyses were performed by atomic absorption spectrophotometry, using lanthanum oxide to prevent phosphate interference.

Reagents

Deionized water was used throughout this experiment including the pilot plant tests. All chemicals were reagent grade with the exception of sodium tripolyphosphate which was the commercial product distributed by "Calgon."

RESULTS & DISCUSSION

THE OCCURRENCE OF heavy precipitates in phosphate curing pickles containing high levels of calcium was investigated by Urbin in 1957. In his first experiment

Table 3–Processing and shrink data for cured ham Tests I and II.[a]

Ham No.	pH	% Pump	Control Pickle: % Pump After 1-1/2 Hr Drain	% Hot Yield	% Yield 16 Hr	Ham No.	pH	% Pump	Calcium Pickle: % Pump After 1-1/2 Hr drain	% Hot Yield	% Yield 16 Hrs
TEST I.											
180	5.5	14.6	12.6	105.2	103.2	280	5.5	16.4	16.0	110.4	109.8
181	5.4	14.5	12.7	107.8	106.4	281	5.4	14.2	12.3	106.2	104.6
182	5.0	14.7	13.7	107.0	105.4	282	5.0	14.4	12.3	106.2	103.2
183	5.5	15.7	14.8	106.0	104.3	283	5.5	14.8	12.8	107.0	105.0
184	5.4	15.0	13.4	108.4	107.0	284	5.5	17.0	15.9	107.2	105.8
185	6.25	14.9	13.0	108.0	106.6	285	6.25	15.3	14.0	110.2	108.8
186	5.2	15.4	14.0	107.3	105.8	286	5.2	15.3	14.4	109.0	107.3
187	5.0	14.2	13.4	104.6	103.2	287	5.3	16.2	14.8	107.6	105.2
188	5.9	15.0	13.2	108.6	107.8	288	5.65	16.2	15.8	110.8	109.0
Average		14.9	13.5	106.9	105.4			15.5	14.2	108.3	106.5
TEST II.											
250	5.4	16.3	15.2	107.8	105.3	150	5.4	16.2	15.0	106.2	102.4
236	5.4	14.2	12.8	102.7	99.5	136	5.4	14.5	13.8	106.0	102.7
218	5.4	14.2	12.6	104.5	102.7	118	5.4	15.0	13.8	106.7	103.8
222	5.5	14.8	14.0	106.5	104.7	122	5.5	15.2	14.4	106.7	103.2
267	5.6	13.8	13.0	104.5	103.0	167	5.6	14.4	13.0	105.6	103.8
259	5.4	14.6	13.8	104.5	100.8	159	5.4	15.0	13.7	104.5	101.4
260	5.4	14.4	13.3	105.0	102.7	160	5.4	14.0	12.8	104.2	101.4
255	5.4	15.4	15.0	107.5	105.0	155	5.4	15.7	14.8	107.6	105.8
257	6.2	14.3	12.8	108.3	107.3	157	6.2	14.2	13.2	108.2	106.0
237	5.3	15.5	15.2	107.0	104.2	137	5.3	14.5	14.0	105.7	103.4
Average		14.8	13.7	105.8	103.5			14.9	13.8	106.1	103.3
Average of both Experiments		14.8	13.6	106.4	104.5			15.2	14.0	107.2	104.9

[a]Control hams and hams cured with high calcium pickle were paired according to pH for Test I and paired hams from the same carcass were used in Test II.

he observed precipitates in all pickles containing 5% tripolyphosphates. He found that the addition of $CaSO_4$ reduced the amount of this precipitate. However in a second experiment he was unable to observe this phenomenon; another type of precipitate was observed which was primarily calcium tripolyphosphate. He reported that this second precipitate could be avoided by increasing the Na-TPP level above 2%. This, undoubtedly, is the same precipitate discussed here. This precipitate occurs occasionally in plants using rock salt for brine generation and where extremely hard water is used. The precipitate in question occurs almost immediately upon mixing of Na-TPP solution with brine.

The precipitate can be resolubilized by reducing the NaCl concentration or increasing the Na-TPP concentration, which would not occur if the precipitate was calcium orthophosphate. This would seem to indicate that the precipitate is primarily calcium tripolyphosphate.

The critical calcium concentration curves for precipitate formation are presented in Figure 1. The range of NaCl and Na-TPP concentrations used in this study covers the range of concentrations used in commercial practice. Any combination of Na-TPP and calcium which falls to the right of a curve representing a particular NaCl concentration would result in precipitate formation. The curves are surprisingly linear and the point at which precipitation occurs is sharp and well defined. These points which were determined in a system consisting of a few ml were confirmed in several instances on a 10 gal basis.

The anion associated with the calcium salt has no effect on the precipitation point as several of the curves in Figure 1 were almost exactly duplicated with either $CaCl_2$ or $CaSO_4$.

Magnesium is reported to be more difficult to chelate than calcium (Chaberek and Martell, 1957), but the critical concentration point for magnesium is shifted considerably to the right of the critical calcium concentration in Figure 2. This indicates that considerably more magnesium than calcium can be tolerated in a phosphate pickle.

The effect of pH on the critical calcium concentration is shown in Figure 3.

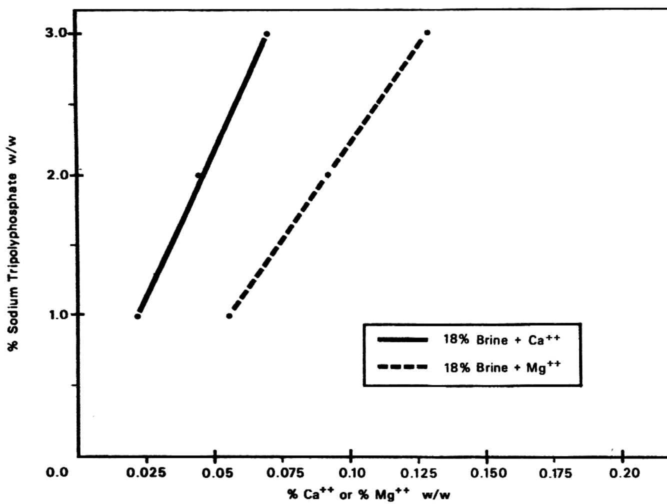

Fig. 2–Critical magnesium concentration curve compared to calcium concentration in 18% NaCl solution.

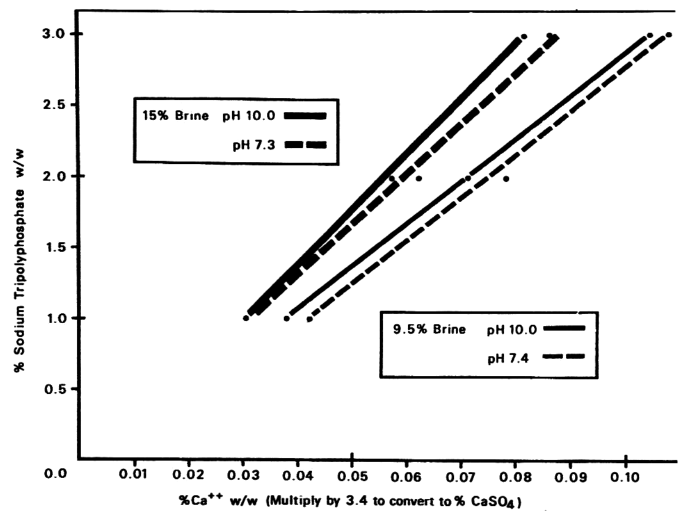

Fig. 3–The effect of pH on critical calcium concentration curves in 15% NaCl and 9.5% NaCl solution.

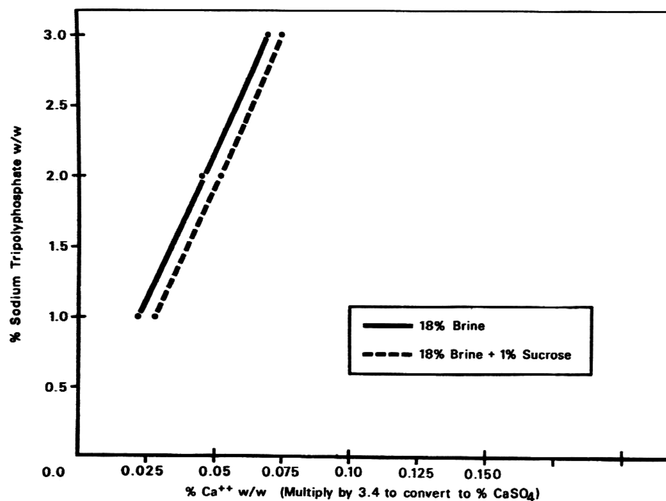

Fig. 4–The effect of sucrose on the critical calcium concentration curve in 18% NaCl solution.

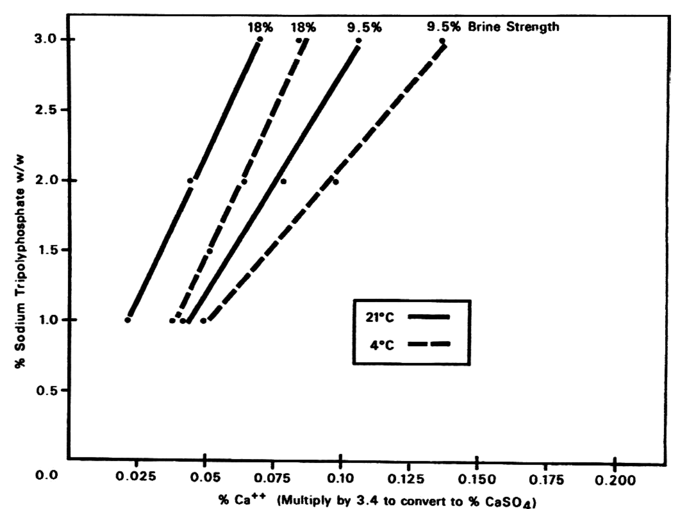

Fig. 5–The effect of temperature on the critical calcium concentration curves at two brine strengths.

Table 4–Percent yield of 2 in. cured ham center slices cooked to 70°C internal tempera-

Control pickle		Calcium pickle	
	Yield	Ham No.	Yield
180	86.2	280	89.3
181	87.0	281	87.3
182	86.0	282	86.7
183	85.1	283	89.8
184	87.9	284	85.2
185	89.8	285	84.2
186	89.1	286	84.4
187	87.3	287	87.1
188	87.7	288	87.0
250	88.3	150	88.1
236	85.4	136	81.7
218	84.8	118	87.6
222	87.4	122	86.9
267	87.6	167	86.7
259	83.2	159	87.5
260	86.6	160	88.3
255	86.9	155	85.7
257	88.1	157	91.8
237	84.2	137	83.2
Average	86.8		86.8

There appears to be small displacement to the left at high pH's. A pH of 10 would be very unlikely to occur in a curing pickle. The normal pH of a phosphate curing pickle is slightly above 7.

The effect of sucrose, which is frequently used in ham curing pickles, is shown in Figure 4. In this case there is a slight shift to the right.

Probably the most significant effect on the critical calcium concentration curves is that of temperature (Figure 5). The calcium tolerance of a phosphate pickle is increased by 0.01–0.015% calcium by reducing the pickle temperature from 21°C to 4°C. The temperature of water with which curing pickles are made at the plant level generally is somewhere between these temperatures depending on the season of the year. Pickles are refrigerated at 4°C–5°C after preparation. It would appear that more problems would be encountered with the precipitate during the summer months when water temperatures are high.

The effect of calcium (as calcium sulfate) on water holding capacity of cooked, cured ground hams was evaluated using the tube method described above. The object of these tests was to investigate the calcium effect on ham shrink under a variety of conditions, i.e., with and without Na-TPP, and at four ham pH's. (Ham pH was not adjusted. Hams were selected with the pH values desired.)

Data from these tests were analyzed by analysis of variance. The individual variables were analyzed by pairs, each pair consisting of a pickle variable with calcium and a corresponding control without calcium. Mean values for each of the tests are in Table 1 and 2. The well-documented effects of pH and polyphosphates on water holding capacity are confirmed by these tests.

It is interesting to note that Na-TPP appears to be effective in increasing yields only when ham pH is less than 6. The analysis of variance showed that the presence of calcium in the curing pickle significantly decreased cooking shrink of cured ground ham.

Two processing tests were conducted to evaluate the effect of a high calcium pickle on cured hams. The pickle was prepared according to a standard commercial formula. The control pickle contained approximately 14% NaCl and 1.8% Na-TPP. The pickle also contained a small amount of brown sugar and the levels of sodium erythorbate and sodium nitrite were within the legal limits. The experimental pickle was identical to the control except it contained calcium in the form of $CaSO_4$. Calcium was added to the pickle at the maximum level allowable without the occurrence of a precipitate in the pickle (this was 0.05% determined from the curves in Figure 1). Assuming a 14% pump, the experimental hams contained about 70 ppm of added calcium.

The results of these tests are presented in Table 3. Analysis of variance showed no significant difference between yields of hams cured with control pickle and hams cured with high calcium pickle.

The cooking shrink of 2 inch center slices from the above hams is outlined in Table 4. Here it is obvious that calcium had no effect on cooking shrink.

The results of this work show that relatively high levels of added calcium sulfate do not reduce the water holding capacity of cured ham and may well result in increased yields under certain conditions. This would indicate that calcium does not interfere with the activity of tripolyphosphate in improving water holding capacity. This agrees with the claims made in the patent of Benckiser (1966) who indicated that significant increases in water holding capacity of sausage could be obtained using polyphosphates which were partially or completely complexed with calcium magnesium or iron.

In any case, it would not be advisable to use softened water or high purity salt for curing pickles for the sole purpose of improving cured ham yields.

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Ms. received 1/30/70; revised 6/20/70; accepted 7/3/70.

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MICROFLORA OF FRESH PORK SAUSAGE CASINGS. 2. Natural Casings

SUMMARY—Aerobic total plate counts on TGE Agar at 28°C varied from a level greater than 30,000 to 59,000,000 microorganisms per gram in salt-packed natural casings and from 180,000 to 23,000,000 organisms per gram in wet-packed natural casings. Both salt-packed and wet-packed casings supported similar growth patterns in selective media. 38 isolates were identified from the salt-packed casings and 53 from the wet-packed. Of the salt-packed isolates, 60.5% were of the genus Bacillus, *7.9%* Pseudomonas, *15.8%* Clostridium, *1.6%* Micrococcus *and 5.3%* Gaffkya. *Those isolates obtained from the wet-packed casings included 62.3%* Bacillus, *7.5%* Pseudomonas, *7.5%* Clostridium, *7.5%* Micrococcus, *5.6%* Proteus, *1.9%* Lactobacillus *and 5.7% unidentified.*

INTRODUCTION

NATURAL casings are made from the intestinal tracts of hogs, sheep and cattle. The small intestines or rounds are used in the manufacture of pork sausage casings. Natural casings are prepared by removing the intestine from the slaughtered animal. The fat and mysentery is then separated from the intestine by a manual knife operation known as "running." More closely adhering fat is removed by passage through a series of brushes. Contents of the intestine are removed by hand or machine under a warm-water spray which washes away the contents as they are expressed and keeps the exterior clean. Various unwanted intestinal layers are then removed by passage through a succession of rollers. Washing follows, after which the casings are stored in a 15–20% salt solution before grading and curing. Casings are cured by rubbing with a medium-fine salt and then allowing the casings to drain in a tub for 1 wk. After curing, the casings are shaken out and prepared as salt-packed or wet-packed (AMIF, 1960).

Salt-packed casings are prepared by rubbing the cured casings with fine salt and packing the casings with salt in tightly sealed barrels. The sealed barrels contain from 40–60% salt. The wet-packed casings are packed in pouches containing a saturated brine solution.

The microflora of the intestines of animals has been reported by several investigators (Ayres, 1955; Willingale and Briggs, 1955; Fewins et al., 1957; Brownlie and Grau, 1967; Williams and Newell, 1967). Ayres (1955) reported that average counts in the rumen on a dry weight basis were 53,000,000 bacteria per gram, 180,000 yeast per gram and 1,600 molds per gram. These counts were found to increase to 90,000,000 bacteria, 200,000 yeasts and 60,000 molds per gram in the excreted feces. Isolates obtained from the rumen were primarily *Sarcina, Diplococcus, Streptococcus, Protozoa* and *C. butyricum*. Willingale and Briggs (1955) found 10^8 to 9.9×10^8 organisms per gram in pig feces. These organisms included *E. coli, Streptococcus, Proteus, Salmonella, Shigella, B. subtilis, C. butyricum* and *Corynebacterium*.

The first article in this series dealt with the quantitative and qualitative analysis of the microflora of regenerated collagen casings. This paper deals with quantitative and qualitative analysis of the microflora of 2 types of natural casings, standard salt-packed and wet-packed.

MATERIALS & METHODS

Casings

3 lots of standard salt-packed casings and 2 lots of wet-packed casings were obtained from the Oppenheimer Casing Company, Chicago, Illinois. All casings were 20 to 25 mm American Sheep of best quality. Each lot was selected at random from a single production day. All casings were sampled within 1 to 4 weeks after original packing except for lot 1 salt-pack which was refrigerated (4°C) for 4 months before examination.

Preparation of inoculum

Slurries were prepared by aseptically removing single casings from each lot, cutting them into 1-in. pieces and placing 20 g of pieces into

Table 1—Growth response from composite samples of standard salt-packed natural casings taken from 3 different days and preflushed wet-packed natural casings taken from 2 production days.

		Pretreatments and incubation conditions											
										Heat shock[2]			
		Aerobic				Anaerobic				Aerobic		Anaerobic	
	Type of	Incubation				Incubation							
Enrichment	natural	temperature (°C)				temperature (°C)				90°C	90°C	90°C	90°C
medium	casing[1]	4	28	37	55	4	28	37	55	30 min	60 min	30 min	60 min
Fluid Thioglycollate	SP					–	+	+	+			+	–
Medium	WP					–	+	+	–			+	+
Tryptone Glucose	SP	–	+	+	+					+	+		
Extract Broth	WP	+	+	+	+					+	+		
APT Broth	SP			+				+					
	WP			+				–					
Buffered Desoxycholate	SP			+				–					
Glucose Broth	WP			+				+					
Selenite-F Enrichment	SP			–									
	WP			–									
Liquid Sabouraud's Medium	SP		+				+						
	WP		+				+						

[1] SP: Salt-packed casings; WP: Wet-packed casings; +: Apparent growth; –: No apparent growth.
[2] Incubation at 37°C.

Table 2–Genera of isolates obtained from salt-packed casings and enrichment medium on which the isolate was originally grown.

Enrichment medium	Incubation temperature (°C)	Incubation atmosphere		Genus isolated					
		Aerobic	Anaerobic	Bacillus	Pseudomonas	Clostridium	Micrococcus	Alcaligenes	Gaffkya
Fluid thioglycollate	55		x	x					
	37		x	x		x			
	28		x		x	x		x	x
	37 h.s.[1]		x			x			
	37 h.s.[2]		x			x			
Tryptone glucose Extract broth	55	x		x					
	37	x		x			x		
	37 h.s.[1]	x		x					
	37 h.s.[2]	x		x					
Liquid Sabouraud's	28	x		x					
Number of isolates				23	3	6	1	3	2
% Total isolates				60.5	7.9	15.8	2.6	7.9	5.3

[1] Heat shocked at 90°C for 30 min.
[2] Heat shocked at 90°C for 60 min.

180 ml of sterile 0.01% collagenase solution in 0.067 M phosphate buffer at pH 7.4. The wet-packed casings were used directly from the package. To simulate industrial practices, 50 g of the salt-packed casings were rinsed in a sterile 99 ml distilled water blank after removal from the package.

The casing pieces were held in the collagenase solution at 37°C for 10 min prior to blending for 4 min in a sterile stainless steel Waring Blendor. This treatment permitted effective grinding and mixing of the casing pieces. This slurry was then used as the inoculum for the qualitative and quantitative analyses. Each lot of salt-packed and wet-packed casing was analyzed twice. The total plate count of the rinse water from 2 lots of salt-packed casings was determined.

Qualitative analysis

The casings were qualitatively analyzed by enrichment techniques followed by streak plating on general purpose and differential media. 5 ml of the slurry was inoculated into 195 ml of enrichment broth. Each enrichment was done in duplicate. Isolates obtained from subsequent streak plates were identified according to the scheme of Skerman (Breed et al., 1957).

Quantitative analysis

To enumerate the microflora of the casings, pour plate analysis was conducted using general purpose and selective plating media. 1 ml of the slurry was plated in duplicate. Where necessary, serial dilutions were made using sterile 0.1% peptone water.

Media and incubation

Media incubation conditions used were previously described in detail by Riha and Solberg (1970) and may be seen in Tables 1 and 4 of this paper.

RESULTS & DISCUSSION

Enrichment analysis and identification of isolates

Table 1 illustrates the growth responses obtained on the enrichment media from both types of casings. The general-purpose media usually supported growth. Tests conducted on the general-purpose media for sporulating microorganisms were positive. Aerobic and anaerobic growth responses were similar on the general-purpose enrichment media for both salt-packed and wet-packed casings, except that the only psychrophiles isolated were from the wet-pack casing enrichment grown aerobically and the only broth not showing thermophilic growth was the wet-packed enrichment grown anaerobically. Liquid Sabouraud's medium supported growth both aerobically and anerobically for both wet-packed and salt-packed casings at 28°C. This may be expected since the pH of the medium may be similar to the pH of the intestine. However, no molds or yeasts were isolated from either type of casing, as shown in Tables 2 and 3.

Aerobic and anerobic sporeformers were present in both types of casings, although highly resistant organisms which would survive exposure to 90°C for 60 min were not isolated from salt-packed casings under anaerobic growth conditions.

The generic distribution of isolates obtained from both types of casing are

Table 3–Genera of isolates obtained from wet-packed casings and enrichment medium on which the isolate was originally grown.

Enrichment medium	Incubation temperature (°C)	Incubation atmosphere		Genus isolated							
		Aerobic	Anaerobic	Bacillus	Pseudomonas	Clostridium	Micrococcus	Proteus	Lactobacillus	Alcaligenes	Unidentified
Fluid Thioglycollate	37		x	x							
	28		x							x	x
	37 h.s.[1]		x			x					
	37 h.s.[2]		x	x							
Tryptone Glucose Extract Broth	55	x		x							
	37	x		x			x				
	28	x			x					x	x
	37 h.s.[1]	x		x							
	37 h.s.[2]	x		x							
Liquid Sabouraud's	28	x						x			
APTBroth	37	x		x					x		
BDG Broth	37	x		x							
No. of isolates				33	4	4	1	3	1	4	3
% Total isolates				62.3	7.5	7.5	1.9	5.7	1.9	7.5	5.7

[1] Heat shocked at 90°C for 30 min.
[2] Heat shocked at 90°C for 60 min.

Table 4–Average pour plate counts of 3 lots of salt-packed casings and the rinse water from lots 1 and 2.

Medium	Incubation[1]	Contamination level (organisms/g)			
		Lot 1	Lot 2	Lot 3	Rinse water
Tryptone Glucose	55°,A	30	40	10	<500
Extract Agar	37°,A	500	71,000	190,000	950,000
	28°,A	>30,000	59,000,000	1,900,000	2,700,000
	4°,A	400	<10	<10	<500
Anaerobic	55°,An	<10	<10	10	<500
	37°,An	190	80,000	1,400	490,000
	28°,An	150	38,000	2,200	720,000
	4°,An	<10	<10	150	<500
APT Agar	37°,A	200	10,000	64,000	200,000
	37°,An	350	84,000	2,300	330,000
Azide Blood	37°,A	80	750	500	38,000
Agar Base	37°,An	50	2,700	200	<500
Desoxycholate	37°,A	60	30	71,000	900
Agar	37°,An	<10	<10	7,100	<500
Staph.	37°,A	180	130,000	2,400	310,000
Medium No. 110	37°,An	90	10	230	3,800
Potato Dextrose	28°,A	> 30,000	2,400,000	30,000	37,000
Agar	28°,An	50	20	1,000	12,000

<= Less than; >= greater than.

[1] Incubation temperature is given in °C; "A" indicates aerobic incubation; "An" indicates anaerobic incubation.

presented in Tables 2 and 3. The isolation frequencies shown in these tables are not representative of the casing microflora distribution, since only colonies which appeared to have different morphologies on streak plates were isolated for identification. The large variety of sporeformers would be expected since Ayres (1955) reported that bacilli and clostridia were found in the intestines and feces of cattle, swine and sheep, and these organisms may be better suited to survive the processing treatments, in particular, the high salt concentrations. Most of the other genera cited are also common fecal contaminants as reported by Breed et al. (1957) and may be expected as naturally occurring contaminants.

Quantitative Analysis

Table 4 shows the average pour plate counts for the 3 lots of salt-packed casings evaluated. Recovery of aerobes was generally greater than anaerobic recovery, as may be expected since most organisms reported isolated from feces by Ayres (1955) and Willingale and Briggs (1955) were aerobes or faculative anaerobes. On the general-purpose media, aerobic recovery indicated the presence of thermophiles and psychrophiles as previously shown during the enrichment analysis. Recovery on Tryptone Glucose Extract Agar (TGE) was much greater at 28 than 37°C. This phenomenon has often been reported in fresh meats and usually indicates the presence of pseudomonads.

Table 4 also illustrates the variability of contamination levels, some of which may be attributed to biological variations. Some of the variation may be caused by changing microflora during casing storage. There were no microflora differences observed during the 3 weeks required to run replicates on each lot. The lower contamination level on the casings stored for 4 months may have been coincidence. There is a need for further study of natural casing microbiology during long-term storage.

The contamination levels of the rinse water presented in Table 4 show that many microorganisms are removed from the casing by the rinsing process. Both the rinse water and the casings yielded similar microbial counts on general-purpose media, indicating approximately a 50% reduction of count due to the rinsing process.

Table 5 presents the average pour plate counts for both lots of wet-packed casings studied. These casings were generally more highly contaminated than the salt-pack casings. Aerobic recovery on the general-purpose media was usually greater than anerobic recovery. No psychrophiles or thermophiles were found on the pour plates, although results obtained from the enrichments indicate the presence of these organisms. Unlike the results obtained from the salt-packed casings, recoveries on TGE were equivalent at 37 and 28°C. Recovery trends on the selective media were similar for both types of casings.

CONCLUSIONS

THE CONTAMINATION levels for natural casings are higher than those previously reported for regenerated collagen casings (Riha and Solberg, 1970). The contamination contributed to fresh pork sausage by the natural casings, which represent approximately 2% of the

Table 5–Average pour plate counts of lots 1 and 2 of wet-packed casings.

Medium	Incubation[1]	Contamination level (organisms/g)	
		Lot 1	Lot 2
Tryptone Glucose	55°,A	<10	<10
Extract Agar	37°,A	120,000	75,000,000
	28°,A	180,000	23,000,000
	4°,A	<10	<10
Anaerobic	55°,An	<10	<10
Agar	37°,An	3,200	23,000
	28°,An	100	290,000
	4°,An	<10	<10
APT Agar	37°,A	22,000	1,200,000
	37°,An	780	580,000
Azide Blood	37°,A	3,500	10,000
Agar Base	37°,An	2,200	94,000
Desoxycholate	37°,A	730	33,000
Agar	37°,An	<100	700
Staph.	37°,A	1,800	1,900
Medium No. 110	37°,An	40	220
Potato Dextrose	28°,A	1,300	2,900,000
Agar	28°,An	200	290,000

<= Less than; >= greater than.

[1] Incubation temperature is in °C; "A" indicates aerobic incubation; "An" indicates anaerobic incubation.

weight of a sausage link, would vary from a low of approximately 600 organisms per gram to a high of approximately 1.5 × 10^6 organisms per gram, since the casings themselves varied from 30,000 to 75 × 10^6 organisms per gram when the highest general-purpose medium total plate count was considered for each lot. These levels are in the range of the contamination levels for fresh sausage reported by Brewer (1925) and Miller (1964) and are 2 log cycles less than the animal feces contamination levels reported by Willingale and Briggs (1955) and Ayres (1955).

Since very low levels of psychrophilic contamination were encountered, it may be concluded that potential shelf-life reduction contributed by the casing material is minimal provided the sausage is processed properly and then stored at refrigerator (4°C) temperature.

From the qualitative studies, the wide variety of sporeformers shown may be attributed to their ability to withstand processing treatments. Of the other microorganisms isolated and identified, many have been previously reported in the rumen and feces of animals (Ayres, 1955; Willingale and Briggs, 1955) and are considered fecal contaminants (Breed et al., 1957). Most of these micro-oganisms have been reported as fresh pork sausage contaminants (Sulzbacher and McLean, 1951).

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Ms. received 5/7/70; revised 5/7/70; accepted 5/11/70.

Paper No. 4003 of the Journal Series, New Jersey Agricultural Experiment Station, Rutgers The State University, Food Science Department, New Brunswick, New Jersey.

This research was supported in part by a grant from Devro, Somerville, New Jersey.

Natural casings were supplied by cooperation of the International Natural Sausage Casing Association, Chicago, Illinois.

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EFFECT OF HEAT TREATMENT ON VISCOSITY OF YOLK

SUMMARY—Apparent viscosity readings at different shear rates for yolk or albumen-containing yolk in the native or heated conditions were obtained by a Wells-Brookfield microviscometer. The viscosity-shear rate curves for these yolk samples are typical for a pseudoplastic, non-Newtonian fluid. The apparent viscosity of native yolk with a solids content of 52.5% dropped gradually from 23 to 18 poises with an increase in shear rate from 1.9 to 76.8 sec^{-1}. The apparent viscosity of pasteurized yolk (65.6°C for 3 min) dropped drastically from 200 to 100 poises with an increase in shear rate from 1.9 to 19.2 sec^{-1}. Addition of thin albumen at levels of 5, 10 and 20% to native yolk brought about a considerable decline in the viscosity. The heat damage to the albumen-containing yolk as reflected by viscosity increase was reduced as the content of thin albumen increased. The viscosity increase of urea-treated yolk and yolk with 5 and 10% thin albumen was related to the temperature of heat treatment prior to urea addition.

INTRODUCTION

EGG YOLK may be regarded as a non-Newtonian fluid since C. M. Chang (1966, unpublished data) found that the shear stress (dynes/cm^2)-shear rate (sec^{-1}) relationship was nonlinear. This result is not surprising since the majority of solids (50 to 52%) in yolk consists of particulate matter, namely granules and low-density lipoprotein micelles (Sugano and Watanabe, 1961). The viscosity of a non-Newtonian fluid depends on the concentration of polymers and particulate matter, the shear rate and temperature of the fluid. Kline et al. (1965) noted that with a rise in yolk solids from 50.8 to 53.2% the viscosity increased markedly from 782 to 1926 cps at 25°C. The viscosity of fresh yolk with a percent solids content of about 52.7 was estimated by Meyer and Woodburn (1965) to be around 27 poises, whereas yolk with 50% solids (from stored eggs) had a viscosity of approximately 9 poises. According to Payawal et al. (1946), the viscosity of native yolk with a solids content of 50.5 to 51% was about 800 cps at 25°C. Moran (1935) reported that yolk with a solids content of 52.5 to 53% had a viscosity of about 2500 cps at 18 to 19°C. So far, no systematic studies have been carried out on the influence of shear rate and temperature on the viscosity of yolk and albumen-containing yolk.

Heat treatment of yolk with subsequent cooling has been found to increase the viscosity. When Payawal et al. (1946) heated yolk instantaneously to temperatures between 62.5 and 70°C, the viscosity rose progressively. At 70°C, the yolk coagulated in their heat exchanger. Moreover, these investigators observed that a holding time for yolk at 62.5°C and higher had an additive viscosity effect. For example, the viscosity of yolk rose from about 850 to a maximum of 10,000 cps within a 200-sec holding period at 62.5°C. Presumably, a further increase in the viscosity of heated yolk may occur upon the addition of urea since the viscosity of frozen yolk, but not native yolk, was increased markedly when 0.166 mole of urea/100 g yolk was added (Powrie et al., 1963). A relationship between degree of thermal damage to proteins in yolk and the viscosity increase of urea-treated yolk could serve as a basis for a test to assess the adequacy of yolk pasteurization.

This study was undertaken to determine the viscosity-shear rate relationship of native and heat-treated yolk and albumen-containing yolk. The influence of urea on the viscosity increase of heat-treated yolk samples was assessed. For most experiments, the Wells-Brookfield cone-plate rotational viscometer was used for viscosity measurements. This viscometer had the feature of a wide selection of shear rates and requires a small volume (about 1.25 ml) of sample. The Brookfield spindle viscometer was used when viscosities of samples (urea-treated yolk without added thin albumen) were beyond the range of the cone-plate viscometer.

EXPERIMENTAL

Materials

Fluid yolk was prepared by the method of Powrie et al. (1963) from infertile eggs not more than 24 hr old. Thin albumen was obtained by filtering albumen through 2 layers of cotton gauze.

Heat treatment of egg samples

Small samples of yolk and yolk containing different amounts of thin albumen were heated in glass tubes, 7.5 cm long with an I.D. of 0.6 cm. Samples were introduced into the tubes (sealed at one end) with the aid of a hypodermic syringe. A copper-constantan thermocouple was inserted through a rubber septum into the center of each glass tube. Tubes filled with egg samples were immersed into a constant-temperature water bath preheated to the desired pasteurization temperature, and the sample temperature was recorded by means of a Leeds and Northrup potentiometer. Each sample, reaching the desired pasteurization temperature within 45 sec, was held at this temperature for 3 min. Egg samples were cooled immediately by placing the glass tubes in an ice-water bath.

Large amounts of yolk for urea studies were heated in a laboratory pasteurizer consisting of a Sigma pump which pumped each egg sample through a tube heat exchanger, then a holding tube and finally a cooling tube. The tube heat exchanger was immersed in a constant-temperature water bath at 65.6°C (150°F). Desired temperatures of 60°C (140°F), 62.7°C (145°F) and 64.4°C (148°F) were obtained by varying the speed of the pump and the length of tube heat exchanger. A copper-constantan thermocouple was inserted at the end of the heat exchange tubing to measure the temperature of the yolk. The comeup time for reaching the desired temperature was within 15 sec. The holding tube (Tygon) was immersed in a water bath at the temperature of the heated yolk. The holding period for all samples was 3 min. Each yolk sample was cooled immediately to about 35°C by passing it through a condenser with ice-water circulating in the jacket.

Addition of urea

With native and pasteurized albumen-containing yolk, 1.5 g of powdered urea were added to a small tube containing 10 g of albumen-containing yolk to produce a urea concentration of 0.25 mole/100 g of egg sample. The contents were mixed thoroughly with a spatula for 1 min while the tube was immersed in a constant-temperature water bath at 25°C. After 3 min, the temperature of egg mass was at 25°C. Zero time means no treatment, and reaction time implies immediately after the urea was added.

Since 100-ml samples were required for viscosity measurement of native and pasteurized yolk without added albumen, a procedure similar to that of Powrie et al. (1963) was used for the addition of 0.25 mole of urea per 100 g yolk.

Viscosity measurement

A Wells-Brookfield microviscometer (Model HBT) was used for the measurement of apparent viscosity of egg samples. Various speeds (rpm) were selected by a clutch connected to a reduction gear system. Shear rate (sec^{-1}) was calculated for each rpm value. The shear rates used in this study were 1.9, 3.8, 9.6, 19.2, 38.4, 76.8 and 192.0 sec^{-1}. The sample to be placed in the sample cup was about 1.25 g. A weight in excess of 1.25 g did not have any influence on the viscosity readings. The temperature of each sample in the water-jacketed cup was maintained at 25°C for viscosity determination. Since samples of yolk and urea-treated yolk had viscosities too high to be measured by the microviscometer, the Brookfield spindle viscometer (Model RVT) with spindle No. 6

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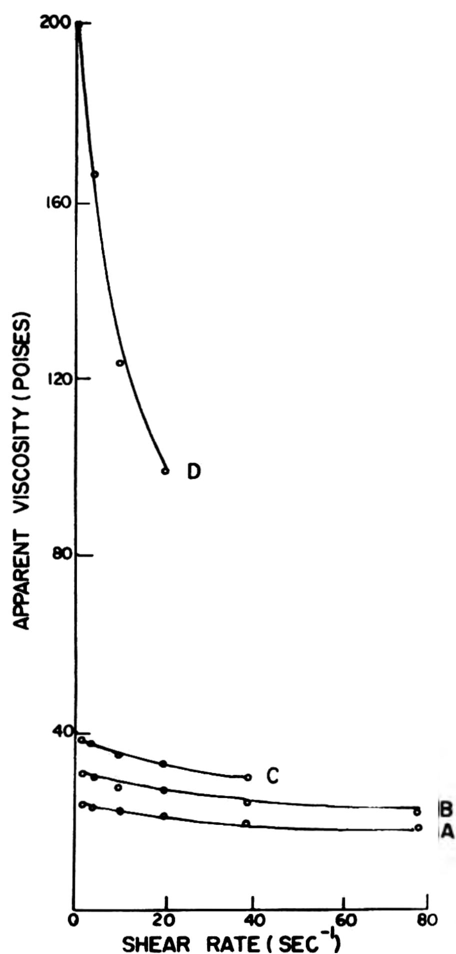

Fig. 1–Viscosity-shear rate relationships of yolk (52.5% solids). A: Native. B: heated at 60°C (140°F) for 3 min. C: Heated at 62.8°C (145°F) for 3 min. D: Heated at 65.6°C (150°F) for 3 min.

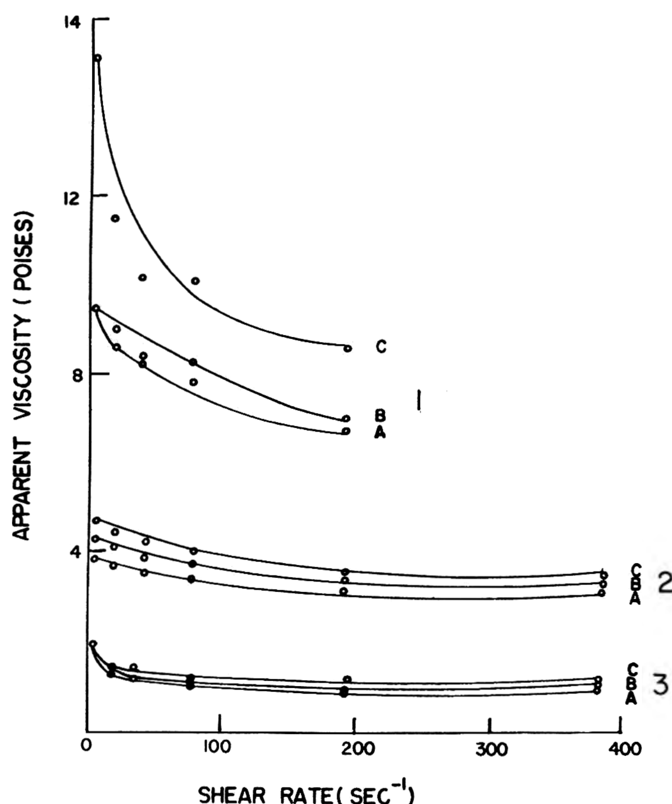

Fig. 2–Viscosity-shear rate relationships of yolk containing 1: 5% (w/w) thin albumen, 50.9% solids. 2: 10% thin albumen, 48.8% solids. 3: 20% thin albumen, 44.7% solids. Albumen-containing yolk was A: unheated, B: heated at 60°C for 3 min, C: heated at 62.8°C for 3 min.

rotating at 10 rpm was used to measure the viscosity of these samples at 25°C. A volume of about 100 ml was required for the viscosity measurement. Silicone oils from the National Bureau of Standards were used as viscosity standards.

RESULTS & DISCUSSION

THE VISCOSITY-SHEAR rate curves for native and pasteurized yolk at 25°C are presented in Figure 1. The data were obtained with the Wells-Brookfield microviscometer. It is evident that the apparent viscosity of the yolk samples was dependent on rate of shear. The apparent viscosity of native yolk with a solids content of 52.5% dropped gradually from 23 to 18 poises with an increase in shear rate from 1.9 to 76.8 sec^{-1} (curve A in Fig. 1). The data indicate that to have a meaningful apparent viscosity value of yolk, the shear rate should be reported. Kline et al. (1965), using a Brookfield spindle viscometer, indicated that yolk at 25°C with solid contents of 52.5 and 50.8% had viscosities of 1,462 and 782 cps, respectively. However, these authors specified neither the spindle size nor the rate of spindle rotation. When Meyer and Woodburn (1965) used the Brookfield viscometer with a spindle No. 5 and a constant spindle speed of 20 rpm, the viscosities of yolk with solids contents of about 52.7 and 50% were estimated as approximately 27 and 9 poises, respectively. Curve A for native yolk is typical for a pseudoplastic, non-Newtonian fluid.

Curves with similar shapes were shown by Rand et al. (1964) for normal human blood. The non-Newtonian behavior of yolk may be attributed to the gradual alignment of particles, such as granules and low-density lipoprotein micelles, with an increase in shear rate. Heat treatment of yolk at temperatures of 60°C (140°F) and above caused a significant rise in the viscosity at each of the shear rates. Payawal et al. (1946), using a capillary viscometer, noted a rise in yolk viscosity from about 8 to 10 poises at 25°C when native yolk was heated at 62.5°C (144.5°F) for 2 min. In our study with a shear rate of 1.9 sec^{-1}, the difference between the viscosities of native yolk and yolk treated at 62.8°C for 3 min was 15 poises (Fig. 1). The viscosity difference was somewhat less as the shear rate increased.

The shapes of viscosity curves B and C in Figure 1 for yolk pasteurized at 60 and 62.8°C were similar to that for native yolk. On the other hand, yolk pasteurized at 65.6°C (150°F) had a very high viscosity value of 200 poises at 1.9 sec^{-1} shear rate, but the viscosity dropped drastically to 100 poises with an increase in shear rate to 19.2 sec^{-1}. Viscosities at higher shear rates were not possible because the readings were off-scale. The abrupt drop of viscosity for the 65.6°C-treated yolk with a small increase in shear rate as shown in curve D may be interpreted as a disruption during shearing of weakly bound aggregates which probably were formed during heat treatment. According to Payawal et al. (1946), the viscosity of yolk heated at 65°C reached a maximum of 100 poises with a 200-sec holding time

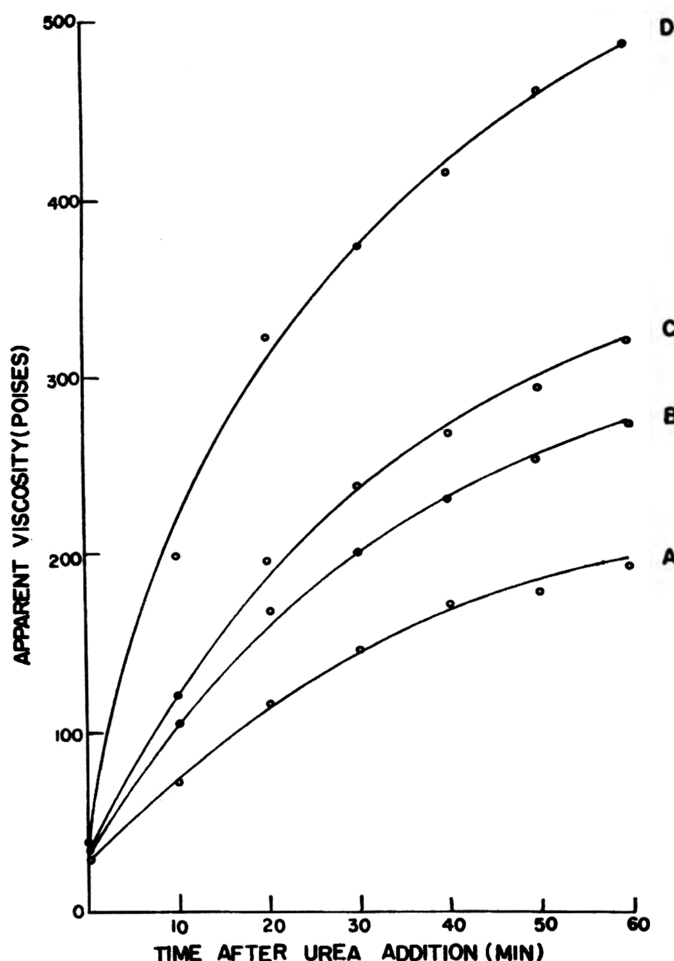

Fig. 3–Viscosity changes after urea addition (0.25 mole/100 g) to yolk, A: native, B: heated at 60°C for 3 min, C: heated at 62.8°C for 3 min, D: heated at 64.4°C for 3 min.

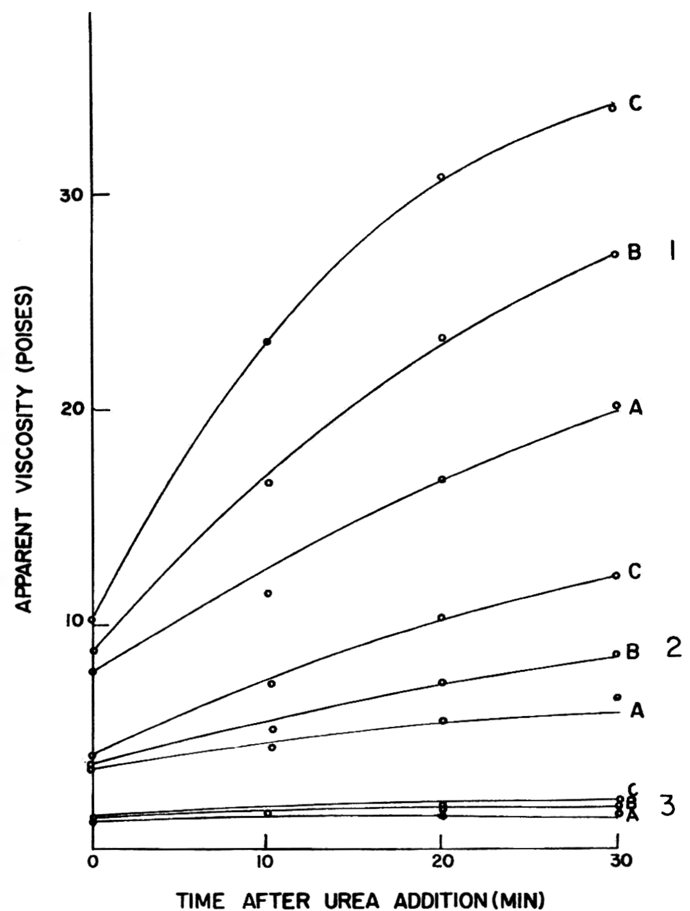

Fig. 4–Viscosity changes after urea addition (0.25 mole/100 g) to yolk containing 1: 5% (w/w) thin albumen, 2: 10% thin albumen, 3: 20% thin albumen; albumen containing yolk was A: unheated, B: heated at 60°C for 3 min, C: heated at 62.8°C for 3 min.

and then dropped to a minimum of 25 poises in 475 sec of heating.

Addition of thin albumen with a solids content of 12% to native yolk brought about a considerable decline in the yolk viscosity. As shown in Figure 2, the viscosity of yolk containing 5% albumen (50.9% solids) had a maximum value of 9.5 poises with a shear rate of 1.9 sec^{-1} as compared with a maximum viscosity value of 23 for native yolk. The viscosities of yolk with levels of 5, 10 and 20% thin albumen differed considerably at each of the shear rates. Yolk with 5, 10 and 20% thin albumen had 50.9, 48.8 and 44.7% solids, respectively. As shown in Figure 2, the viscosities of both unheated and heated albumen-containing yolk samples were still dependent on shear rate. Heat treatment of yolk with 10 and 20% thin albumen did not have any appreciable influence on the viscosity at specific shear rates. When Payawal et al. (1946) heated whole egg having about 60% albumen to temperatures of 62.5°C and higher, the viscosity increased with heating time. In our study, a considerable viscosity difference existed between the 62.8°C-treated yolk with 5% albumen and corresponding unheated yolk-albumen mixture (Fig. 2).

It is evident that upon dilution of yolk by thin albumen having 88% water, the heat damage to the yolk system as reflected by viscosity increase was reduced. With a lower concentration of particulate matter in the yolk with 10 and 20% albumen, there would be less chance for particle-particle interaction during the heat treatment.

According to Powrie et al. (1963), addition of urea to frozen-thawed yolk caused a marked increase in viscosity during a short holding period. Apparently the freezing process brought about structural changes of yolk constituents, presumably lipoproteins, to the extent that they were rendered more susceptible to urea damage than constituents in unfrozen yolk. Yolk, becoming more viscous with heat treatment at 60°C and above, must undergo microstructural changes at these temperatures. It was of interest to examine the influence of urea, a hydrogen-bond breaker, on the viscosities of native and heat-treated yolk samples. Preliminary studies indicated that upon addition of 0.25 mole urea per 100 g native or heat-treated yolk, the viscosities were far too high to obtain a reading on the Wells-Brookfield microviscometer. Thus, the Brookfield spindle viscometer with spindle No. 6 rotating at 10 rpm was employed for determining the viscosity of about 100 ml of a yolk-urea sample at 25°C at various holding times.

Large amounts of heat-treated yolk for these urea studies were prepared with the laboratory pasteurizer. A concentration of 0.25 mole of urea per 100 g of yolk was selected because, at this concentration, Powrie et al. (1963) had found that the viscosity of the urea-treated native yolk increased only gradually with treatment times up to 120 min and, thus, a more rapid viscosity change attributable to thermal damage to yolk constituents could be measured.

As shown in Figure 3, native and pasteurized yolk with no urea treatment (zero time) had viscosities in the range of

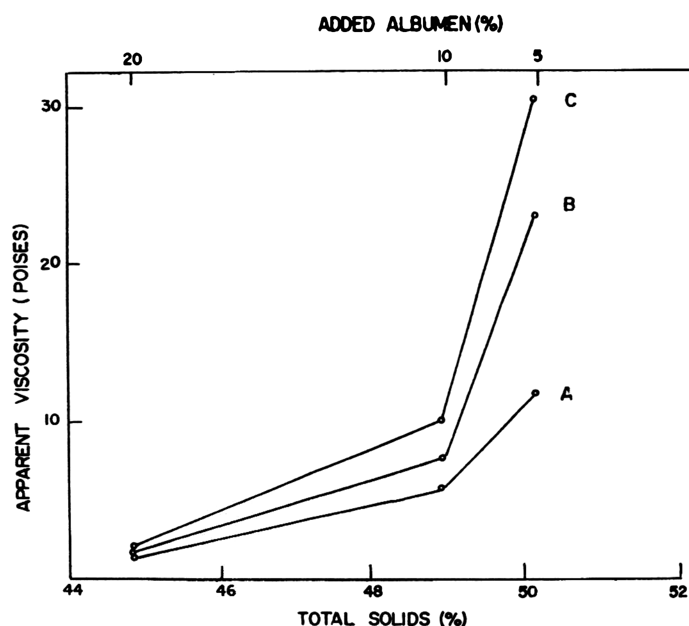

Fig. 5–Relationship of percent of solids content of native and heated yolk containing different percents of thin albumen to viscosity 20 min after addition of urea (0.24 mole/100 g). A: unheated, B: heated at 60°C for 3 min, C: heated at 62.8°C for 3 min.

between about 28 and 40 poises. Urea treatment of these samples caused a marked increase in the sample viscosities, differing widely from each other. With a higher temperature of heat treatment of yolk, the viscosity of the urea-yolk system at any given treatment time was higher. As the holding time progressed, the divergence of viscosity values for the various urea-yolk samples was increased. At 60-min holding time, the viscosities for native yolk and yolk pasteurized at 60, 62.8 and 64.4°C were 192, 269, 317 and 484 poises, respectively. Apparently, the susceptibility of the yolk components to urea damage was increased as the heat-treatment temperature increased.

Since commercial yolk contains some albumen, experiments were carried out with yolk containing 5, 10 and 20% thin albumen. The Wells-Brookfield microviscometer was used for these experiments, since the viscosities were within the range of the instrument. Data on the viscosity increase of albumen-containing yolk with added urea is presented in Figure 4.

Since the small viscosity differences between native and heat-treated yolk samples with 20% albumen (Fig. 2) were indicative of insignificant microstructural changes, one would not expect any appreciable viscosity increases in the respective samples with added urea. Indeed, the viscosity curves of these urea-yolk samples (curves III A, B and C in Fig. 4) remained almost level. On the other hand, the viscosity increase of the urea-treated yolk with 5 and 10% albumen was related to the temperature of heat treatment. As the urea treatment time increased to 20 min, the curves for the 5% and 10% albumen samples diverged from each other.

Figure 5 shows the relationship of the viscosity of the heat-treated albumen containing yolk at 20 min after urea addition versus percent total solids of these albumen-yolk samples. It is obvious from this figure that a more meaningful relationship was obtained when the total solids of the albumen-containing yolk increased towards 50%.

On the basis of the above findings, a rapid method may be developed for testing the adequacy of pasteurization of yolk and yolk with 10% albumen or less. The ratio of the viscosity of a urea-treated sample to the viscosity of the native sample with a known solids content may be useful for obtaining an arbitrary pasteurization value.

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Ms. received 4/11/69; revised 5/11/70; accepted 5/12/70.

This investigation was supported in part by Public Health Service Grant No. UI-136-05 from the National Center for Urban and Industrial Health.

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TEMPERATURES IN EGG PRODUCT FOAMS DURING VAT PASTEURIZATION

SUMMARY—Most egg products are pasteurized in continuous-flow equipment, but small quantities are more economically processed in a vat pasteurizer. By insulating itself, the surface foam in the vat process may be below pasteurization temperature for a significant part of the holding time and salmonellae in the foam may survive. Using a 100-gal vat, we generated foams on 5 egg products and measured foam temperatures during pasteurization. Maximum jacket temperature was held to 160°F and no product burn-on was observed in the vat. All products were held above their respective pasteurization temperatures for 30 min. The minimum temperature of a 6.5-in. liquid whole egg foam was below pasteurization temperature for 11 min after start of pasteurization. The delayed heating effect of a 1-in. foam on sugared yolk (10% sucrose by weight) was 13 min. Yolk and salted yolk (10% salt by weight) foams were below liquid temperature during heating; however, in both cases liquid and foam reached pasteurization temperature simultaneously. Minimum temperature of plain egg white foam (pH 9.0) did not reach pasteurization temperature during the holding period. In vat pasteurization, the holding time begins when the liquid reaches pasteurization temperature, and the holding time should be long enough for both foam heating and pasteurization of foam. Except for egg white, about 15 min was required to heat foam to pasteurization temperature. Adding 15 min for pasteurization of the foam yielded a total holding time of 30 min, with the temperature of pasteurization established on the basis of the 15-min holding time for the foam as follows: liquid whole egg, 133°F; yolk and yolk blends, 135°F; sugared and salted yolk, 139°F. With egg white, foam heating times were in excess of 30 min, and vat pasteurization, with a holding time of 30 min, was not effective.

INTRODUCTION

DEVELOPMENT of processes for pasteurization of liquid egg products has been limited to high-temperature, short-time (HTST) pasteurizers. These operate in continuous flow, with capacities ranging from 300–10,000 gal/hr. Because of product losses, the smallest HTST unit cannot be operated efficiently on egg lots of less than 300 gal. Small quantities are more economically processed in a vat pasteurizer.

The vat pasteurizer is operated at lower temperatures and longer holding times than HTST units, and will produce an equivalent lethal effect on the liquid whole egg without harming functional properties (Brant et al., 1968). Heating and cooling liquid whole egg in the vat is feasible (Walters et al., 1968), and at least 1 commercial unit is available (Ciani, 1967).

The potential problem in vat pasteurization is foam heating, and Brant et al. (1968) found that air-space heating with steam was necessary for acceptable inactivation levels of salmonellae in liquid product. With dye tests, they demonstrated that foam was continuously forming, decaying and inoculating the liquid product with viable salmonellae.

Egg foams can cover ¾ths of the liquid surface in depths up to 3 in. (Patterson, 1967) and the only way a foam can be heated is by conduction of heat through the foam from the hot product below and the hot air space above. The coolest portion of the foam may occur about midway between the top and bottom foam surfaces. This part of the foam may be below pasteurization temperature for a significant part of the holding time, despite air-space heating. If this occurs, salmonellae in the foam may survive the process.

Our objective was to measure temperature distributions in foam during vat pasteurization of egg products and to develop process times and temperatures to ensure that every particle of liquid and foam receives a lethal treatment equivalent to the HTST processes.

MATERIALS & METHODS

Products

All egg products were unpasteurized with no history of freezing and were obtained from Ballas Egg Products Corporation, Zanesville, Ohio. The salted yolk (10% salt by weight) and sugared yolk (10% sucrose by weight) were prepared by the same organization. The liquid whole egg, yolk and white were unaltered.

Pasteurizer

The pasteurizer was a 100-gal vat (Cherry-Burrell, Model No. SF 100) with air space heater, recirculating hot-water jacket and automatic temperature control. The agitator operated at 48 rpm and the blade was 3 in. wide and 21 in. long. The agitator shaft was mounted in a position 8° from vertical and the bottom edge of the blade was about 1 in. from the bottom of the tank.

Test procedure

About 70 gal of raw product were pumped from refrigerated storage to the pasteurizer. A foam was generated on the liquid surface by pumping product from the pasteurizer, metering air into the suction side of the pump and discharging the aerated product into the pasteurizer (Fig. 1). A 2-hp centrifugal pump was used (Cherry-Burrell Corp., Model VAH). Foam thickness and liquid depth were recorded and a sample of foam (100 ml) taken for measurement of density. Jacket temperature was held to 160°F to prevent burn-on of product (Patterson, 1967). Air space steam flow was adjusted to maintain air space temperature at least 5°F higher than product temperature during heating and holding. When the temperature of the liquid reached pasteurization temperature, a holding period of 30 min was begun. At the end of holding, the vat lid was raised for measurements of foam thickness and foam density.

Pasteurization temperatures were as follows: liquid whole egg, 133°F; plain egg white (pH 9.0), 127°F; yolk, 135°F; sugared yolk (10% sucrose by weight), 139°F; salted yolk (10% salt by weight), 139°F. In each case, the product was heated to a temperature slightly higher than these, to ensure that the liquid temperature did not fall below pasteurization temperature during the holding period.

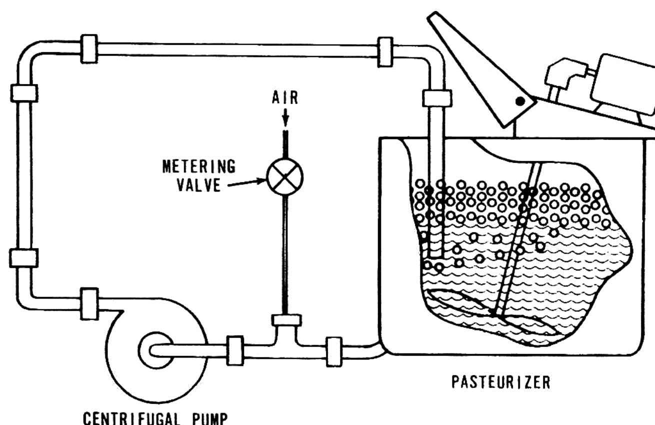

Fig. 1 Apparatus for generating foams.

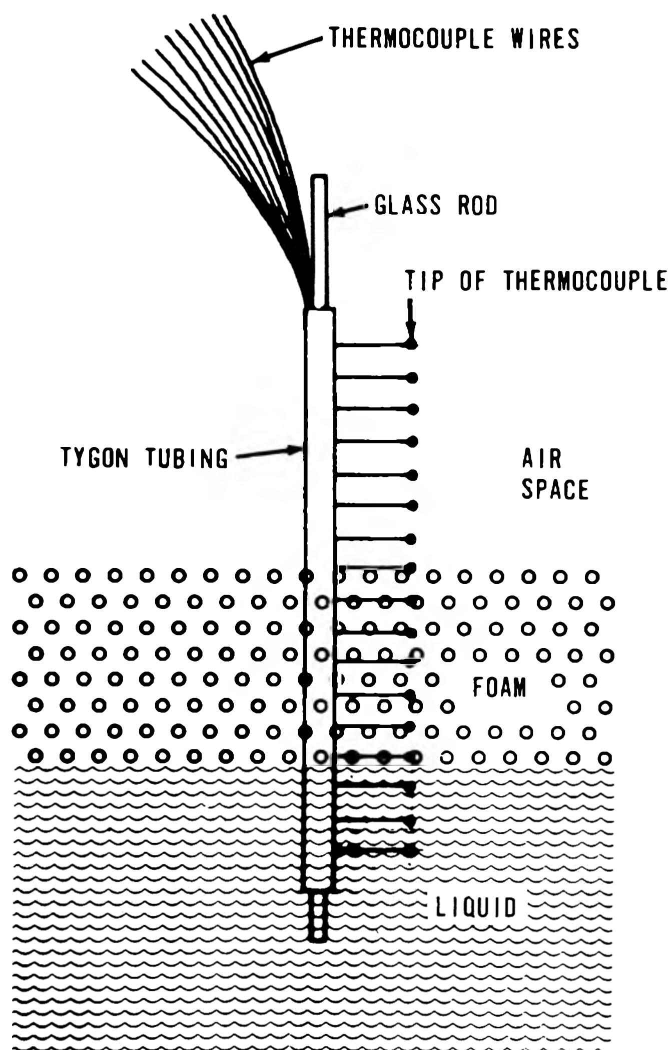

Fig. 2—Rake of thermocouples positioned to measure temperature of the air space, foam and liquid product.

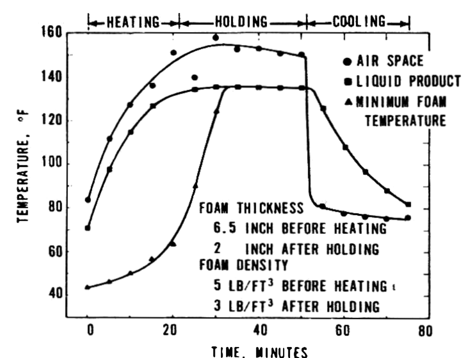

Fig. 3—Minimum temperature in foam during vat pasteurization of liquid whole egg.

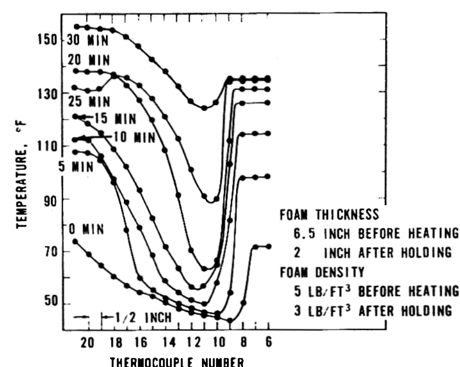

Fig. 4—Temperature distributions in foam during vat pasteurization of liquid whole egg.

Analytical procedures

A rake of 17 thermocouples spaced 0.5-in. apart was installed in the vat and adjusted to measure temperature of the heated air space, foam and liquid product (Fig. 2). The thermocouples were fabricated from No. 20 (American wire gauge) copper-constantan wire (0.032-in.-diamater) and were installed on the rake so that the temperature-sensitive tip was at least 2 in. away from the supporting structure. The supporting structure was a borosilicate glass rod, ¼-in.-diameter, and the thermocouple wires were held in place with vinyl tubing. Temperatures were recorded with a 24-point strip-chart recorder (the Bristol Company) at the rate of 1 point per sec. The thermocouples were calibrated with a National Bureau of Standards certified thermometer, at a temperature of 135°F, and differed by less than ± 0.5°F.

Foam densities were obtained from the weight of a known volume of foam. Foam thickness was obtained by measuring the distance from the top rim of the vat to the top of the foam and to the liquid product surface. The liquid surface was completely covered with foam and its location measured with a rubber ball that sank through the foam and floated on the liquid. The measurement was verified by observing the depth of liquid in a 1.5-in.-diamater glass standpipe connected to the discharge port at the bottom of the vat.

RESULTS

THE MOST prominent characteristic of egg product foam was a lack of reproducibility of foam thicknesses and heating rates. Using liquid whole egg that had been pasteurized in a previous unsuccessful attempt to generate a stable foam, we obtained a 6.5-in. foam that was very stable. The liquid whole egg and foam were pasteurized (Fig. 3) and minimum temperature of the foam was below pasteurization temperature for 11 min after start of pasteurization. Using a fresh supply of raw liquid whole egg, we developed only a 1.2-in. foam, despite our efforts to generate more. The delayed heating effect of the 1.2-in. foam was 2.5 min. In contrast to this, Patterson (1967) made no attempt to generate foams during his processes and observed foams on liquid whole egg up to 3 in. in depth. Deeper foams occurred with the raw product.

The minimum temperature in foam from liquid whole egg was significantly lower than the temperature of the liquid product or the air space (Fig. 4). The location of minimum foam temperature was closer to the liquid, and this was consistent with our observation of dense foam near the liquid surface and lighter foam near the air space (dense foams are more difficult to heat). During processing, foam thickness decreased from 6.5 to 2 in., and density decreased from 5–3 lb/ft³, suggesting considerable liquid drainage from the foam. This is demonstrated in Figure 4, where only 2 thermocouples (No. 6 and 7) were submerged in liquid at the start of heating and 4 (No. 6, 7, 8 and 9) submerged at start of holding, as evidenced by a constant temperature.

A 3-in. foam on egg yolk exhibited some delayed heating (foam temperature was 20°F below liquid temperature after 20 min of heating), but liquid and foam reached pasteurization temperature simultaneously.

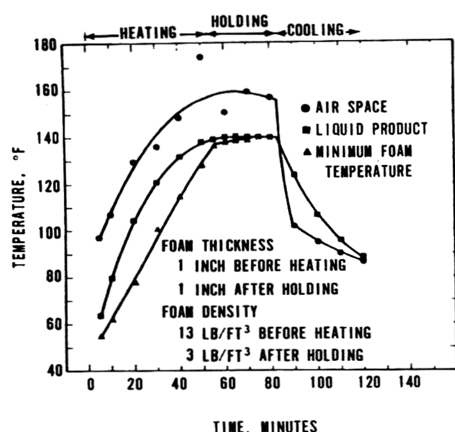

Fig. 5–Minimum temperature in foam during vat pasteurization of sugared yolk (10% sucrose by weight).

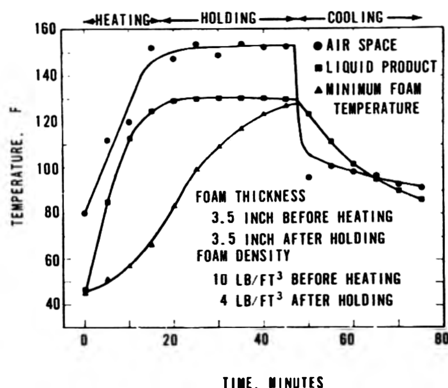

Fig. 6–Minimum temperature in foam during vat pasteurization of plain egg white (pH 9.0).

Table 1–Holding times and temperatures for vat pasteurization of egg products.

Product	Temperature (°F)	Time (min)
Whole egg	133	30
Yolk	135	30
Yolk blends	135	30
Sugared yolk (10% sucrose by weight)	139	30
Salted yolk (10% salt by weight)	139	30

Salted yolk (10% salt by weight) foamed less readily than yolk or liquid whole egg; we obtained only 0.5 in. with our foaming procedure. There was a slight delay in heating of foam (foam temperature was 5°F below liquid temperature after 30 min of heating), but liquid and foam reached pasteurization temperature simultaneously.

Sugared yolk (10% sucrose by weight) foamed more readily than salted yolk, and we obtained a 1-in. foam. While the foam thickness was not great, the minimum foam temperature was below pasteurization temperature for 13 min after start of pasteurization (Fig. 5). Unlike liquid whole egg, which achieved pasteurization temperature precipitously, the sugared yolk foam approached pasteurization temperature very slowly over the last 2°F. If pasteurization temperature had been 2°F lower, the delayed heating effect would have been only 6 min instead of 13 min, further evidence of the inconsistent nature of egg foams.

The foam from plain egg white (pH 9.0) was the most stable. Minimum temperature of a 3.5-in. foam did not reach pasteurization temperature during the entire 30-min holding period (Fig. 6). At the start of the holding period, foam temperature was 61°F below pasteurization temperature and, at the end of the holding period, it was 1°F below pasteurization temperature. Based on inactivation data for *Salmonella typhimurium* TM-1 (unpublished), the lethal effect of the process was 13 D in the liquid but only 1.2 D in the foam. (D = one log reduction of survivors.)

Heating and cooling times for liquid whole egg were about the same as those of Walters et al. (1968), who reported heating times from 23 min for a horizontal-coil pasteurizer to 32 min for a swept-wall pasteurizer. Heating times with our impeller-agitated pasteurizer were 22 min for liquid whole egg, 44 min for yolk, 53 min for sugared yolk (10% sucrose by weight) and 81 min for salted yolk (10% salt by weight). Temperature of the water jacket was maintained at 160°F and no product burn-on was observed in the vat.

DISCUSSION

BECAUSE of the unpredictable nature of egg foams, we were unable to reproduce foam heating results; consequently, no single result can be taken as the worst possible delayed heating effect of foam. In practice, foams on liquid whole egg in the vat are rarely greater than 3 in. (Patterson, 1967) (K. R. Girton, personal communication, 1968), and the results for our 6-in. foam are believed to be conservatively applicable to the occasional severe condition for liquid whole egg. With the exception of egg white, foams on all the products were heated to pasteurization temperature within 15 min after the liquid had achieved pasteurization temperature.

Because of the delay in heating foam, holding times for products other than egg white should be at least 30 min, to allow time for pasteurization of the foam after it has reached holding temperature. For egg white, more than 30 min is required to heat the foam to pasteurization temperature. The holding time would be 50–60 min and, consequently, vat pasteurization of egg white, with a holding time of 30 min, is not effective and should not be used.

In vat pasteurization, the holding time begins when the temperature of the liquid reaches pasteurization temperature. Therefore, the holding time should be long enough for both foam heating and pasteurization of foam. The first 15 min of the holding time is assigned to foam heating, to ensure that every particle of foam is heated to pasteurization temperature. The remaining 15 min of holding satisfies the pasteurization requirement. On this basis, processing temperatures for vat pasteurization of egg products were obtained (Table 1) by interpolating the existing HTST standards (U.S. Public Health Service, 1970) from holding times of 1.75–15 min, using a z value of 8°F (Anellis et al., 1954). The z value is the number of °F change in process temperature that produces a 10-fold change in process time.

The 30-min holding time could be increased or decreased by changing process temperature, but the first 15 min of the process is required to heat the foam to pasteurization temperature and cannot be shortened without endangering the public health value of the process. Therefore, the total time that the liquid is held at pasteurization temperature should never be less than 15 min plus the holding time required to inactivate salmonellae in the foam.

Effect of liquid heating rate

The egg product foams are heated by conduction heat transfer, and the heating rate is determined by thermal properties and thickness of the foam. The rate of liquid heating has a small effect, but for a given foam, total heating time will not vary appreciably. This means that a reduction in heating time of the liquid must be compensated by an increased portion of the holding time required for foam heating. Consequently, the lethal effect on foam, of any vat process, is dependent upon the rate of heating the liquid product and a minimum liquid heating time must be identified.

Using the foam-heating rates reported here, and thermal inactivation data for *Salmonella typhimurium* TM-1 in the various egg products (unpublished), we computed the minimum acceptable liquid-heating time for each product. The heat treatments shown in Table 1 will ensure that every particle of foam is exposed to at least a 6 D process, provided that heating times of the liquid are no shorter than 2 min for liquid whole egg, 28 min for yolk and 36 min for sugared yolk. The heating time of salted yolk is not significant, because of

the lack of foam. Any holding time could have been selected for salted yolk; to be consistent with the other products we recommended 30 min. We concluded that the computed minimum liquid-heating rates represent a practical limitation of equipment available for vat pasteurization. Accordingly, a requirement of minimum heating time of the liquid is not necessary, provided the holding times and temperatures of Table 1 are used.

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Ms. received 5/21/69; revised 7/17/70; accepted 7/20/70.

Mention of company or product names implies neither endorsement nor criticism by the Department of Health, Education, and Welfare.

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RHEOLOGY OF FRESH, AGED AND GAMMA-IRRADIATED EGG WHITE

SUMMARY—Rheology of unmixed egg white at 2°C was studied with a narrow-gapped rotational viscometer over a 20-fold range of shear rates. For a constant shear rate, egg white consistency decreases with time and approaches an equilibrium value in a few minutes. Flow behavior is pseudoplastic at 2°C between shear rates of 8.1–147 sec^{-1} and the shear stress-shear rate relation is accurately described by the power law and Casson models. The effects of gamma irradiation dose on Haugh unit score and equilibrium shear stress are discussed.

INTRODUCTION

VISCOUS properties of fluid food materials govern the design of flow systems, heat exchangers and mixing equipment. Most liquid foods are non-Newtonian (Charm, 1960, 1963; Harper and Lebermann, 1964; Harper and El Sahrigi, 1965; Longree et al., 1966; Watson, 1968); that is, the consistency changes as the shearing rate is varied. In such cases single-point viscosity measurements provide little useful information. Modern viscometers (Van Wazer et al., 1963) can characterize liquid foods over a wide range of shear rates to provide data needed for engineering design of food-handling equipment.

Three hundred million pounds of egg white are processed annually on this continent (Forsythe, 1968). Processing operations include pumping, mixing, heating, cooling and spray drying—all influenced by the viscous properties of egg white. Despite the apparent need for rheological data on egg white, few are available. Smith (1934) used a capillary viscometer to compare consistency of egg white samples subjected to different storage conditions. A torsion pendulum was used by Atanasoff and Wilcke (1937) and later by Rowan et al. (1958) to estimate viscous behavior of the contents of intact eggs. Kaufman et al. (1968) cited unpublished data of another worker in which viscosity of commercial unfrozen egg products at pasteurization temperatures did not change greatly with changes in shear rate. However, in a recent study of egg white between 10 and 40°C at shear rates of 220–3140 sec^{-1}, Tung et al. (1970) found that apparent viscosity decreased with increasing shear rates.

Shell eggs and liquid egg white have been subjected to ionizing irradiation for pasteurization or sterilization without thermal degradation of the egg white proteins. Apart from the destruction of micro-organisms, high-energy irradiation is reported to cause thinning of the egg white as indicated by a reduction of Haugh units (McArdle et al., 1954; Desrosier et al., 1955; Parsons and Stadelman, 1957) and a decrease in viscosity measured by a capillary viscometer (Ball and Gardner, 1968). In view of the possible non-Newtonian nature of egg white, a single-point viscosity determination may fail to characterize the change in rheological behavior brought about by irradiation.

Although thinning of egg white due to aging is well known, rheological changes in egg white during storage have not been documented. This paper describes a study on the viscous properties of egg white from fresh, aged and gamma-irradiated eggs.

EXPERIMENTAL

FRESH eggs were cooled to 4°C, weighed, broken out on to a level glass plate and the albumen height measured with a tripod-mounted dial micrometer. Haugh units were determined using an interior quality calculator (Brant et al., 1951). Yolks were discarded and the egg whites saved in plastic containers labelled with the corresponding Haugh score. When several eggs had been processed in this manner, egg whites of similar Haugh score were paired up and combined to provide a sample of sufficient volume for viscometric study. The average of the 2 Haugh units was taken to represent the quality of the sample. No attempt was made to blend thick and thin fractions of the egg white.

Another lot of eggs was stored at room temperature for 2 to 4 weeks, then cooled, measured for Haugh units and the egg whites saved as previously described.

A third group of fresh eggs was subjected to gamma irradiation at a dose rate of about 1.1 Mrad per hour in a cobalt-60 gamma cell. 3 samples were taken from eggs receiving each of the following radiation doses: 0.02, 0.04, 0.06, 0.08, 0.10, 0.15, 0.20, 0.25 Mrad, and 5 samples from eggs receiving 0.50 Mrad. The eggs were packed in crushed ice to maintain consistent temperature conditions during irradiation. Following this treatment, Haugh units were determined and egg whites separated and saved. The objectives in testing egg white from irradiated eggs were to determine the effect of irradiation on egg white rheology and to compare their viscometric behavior with eggs whose quality (as measured by Haugh units) has been lowered by aging.

The entire experiment consisted of 22 fresh, 25 aged and 29 irradiated egg white samples.

Viscosity measurements

Viscous behavior of all egg white samples was measured with a Haake Rotovisco (described by Van Wazer et al., 1963) at 6 different shear rates ranging from 8.1–147 sec^{-1} using an MVI spindle. Calibration constants for the spindle were established during the experiment, using oil viscosity standards. The shear stress signal from the viscometer was measured on a 10-in. strip-chart recorder with a full-scale deflection corresponding to a shear stress of 152 dynes/cm^2. The sample temperature was held at 2.0 ± 0.5°C during the test by an ice-water supply circulating through the water jacket surrounding the sample cup.

Table 1—Shear stress decay studies. Mean values and "t" test results.

	Fresh (n = 20)	Aged (n = 15)	Irradiated (n = 29)
Haugh units	80.32^{a} [1]	43.47^{b}	34.98^{b}
τ_e, dyne/cm^2	28.18^{a}	26.27^{a}	17.60^{b}
A_1, dyne/cm^2	181.9^{a}	56.82^{b}	33.97^{c}
B_1, dyne/cm^2	81.94^{a}	16.14^{b}	8.761^{b}
A_2	1.887^{a}	0.967^{b}	0.389^{c}
B_2, sec^{-1}	0.0170^{a}	0.0120^{b}	0.0145^{b}

[1] Means with different superscripts are different ($P \leq 0.05$).

Table 2—Flow behavior studies. Mean values and "t" test results.

	Fresh (n = 22)	Aged (n = 25)	Irradiated (n = 29)
Haugh units	81.05^{a} [1]	47.60^{b}	34.98^{c}
K, dyne sec^n/cm^2	1.908^{a}	1.586^{a}	1.197^{b}
n	0.563^{a}	0.599^{a}	0.558^{a}
k_0, dyne$^{1/2}$/cm	1.588^{a}	1.404^{a,b}	1.259^{b}
k_1, (dyne sec)$^{1/2}$/cm	0.324^{a}	0.338^{a}	0.248^{b}

[1] Means with different superscripts are different ($P \leq 0.05$).

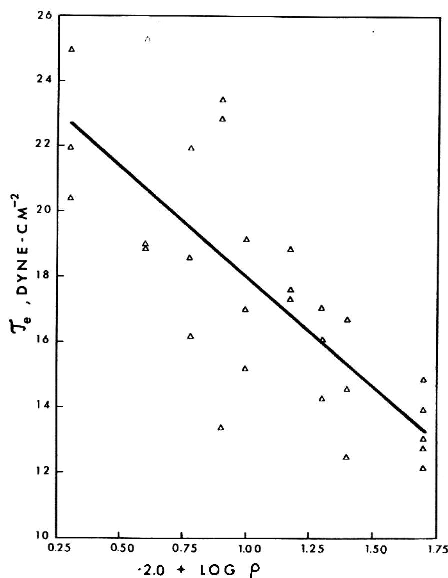

Fig. 1—Equilibrium shear stress as a function of gamma irradiation dose.

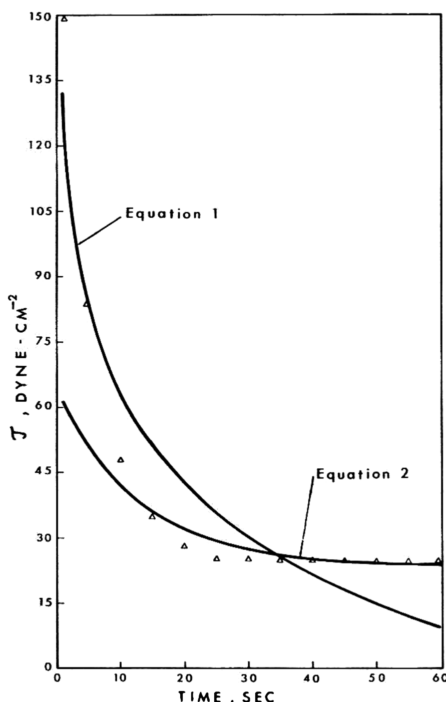

Fig. 2—Shear stress decay data for 1 sample showing least-squares fits of Equations [1] and [2].

Preliminary tests (Tung et al., 1970) at a constant rate of shear revealed a decreasing shear stress that reached an equilibrium value after a few minutes. Subsequent tests on the same sample produced similar results, hence egg white appears to be a thixotropic material. This time-dependent property was studied by recording the shear stress decay curve at the highest rate of shear (147 sec^{-1}). After the equilibrium value was reached, shear stresses were recorded for each of the 6 shear rates in a decreasing series, followed by the increasing series such that 2 readings were made at each rate of shear.

Analyses of data

Shear stress decay during the initial minute of shearing was studied by means of the shear stresses calculated from strip-chart readings taken at 5-sec intervals. The initial reading was made 1 sec after shearing began because of the limitation imposed by recorder response time. This measurement also required a correction due to the inertia of the spindle. Equilibrium shear stress was taken to be the value at which the stress remained constant for at least 5 min. Complete sets of shear stress decay data were obtained for 64 egg white samples sheared at 147 sec^{-1}. 2 mathematical models were fitted to each set of data:

$$\tau = A_1 - B_1 \log t \quad [1]$$

$$\text{and } \log(\tau - \tau_e) = A_2 - B_2 t \quad [2]$$

where τ is shear stress in dynes/cm^2, τ_e is equilibrium shear stress in dynes/cm^2, t is time in seconds and A_1, A_2, B_1 and B_2 are constants with the appropriate units. These relationships were proposed by Weltmann (1943) and Hahn et al. (1959), respectively, to describe thixotropic behavior of several fluid systems.

2 models were fitted to the shear stress-shear rate data of all 76 egg white samples:

$$\tau = K \dot{\gamma}^n \quad [3]$$

$$\text{and } \sqrt{\tau} = k_o + k_1\sqrt{\dot{\gamma}} \quad [4]$$

where $\dot{\gamma}$ is shear rate in sec^{-1} and K, n, k_o and k_1 are material constants with appropriate units. Equation [3] is the well-known power law in which the constants, K and n are called the consistency index and flow behavior index, respectively. Casson (1959) proposed the relation in Equation [4] for the rheological behavior of suspensions containing particles capable of forming chain-like aggregations. These equations have been used to describe the flow of food materials (Charm, 1963; Harper and El Sahrigi, 1965) and blood (Merrill et al., 1965; Fukada and Kaibara, 1968) over a moderate range of shear rates.

Coefficients in Equations [1] to [4] were evaluated for each sample and also for pooled data of the fresh, aged and irradiated groups. Data from the 3 groups were subjected to "t" tests to determine whether mean values of the various parameters differed significantly.

The relation between egg quality and irradiation dose was studied in the 29 gamma-irradiated samples. Simple regressions of all combinations of linear, logarithmic and hyperbolic functions of Haugh units and irradiation dose were computed to find the model most accurately describing this relation. The equlibrium shear stress-gamma dose data were also examined in this manner.

RESULTS & DISCUSSION

MEAN values of the coefficients in Equations [1] and [2] are presented along with results of "t" tests in Table 1. Egg white quality of aged and irradiated samples as measured by Haugh units did not differ significantly ($P \leqslant 0.05$), although both were of lower quality than the fresh egg samples. Coefficients A_1 and A_2 reflect initial shear stresses as shearing begins. It is evident that aging and gamma irradiation caused a breakdown in egg white structure and that under the conditions of this experiment, the irradiation effect is more severe. Rates of structural breakdown, indicated by B_1 and B_2, prove to be greater for fresh egg white than for either aged or irradiated samples.

Equilibrium shear stresses in fresh and aged samples were essentially the same, whereas the lower value for irradiated samples indicates a breakdown in excess of that caused by viscometric shearing. Increasing levels of gamma irradiation significantly ($P \leqslant 0.01$) reduce the equilibrium shear stress (Fig. 1) according to the relation:

$$\tau_e = 11.31 - 6.73 \log \rho$$
$$(r^2 = 0.60, n = 29) \quad [5]$$

where ρ is the radiation dose in Mrad. This result is not unexpected, in view of the thinning effect of high-energy irradiation discussed in the Introduction.

Shear stress decay data were more accurately described by Equation [1] than by Equation [2] in 48 of the 64 samples. The average coefficients of determination of 0.81 and 0.70, respectively, were significantly different ($P \leqslant 0.05$). In general, both models fitted many of the data reasonably well, either in the initial stage of rapidly falling shear stress or as the equilibrium shear stress was approached. This is illustrated by the curves in Figure 2. Perhaps a more valid method of sampling data from the recorder chart would be that of Cramer and Marchello (1968), in which data were sampled at an interval such that the shear stress changed by no more than a specified fractional amount. Such a sampling procedure would preserve data at all positions in the curve more effectively than the method of the present study, which used equal intervals of the independent variable (time) as the sampling basis.

Mean values of flow behavior coefficients are shown in Table 2. For the power law function, the average flow behavior index (n) is essentially the same for fresh, aged and irradiated egg white. The consistency index (K) may be used to compare apparent viscosities of fluids with equal flow behavior indices. Average values of K for fresh and aged egg white

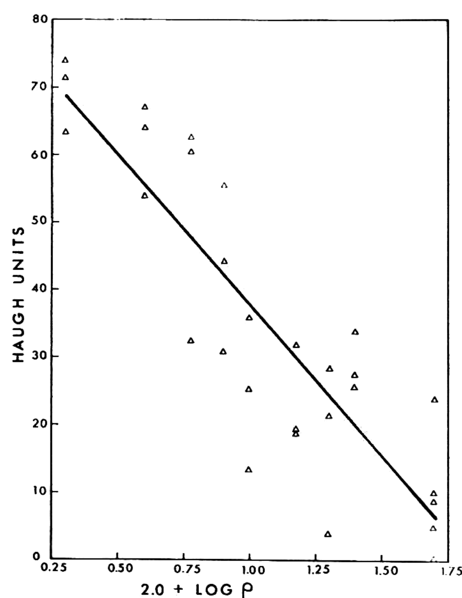

Fig. 3–Haugh unit score as a function of gamma irradiation dose.

were not significantly different ($P \leqslant 0.05$), although both were higher than those of the irradiated samples.

Coefficients of determination for Equations [3] and [4] fitted to the 76 individual sets of data average 0.96 and 0.97, respectively; hence, the power law and Casson models fit these data equally well. It should be pointed out that the rheological data presented herein are based on tests of unmixed egg white at 2°C over a moderate range of shear rates and that extrapolation to dissimilar conditions is not recommended. Additional study is required to characterize viscous behavior over the wide range of conditions encountered in commercial egg white processing.

Gamma irradiation was observed to weaken yolk membranes and reduce interior egg quality as measured by Haugh units (Fig. 3). The equation describing the effect on Haugh score is:

$$HU = -6.73 - 44.6 \log \rho$$

$$(r^2 = 0.76, n = 29) \qquad [6]$$

where HU represents Haugh units. The average Haugh score for 14 eggs randomly selected from the group to be irradiated was 80.0. Using USDA standards, the quality of these eggs would be reduced to grades B and C by gamma doses of 0.04 and 0.15 Mrad, respectively.

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Ms. received 8/15/69; revised 1/9/70; accepted 6/29/70.

The authors acknowledge financial support of this project by the National Research Council of Canada and Atomic Energy of Canada Ltd.

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CHEESE COLORS FROM PLANT SOURCES. 1. Preparation and Properties of Color from Pepper and Safflower

*SUMMARY—A cheese color was prepared from the extract of pepper (*Capsicum frutescens *var. California wonder) and safflower* (Carthamus tinctorius) *pigments. The prepared color had a good keeping quality, slightly affected by temperature and sunlight. The prepared color proved successful in cheese coloration and resisted the biochemical changes in cheese during ripening. However, a slight loss in the color of cheese was observed during storage.*

INTRODUCTION

ANNATTO colors have been used in dairy products since the early days of the dairy industry. According to food regulations in the U.A.R., annatto colors and carotene are the only coloring which may be added to milk products. However, this does not exclude the possibility of other coloring matters from natural sources proving their safety as food additives.

Previous work by Said and Nada (1946) and El-Shibiny (1964) has shown the possibility of using some local plants, e.g., *Calendula officinalies, Carthamus tinctorious* (safflower) and *Capsicum annuum* var. *grossum* (pepper), as sources of suitable coloring matter for dairy products. These studies, however, were mainly concerned with the preparation of butter colors.

The present paper describes the preparation and properties of a cheese color extracted from pepper and safflower.

MATERIAL & METHODS

Preparation of samples

Fully ripened pepper pods were collected, cleaned and sliced into pieces. They were dried in an oven provided with a forced stream of air and maintained at a constant temperature of 60°C. The dried pepper was then ground to a fine powder.

Air-dried flowers of safflower were obtained from the market. The good flowers were selected, dried and ground as described for pepper.

Color extraction

The color of the fine powder of pepper was completely extracted with ether in Soxhlet extraction apparatus. The ether was removed by distillation, leaving the viscous residue of the coloring matter.

The coloring matter of safflower was extracted several times from the fine powder with distilled water at room temperature. The concentrated extract was kept overnight in a refrigerator and the formed precipitate removed by filtration.

Determination of the color

In the color solution. 0.02 ml of the prepared color was pipetted into a 50-ml volumetric flask and made up to volume with methanol. The optical density was measured at 410 and 510 mμ wavelengths against methanol as a blank.

In cheese. 5 g cheese were ground in a mortar with 10 ml methanol and then filtered into a 50-ml volumetric flask. Extraction was repeated with successive portions of methanol until the extract was free from color. The combined extract was made up to 50 ml with methanol and the optical density measured at 410 and 510 mμ wavelengths, respectively.

Separation and identification of carotenoids from pepper extract

1 g of the dried powder was extracted with ether as previously described and the ether removed by distillation. The residue was hydrolyzed with 5 ml of 1 N alcohol KOH solution at room temperature for 2 days, distilled under vacuum until dried and then extracted with ether. The extract was washed with distilled water until free from alkali, then dried with anhydrous sodium sulfate and ether removed by distillation. The residue was dissolved in 2 ml of 15% ethyl acetate in hexane and then applied to a chromatographic column of alumina 2.5 by 40 cm. Elution was carried out by using 50% ethyl acetate in hexane.

The absorption spectra of the separated fraction in hexane, petroleum ether 70–80°C, chloroform and carbon disulfide were recorded.

Chemical analysis of cheese

The analyses of cheese for moisture and acidity were carried out according to Ling (1956).

EXPERIMENTAL

TO ASSESS the properties of the coloring matter in pepper, separation and identification of its carotenoids were attempted.

7 carotenoids were separated from the ether extract of pepper by column chromatography on alumina, using 50% ethyl acetate in hexane as an eluting system. The separated carotenoids were identified by comparing their absorption spectra in different solvents; namely, hexane, petroleum ether 70–80°C, chloroform and carbon disulfide with those quoted in the literature (Karrer and Jucker, 1950). The following carotenoids were identified according to their descending order from the chromatogram: β-carotene, cryptoxanthin, zeaxanthin, antheroxanthin, violaxanthin, capsanthin and capsurbin.

Out of the separated carotenoids from pepper, capsanthin and capsurbin were present in relatively high concentration, zeaxanthin and β-carotene in moderate concentration and the rest of the carotenoids present as minor constituents.

It is obvious from these results that the carotenoids of pepper were largely hypophasic, as they favor the solubility in alcohols miscible with water. Glycerol was found to be the best solvent to dissolve the pepper pigments, and it had no harmful effect.

As the carotenoids were mainly present as esters (Karrer and Jucker, 1950), they were dissolved in alkaline glycerol which hydrolyzed the esters and made them soluble in water. Therefore, the ether extract of pepper was evaporated to dryness and then a suitable quantity of 2 N KOH in glycerol, about 15 ml, added to 1 ml of the residue. The mixture was left at room temperature for 2 days, after which a clear color solution was obtained.

Preliminary experiments on the coloration of cheese with the prepared color from pepper, however, gave unsatisfactory results. It gave the curd a reddish taint far from the normal golden color given by annatto to cheese.

This could be explained on the basis of the difference in the absorption spectra of the prepared color and those of annatto or the standard cheese color solution (B.S.I., 1954). As the yellow and red colors are absorbed strongly around 410 and 510 mμ wavelengths, the ratio between the absorbance at these 2 wavelengths will be taken as indicative of the ratio of yellow to red shades in the colors used.

The absorbance of standard cheese color solution at 410 and 510 mμ wavelengths had a ratio of 4.0:1, similar to that given in the literature (B.S.I., 1954) for the yellow and red units of the same solution by tintometric measurements; namely, 2.8 and 0.6 units, respectively. The same results were obtained with commercial annatto solution. The absorbance of pepper color at the preceding wavelengths had a ratio of 1:1, which explains the reddish shade (Fig. 1). Therefore, the ratio of red to yellow shade in pepper had to be adjusted to that of annatto, to be suitable for cheese coloration. This was tried by adding to the pepper solution the concentrated extract of safflower characterized by its yellowish taint (El-Shibiny, 1964).

The extract of safflower has negligible absorbance at 510 mμ wavelength (Fig. 1). Thus,

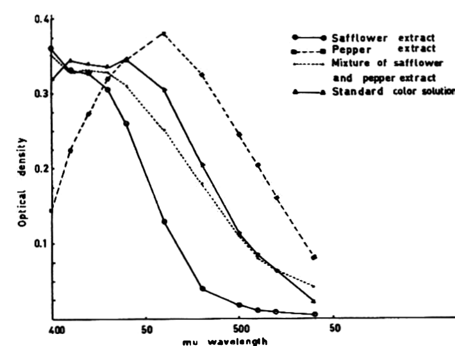

Fig. 1—Absorption spectra of standard color, pepper, safflower, and mixture of pepper and safflower in visible region.

Table 1–Effect of storage on color losses of the prepared cheese color.

	Transparent bottle				Brown bottle			
	At room temperature		In refrigerator		At room temperature		In refrigerator	
	410 mμ (%)	510 mμ (%)	410 mμ (%)	510 mμ (%)	410 mμ (%)	510 mμ (%)	410 mμ (%)	510 mμ (%)
After 7 days	84.5	73.1	90.9	87.5	85.1	74.1	95.0	93.1
After 14 days	72.8	59.9	88.2	83.2	80.8	67.3	85.7	80.9
After 21 days	69.1	66.4	87.0	82.6	74.3	68.6	85.6	80.3
After 28 days	67.4	58.3	86.2	79.6	69.4	60.8	82.0	73.6
After 43 days	60.8	54.6	76.3	67.5	60.1	53.3	76.5	67.2
After 58 days	63.2	59.5	72.0	65.4	58.1	55.9	75.9	67.8
After 88 days	56.7	50.7	68.3	72.5	55.5	50.4	69.6	60.1
After 118 days	47.8	43.4	58.3	50.7	48.1	44.7	59.5	52.2
After 148 days	53.1	50.6	59.5	54.6	50.4	48.9	61.2	53.3
After 178 days	50.7	55.7	59.0	56.3	49.6	50.4	60.7	58.3
After 208 days	41.3	50.7	52.8	50.4	46.3	47.6	55.4	53.9

Table 2–Effect of pH on the prepared color content.

pH Value	Color percent at 410 mμ wavelength	Color percent at 510 mμ wavelength
4	66.0	63.4
5	69.5	75.1
6	72.3	76.2
7	72.7	76.3
8	75.7	76.3
9	83.7	85.5

pH of the original solution was 11.0.

pepper and safflower colors with absorbance of 0.270 and 0.990, respectively, at 410 mμ wavelength were mixed in a ratio of 1:1.5. The mixed color solution had a spectrum similar to that of standard color as shown in Figure 1. The prepared cheese color was slightly opaque; however, it was homogeneous during the storage period.

Effect of storage on stability of the prepared color

Color loss in the new color was slightly affected by both temperature and light. However, the effect of temperature was the more pronounced (Table 1). The rate of loss in yellow and red taints was nearly the same and their ratio was almost constant during storage.

As the pepper pigments form the unsaturated compounds in the prepared color, one would suspect they were responsible for the color loss. However, the rate of color loss in the new color followed a trend similar to that reported by Chen and Gutmanis (1968), for extractable color in Chili pepper. They reported that the rate of color deterioration followed a second-order reaction, which seemed to apply to data of the present work.

Color loss in the different treatments was nearly the same. This may be explained on the basis that pigments of pepper deteriorated much faster in an oxygen-containing atmosphere, irrespective of storage conditions (Chen and Gutmanis, 1968).

Effect of pH

The color content of the prepared color was affected by the pH of the media. Thus, at low pH values the color content decreased, especially in the yellow taint (Table 2).

Use of the new color in cheese manufacture

Suitability of the new color was tested in the manufacture of Derby cheese. In cheese processing, the color was neither affected nor did it interfere with the manufacturing steps. It was well distributed in the curd and it did not impart any disagreeable flavor to the cheese, but there was a small loss of color in whey. The produced cheese had an orange-yellow color comparable to that of annatto.

Persistency of the color in cheese during ripening was followed and recorded in Table 3. Losses in yellow and red taints in cheese colored with new color were slight and consistent.

Table 3–Change in the color of Derby cheese during ripening and storage.

	With new color		With annatto	
	At 410 mμ (%)	At 510 mμ (%)	At 410 mμ (%)	At 510 mμ (%)
After 2 weeks	14.8	8.2	+46.2	+38.7
After 6 weeks	20.8	11.5	+38.5	+33.9
After 10 weeks	24.7	12.1	+18.7	15.8
After 14 weeks	22.1	10.2	+10.4	30.3
After 18 weeks	22.4	9.6	+13.7	28.9
After 22 weeks	32.2	11.9	+ 5.5	53.9

These losses might be explained on the basis of the changes in the developed acidity in the cheese. The cheese colored with annatto showed diversity in the losses of yellow and red taint. Thus, the cheese lost appreciably in its red shade with an increase in the yellow one. Similar results were given by Barnicoat (1937).

DISCUSSION

THE SOLUBILITY of pepper pigments in alkaline glycerol resulted in a color solution easily soluble in an aqueous system. To bring about a yellowish taint in the pigment, the extract of safflower was added.

The new color showed a fairly good keeping quality, since it retained more than half of its color content for 7 months at room temperature. It did not give any precipitate, which was contrary to the annatto colors (Barnicoat, 1937). Data on the color loss suggested that the reaction followed the course of a second order (Chen and Gutmanis, 1968). The rate of color loss depended on its color concentration, being high in the early weeks of storage when the preparation had a high color density and relatively low in the last weeks.

Temperature and sunlight had a slight effect on the rate of color losses. Color losses in the prepared color were largely due to pepper pigments and the spontaneous autoxidation of its color pigments (Chen and Gutmanis, 1968). Therefore, the shelf life of this new color could be improved by hindering the oxidation process by adding antioxidants.

Use of this color in cheese coloration was proven successful. It was evenly distributed in the curd. Besides, the color did not impart any disagreeable flavor or odor to the cheese. The color seemed to resist the biochemical changes occurring during ripening, and the slight loss in its intensity during this period was probably due to changes in the pH of the cheese.

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Ms. received 1/5/70; revised 6/19/70; accepted 7/3/70.

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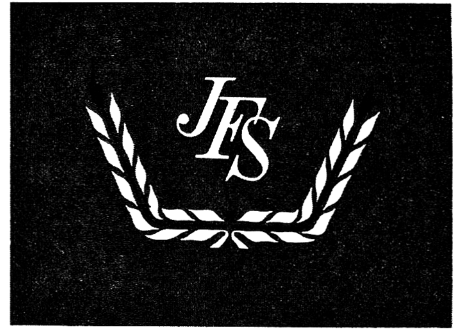

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AN OFFICIAL PUBLICATION OF THE

Institute of Food Technologists

221 North LaSalle Street / Chicago, Illinois 60601 U.S.A.

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Printed in U.S.A.

Institute of Food Technologists

The Institute of Food Technologists is a professional society of scientists, engineers, educators and executives in the field of food technology. Food technologists are professionals who apply science and engineering to the research, production, processing, packaging, distribution, preparation, evaluation and utilization of foods. Individuals who are qualified by education, special training or experience are extended an invitation to join in professional association with the select group of the food industry's scientific and technological personnel who are Institute members. Membership is worth many times its modest cost, reflecting positive benefits, stimulation and opportunities for the individual in his business or profession.

OBJECTIVES

The Institute, as a non-profit, professional, educational society, has several major aims: to stimulate investigations into technological food problems; to present, discuss and publish the results of such investigations, to raise the educational standards of Food Technologists; and to promote recognition of the scientific approach to food and the basic role of the Food Technologists in industry. All of these activities have the ultimate objective to provide the best possible foods for mankind.

ORGANIZATION AND PROGRESS

Organized July 1, 1939, at Cambridge, Mass.. with a membership of less than 100, the Institute has grown to more than 10,000. It is worldwide in scope with almost 2,000 of its membership overseas.

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Members. Any qualified person active in any aspect of food technology.

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Fellows. Any Professional Member who has been active for at least ten years and who has been nominated by the IFT Committee on Fellows for outstanding contributions to the field of food science/technology is eligible to be elected a Fellow of the Institute by the IFT Council.

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Where 25 or more members live within commuting distance of a given point, a regional section may be established. Meetings can be held at more frequent intervals by such groups. Presently, there are 41 regional sections.

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Erratum Notice

Page 312: Summary, 6th line, change to: 0.059 inch
Summary, 7th line, change to: 0.264/1.0 inch taper

Page 313: Second column, 6th line from bottom, change to: at 0.059 inch, varying the taper from

Second column, 5th line from bottom, change to: 0.236/1.000 to 0.500/1.000 inch seemed neither

Second column, last line, change to: needle with a 0.059 inch diameter land and

Third column, 1st line, change to: an intermediate taper of 0.264/1.000 inch of

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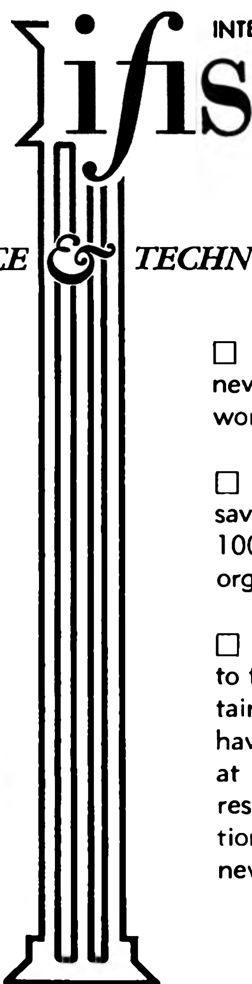

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