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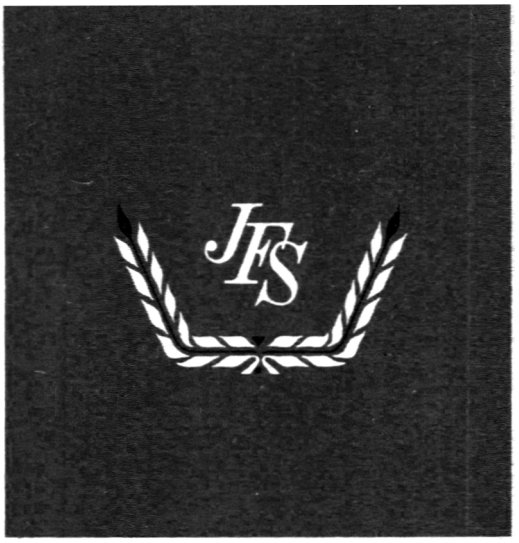

JOURNAL of FOOD SCIENCE

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ABSTRACTS:
IN THIS ISSUE

EFFECT OF DRY AND MOIST HEAT TREATMENTS ON SELECTED BEEF QUALITY FACTORS. D. R. SCHOCK, D. L. HARRISON & L. L. ANDERSON. *J. Food Sci.* 35, 195–198 (1970)–Pieces of SM muscle were deep-fat fried, oven-roasted, oven-braised, pressure-braised to 70°C. Shear, pH, color, flavor, tenderness and over-all acceptability were similar for all treatments. Oven-roasted pieces had slowest heat penetration, longest cooking time, and highest total moisture content, press fluid, water-holding capacity and juiciness, and appeared less well-done than pieces given the other treatments. Rate of heat penetration was correlated with cooking time for all treatments and with cooking losses, moisture, juiciness and apparent degree of doneness for pressure-braising. Tenderness and flavor apparently influenced over-all acceptability scores more than juiciness or apparent degree of doneness.

ON THE NATURE OF ALTERED PROTEIN IN COD MUSCLE STORED AT −29°C AFTER AGING IN ICE. M. L. ANDERSON & E. M. RAVESI. *J. Food Sci.* 35, 199–206 (1970)–Up to 40 percent of protein in addition to that readily extractable was extracted from cod muscle stored in ice and then at −29°C. Ultracentrifugal patterns showed that additional protein extracted was aggregated, polydisperse, and similar in sedimentation behavior to altered protein in cod muscle stored for long periods in ice. Extraction conditions, ultracentrifugal patterns of altered proteins stored in salt solution and microscopic appearance of inextractable residues suggest that some of the linkages responsible for decrease in readily extractable protein were salt-sensitive.

OXIDATION OF PYRUVATE-2-C^{14}, AND LACTATE DEHYDROGENASE ACTIVITY AND ISOENZYME DISTRIBUTION OF FIVE PORCINE MUSCLES. P. B. ADDIS & E. ALLEN. *J. Food Sci.* 35, 207–210 (1970)–Factors associated with oxidative energy metabolism in porcine muscle may influence the rate of postmortem glycolysis. A comparison was made of biochemical parameters of aerobic and anaerobic energy metabolism in dark and light porcine muscle. Oxidative metabolism was estimated by measuring the conversion of pyruvate to CO_2. Results give the following ranking from most to least aerobic: trapezius, gluteus medius, dark biceps femoris, light biceps femoris, longissimus dorsi, dark semitendinosus and light semitendinosus. Activities of lactate dehydrogenase (LDH) and LDH isoenzyme V in light muscle exceeded dark muscle. The results implicated LDH as a factor contributing to rapid postmortem lactate accumulation in light muscles of some porcine animals.

APPLYING PROTEOLYTIC ENZYMES ON SOYBEAN. 2. Effect of Aspergillopeptidase A Preparation on Removal of Flavor from Soybean Products. M. NOGUCHI, S. ARAI, H. KATO & M. FUJIMAKI. *J. Food Sci.* 35, 211–214 (1970)–Applying Aspergillopeptidase A preparation to either soybean curd or defatted soybean flour was effective in removing the flavor compounds and related fatty materials. Significantly larger amounts of *n*-hexanal, *n*-hexanol and volatile reducing substances were removed when the soybean flour was treated with the enzyme at pH 2.8 for 2 hr at 50°C than when it was treated similarly without the enzyme. Larger amounts of the residual fatty materials in the soybean flour, including lipids, genistein, saponins and the like were also removed, especially when the enzyme constituted 1% of the substrate protein. The resulting enzymic digestion product had less odor, taste and color, having more resistance to autoxidation during preservation. Sensory tests supported these results.

APPLYING PROTEOLYTIC ENZYMES ON SOYBEAN. 3. Diffusable Bitter Peptides and Free Amino Acids in Peptic Hydrolyzate of Soybean Protein. M. FUJIMAKI, M. YAMASHITA, Y. OKAZAWA & S. ARAI. *J. Food Sci.* 35, 215–218 (1970)–The bitter flavor which appeared by peptic hydrolysis of soybean protein at 37°C for 24 hr was due to some free amino acids and diffusable bitter peptides such as H·Gly-Leu·OH, H·Leu-Phe·OH, H·Ser-Lys-Gly-Leu·OH, H·Leu-Lys·OH, H·Phe-(Ile, Leu_2)-Gln-Gly-Val·OH, H·Arg-Leu-Leu·OH and H·Arg-Leu·OH. Applying a carboxypeptidase A or a bacterial neutral proteinase on the peptic hydrolyzate of soybean protein was effective in reducing the bitterness.

CORRELATION BETWEEN GAS-CHROMATOGRAPHIC PATTERNS AND FLAVOR EVALUATION OF CHEMICAL MIXTURES AND OF COLA BEVERAGES. L. L. YOUNG, R. E. BARGMANN & J. J. POWERS. *J. Food Sci.* 35, 219–223 (1970)–Two methods of discriminant analysis, Chi-square test, "t" tests and analysis of variance were applied to gas-liquid chromatographic (GLC) data to compare GLC measurements of food volatiles with organoleptic evaluation of flavor. Model systems composed of ethyl butyrate, ethyl heptylate and benzaldehyde were used for one set of trials. A second system consisted of blends of Pepsi-Cola® and Coca-Cola®. The stepwise discriminant analysis (SDA) procedure described by Powers et al. (1968) was only moderately successful in classifying the mixtures when applied to the GLC data for the model systems, but the cola blends could be classified readily. From GLC data, cola blends differing from each other by only 7–10% could be distinguished. A new discriminant analysis program is more useful for predictive purposes. The analysis yields the correlation coefficients for each peak area with sample differences and the weighting factors. Discriminant equations can thus be written. By substitution of measurement values for unknowns, their identity may be predicted. Organoleptic subthreshold additive effects could not be demonstrated with binary mixtures of flavor substances, but judge-compound interaction effects were observed.

COMPARISON OF FLAVOR AND VOLATILES OF TOMATO PRODUCTS AND OF PEANUTS. L. MILUTINOVIĆ, R. E. BARGMANN, K-Y CHANG, M. CHASTAIN & J. J. POWERS. *J. Food Sci.* 35, 224–228 (1970)–The volatiles of canned tomatoes acidified with citric and with malic acid differed significantly from those of control, nonacidified tomatoes and from each other. Tomatoes treated with sugar and acid also differed significantly in volatiles. Blends of tomato juice prepared from various proportions of canned juice and juice reconstituted from tomato powder differed significantly in flavor. Through the application of discriminant analysis to gas-liquid chromatographic data, blends could usually be predicted correctly from the discriminant equations. Peanuts roasted for 12, 16, 20 and 24 min were similarly evaluated organoleptically and gas chromatographically. For the 3 products above, one or a few peaks were generally not sufficient to characterize the sample. When 10–15 peak areas were used to make the discrimination, each of the 3 products could be categorized readily according to treatment.

EFFECT OF FREEZING RATE AND FREEZE DRYING ON THE SOLUBLE PROTEINS OF MUSCLE. 1. Chicken Muscle. C.S. HUBER & W. J. STADELMAN. *J. Food Sci.* 35, 229–232 (1970)–The pectoralis major and the pectoralis minor muscles were obtained from mature White Leghorn hens. 2 freezing methods were employed, immersion in liquid nitrogen and a −10°C air freezer. Proteins were extracted from both raw and cooked muscle tissue. Total protein and the sarcoplasmic proteins were extracted from frozen and freeze-dried samples. Cooking decreased the amount of extractable protein. Freezing rate also influenced the amount of protein extracted from freeze-dried cooked tissue. Protein solubility was slightly higher for samples frozen at −10°C compared to those immersed in liquid nitrogen. Only small changes in protein extractability were observed for freeze-dried raw tissue.

EFFECT OF FREEZING RATE AND FREEZE-DRYING ON THE SOLUBLE PROTEINS OF MUSCLE. 2. Turkey Muscle. C. S. HUBER, R. B. HARRINGTON & W. J. STADELMAN. *J. Food Sci.* 35, 233–236 (1970)–Samples were obtained from the pectoralis major and the pectoralis minor of Large White turkeys. Two freezing methods were employed: immersion in liquid nitrogen and a −10°C plate freezer. Proteins were extracted from both raw and cooked tissue. Total soluble and sarcoplasmic proteins were extracted from frozen and freeze-dried samples. A Stokes Model 21 unit was used to freeze dry the samples. The platen temperature was 52°C. A modification of the biuret test was used for protein determination. Cooking decreased the amount of extractable protein. Freezing rate also influenced the amount of protein extracted from freeze-dried cooked tissue. Protein solubility was slightly higher for samples frozen at −10°C than for those immersed in liquid nitrogen. Only small changes in protein extractability were observed for freeze-dried raw tissue. The amount of extractable protein was slightly higher in samples frozen at the slower rate. This was more evident in the water-soluble fraction.

ANTHOCYANIN PIGMENTS IN RED TART CHERRIES. E. D. DEKAZOS. *J. Food Sci.* 35, 237–241 (1970)–The anthocyanin pigments of red tart cherries (*Prunus cerasus* L., var. Montmorency) were extracted with 0.1% methanolic HCl and partially purified by adsorption on cation-exchange resin, then separated into 7 bands by paper chromatography. The anthocyanins were identified by their R_f values, aglycones, sugar moieties, partial acid hydrolysis and spectral properties. The major pigment was a branched triglycoside, cyanidin 3-glucosylrhamnosyl-glucoside, followed by cyanidin 3-rutinoside. Small amounts of cyanidin, peonidin, cyanidin 3-glucoside, peonidin 3-rutinoside and cyanidin 3-sophoroside were present. No peonidin derivative has been previously isolated from sour cherries. It is the first time that a branched triglycoside has been reported for Montmorency cherries.

QUANTITATIVE DETERMINATION OF ANTHOCYANIN PIGMENTS DURING THE MATURATION AND RIPENING OF RED TART CHERRIES. E. D. DEKAZOS. *J. Food Sci.* 35, 242–244 (1970)–The anthocyanins of red tart cherries *(Prunus cerasus* L. var. Montmorency), at different maturity levels, were extracted with 1% methanolic HCl and the total anthocyanin content of each stage calculated with the aid of a molar-extinction coefficient at 530 mμ. The pigment solution for each stage of maturity was purified by adsorption on AG 50W X 4 cation-exchange resin, concentrated and separated by descending chromatography. The relative intensities of the 7 well-defined pigments were measured photodensitometrically. The individual pigment content was calculated from the total anthocyanin content and the ratio of individual anthocyanin for each stage of maturity. The stage of maturity of the red tart cherry is an important factor influencing the total and individual anthocyanin content in cherries. This work also helps to give a greater insight into the development of anthocyanins in the maturing red tart cherry.

EFFECT OF SMOKING PROCESS ON SOLUBILITY AND ELECTROPHORETIC BEHAVIOR OF MEAT PROTEINS. C. J. RANDALL and L. J. BRATZLER. *J. Food Sci.* 35, 245–247 (1970)–The solubility characteristics and starch gel electrophoretic properties of untreated, heated and heated and smoked pork longissimus dorsi muscle samples were investigated. The percentages of the low ionic strength fraction, the sarcoplasmic and the soluble fibrillar protein nitrogen fractions decreased in the heated and heated and smoked samples when compared to the untreated samples. The myofibrillar protein nitrogen fraction increased in the heated samples and decreased in the heated and smoked samples. The stroma fraction from the untreated to the heated state remained almost constant, but increased considerably in the heated and smoked samples. Electrophoretic studies indicated numerous changes in the sarcoplasmic fraction in the heated and heated and smoked samples.

CHANGES IN VARIOUS PROTEIN PROPERTIES OF PORK MUSCLE DURING THE SMOKING PROCESS. C. J. RANDALL & L. J. BRATZLER. *J. Food Sci.* 35, 248–249 (1970)–Changes in the pH, free sulfhydryl groups, amino nitrogen content and total free amino acids of untreated, heated, and heated and smoked pork longissimus dorsi muscle samples were investigated. Heating and heating and smoking caused changes in the pH, free sulfhydryl and amino nitrogen content of pork samples. An interesting observation was the increase in the myofibrillar protein nitrogen fraction, pH and free sulfhydryl groups of the heated samples, and the decrease of these values in the heated and smoked samples. Results of this study indicated that smoke constituents react with the functional groups of meat proteins.

EFFECT OF SMOKE UPON ACID PHOSPHATASE ACTIVITY OF SMOKED MEAT. C. J. RANDALL & L. J. BRATZLER. *J. Food Sci.* 35, 250 (1970)–The effect of heating and heating and smoking on the acid phosphatase activity of smoked meat was investigated. It was found that heating markedly decreased acid phosphatase activity and that if smoking accompanied heating, a further decrease in activity was noted.

RADIO RESISTANCE OF Byssochlamys fulva ASCOSPORES AS SHOWN BY STORAGE TESTS. G. PARTSCH & H. ALTMANN. *J. Food Sci.* 35, 251–252 (1970)–The radiosensitivity of the ascospores of *Byssochlamys fulva*, strain A 3849, was tested in different fruit juices. The results obtained showed that for grape and apple juice a dose of 220 krads is required to guarantee a minimum storage time of 3 months; for orange juice 250 krads is necessary.

ANTIGENICITY OF SALT-SOLUBLE BEEF MUSCLE PROTEINS HELD FROM FRESHNESS TO SPOILAGE AT LOW TEMPERATURES. S. MARGITIC & J. M. JAY. *J. Food Sci.* 35, 252–255 (1970)–Ground semitendinosus beef muscle was allowed to undergo normal spoilage at 7°C for 30 days. Changes in the antigenicity of the muscle proteins were determined periodically by injecting rabbits with 3% NaCl extracts of beef from freshness to spoilage. According to the serological methods employed, the salt-soluble muscle proteins were highly antigenic in all meat extracts. Although the anti-putrid and anti-fresh extract antibodies cross reacted they were, nevertheless, serologically distinct, in that the former always yielded a greater amount of precipitate and a larger number of precipitating bands than did the latter, when the two were tested against their homologous antigens. This study revealed that spoiled meat extracts contained at least 2 new antigenic species not demonstrable in fresh meat extracts, in addition to the antigens serologically common to both extracts. These findings indicate a lack of complete breakdown of salt-soluble beef proteins by the spoilage flora.

LYSOSOMAL-TYPE ENZYMES IN BEEF LONGISSIMUS DORSI MUSCLE. K. ONO. *J. Food Sci.* 35, 256–257 (1970)–Biochemical evidence for the presence of lysosomes in bovine longissimus dorsi muscle has been presented. Comparisons of enzymatic activities among lysosomal fractions from rat liver, bovine liver and bovine l. dorsi muscle indicate that there are specific as well as organic differences with respect to the activities of some of the enzymes.

EFFECT OF POST-MORTEM AGING ON ISOLATION OF INTRAMUSCULAR CONNECTIVE TISSUE. P. E. McCLAIN, G. J. CREED, E. R. WILEY & I. HORNSTEIN. *J. Food Sci.* 35, 258–259 (1970)–The yield of bovine and porcine intramuscular connective tissue (IMCT) was quantitated at various time intervals post-mortem. The pH of the muscle tissue was followed through the aging period, and the heat absorbed during hydrothermal shrinkage (ΔHs) and the temperature of the transition (Ts) determined on the IMCT. The yield of bovine IMCT at 72 hr post-mortem was 50% lower than that at 0 hr, whereas 34% lower yields were found for the porcine tissue ($P < 0.01$). Loss in the yield of IMCT was paralleled by the drop in pH post-mortem. Recovery of IMCT reached a minimum in the pH range 5.43–5.53. No marked differences in ΔHs or Ts were observed in any of the samples studied.

SOME POST-MORTEM CHANGES IN PORCINE MUSCLE HELD AT 25°C. E. D. CAGLE & R. L. HENRICKSON. *J. Food Sci.* 35, 260–262 (1970)–Differences in shear force due to treatment were found to be highly significant for the left side and approached significance for the right side. The pH declined at a constant rate to 8 hr post-mortem but had not reached an ultimate at 8 hr. Shear force values decreased at a rate closely paralleling the pH decline beyond 4 hr after death. Fiber diameter and percent fiber kinkiness followed essentially the same pattern, in that both decreased beyond 4 hr. Press fluid did not significantly change over the period of this study. An interaction was found for samples from the left side and was attributed to the method of suspending the animals during exsanguination.

CAROTENOIDS IN 3 STAGES OF RIPENING OF MANGO. J. JOHN, C. SUBBARAYAN & H. R. CAMA. *J. Food Sci.* 35, 262–265 (1970)–There was an increase in content as well as in number of carotenoids during ripening. The present study showed there were 15, 14 and 17 different carotenoids in the unripe, partially ripe and fully ripe mangoes, respectively. Even though phytofluene (39.26%) was the major carotenoid in the partially ripe mango, β-carotene constituted the major carotenoid in the unripe (37.47%) and fully ripe mango (50.64%). *cis*-β-Carotene was present only in the fully ripe mango. Only the unripe mango contained ξ-carotene, whereas γ-carotene was present in all the 3 stages of ripening. The major xanthophyll present in the unripe mango was mutatoxanthin (9.44%), whereas auroxanthin constituted the major hydroxylated carotenoid of the partially ripe (5.07%) and fully ripe (10.40%) mangoes.

EFFECTS OF PRE- AND POSTMORTEM GLYCOLYSIS ON POULTRY TENDERNESS. A. W. KHAN & R. NAKAMURA. *J. Food Sci.* **35**, 266–267 (1970)–To study the effects of glycolysis on tenderness of poultry breast meat, premortem and postmortem glycolysis was controlled either by epinephrine administration at suitable intervals of time before slaughtering or by allowing the birds to struggle freely just before and during slaughtering. The results showed that extensive glycolysis occurring immediately before and during slaughtering and bleeding caused low post-slaughter pH and toughness. In practice, the premortem glycolysis can be controlled by minimizing the physical activity immediately before slaughtering, and by restricting the free movement of limbs during slaughtering and bleeding.

EFFECT OF TREATMENT OF PRE- AND POST-RIGOR PORCINE MUSCLES WITH LOW SODIUM CHLORIDE CONCENTRATIONS ON THE SUBSEQUENT EXTRACTABILITY OF PROTEINS. R. G. JOHNSON & R. L. HENRICKSON. *J. Food Sci.* **35**, 268–270 (1970)–Comparisons were made of the extractable salt-soluble protein content between pre- and post-rigor normal- and low-pH muscles. Pre-rigor normal-pH muscle was found to contain 69.6% greater extractable salt-soluble protein than post-rigor normal-pH muscle, whereas pre-rigor low-pH muscle contained only 7.3% greater extractable salt-soluble protein than post-rigor low-pH muscle. Addition of 3 levels of sodium chloride to meat samples 24 hr before protein extraction increased the extractable salt-soluble protein content in pre-rigor normal-pH muscle and decreased the extractable salt-soluble protein content in post-rigor normal-pH muscle. Addition of sodium chloride decreased the extractable salt-soluble protein content in pre- and post-rigor low-pH muscle.

INFLUENCE OF SLICING WARM PORCINE MUSCLE ON FIBER DIAMETER, KINKINESS AND SHEAR FORCE. E. D. CAGLE & R. L. HENRICKSON. *J. Food Sci.* **35**, 270–271 (1970)–Ten market-weight Hampshire pigs were used to test 2 methods of fabricating hot-processed pork loins. Longissimus dorsi muscle sliced before the removal of body heat had significantly higher shear values than the corresponding muscle sliced after a 24-hr chill. This difference was believed to be due to the contraction of the muscle fibers caused by slicing the muscle soon after death. Fiber diameter and percent kinkiness were also significantly affected by method of slicing, but the larger fiber diameter and greater percent kinkiness were found for the cold-sliced muscle. An interaction between side and treatment was attributed to the manner in which the animal was suspended during death.

EFFECT OF PSYCHROTOLERANT BACTERIA ON THE AMINO ACID CONTENT OF CHICKEN SKIN. M. ADAMČIČ, D. S. CLARK & M. YAGUCHI. *J. Food Sci.* **35**, 272–275 (1970)–Changes induced by strains of *Achromobacter* and *Pseudomonas* in the amino acid content of chicken skin were studied during storage at 5°C. The *Achromobacter* and nonpigmented *Pseudomonas* cultures reduced the amounts of all amino acids to below detectable levels during the early log phase and produced no detectable change subsequently. The pigmented *Pseudomonas* caused no appreciable change initially but produced an increase in most amino acids during the late log phase; an increase in proline and hydroxyproline indicated that collagenous proteins were attacked. The study indicated that pigmented pseudomonads are the most proteolytic of the common types of psychrotolerant spoilage bacteria and that they are collagenolytic.

PROTEOLYTIC ACTIVITY OF Cucumis trigonus Roxb. EXTRACTION, ACTIVITY, CHARACTERISTICS. S. HUJJATULLAH & A. K. BALOCH. *J. Food Sci.* **35**, 276–278 (1970)–An enzymic fraction, found to possess considerable proteolytic activity, was obtained from the fruit of *Cucumis trigonus* Roxb. Chemical and physical properties of the enzyme were investigated. The proteolytic principle, provisionally called "Cucumin," exhibits strong meat-tenderizing action but does not clot milk. Accumulated evidence indicates that the enzyme is an –SH containing protease. The values obtained from experimentation on the enzyme isolate are: protease units/g of the powdered enzyme; 18,000, specific rate constant K; 0.98×10^{-3}, Km; 4.8×10^{-5} g mole/liter, maximum velocity V; 5.56×10^{-7} g mole/minute, isoelectric point; 3.4, optimum pH; 5.0, optimum temperature; 40°C. The proteolytic properties of Cucumin have been exploited in the formulation of an active meat tenderizer.

EFFECT OF TEMPERATURE ON STABILITY OF ORANGE AROMA SOLUTION. D. G. GUADAGNI, J. L. BOMBEN & H. C. MANNHEIM. *J. Food Sci.* **35**, 279–281 (1970)–Aroma strength as measured by threshold determinations remained fairly constant at 0 to 20°F for up to 1 year. Aroma character, however, changed significantly within 6 months and 1 year at 20 and 10°F, respectively. At 0°F, fresh orange aroma was maintained without significant change for up to 88 weeks. Aroma strength and character during storage at 70°F were preserved by sealing in glass tubes and storing in the dark.

AVOCADO POLYPHENOLOXIDASE: PURIFICATION, AND FRACTIONATION ON SEPHADEX THIN LAYERS. N. S. DIZIK & F. W. KNAPP. *J. Food Sci.* **35**, 282–285 (1970)–Polyphenoloxidase (PPO) was purified 28-fold by ammonium sulfate precipitation, dialysis and gel filtration. Enzyme constants were determined with a variety of substrates. Thin-layer gel filtration resolved the crude PPO into 5 fractions with molecular weights estimated at 14-, 28-, 56-, 112- and over 400-thousand by comparison with standard proteins. Subsequent electrophoresis at 90° to the direction of gel filtration resolved the 28×10^3 MW fraction into 4 to 6 components. One of these was by far the most active of all the isoenzymes toward all the substrate sprays tested. It is probably the activity of this isoenzyme which is reflected in kinetic data, even those obtained with relatively crude avocado PPO preparations.

MAJOR VOLATILE NEUTRAL AND ACID COMPOUNDS OF HYDROLYZED SOY PROTEIN. C.H. MANLEY & I.S. FAGERSON. *J. Food Sci.* **35**, 286–291 (1970)–Interest in the use of hydrolyzed vegetable protein in many food products has led to the need for more basic knowledge of the types of compounds present. The present study shows that a number of aldehydes and furan-type compounds are the major compounds of significance in a neutral fraction. An acid fraction, which appeared to carry most of the odor associated with the hydrolyzed soy protein, has levulinic acid as a major component. Thirteen other acids, 2 lactones, 4 phenols plus other compounds were also isolated and identified in the acid fraction. The mechanisms for the formation of some of the compounds are discussed.

EFFECT OF AGING ON PALATABILITY AND SELECTED RELATED CHARACTERISTICS OF PORK LOIN. D. L. HARRISON, J. A. BOWERS, L. L. ANDERSON, H. J. TUMA & D. H. KROPF. *J. Food Sci.* **35**, 292–294 (1970)–Left pork loins from the 4th thoracic to the 5th lumbar vertebra were used to study the effect of 4 aging periods (1, 4, 8 and 12 days) on pork longissimus dorsi and the fat covering. Aging 12 days increased cooking time in min/lb ($P < 0.05$), dripping cooking losses ($P < 0.05$), acid numbers for fat ($P < 0.01$), pH of raw ($P < 0.05$) and cooked ($P < 0.01$) muscle, TBA value of raw muscle ($P < 0.05$), tenderness score ($P < 0.05$) and free amines ($P < 0.01$). Aging decreased ($P < 0.05$) percentage total moisture of raw muscle and shear values. Few changes occurred until after 4 days' aging. Changes in palatability and pH were slight.

URIC ACID LEVELS IN MEN FED ALGAE AND YEAST AS PROTEIN SOURCES. C.I. WASLIEN, D.H. CALLOWAY, S. MARGEN & F. COSTA. *J. Food Sci.* **35**, 294–298 (1970)–Microorganisms grown on human or industrial waste may be economical, nutritious food sources. Algae (*Chlorella sorokiniana)* and yeast *(Torulopsis utilis)* were compared with casein at 2 nitrogen levels as sole sources of protein for men. Biological value of algal protein was superior to casein, but yeast protein was not quite as good as algae, in diets containing 25 g protein. When 50 g of algae protein was consumed, true nitrogen digestibility was reduced, but nitrogen balances indicated that 50 g of any of these proteins met or exceeded dietary requirements. Urinary uric acid increased considerably with algae and yeast. Renal clearances were not sufficient to prevent abnormally high plasma uric acid levels, comparable to those found in gout.

DEVELOPMENT OF A MICRO-EMULSIFIER. R. Y. T. TSAI, R. G. CASSENS & E. J. BRISKEY. *J. Food Sci.* **35**, 299 (1970)–A micro-emulsifier is described. The emulsifying capacity of very small quantities of protein can be measured in an essentially air-free condition.

COLORIMETRIC ESTIMATION OF TOTAL ALDEHYDES IN AQUEOUS ORANGE ESSENCE USING N-HYDROXYBENZENESULFONAMIDE. M. A. ISMAIL & R. W. WOLFORD. *J. Food Sci.* 35, 300–301 (1970)–A spot-test method for detection of aldehydes has been adapted colorimetrically for quantitative determination of total aldehydes in aqueous solutions. The final colored complex has an absorption maximum at 510–525 mμ. The technique is simple, and the color produced is linear with aldehyde concentrations up to 300 mg/liter using n-octanal and trans-2-hexenal as a mixed standard. The reaction is specific to aldehydes and no interference by ketones, alcohols, esters or terpenes has been observed. The test is applicable to aldehyde determinations in aqueous orange essences provided proper sample dilution is made before analysis.

PHYSICAL AND CHEMICAL STABILITY OF SOYBEAN OIL-FILLED MILK. H. W. MODLER, A. L. RIPPEN & C. M. STINE. *J. Food Sci.* 35, 302–305 (1970)–Filled milks were formulated from fresh skimmilk, vegetable oils and emulsifiers. The filled milks were pasteurized at 170°F (except in off-flavor studies), homogenized at 500/2,500 psi and cooled to 36°F. Lightly hydrogenated salad oil, prepared from soybean oil, was quite acceptable when evaluated organoleptically at 24-hr intervals of approximately 1 wk. Thiobarbituric acid and peroxide values revealed that very slight oxidation had occurred during storage for approximately 1 wk at 40°F. 4 monoglyceride emulsifiers with varying degrees of saturation were used to stabilize the emulsion of soybean oil in skimmilk. 2 of the more unsaturated monoglycerides tended to impart a bitter flavor to the milk when used at 0.1% (based on weight of product) and also were less efficient emulsifiers when compared to saturated monoglycerides. Development of a very undesirable sulfide-like odor and taste occurred under extremely high pasteurization temperatures. The degree of off-flavor was directly proportional to time and temperature of heating.

LIPID AND OTHER COMPOSITIONAL CHANGES IN 9 VARIETIES OF SWEET POTATOES DURING STORAGE. T. S. BOGGESS JR., J. E. MARION & A. H. DEMPSEY. *J. Food Sci,* 35, 306–309 (1970)–9 Varieties of sweet potatoes (*Ipomoea batatas*) were harvested from controlled experimental plots, graded, cured 5 to 7 days and stored for 5 months at 15.5°C and 80–85% r.h. Centennial, Canbake, Georgia Red, Copperskin Goldrush, Gem, Coastal Sweet, Julian, Nuggett and Puerto Rico were analyzed after curing and after storage for °Brix, acidity, solids, total lipids, levels of nonphospholipids, cephalin, lecithin and fatty acid composition of each lipid. Variations in mean measurements were °Brix 13.5–15.7, solids 24.6–31.8, pH 5.92–6.06; acidity 0.134–0.178 and lipids 1.21–2.50. Each of these was significantly influenced by variety of potato and storage. Most of the fatty acids in total lipids and lipid fractions were influenced significantly by variety and storage of potatoes.

QUANTITATIVE METHODS FOR ANTHOCYANINS. 5. Separation of Cranberry Phenolics by Electrophoresis and Chromotography. P. E. CANSFIELD & F. J. FRANCIS. *J. Food Sci.* 35, 309–311 (1970)–Anthoxanthins and colorless phenolics present in extracts of cranberries were separated from anthocyanins by paper electrophoresis. Anthoxanthins were separated from other phenolics and from one another, by 2 dimensional chromatography on a portion cut from the electropherogram.

A PENETROMETER TEST TO MEASURE MEAT TENDERNESS. L. C. HINNERGARDT & J. M. TUOMY. *J. Food Sci.* 35, 312–315 (1970)–Attempts to relate mechanical measures of raw meat tenderness to cooked meat tenderness have generally been unsuccessful. Therefore, a study was designed to evaluate the use of a simple penetration test to predict cooked meat tenderness from the force required to penetrate the raw meat sample. An Allo-Kramer Shear Press was modified to function as a penetrometer by replacing the Standard Shear-Compression cell and shearing blades with a plate containing 5 needles. The needles were made from 1/8-in.-diameter drill rod and were semiblunt, having 0.007-in.-diameter land and 0.472/1.0-in. taper. Using slices from the longissimus dorsi muscle of pork, various factors of the time-force curve were analyzed to determine which would give the best indication of tenderness. It was found that the maximum force value gave the best results. Regression analysis showed correlations significant at the 1% level between the force necessary to penetrate the raw sample versus both force necessary to penetrate the cooked sample and trained technological panel evaluations.

COLORIMETRY OF FOODS. 2. Color Measurement of Squash Puree Using the Kubelka-Munk Concept. I-LO HUANG, F.J. FRANCIS & F.M. CLYDESDALE. *J. Food Sci.* 35, 315–317 (1970)–A series of squash purees was prepared with added canthaxanthin in 0.3-ppm increments. They were ranked for color differences visually under 7400°K light and instrumentally with a G.E. spectrophotometer and a Hunterlab colorimeter. Plexiglass cells varying from 2 to 8 mm in thickness and a black and white background were used in all cases. Optimum visual rankings were performed with 5 mm or greater thickness and a black background. Samples 0.2 delta E units could be ranked visually. Adequate instrument rankings required all three color parameters. Calculation of X Y Z or L a b data in terms of K/S ratios did not improve the correlations with visual or theoretical rankings.

COMPARATIVE STUDIES ON THE NITROGEN SOLUBILITY OF MUNG BEANS, PEA BEANS AND RED KIDNEY BEANS. Y. D. HANG, K. H. STEINKRAUS & L. R. HACKLER. *J. Food Sci.* 35, 318–320 (1970)–The effect of pH and some salts on the extraction of nitrogenous matter from mung beans, pea beans and red kidney beans has been studied. The nitrogen solubilities of these beans were found to be strongly pH-dependent. The amount of nitrogen extracted at alkaline pH is greater than that produced at either neutral or acidic pH. Minimum points of nitrogen dispersion occurred at pH 4.0. Salts dispersed more nitrogenous constituents from the beans than did water. Dilute solutions of sodium chloride, sodium sulfate, calcium chloride and magnesium chloride were found to have an inhibitory effect on the dispersion of the nitrogenous matter of the beans. Alkaline salts such as sodium carbonate, disodium phosphate and sodium citrate appeared to be fairly effective dispersing agents, and the exact amount of nitrogen extracted was dependent upon the concentration of the salt solution.

STUDIES ON AROMA OF CURED HAM. E. G. PIOTROWSKI, L. L. ZAIKA & A. E. WASSERMAN. *J. Food Sci.* 35, 321–325 (1970)–Cured and uncured hams (raw, cooked or cooked-smoked) were analyzed for free amino acids. The total amino acid content of hams decreased on curing, increased on cooking and was relatively unaffected on smoking. Changes in concentrations of individual amino acid as a result of the treatments of hams were noted. A trained panel differentiated cured and uncured hams by aromas produced on heating aqueous extracts and diffusates. Smoke aroma was more readily detected in cured samples. Extraction of hams with water yielded precursors of the basic meaty aroma; extraction with chloroform-methanol yielded precursors or components of cured and smoky notes. Profiles of volatile compounds developed on heating of ham diffusates and lipid extracts were compared using gas chromatography. No individual component separated had a meaty or cured aroma.

GROWTH OF SALMONELLA AT LOW pH. K. C. CHUNG & J. M. GOEPFERT. *J. Food Sci.* 35, 326–328 (1970)–The growth of salmonellae was observed to occur at pH values as low as 4.05 ± 0.05. The growth-limiting pH was dependent on several factors, most important the acid molecule itself. Additionally, the effect of temperature, relative oxygen supply and level of inoculum was studied. The salmonellae could not be "trained" to grow at a lower pH by sequential transfer at near-optimum pH values.

SUGAR-AMINO ACID INTERACTION IN THE DIFFUSATE OF WATER EXTRACT OF BEEF AND MODEL SYSTEMS. A. E. WASSERMAN & A. M. SPINELLI. *J. Food Sci.* 35, 328–332 (1970)–There is no pattern of amino acid losses in a diffusate of a water extract of beef that accounts for the brothy aroma on boiling and the roast beef aroma on pyrolysis. Amino acid concentration declines 50 to 60% and sugars disappear completely on pyrolysis. A similar situation exists in a model system of amino acids and sugars in concentrations simulating beef diffusate, but greater losses in amino acids are observed. Some decrease in concentration of amino acids and sugars occurs when each is pyrolyzed separately. In the presence of excess sugar the amino acids are completely utilized and there is a ratio of 4:1 for glucose used to amino acids used.

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EFFECT OF DRY AND MOIST HEAT TREATMENTS ON SELECTED BEEF QUALITY FACTORS

SUMMARY—Pieces of beef semimembranosus muscle, relatively uniform in weight and shape, were deep-fat fried (DF), oven-roasted (OR), oven-braised (OB) and pressure-braised (PB) at 10 psig to an internal temperature of 70°C to investigate the effects of dry and moist heat on selected characteristics of beef. Rate of heat penetration, cooking time, cooking losses, total moisture, press fluid, water-holding capacity and juiciness varied ($P < 0.01$) and apparent degree of doneness varied ($P < 0.05$) among the 4 heat treatments. Warner-Bratzler shear, color-difference, flavor, tenderness and over-all acceptability were not affected significantly by treatment. OR pieces had the slowest rate of heat penetration and the longest cooking time, highest values for total moisture, press fluid, water-holding capacity and juiciness. For those measurements, OB pieces always ranked next to OR pieces, followed by DF and PB pieces. OR pieces appeared less well-done than meat given the other treatments. For every heat treatment, there was an excellent relationship between rate of heat penetration and cooking time. For PB pieces, rate of heat penetration was moderately related to total moisture, press fluid, juiciness and apparent degree of doneness, and highly related to cooking losses. It appeared that tenderness and flavor influenced over-all acceptability scores more than juiciness or apparent degree of doneness. Differences between values for selected characteristics of raw muscle and of muscle subjected to each treatment were not significantly different from each other.

INTRODUCTION

DIFFERENCES among data from 2 or more laboratories studying similar problems related to the production or processing of beef, or both, often are reported in the literature. Ramsbottom et al. (1945) suggested that lack of uniformity in cooking methods, temperature and time, as well as differences in muscles used, degree of fatness of the meat and age of the meat, may account for the varied results obtained by different investigators.

Ramsbottom et al. (1945), Harrison et al. (1949) and Paul et al. (1955) reported the use of deep fat as a cooking medium for experimental work on beef. Visser et al. (1960) discussed some of the problems encountered in studying degree of doneness of oven-roasted beef and beef cooked in deep fat.

Clark et al. (1955) braised top and bottom round steaks under 10 and 15 psig and at atmospheric pressure to 80 and 112.2°C. The end point temperature to which the steaks were cooked, rather than the method, was important in determining palatability and cooking losses. Rate of heat penetration and cooking time were important when the tenderness of deep-fat fried beef steaks and over-roasted beef roasts was compared (Paul et al., 1952).

None of the studies reviewed was designed specifically to study the effects of heat treatment (cooking method) on laboratory measurements of the quality of cooked beef. Researchers should know whether there are significant effects specifically attributable to type of heat treatment and the nature of those effects on the characteristics of cooked beef. The objective of this study was to investigate the effects of dry and moist heat treatments on selected characteristics of cooked beef.

EXPERIMENTAL

U.S. GOOD beef top rounds (18–22 lb) were purchased from a local wholesale meat company. The fat was trimmed from each top round and the semimembranosus muscle (SM) cut into 4 pieces as nearly alike as possible in size and shape. Each piece was wrapped in aluminum foil (gauge 0.0015), frozen and stored at 0°F from 2 to 14 wk. Before cooking each piece was thawed for approximately 5 hr at room temperature (approximately 78°F) and an additional 19 hr at 45°F. The internal temperature of thawed pieces was 5 ± 2°C.

Experimental design and analysis of data

The design followed to evaluate the effect of the 4 heat treatments on pieces of SM was an incomplete block (Cochran et al., 1957, p. 471). There were 24 evaluation periods with 2 pieces, randomized by section of muscle, cooked at each period. Data for each measurement were analyzed by analysis of variance (Cochran et al., 1957, p. 449) to study differences attributable to heat treatment. When significant F-values occurred, least significant differences (LSD) at the 5% level were calculated. Also, correlation coefficients were determined to study relationships among measurements within each heat treatment.

Heat treatments

All pieces were cooked to an end point temperature of 70°C in the center. One-fourth of the pieces were deep-fat fried (DF). Each piece was placed on a rack in an electric deep-fat cooker, covered with cottonseed oil preheated to 105°C, and held at 100 ± 2°C until the end point was reached. One-fourth of the pieces were oven-roasted (OR), uncovered on a rack in a shallow roasting pan, in a rotary hearth gas oven at 300°F. One-fourth of the pieces were oven-braised (OB) at atmospheric pressure, on a rack in a Pyrex baking dish containing 30 ml water and covered with aluminum foil, in a rotary gas oven maintained at 300°F. The remaining one-fourth of the pieces were braised, with 30 ml water in a pressure saucepan (PB) equipped with both a thermocouple and a thermometer, at 10 psig (115°C).

Rate of heat penetration

A centigrade thermometer was inserted into the midportion of each piece of SM muscle for treatments DF, OR and OB. An iron-constantan thermocouple was inserted into the midportion of the PB pieces, the cover of the pressure saucepan adjusted, and the thermocouple then connected to a potentiometer that gave a direct temperature reading in degrees Fahrenheit. Steam was allowed to escape from the saucepan for 1 min to evacuate the air before the weight was placed on the vent, and the pressure brought to psig. For all 4 treatments the time required for each 5°C (9°F) rise in internal temperature of the muscle from initial to end point temperature was recorded.

Cooking losses, pH and shear values

Percentage total cooking losses were determined for all pieces of SM. In addition, volatile and dripping losses were measured for OR pieces.

The method described by Rogers et al. (1967) was used to determine pH of cooked muscle. To obtain shear values, 3 ½-in. cores were cut with the grain of the tissue and sheared on a Warner-Bratzler shearing apparatus with a 25-lb dynamometer. Duplicate readings were made on each core.

Press fluid yield, water-holding capacity and total moisture

Duplicate samples (25-g) of ground meat were pressed in a Carver laboratory press according to a standardized time-pressure schedule with a maximum pressure of 4,000 psig. The press fluid was collected in centrifuge tubes and the volumes of total fluid, serum and fat measured to the nearest 0.1 ml.

Water-holding capacity (WHC) was measured according to the method described by Miller et al. (1965). Percentage total moisture (TM) was determined by drying 10-g samples in a C.W. Brabender Semi-Automatic Rapid Moisture Tester for 60 min at 121°C.

Gardner color-difference values and sensory analysis

RD (reflectance), a+ (redness) and b+ (yel-

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lowness) of ground muscle were measured on a Gardner Color Difference Meter standardized by using a satin-finish ceramic tile with calculated values of 15.53 (Rd), +9.33 (a+) and +13.10 (b+).

Desirability of flavor, intensity of juiciness and tenderness and over-all acceptability of each piece were scored by an experienced laboratory panel of 9 members on a 1- to 7-point scale. Each judge selected at random and evaluated ½-in. cubes of muscle. One cube was used to evaluate flavor, juiciness and over-all acceptability, and one cube to evaluate tenderness. Each judge also observed and rated a slice of muscle placed under a MacBeth Skylight as rare (1), medium (2) or well-done (3).

RESULTS & DISCUSSION

Initial weight, initial temperature and rate of heat penetration

Analysis of variance indicated no significant differences among weights of the pieces (means 809.7–826.1 g). However, there were significant ($P < 0.05$) differences in initial internal temperature among the pieces assigned to the 4 heat treatments. The initial temperature of PB pieces averaged approximately 2 to 4°C higher than other pieces (Table 1).

Rate of heat penetration to the center of the pieces of muscle was affected significantly ($P < 0.01$) by the heat treatment (Table 1). Mean values for the minutes required for the temperature at the center of the pieces of muscle to rise 5°C indicated that each heat treatment differed ($P < 0.05$) from every other treatment, except that OR and OB did not differ significantly from each other. The average rate of heat penetration was faster ($P < 0.05$) in PB pieces than in pieces given any of the other treatments. The order of the average rate of heat penetration in pieces of muscle given the other treatments was DF > OB > OR.

Time-temperature curves (Fig. 1) indicate that in treatments PB and DF the heat penetrated the muscle at a fairly constant rate after an interval temperature of 10°C was reached; above 10°C the rate of heat penetration was faster than it was below that temperature. In OB pieces the rate of heat penetration was relatively constant throughout the cooking period. Heat penetrated OR pieces most rapidly between internal temperatures of approximately 12 and 50°C, but slowed down slightly between 40 and 50°C. After 88 min of cooking, the internal temperature of both OB and OR pieces was approximately 65°C. Thereafter, the rise in temperature of OR pieces slowed down.

Other workers have reported data on heat penetration in beef similar to those obtained in this study. Clark et al. (1949) found that heat penetration in pressure-braised beef was more rapid and more constant than in oven-roasted beef. Harrison (1943) stated that in mediums where heat penetration was rapid, the rate of temperature rise at the center of a piece of beef was approximately the same throughout the entire cooking period.

Time-temperature curves for beef deep-fat fried at 100°C (212°F) and oven-roasted at 148.9°C (300°F) presented by Visser et al. (1960) were similar to those of this study. Both Harrison (1943) and Visser et al. (1960) reported a decrease in rate of heat penetration in oven-roasted beef at an internal temperature of approximately 50–55°C.

Table 1—Means, F-values and LSD's attributable to heat treatment for objective and sensory measurements.

Measurements	Heat treatment (N = 12) DF	OR	OB	PB	F-value	LSD[1]
Initial weight (g)	818.1	818.9	826.1	809.7	.0.56	–
Initial temperature (°C)	4.8	3.2	4.3	7.1	4.04*	1.49
Rate of heat penetration (min/5°C)	4.6	7.2	6.8	3.0	122.60**	0.49
Cooking time						
Total, min	65.6	101.2	94.7	42.9	115.19**	7.08
min/lb	36.6	56.0	52.0	24.1	97.45**	4.25
Cooking losses, total (%)	28.7	23.5	27.9	33.2	35.84**	2.23
Total moisture (%)	60.9	64.0	61.1	59.6	9.83**	1.87
Press fluid yield (ml/25 g)	7.5	8.5	7.5	6.6	11.71**	0.73
WHC[2]	0.57	0.64	0.61	0.55	6.28**	0.04
Shear value (lb/½-in. core)	9.9	8.8	9.7	8.8	1.10	–
pH	5.64	5.63	5.61	5.66	0.02	–
Gardner color-difference meter						
Rd	21.03	22.00	21.08	20.26	0.41	–
a+	6.75	7.31	6.92	6.81	0.20	–
b+	12.25	12.77	11.79	12.13	0.09	–
Sensory scores						
Apparent degree of doneness[3]	2.8	2.2	2.9	2.7	4.40*	0.42
Juiciness[4]	5.0	5.8	5.3	4.5	6.49**	0.66
Flavor[4]	5.7	5.8	5.6	5.6	0.76	–
Tenderness[4]	5.0	5.2	5.2	5.4	0.83	–
Over-all acceptability[4]	5.2	5.6	5.4	5.1	1.72	–

[1] LSD, least significant difference at 5% level.
[2] WHC, water-holding capacity (1.0–expressible moisture index).
[3] 1-rare, 2-medium-done, 3-well-done.
[4] Range, 7 (extremely desirable, tender or juicy) to 1 (extremely undesirable, tough or dry).
DF, Deep-fat fried; OR, Oven-roasted; OB, Braised, atmospheric pressure; PB, Braised, 10 psig.
*$P < 0.05$.
**$P < 0.01$.

Cooking time

Cooking time, on the basis of both total minutes and min/lb, was affected ($P < 0.01$) by heat treatment (Table 1). As might be expected, the effect of heat treatment followed the same pattern on

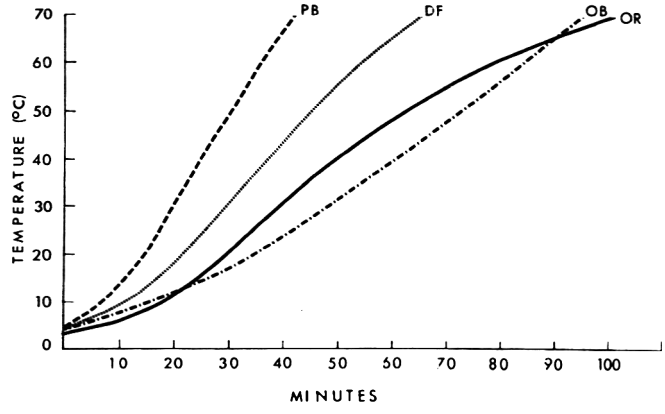

Fig. 1—Rate of heat penetration in semimembranosus muscle heated to 70°C in four cooking mediums: DF—Deep-fat fried; OR—Oven-roasted; OB—Braised, atmospheric pressure; PB—Braised, 10 psig.

Table 2–Mean values for selected measurements on raw and heated muscle.

Measurements	Raw muscle	Cooked muscle[1] (N=12)							
		(N=12) DF	Diff	OR	Diff	OB	Diff	PB	Diff
Total moisture (%)	72.8	60.9	11.9	64.0	8.8	61.1	11.7	59.6	13.2
Shear value (lb/½-in. core)	7.4	9.9	2.5	8.8	1.4	9.7	2.3	8.8	1.4
pH	5.61	5.64	0.03	5.63	0.02	5.61	0.00	5.66	0.05
Gardner color-difference									
Rd	8.76	21.03	12.27	22.00	13.24	21.08	12.32	20.26	11.50
a+	12.30	6.75	5.55	7.31	4.99	6.92	5.38	6.81	5.49

[1] Diff–Difference between the value for raw and cooked muscle.
DF, Deep-fat fried; OR, Oven-roasted; OB, Braised, atmospheric pressure; PB, Braised, 10 psig.

cooking time as on rate of heat penetration. Cooking time for each heat treatment differed ($P < 0.05$) from that of every other treatment with the exception of OR and OB, which were not significantly different from each other. Also, PB pieces of muscle required less ($P < 0.05$) time to cook than pieces given any of the other treatments; the order of cooking time for the other treatments was DF < OB < OR (Table 1, Fig. 1).

Differences in rate of heat penetration and cooking time attributable to treatment may be explained, in part, by differences in the heat conductivity of the 4 cooking mediums.

Cooking losses

Cooking losses for a given method of cooking usually are related to cooking time, with losses increasing as time increases. As would be expected, in this study cooking losses as well as cooking time were affected ($P < 0.01$) by heat treatment (Table 1). However, losses from OR pieces were lower ($P < 0.05$) than losses from any of the other pieces, although cooking time was longer ($P < 0.05$) for OR pieces than for any of the others. Losses from PB pieces were greater ($P < 0.05$) than those from DF and OB pieces, with no significant difference between DF and OB pieces.

Total moisture, press fluid yield and water-holding capacity

TM, press fluid yield and WHC were affected ($P < 0.01$) by heat treatment. TM was higher ($P < 0.05$) in OR pieces than in pieces given any of the other treatments. No significant differences occurred between PB and OB or DF, nor between DF and OB treatments (Table 1).

Press fluid yield from OR pieces was greater ($P < 0.05$) than that from pieces given the other heat treatments. Press fluid yield differed ($P < 0.05$) between PB and both DF and OB, whereas there was no difference between DF and OB (Table 1).

Similar to press fluid yield, values for WHC were higher ($P < 0.05$), i.e., more moisture was expressed under pressure, for OR pieces than for PB and DF pieces. However, there was no significant difference in WHC between OR and OB pieces. Values for PB pieces were not significantly different from values for DF pieces, but were significantly lower ($P < 0.05$) than those for OB pieces.

Shear values, pH and Gardner color-difference values

No significant differences were attributable to heat treatment for shear values, pH and color-difference of the muscle pieces (Table 1). In contrast, Harrison (1943) found that cooking medium significantly ($P < 0.05$) affected shear value.

As expected, total moisture, shear value, pH and Gardner color-difference of raw beef changed when any one of the 4 heat treatments was applied. To study further the changes attributable to the specific heat treatments, the difference between values for selected characteristics of raw muscle and of muscle subjected to each heat treatment was calculated. None of the calculated differences was significantly different from any other (Table 2).

Sensory measurements

All pieces were cooked to an end point temperature of 70°C, a temperature usually considered in the range for medium-done beef. Differences in mean scores for apparent degree of doneness were significant ($P < 0.05$) only between treatment OR and every other treatment. Scores for muscle from all treatments averaged between medium- and well-done, with the mean value for OR pieces closest to well done. Also, Gardner a+ values for OR pieces were highest (most pinkness), Table 1.

Differences in juiciness scores were significant ($P < 0.05$) between treatment OR and both treatments DF and PB, and between treatments OB and PB. Differences between OR and OB and between DF and PB approached significance at the 5% level (Table 1).

No differences attributable to heat treatment were found for flavor, tenderness or over-all acceptability (Table 1). The mean over-all acceptability score for OR pieces was slightly higher than that for pieces given any other treatment. The descending order of acceptability for the other treatments was OB, DF and PB.

Relationships between selected measurements

Correlation coefficients for selected paired variates were calculated on the basis of heat treatment. With all 4 treatments there was an excellent relationship between rate of heat penetration and cooking time. For PB pieces there were high or moderate relationships for rate of heat penetration vs. cooking losses ($r = 0.91^{**}$), total moisture ($r = -0.66^{*}$), press fluid yield ($r = -0.78^{**}$), water-holding capacity ($r = -0.51$) and panel scores for juiciness ($r = -0.63^{*}$) and apparent doneness ($r = 0.79^{**}$). Those relationships were low (r between 0.00 and 0.40) for the other heat treatments except OR for apparent doneness ($r = -0.63^{*}$). Relationships were low or slightly above for rate of heat penetration vs. pH, shear value, color-difference values and panel scores for flavor, tenderness and over-all acceptability. Low relationships were found for apparent degree of doneness and the color-difference values (Table 3).

Correlation coefficients indicated that, in general, of the 4 palatability factors studied, tenderness had the most influence on the over-all acceptability score. Flavor had the next greatest influence and juiciness and apparent degree of doneness the least. A survey of the palatability panel also indicated that the

judges based their over-all acceptability scores mostly on tenderness and flavor. Some judges stated that flavor was most important in assignment of the over-all scores. Juiciness seemed to be an important factor to most judges only when extremely low levels were noted.

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Table 3—Correlation coefficients for selected paired variates on basis of heat treatment.

Paired variates d/f = 10	r-Values[1] Heat treatment DF	OR	OB	PB
Rate of heat penetration, min/5°C vs.:				
Cooking time				
total (min)	1.00**	0.98**	1.00**	0.95**
min/lb	0.94**	0.96**	0.91**	0.96**
Cooking losses (total %)	0.40	0.36	0.17	0.91**
Total moisture (%)	−0.37	0.22	−0.21	−0.66*
Press fluid yield (ml/25 g)	−0.26	−0.18	−0.20	−0.78**
WHC[2]	−0.38	−0.32	−0.07	−0.51
pH	−0.24	−0.06	−0.29	0.06
Shear (lb/½-in. core)	−0.04	−0.16	−0.27	0.10
Color-difference				
Rd	−0.28	−0.20	0.05	0.25
a+	0.42	0.25	0.01	0.18
b+	−0.13	−0.55	−0.19	0.24
Flavor	0.30	0.10	−0.48	0.02
Tenderness	0.40	0.32	0.30	−0.33
Juiciness	−0.37	−0.21	0.09	−0.63*
Over-all acceptability	0.44	0.19	0.08	−0.46
Apparent degree of doneness	−0.27	−0.63*	−0.14	0.79**
Apparent degree of doneness vs.:				
Color-difference				
Rd	−0.23	0.11	−0.07	0.22
a+	−0.46	0.17	0.42	0.26
b+	−0.10	0.09	0.34	0.38
Over-all acceptability vs:				
Flavor	0.54	0.73**	0.72**	0.24
Tenderness	0.94**	0.70*	0.47	0.86**
Juiciness	−0.03	−0.14	0.39	0.86**
Apparent degree of doneness	−0.40	0.02	0.17	−0.54

[1] Levels of significance: * $P < 0.05$, $r = 0.567$; ** $P < 0.01$, $r = 0.708$.

[2] WHC, 1.0—expressible moisture index; ml/25 g.

[3] DF, Deep-fat fried; OR, Oven-roasted; OB, Braised, atmospheric pressure; PB, Braised, 10 psig.

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ON THE NATURE OF ALTERED PROTEIN IN COD MUSCLE STORED AT −29°C AFTER AGING IN ICE

SUMMARY—Additional protein, inextractable with the usual technique, was extracted from cod muscle which had various free fatty acid contents and had been stored at −29°C after aging in ice. Ultracentrifugal patterns showed that the extracted protein was aggregated and polydisperse. Sedimentation behavior was similar to that of aggregated protein in extracts of muscle in which protein-free fatty acid interaction occurred during prolonged aging in ice. Conditions under which altered protein was extracted, overnight exposure of homogenized muscle to neutral salt solution followed by rehomogenization; phase contrast micrographs of residues of homogenates before and after rehomogenization; and ultracentrifugal patterns of extracts containing aggregated protein that had been stored in extractant suggested that some of the linkages resulting from reactions in the frozen state were salt-sensitive. The association that occurred in the muscle after storage at −29°C appeared to be more stable in a neutral salt environment than that occurring in muscle aged in ice.

INTRODUCTION

DECREASE in protein extractability of cod muscle aged in ice involves protein-free fatty acid (FFA) complex formation (Anderson et al., 1968). Interaction of protein with FFA formed during aging in ice is favored when the muscle is in the frozen state (Anderson et al., 1969). Decrease in protein extractability resulting from this interaction, as well as that observed in muscle in which the FFA is formed largely during frozen storage (Dyer et al., 1950; King, 1966), is largely at the expense of actomyosin. Investigation of the changes that cause this component to become inextractable in neutral salt solution have been hampered for want of a technique to extract altered protein, since most physico-chemical techniques for studying protein require extraction. Altered protein can be extracted from muscle which has undergone prolonged aging in ice (Anderson et al., 1968) if the homogenized muscle is exposed to neutral salt solution overnight and rehomogenized.

Using this technique in the present study to extract protein from muscle held at −29°C after aging in ice for various periods, we have studied both the composition of extractable protein in ultracentrifugal patterns of protein extracts and the nature of fragments in the residues of the centrifuged muscle-extractant homogenates as revealed in phase contrast micrographs.

EXPERIMENTAL

Muscle tissue sampling and storage

Muscle tissue for freezing consisted of 10-g samples of pooled myotomes dissected in a 1°C room (Anderson et al., 1968) from gutted and beheaded 15–25-lb cod, *Gadus morhua*, aged in ice 1–35 days in a 4°C room. The muscle iced 1 day had passed through rigor at the time of dissection. Each pool of myotomes comprised muscle from 6 fish. Myotome samples were packaged in three's in Cryovac pouches, vacuum sealed, and frozen (Anderson et al., 1969) at −29°C. They were examined before freezing, after 2 hr, and after 11 months' storage, and in some instances after 17 months' storage. Myotome samples from another lot of cod aged in ice 25, 33 and 39 days were packaged, frozen and stored at −29°C in the same way. They were examined before freezing, after 2 hr, 1 and 2 days and periodically for 11 weeks.

Preparation of extracts

Muscle-extractant homogenates were prepared in triplicate using a 90-second blending technique (Anderson et al., 1968). Extraction was carried out in a 1°C room. The extractant was 0.5 ionic buffed KCl (Connell, 1958). Usually, 10-g samples of myotomes were homogenized in 390 ml of extractant. To obtain extracts of higher protein content, the tissue-to-extractant ratio was increased to 20:380, and in a few instance to 30:370.

For determination of readily extractable protein, a portion of muscle-extractant homogenate was centrifuged at 1,100 g for 20 min in a refrigerated centrifuge at 1°C. In a few instances, preparative centrifugation was carried out at 4,000 g and 15,000 g in a Spinco Model L ultracentrifuge, rather than at the usual 1,100 g. The remainder of the homogenate was stored overnight (20 hr) at 1°C for determination of protein extractable after prolonged exposure to neutral salt solution (extractant). The stored homogenate was stirred magnetically for 5 min and a portion was centrifuged as above. The remainder of the homogenate was reblended (two, 5-sec blending periods separated by a 5-sec interval to allow for settling) and stirred as above, and a portion was centrifuged for determination of protein extractable after overnight extraction plus reblending.

Determination of protein content of extracts

Percent protein extractable was determined by relating protein contents of supernatant fluids of centrifuged homogenates, assayed either by a biuret method (Layne, 1957) or an automated method (Failing et al., 1960), to the total protein contents of the myotomes before freezing, assayed on portions of freshly prepared homogenates.

Compostion of protein extracted

Sedimentation patterns of extracts prepared with overnight extraction plus reblending, and in a few cases before reblending, were obtained with a Spinco Model E analytical ultracentrifuge using schlieren optics. Ultracentrifugation was carried out at a constant temperature of 1°C and a rotor speed of 50,740 rpm, using single-sector cells with an optical light path of 12 mm unless otherwise stated.

Structure of fragments in inextractable muscle residues

Phase contrast micrographs were obtained (Anderson et al., 1968) of wet preparations of residues of muscle-extractant homogenates prepared with overnight extraction with and without reblending.

RESULTS & DISCUSSION

Protein extractability

Decrease in readily extractable protein in muscle stored at −29°C after aging in ice has been reported (Anderson et al., 1969). Contents of protein readily extractable (immediate extraction) from this type of muscle were included here (Table 1) only for comparison with percentage protein extractable with other techniques. For muscle iced 1–35 days and stored at −29°C for 2 hr and for 11 months, an additional 9% of the total

Table 1—Percentage protein extracted with 3 techniques from cod muscle stored at −29°C after aging in ice for 1–35 days.[1]

Length of storage at -29°C	Extraction technique	Days in ice									
		1	5	8	12	15	19	22	27	29	35
2 hr	Immediate	84	89	83	93	85	82	77	71	71	43
	Overnight	94	93	90	98	94	90	99	80	81	55
	Overnight plus reblend	96	96	94	99	97	99	104	96	94	86
11 months	Immediate	61	70	63	54	48	37	42	41	36	25
	Overnight	75	79	73	66	57	44	48	50	43	33
	Overnight plus reblend	91	97	100	96	90	74	78	78	67	54

[1]The data are averages of triplicate samples.

Table 2—Percentage protein extracted with 3 techniques from cod muscle stored at −29°C for up to 11 weeks after aging in ice for 25, 33 and 39 days.[1]

Days in ice	Extraction technique	Length of storage at −29°C														
		Hr		Days		Weeks										
		0	2	1	2	1	2	3	4	5	6	7	8	9	10	11
25	Immediate	70	57	56	61	46	51	42	44		35		49			32
	Overnight	83	72	74	77	61	65	59	60		50		57			40
	Overnight plus reblend	97	96	102	96	93	94	83	83		86		86			71
33	Immediate	54	39	46	42	43	29			33		29				
	Overnight	63	51	57	52	50	36			40		35				
	Overnight plus reblend	90	88	91	82	88	70			75		60				
39	Immediate	51	30	39		31	18		19		18			18		19
	Overnight	61	41	47		38	26		25		25			24		23
	Overnight plus reblend	81	77	68		58	47		51		51			37		40

[1] The data are averages of triplicate samples.

protein, on the average, was extractable with overnight extraction (Table 1). Some additional extractability is to be expected, since time is required to break salt-sensitive linkages even in very fresh muscle, especially in 0.5 ionic extractant (Anderson et al., 1965; Cowie et al., 1968). However, for muscle iced 19–35 days and stored at −29°C for 2 hr, additional protein extractable due to overnight exposure of muscle to extractant averaged 14% of the total protein. When muscle aged for prolonged periods in ice prior to freezing was examined at intervals early in frozen storage, additional protein extractable with overnight extraction was about 14% of the total protein for 25-day muscle, and 8 and 7%, respectively, for 33- and 39-day muscle (Table 2). The results suggest that when there is the potential for additional protein extractability in excess of 9% with overnight extraction, it will occur in muscle of moderate free fatty acid content, such as 25-day muscle (Anderson et al., 1968) stored at −29°C for up to six weeks and muscle of moderate to high FFA content stored for 2 hr. Additional protein extractability greater than 9% may be due to: (1) higher percentages of salt-sensitive linkages; (2) slower dissolution of altered protein; (3) slower dissolution of unaltered protein from areas in the microstructure not involved in the deterioration process at the time of examination, because of the inaccessibility of this protein to the extractant due to resistance of surrounding altered protein to homogenization. The greater extractability on overnight extraction of muscle iced 1 day and stored at −29°C for 11 months (Table 1) was consistent with the third possibility.

The results also suggest that the linkages responsible for the altered protein which remains inextractable even with overnight extraction have low sensitivity to salt in muscle, (1) iced 1–35 days and stored at −29°C for 11 months (Table 1); (2) iced 25 days and frozen 56 days and more (Table 2) and (3) iced 33 and 39 days and stored up to 11 weeks.

Additional blending after overnight exposure of the blended muscle to extractant resulted in what is considered about maximum extractability from fresh cod muscle, 95% (Dyer et al., 1950), in (1) all but the 35-day-iced samples of muscle stored at −29°C for 2 hr and (2) the 1–12-day-iced muscle frozen-stored 11 months (Table 1). When protein extractability with this technique fell below 95%, the amount of protein extracted due to reblending averaged 35% of the total muscle protein. For muscle iced for prolonged periods and examined at various times during 11 weeks' storage at −29°C, the total amount of protein extractable with the reblending technique was 95% or more only in the 25-day samples stored 2 days and less (Table 2). In remaining samples, the amount of protein extracted after reblending averaged close to 38% of the total protein.

The data show that the patterns for decrease in protein extractability with the

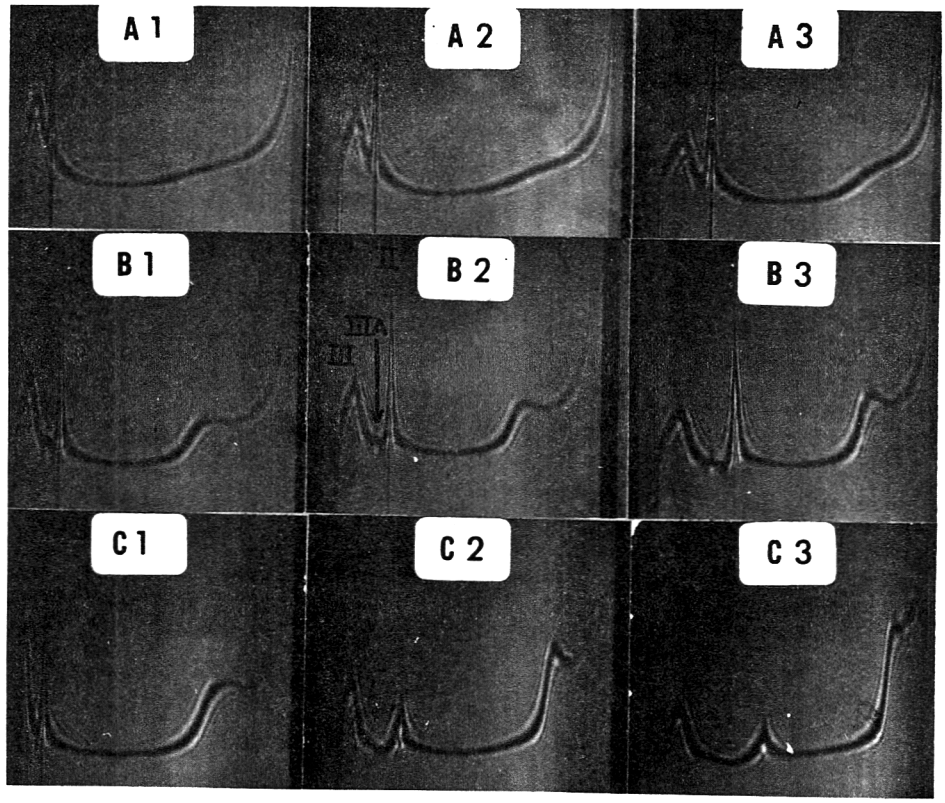


Fig. 1.—Sedimentation patterns of double-strength protein extracts prepared with overnight extraction plus reblending from cod muscle frozen for less than 2 hr after aging in ice for 25 days (A), 33 days (B) and 39 days (C). Protein concentrations (mg protein N/ml) were: A, 1.21; B, 1.33; C, 1.21. Photographs were taken at a phase plate angle of 55° and after reaching 50,740 rpm: A: (1) 16 min; (2) 20 min; (3) 28 min. B: (1) 8 min; (2) 12 min; (3) 20 min. C: (1) 4 min; (2) 12 min; (3) 20 min. The components are identified by Roman numerals in the order of decreasing sedimentation rates (Anderson et al., 1968).

reblending technique are similar to those with the immediate extraction technique. The differences are mainly in the levels of extractability. They are interpreted simply as reflections of difference in extraction technique.

Protein extractability in reblended homogenates after prolonged exposure of the fragmented muscle to extractant was not determined. There is the possibility that additional protein would be extracted due to exposure of salt-sensitive linkages to extractant after reblending.

Ultracentrifugal patterns of protein extracts

Ultracentrifugal patterns of protein extracts prepared with prolonged extraction plus reblending from muscle stored at −29°C after aging in ice (Fig. 1–5) indicated that additional protein extracted following the overnight exposure and the reblending phase of this technique was largely altered protein which sedimented very rapidly. Double-strength extracts (muscle-to-extractant ratio 20:380) of muscle iced for prolonged periods and stored at −29°C for 2 hr showed (Fig. 1) much rapidly sedimenting material (fast peak or peaks plus pellet material) similar to that seen in patterns of comparable extracts from muscle iced 39 days and not frozen (Anderson et al., 1968). During centrifugation, the fast peak was resolved sooner in the 33-day (B1–3; see centrifugation times) than in the 25-day pattern (A1–3). Since protein concentrations were not too different, this is an indication that the concentrations of components resolved in this peak were higher in the 33- than in the 25-day muscle. In the pattern for 39-day muscle (C1–3), peaks II and IIIA were much smaller than in the 25- and 33-day patterns and there was multiple fast peak formation (C3). Whether the fast peak could be demonstrated with this technique for muscle iced less than 25 days before freezing is not known, since patterns of extracts from muscle iced 1–35 days and stored at −29°C for 2 hr were not obtained.

For muscle iced 1–19 days and frozen-stored 11 months (Fig. 2), the fast peak was seen in patterns of single-strength (muscle-to-extractant ratio 10:390) extracts prepared with overnight extraction plus reblending (A-D). Peak II, which decreased with increasing storage of the muscle in ice, was somewhat larger in these patterns than in patterns of protein readily extractable from this type of muscle (Anderson et al., 1969). This may be an indication that with the more effective extraction procedure, some additional native protein as well as altered protein is extracted. However, the increase may be more apparent than real and explainable as an effect of increased protein concentration (Anderson et al., 1969).

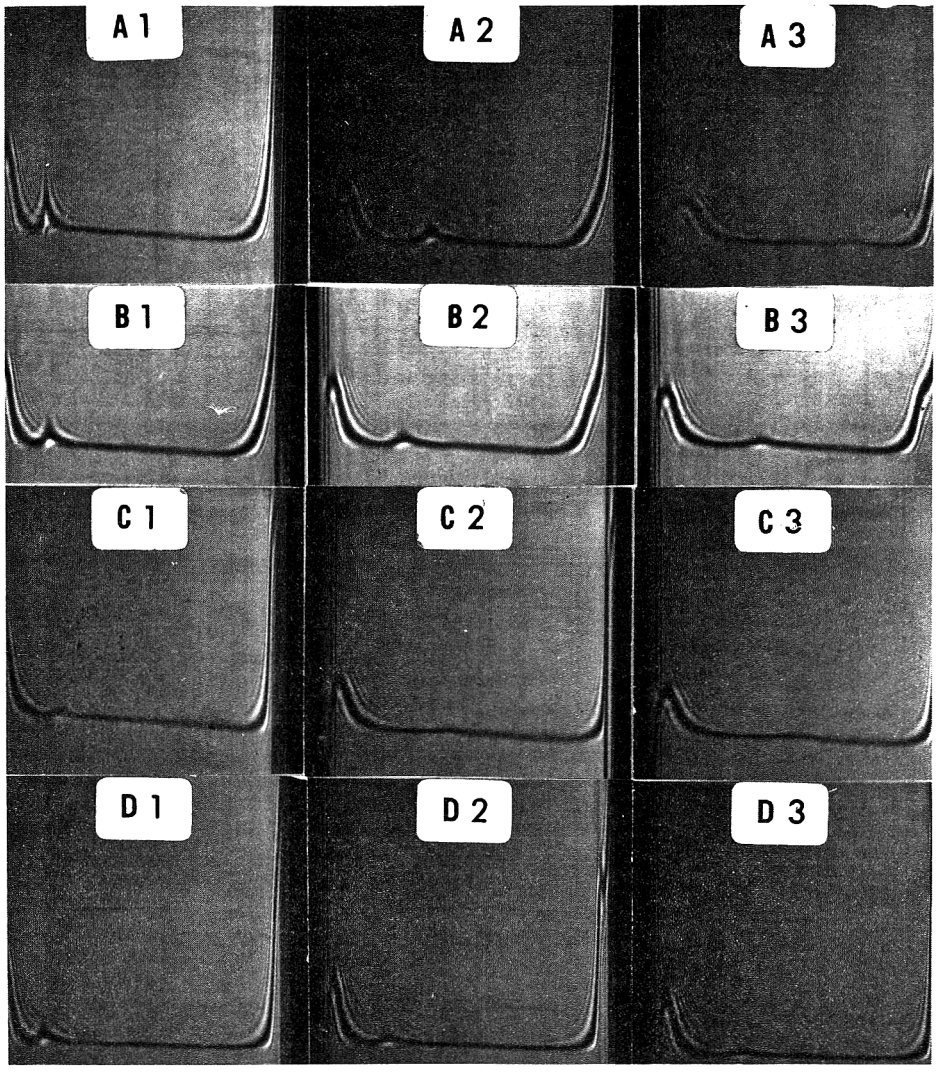


Fig. 2.–Sedimentation patterns of single-strength protein extracts prepared with overnight extraction plus reblending from cod muscle frozen stored 11 months after aging in ice for 1 day (A), 12 days (B), 19 days (C) and (D). Protein concentrations (mg protein N/ml) were: A, 0.586; B, 0.579; C and D, 0.443. Photographs were taken at a phase plate angle of 55°, A and B: (1) 4 min after reaching 50,740 rpm; (2) 8 min; (3) 12 min. C: (1) on reaching 50,740 rpm; (2) on reaching 39,460 rpm 4 min later; (3) 4 min after reaching 34,460 rpm. D: (1) 5 min after reaching 39,460; (2) 9 min; (3) 12 min.

The size of peak II and the fast peak could be increased by reducing the speed of the rotor during centrifugation or using a reduced speed throughout (D1–3). The fast peak was not seen in 22–35-day patterns, but the pellet area was large, except in the 35-day pattern (not illustrated).

Doubling the concentration of all components in extracts prepared with prolonged extraction plus reblending favored the resolving of fast-sedimenting components (Fig. 3–5). The patterns for extracts prepared with and without reblending for muscle iced 1 day and frozen-stored 11 months (Fig. 3) show that reblending causes the extraction of aggregated protein.

The pellet area was large in the pattern for the reblended preparation from muscle iced 1 day and frozen-stored 11 months. It was larger in similar patterns for muscle iced 5 to 15 days prior to freezing (Fig. 7 and 4). Illustrated in the patterns for 12-day muscle (Fig. 5), preparative centrifugation at 4,000 g (A) rather than at the usual 1,100 g resulted in a sharper fast peak and some reduction of the pellet area. At 15,000 g (B), however, the pellet was reduced to a greater extent, and the fast peak was no longer discernible.

For muscle iced up to 19 days and frozen-stored 11 months, fast peak formation and large pellet areas were seen in patterns of double-strength, reblended extracts. Muscle iced more than 19 days was not examined after 11 months' frozen storage.

Examination of a few samples of mus-

cle stored for 17 months at −29°C (Fig. 6) showed that with the additional 6 months' storage, more association occurred in the muscle. The fast peak and large pellet area were still seen in 8-day patterns (A), but they were smaller than in patterns of 12- and 15-day iced muscle frozen stored 11 months (Fig. 4). In patterns of 12- and 19-day iced muscle frozen stored 17 months (Fig. 6B–C), the only evidence for components other than peak III was the small but significant pellets and the absence of true plateau areas.

Changes in peak and pellet areas when either the reblended muscle-extractant homogenate or its centrifuged supernate was stored at 1°C suggest that dissociation occurred during their storage. The effects are illustrated for 5-day muscle (Fig. 7). Superimposed pellet areas complicated the picture. Rapid changes in concentration toward the bottom of the cell (Anderson et al., 1968) and their effects on sedimentation rates precluded any further evaluation of the patterns.

Sedimentation patterns of double-strength extracts from muscle iced 25, 33 and 39 days and examined early in the frozen storage period (Fig. 8) were consistent with the patterns for protein extraction shown in Table 2. Comparison of the ultracentrifugal patterns for muscle frozen-stored 1 week (A1–3), typical for muscle stored 1–2 weeks, with those for muscle stored at −29°C for 2 hr (Fig. 1) shows the marked changes in peak II and rapidly sedimenting components that occurred in the muscle during the first week of frozen storage. The shift toward greater association in the muscle itself was reflected in the larger amounts of altered protein in these extracts. While isolated photographs taken during centrifugation give some indication of the composition of these extracts, amounts of rapidly sedimenting, polydisperse aggregates are best assessed by the time of centrifugation required to resolve the fast peak and the speed with which this peak moves into the pellet area and disappears as part of the pellet. In patterns shown in Figure 8, for example, the time of centrifugation required to obtain peak areas illustrated was much greater for muscle iced 25 days prior to freezing (A1) than for either the 33- (A2) or 39-day (A3) muscle. For muscle frozen-stored for 4–5 weeks (B1–3), the patterns and centrifugation times required for build-up of components responsible for the fast peak when compared with patterns for muscle stored 1 week (A1–3) are consistent with a somewhat greater degree of association in the muscle. With further storage (C1–3), the association process was extensive enough so that in the patterns of protein that was extractable, the fast peak was seen only for 25-day muscle (1). Rapidly sedimenting material was seen only in the pellet in patterns for 33-day (2) muscle and was virtually absent in those for 39-day muscle (3). Thus, changes in composition in protein extracts prepared with reblending after overnight extraction followed the same rate pattern noted for changes in readily extractable protein (Anderson et

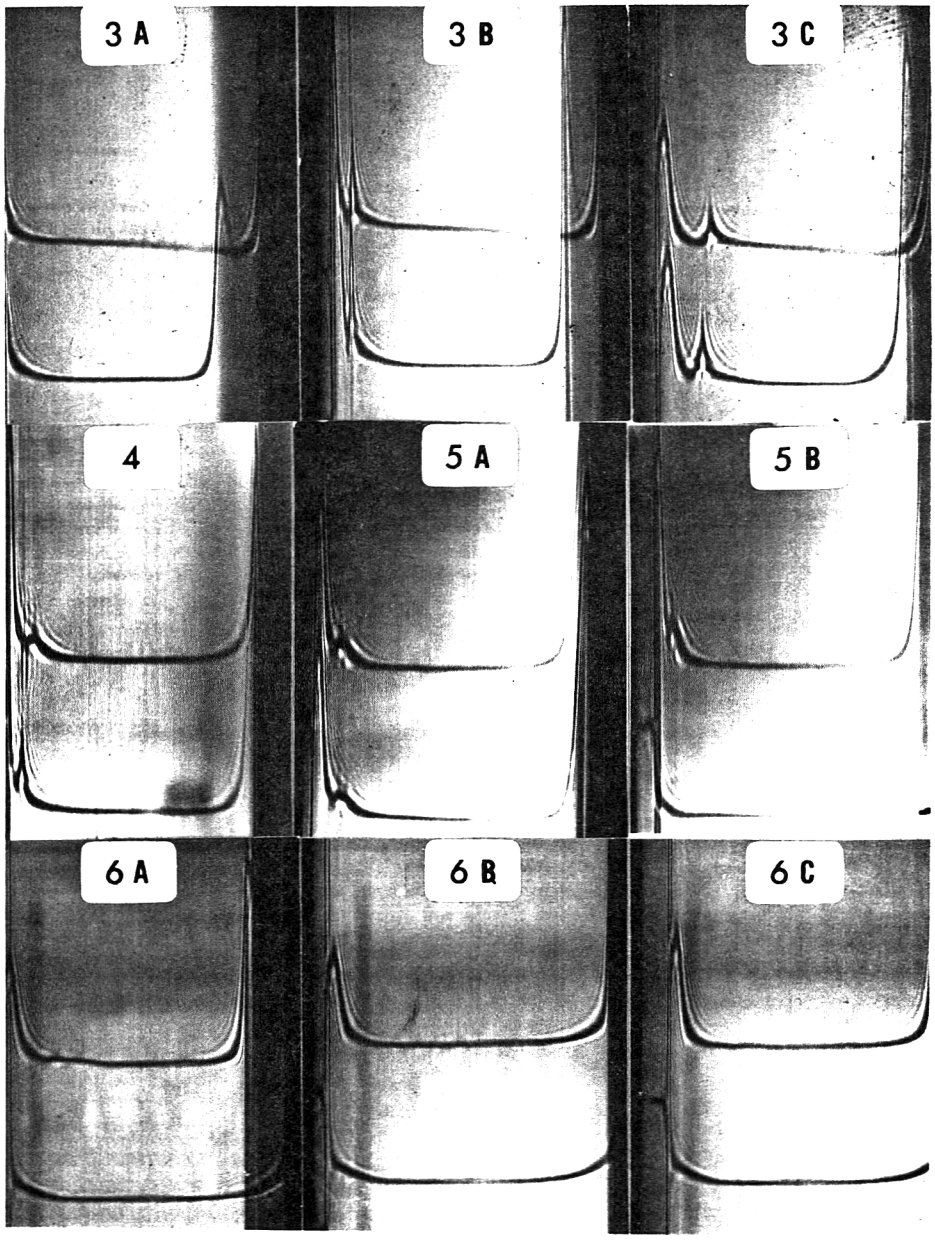
Fig. 3–6.–Sedimentation patterns of double-strength protein extracts of frozen-stored cod muscle.

Fig. 3.–Patterns of extracts prepared with overnight extraction with (lower pattern) and without (upper pattern) reblending from cod muscle frozen stored 11 months after aging in ice for 1 day. Photographs were taken at a phase plate angle of 55° and A, on reaching 50,740 rpm; B, 2 min; and C, 8 min after reaching speed.

Fig. 4.–Patterns of extracts prepared with overnight extraction plus reblending from cod muscle frozen stored 11 months after aging in ice for 12 days (upper pattern) and 15 days (lower pattern). Photograph was taken at a phase plate angle of 55° 3 min after reaching 50,740 rpm.

Fig. 5.–Patterns of extracts from muscle frozen stored 11 months after aging in ice for 12 days prepared with overnight extraction plus reblending and preparative centrifugation: A, upper pattern, at usual 1,100 g; A, lower pattern, at 4,000 g; B, upper pattern, at 15,000 g; B, lower pattern, solvent base line. Photographs were taken at a phase plate angle of 55° 3 min after reaching 50,740 rpm.

Fig. 6.–Patterns of extracts prepared with overnight extraction plus reblending (upper patterns) from muscle frozen stored 17 months after aging in ice for: A, 8 days; B, 12 days; C, 13 days. Lower patterns are solvent base lines. Photographs were taken at a phase plate angle of 55° 9 min after reaching 50,740 rpm.



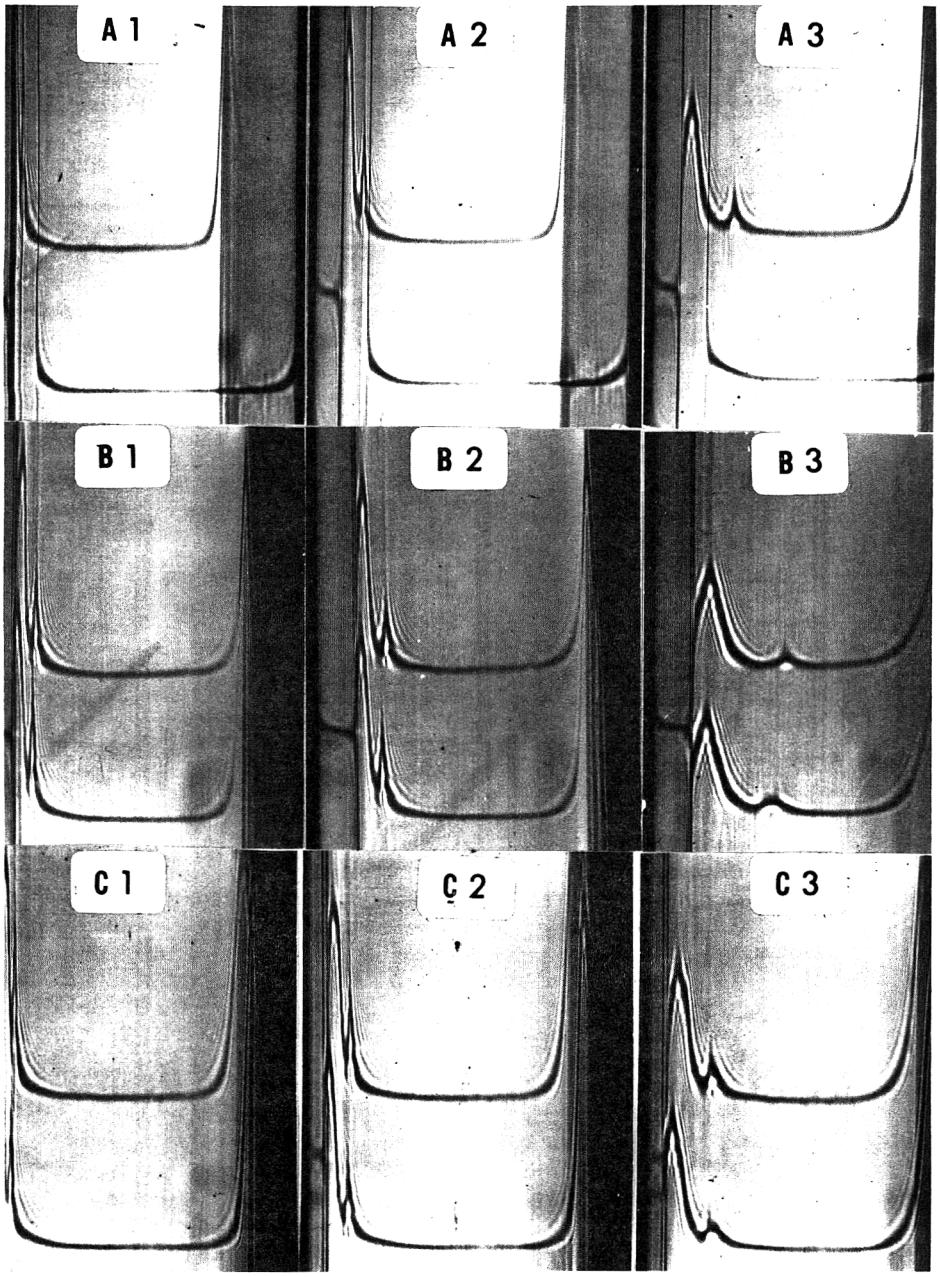


Fig. 7.–Patterns of double-strength protein extracts from muscle frozen stored for 11 months after aging in ice for 5 days prepared (A), with usual overnight extraction plus reblending, upper pattern; lower pattern, solvent base line; (B), with 24-hr additional exposure of protein in the reblended homogenate, lower pattern, or its centrifuged supernate, upper pattern, to extractant and (C), with 48 hr additional exposure of protein in the reblended homogenate, upper pattern, or its centrifuged supernate, lower pattern, to extractant. Photographs were taken at a phase plate angle of 55° (1) 1 min; (2) 3 min; (3) 7 min after reaching 50,740 rpm.

al., 1969), i.e., a phase of very rapid decrease early in storage, followed by a phase of slower decrease, and then little further change.

Phase contrast micrographs of residues of homogenates

When muscle samples were stored at −29°C for various periods after aging in ice, fragments in residues after overnight exposure of the homogenized muscle to extractant differed little from those seen in residues immediately after homogenization, i.e., they were predominantly transversely severed fibers early in the storage period (Anderson et al., 1969).

When homogenates were reblended after overnight storage at 1°C, however, fragments were small when compared with those in homogenates examined immediately after the first blending, indicating that brief reblending after overnight exposure of the homogenized muscle to extractant resulted in much additional fragmentation of the muscle. For muscle iced 25 days (Fig. 9), fragments were small in residues of muscle frozen for 1 week (A). With storage at −29°C, somewhat larger fragments with perceptible banding pattern were found along with increasing numbers of transversely severed fibers against a background of very small fragments having little morphology and practically no discernible banding pattern (B).

Fragments in comparable residues of muscle iced 33 and 39 days prior to freezing were somewhat larger. However, the fragments were at no time predominantly chopped-off fibers. Rather, they were small, clumped and smudged (Fig. 10 and 11).

In muscle iced 1–8 days and frozen-stored 11 months, fragments in residues of reblended homogenates were small (not illustrated). Fragments from muscle iced 12–27 days in general became larger as the duration of storage in ice increased, but many very small amorphous fragments in which there were occasional areas showing a banding pattern were also observed (Fig. 12 A and B). Residues from muscle iced 29 and 34 days before frozen storage contained many transversely severed fibers, 2 to 5 diameters in length, along with many small amorphous fragments (not illustrated).

The protein in these small fragments must be very accessible to the extractant. Its failure to go into solution is an indication that it has undergone alteration. The alterations may be extensive enough to bring about changes in microstructure detectable with phase microscopy. On the other hand, the smudged appearance may be due to (1) irregular cleavage across the cylindrical fiber in which there is increased cohesion of myobibrils due to cross-linking or (2) the effects of partial dissolution of protein.

Just as it cannot be concluded that the lack of normal banding pattern in fragments is evidence for changes in microstructure, the presence of protein aggregates in reblended extracts is not evidence that the aggregates exist as such in the frozen-stored muscle. Change in state of aggregation of altered protein may occur on exposure to extractant.

The low sensitivity to salt of linkages responsible for the aggregated proteins is evidence for involvement of hydrophobic bonding in the formation of aggregates. Ionic forces have been shown, however, to be involved in the complex formation (Anderson et al., 1968) that probably leads to aggregation.

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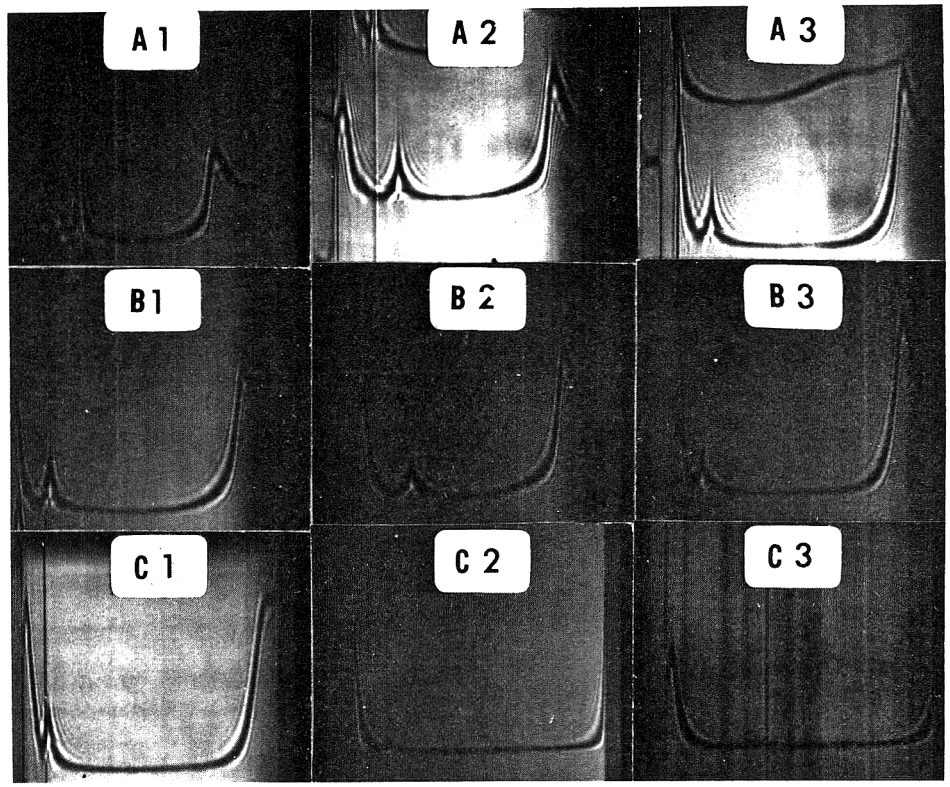

Fig. 8—Sedimentation patterns of double-strength protein extracts prepared with overnight extraction plus reblending from cod muscle frozen stored 1 week (A); 4 weeks (B); 11 weeks (C1); 7 weeks (C2); 9 weeks (C3) after aging in ice for 25 days (A1—C1); 33 days (A2—C2); 39 days (A3—C3). Protein concentrations (mg protein N/ml) were: A1, 1.35; A2, 1.19; A3, 0.894; B1, 1.13; B2, 1.19; B3, 0.842; C1, 1.01; C2, not determined; C3, 0.488. Photographs were taken at a phase plate angle of 55 °, and after reaching 50,740 rpm; A: (1) 28 min; (2) 11 min; (3) 8 min. B: (1) 6 min; (2) 11 min; (3) 2 min. C: (1) 3 min; (2) 2 min; (3) 6 min.

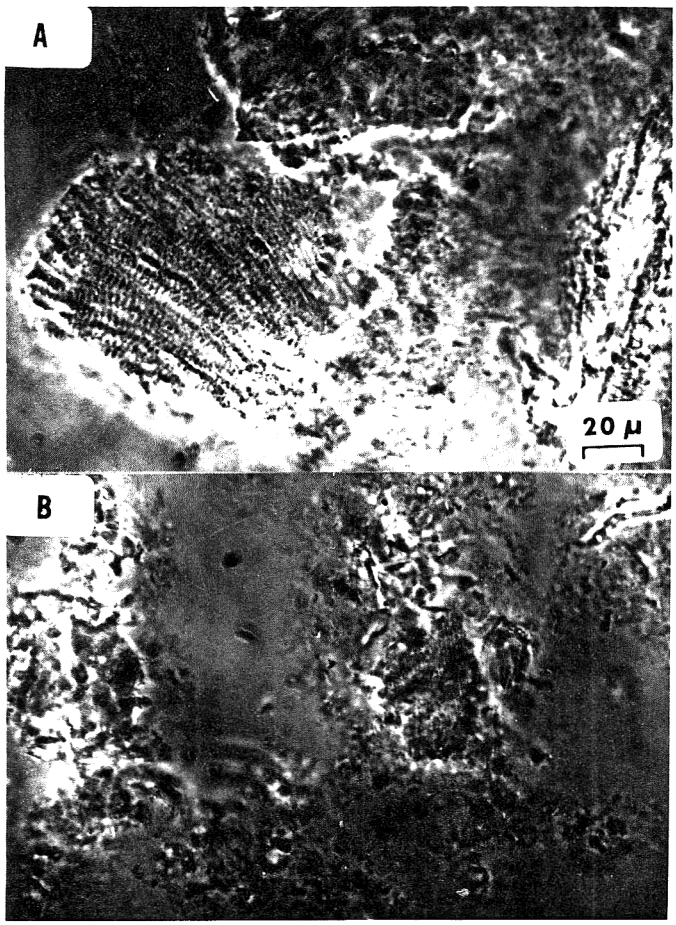

Fig. 9.—Phase contrast micrographs of residues of muscle homogenates prepared with overnight extraction plus reblending from cod muscle iced for 25 days and then frozen stored for 3 weeks (A) and 11 weeks (B).

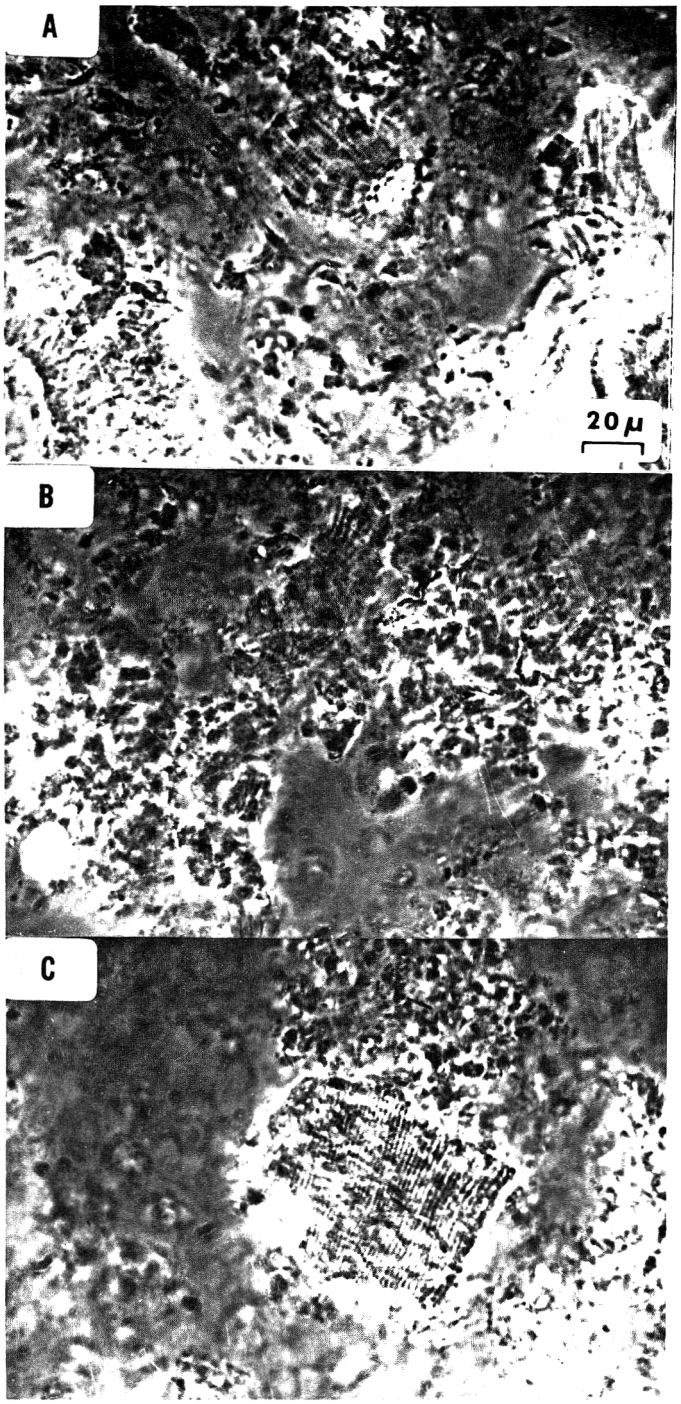

Fig. 10.–Phase contrast micrographs of residues of muscle homogenates prepared with overnight extraction plus reblending from cod muscle iced for 25 days and then frozen stored for 2 days (A); 2 weeks (B); 7 weeks (C).

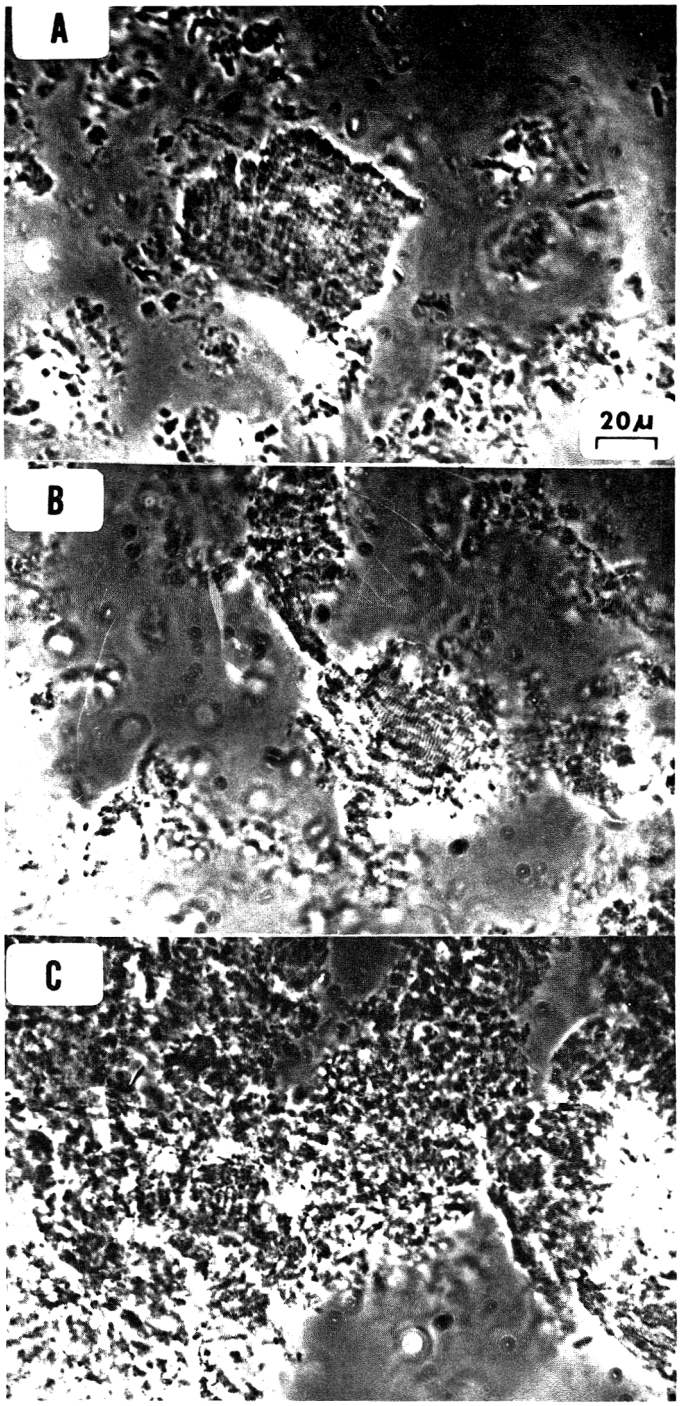

Fig. 11.–Phase contrast micrographs of residues of muscle homogenates prepared with overnight extraction plus reblending from cod muscle iced 39 days and frozen stored 1 week (A); 6 weeks (B); 9 weeks (C).

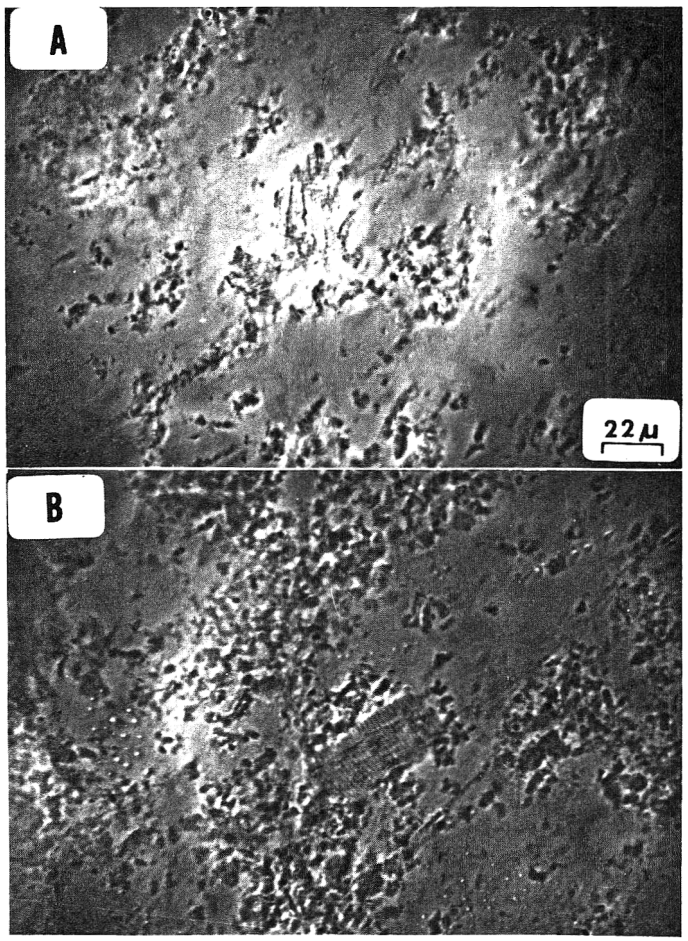

Fig. 12.—Phase contrast micrographs of residues of muscle homogenates prepared with overnight extraction plus reblending from cod muscle frozen stored for 11 months after aging in ice for 12 days (A) and 27 days (B).

cod muscle frozen and stored at −29°C after aging in ice. J. Fish. Res. Bd. Canada 26:2727—2736.

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OXIDATION OF PYRUVATE-2-C^{14}, AND LACTATE DEHYDROGENASE ACTIVITY AND ISOENZYME DISTRIBUTION OF FIVE PORCINE MUSCLES

SUMMARY—A study was conducted to compare parameters of aerobic and anaerobic metabolism in light and dark porcine muscles. Muscle samples were obtained from six Hampshire-Yorkshire gilts and frozen in liquid nitrogen five min post-exsanguination. Oxidative metabolism was estimated by measuring the conversion of pyruvate-2-C^{14} to $C^{14}O_2$ in homogenates. The lactate dehydrogenase (LDH) activity of each of the five isoenzymes, separated electrophoretically on cellulose acetate, was ascertained as a measure of anaerobic metabolism. Comparison of the pyruvate oxidation data suggested the following ranking, from most to least aerobic: trapezius, gluteus medius, dark biceps femoris, light biceps femoris, longissimus dorsi, dark semitendinosus and light semitendinosus. Variance analysis of the data indicated trapezius exhibited greater capacity to oxidize pyruvate than all other muscles ($P < .05$) and gluteus medius and dark biceps femoris exceeded the light semitendinosus ($P < .05$). LDH comparison among the muscles revealed that light muscles displayed higher ($P < .05$) activity than dark muscles (trapezius, dark biceps femoris and dark semitendinosus). The results implicated high lactate dehydrogenase activity—particularly the muscle-type isoenzyme—as a factor contributing to rapid postmortem lactate accumulation in light muscles of some porcine animals.

INTRODUCTION

PORCINE MUSCLES may be classified as light or dark, depending upon their proportion of light and dark muscle fibers. Beecher et al. (1965) reported that the dark muscles of the pig have higher levels of myoglobin and longer post-rigor sarcomeres than light muscles. These findings support the observation of Briskey (1964) that dark muscles are resistant to the development of the pale, soft, exudative (PSE) muscle condition. Data suggest that muscles with a highly developed aerobic mechanism exhibit moderate or slow postmortem glycolytic rates. This observation does not explain some recorded differences in PSE incidence; for example, differences due to stress-susceptibility variations in porcine animals. Briskey and Lister (1968) demonstrated that the longissimus dorsi muscle of Poland China pigs, though the muscles have a propensity to develop the PSE condition, tend to be more aerobic than muscles of a stress-resistant (Chester White) breed.

In spite of the importance of lactate dehydrogenase (LDH) in anaerobic glycolysis, the enzyme has received limited attention. Cassens et al. (1963) suggested that trapezius muscle may have slightly more LDH activity, but higher ultimate pH than the longissimus dorsi. In contrast, Briskey and Wismer-Pederson (1961) reported high methylene-blue reducing capacity in fast-glycolyzing muscle. Discovery of the isomeric nature of LDH has led to a re-evaluation of the role of the enzyme in cellular metabolism. Markert (1963) demonstrated five distinct isoenzymes by electrophoresis. He ascertained two basic subunits, designated heart (isoenzyme I) and muscle (isoenzyme V) type, associate to form an active tetramer. Investigations by Cahn et al. (1962) and Dawson et al. (1964) indicated that the heart type isoenzyme predominates in tissues where oxidative metabolism prevails. Anaerobic tissues contain a large percentage of their LDH activity as the muscle type enzyme. It was also reported that the heart type isoenzyme was inhibited by pyruvate concentrations which are less than saturating for the muscle isoenzyme. The implication of this to the postmortem accumulation of lactic acid in muscle has not been investigated.

These findings prompted Beecher et al. (1969) to study the interrelationships between citrate and glycolysis in postmortem muscle. Their studies showed light muscles exhibited greater LDH activity than dark muscles. Comparison of two light muscles with two dark muscles demonstrated an inverse relationship between LDH activity and parameters of oxidative metabolism. Citrate did not appear to inhibit glycolysis in postmortem muscle.

Use of radioactive tracers has not been widely applied for the estimation of aerobiosis in porcine muscle. Accordingly, an experiment was designed to investigate the relationship between parameters of aerobic (oxidation of pyruvate) and anaerobic (LDH activity and isoenzyme distribution) metabolism in light and dark porcine muscles.

EXPERIMENTAL

SIX HAMPSHIRE-YORKSHIRE gilts of approximately 125 kg weight were used. Samples of the longissimus dorsi, trapezius, gluteus medius, and light and dark semitendinosus and biceps femoris muscles were secured and frozen in liquid nitrogen within 5 min postmortem. Samples were stored at −20°C for less than 3 months before analysis.

Aerobic metabolic activity was estimated by measuring the capacity of muscle homogenates to convert pyruvate-2-C^{14} to $C^{14}O_2$. This is analogous to the determination of succinate dehydrogenase activity (Beecher et al., 1969; Briskey and Lister, 1968) in porcine muscle. The isotopic method, however, has the advantage of measuring the activity of the complete tricarboxylic acid cycle, instead of only one component enzyme. (This is not true if pyruvate-1-C^{14} is used.)

Homogenization buffer (Haslam and Krebs, 1963) was prepared by combining the following solutions: 1) 0.02M $MgCl_2$ (75 ml); 2) 0.1M KPO_4 buffer, pH 7.4 (187.5 ml); and, 3) 0.154M KCl (to 1000 ml). The final mixture was oxygenated for 10 min. The isotopic solution was prepared by dissolving 1.6 mg (0.05 mc) sodium pyruvate-2-C^{14} (New England Nuclear Corp.) in 50 ml deionized, distilled water.

Muscle samples were thawed overnight at 2°C and homogenized in 5 volumes (v/w) homogenization buffer. Homogenate (3 ml) and 0.5 ml isotopic solution were transferred to pre-chilled Erlenmeyer flasks (25 ml). A small vial containing 0.5 ml Nuclear Chicago Solubilizer and a rectangle of filter paper were placed in each flask. Reaction flasks were sealed with serological-type rubber stoppers and flushed with 100% oxygen for one min. Homogenates were incubated by agitating the reaction flasks in a 38–39°C water bath for one hr. The reaction was stopped by the addition of 0.5 ml conc H_2SO_4, followed by agitation for one hr at 38–39°C. The vials were subsequently removed from the flasks, cleaned of homogenate debris, and placed in a scintillator vial with 20 ml scintillator fluid (Buhler, 1963). The samples and standards were counted by liquid scintillation spectroscopy. Results are expressed as counts per min (cpm) per g Kjeldahl protein in the 3-ml aliquot of homogenate (counting efficiency = 85%). The % pyruvate to CO_2 conversion ranged from 0.25% (a semitendinosus muscle) to 5% (a trapezius muscle).

Anaerobic metabolic activity of muscle was assessed by determining total LDH activity and LDH isoenzyme distribution. Ten volumes (v/w) of cold 0.03M KPO_4 buffer, pH 7.4, were used to homogenize and extract muscle samples which had been thawed overnight at 2°C. The extracts were centrifuged at 12,000 g for 20 min (2°C) and filtered through glass wool. Supernatants were assayed for LDH activity (Reeves and Fimognari, 1963) and isoenzyme distribution (Addis and Kallweit, 1969). Supernatants for total activity were diluted 1/1000 with diluent of 0.067M KPO_4 buffer pH 7.4 and 0.1% albumin. One unit of activity was

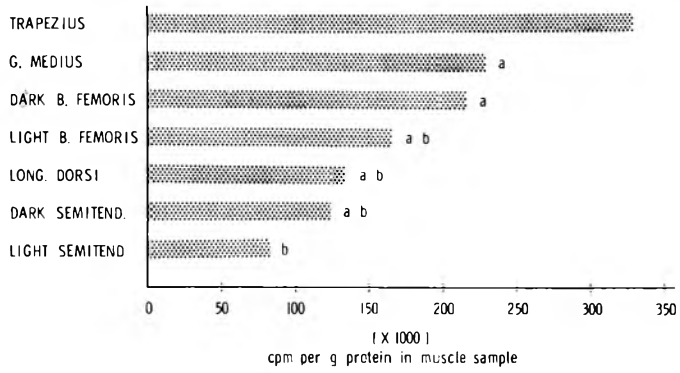

Fig. 1—Conversion of pyruvate-2-C^{14} to $C^{14}O_2$ in light and dark porcine muscle homogenates.

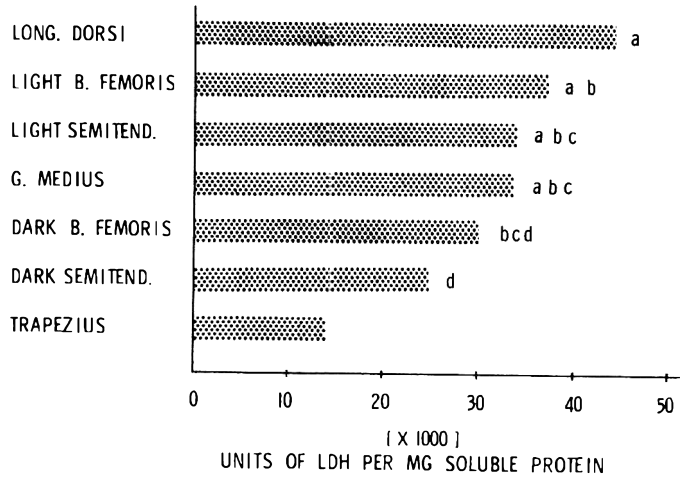

Fig. 2—Lactate dehydrogenase activity in light and dark porcine muscle.

defined as a decrease in absorbance of 0.001 per min at 340 mμ wavelength at 25°C. Protein concentration was determined by the biuret method. Activity was expressed as units of LDH per mg soluble (biuret) protein.

Electrophoretic (Beckman Microzone) separation of the isoenzymes was accomplished on cellulose acetate membranes in Tris-HCl-barbital buffer, pH 8.4. The location of the isoenzymes was determined by staining the strips by the method of Fisher and Nixon (1967). Staining gel (100 ml) was made by combining the following reagents: 1) 1.5g Difco Special Agar, Difco Laboratories, Detroit, Michigan; 2) 70 ml Tris-HCl buffer (0.2M, pH 8.3); 3) 2 ml lactic acid (60%) syrup; 4) 20 ml nitroblue tetrazolium (mg/ml); 5) 2 ml phenazine methosulfate (mg/ml); and, 6) 100 mg nicotinamide adenine dinucleotide dissolved in 6 ml water. The muscle enzyme (isoenzyme V) was the least anodic band. Isoenzyme I (the heart enzyme) was the most anodic. After electrophoresis, the LDH isoenzyme bands were densitometrically quantitated and the activity for each zone was calculated by dividing the total enzyme activity proportionately to the optical density of each zone. Results were expressed as activity per mg soluble protein.

Confirmation of the cellulose polyacetate isoenzyme patterns was accomplished by using acrylamide gel as a supporting medium for electrophoresis. Supernatant (75 μl) was applied to a regular 8% polyacrylamide gel (6 mm thickness) in an E-C Vertical Gel apparatus. The bands were located using a staining procedure similar to that used for the polyacetate membranes.

Data were analyzed by analysis of variance and mean difference tested with the Newman-Keuls's sequential range test as outlined by Steel and Torrie (1960).

RESULTS & DISCUSSION

KNOWLEDGE gained from experience and previous observations (Briskey, 1964; Beecher et al., 1965, 1969) permit these muscles to be classified into two groups according to their relative lightness or darkness. The dark muscles, which rarely exhibit the PSE condition, include the trapezius, dark semitendinosus and dark biceps femoris. The light muscles, which may frequently incur the PSE condition, include the longissimus dorsi, gluteus medius, light semitendinosus and light biceps femoris.

The trapezius muscle exhibited the highest ($P < .05$) capacity for pyruvate oxidation of the muscles studied (Fig. 1). Beecher et al. (1965) concluded that the trapezius muscle may be classified as highly aerobic by several criteria—red fiber content, succinic dehydrogenase activity, myoglobin and fat levels. In the present study, the gluteus medius and dark biceps femoris muscles exceeded ($P < .05$) the pyruvate-oxidizing activity of the light semitendinosus (Fig. 1). The results of Beecher et al. (1965) categorized the dark biceps femoris muscle as aerobic; yet, they classified the gluteus medius as an anaerobic muscle. That the PSE condition frequently occurs in the gluteus medius substantiates their findings. Nevertheless, Allen et al. (1967) demonstrated that the gluteus medius muscle contains a greater proportion of β-hydroxybutyric-dehydrogenase-positive fibers than the longissimus dorsi and light biceps femoris.

Lactate dehydrogenase data, in contrast to the pyruvate oxidation results, demonstrate a definite difference ($P < .05$) in the activity of this enzyme between light and dark muscles (Fig. 2). The finding of greater LDH activity in light muscle than dark muscle is in agreement with the results of Beecher et al. (1969), but does not agree with those

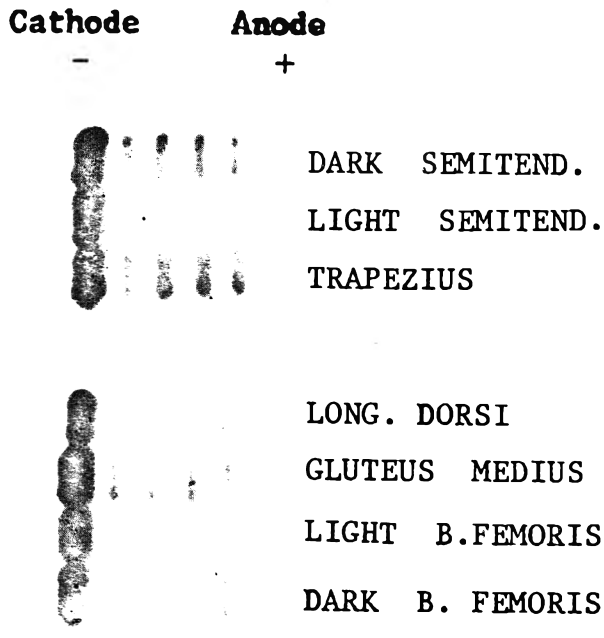

Fig. 3—Typical LDH isoenzyme distribution patterns as determined by cellulose acetate electrophoresis.

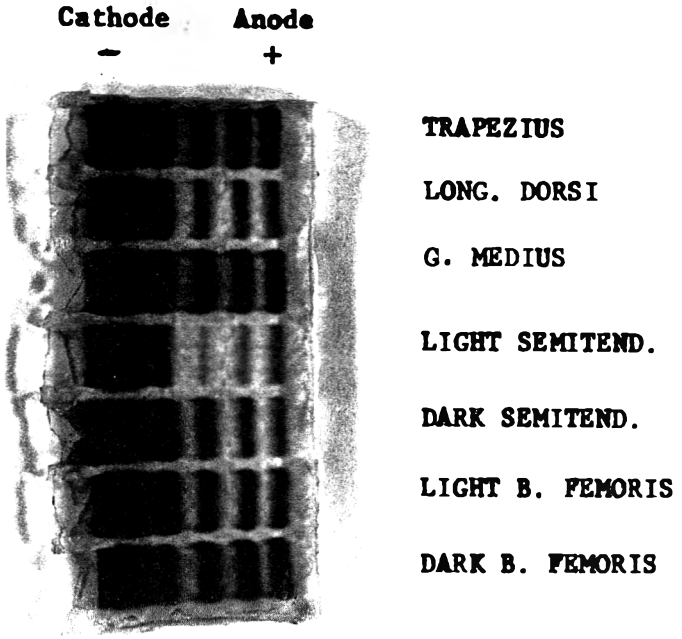

Fig. 4–Typical lactate dehydrogenase isoenzyme distribution patterns as determined by polyacrylamide gel electrophoresis.

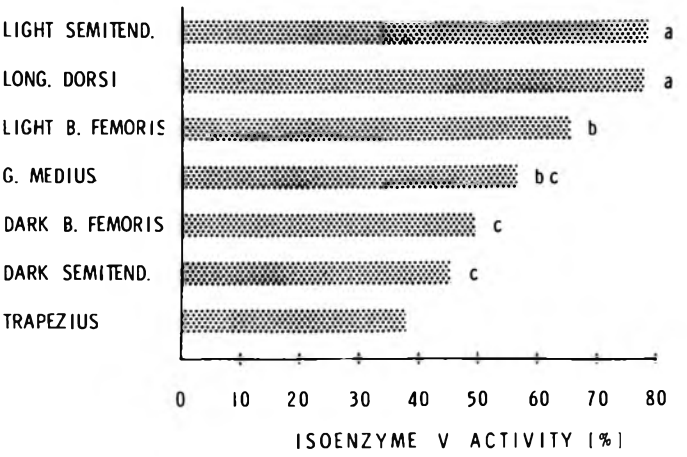

Fig. 5–Percent of total lactate dehydrogenase activity as LDH V.

of Cassens et al. (1963). The findings of high LDH activity in muscle prone to postmortem myolactosis appears to be logical. The trapezius muscle contained the lowest ($P < .05$) LDH activity of the muscles studied. Several other significant ($P < .05$) differences in LDH activity were recorded (Fig. 3). The observed differences in LDH activity and the results of the pyruvate oxidation experiment emphasize the reciprocal relationship between aerobic and anaerobic energy metabolism in most of the muscles studied. This conclusion agrees with previous studies (Beecher et al., 1965, 1969).

Figures 3 and 4 are representative LDH isoenzyme patterns as determined by cellulose acetate and polyacrylamide gel electrophoresis. The two methods gave very similar patterns. All muscles studied (as routinely determined on cellulose polyacetate) exhibited a typical "skeletal muscle" pattern of LDH isoenzyme distribution. Differences were recorded (Fig. 5) for the percent isoenzyme V parameter which closely follow the differences observed in total LDH activity. The light semitendinosus and longissimus dorsi muscles exhibited a greater ($P < .05$) percentage of the LDH activity as isoenzyme V than all other muscles. In addition, the light biceps femoris exceeded the dark biceps femoris, dark semitendinosus and trapezius; the gluteus medius, dark biceps femoris and dark semitendinosus exceeded the trapezius (all differences significant, $P < .05$). These findings suggest that light porcine skeletal muscles contain high LDH activity and a high percentage of their total LDH activity as LDH isoenzyme V. And, since light porcine muscles are susceptible to postmortem myolactosis, these findings implicate isoenzyme V as a factor contributing to the PSE condition.

Further evidence for this conclusion is provided by the difference recorded between the individual activities of each isoenzyme fraction (Table 1). The differences noted in fraction V, the muscle-type isoenzyme, are the most important. The trapezius contained the lowest ($P < .05$) isoenzyme V activity. With the

Table 1–Lactate dehydrogenase isoenzyme distribution in five porcine muscles.[1]

Isoenzyme	Significant mean differences[2]							F
I	LST[3]	LD	LBF	GM	T	DST	DBF	
	0	121	1447	1910	1936	2265	3400	10.3**
II	LST	LD	T	LBF	GM	DST	DBF	
	79	312	2044	2424	2566	3469	3698	8.4**
III	LST	T	LBF	LD	GM	DBF	DST	
	707	2800	3138	3187	3983	4337	4778	6.6*
IV	T	DST	LD	DBF	GM	LBF	LST	
	2178	3847	4003	4823	5187	5613	6222	7.6**
V	T	DST	DBF	GM	LBF	LST	LD	
	5457	11,612	15,111	19,248	24,878	27,298	36,761	40.3**

[1] Enzyme units per mg soluble protein.
[2] Any means not underlined by the same line are significantly ($P < .05$) different.
[3] Abbreviations: LD, longissimus dorsi; GM, gluteus medius; T, trapezius; LST, light semitendinosus; DST, dark semitendinosus; LBF, light biceps femoris and DBF, dark biceps femoris.

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

exception of the gluteus medius muscle, all light muscles exceeded ($P < .05$) all dark muscles, with respect to isoenzyme V activity. The gluteus medius significantly ($P < .05$) exceeded the trapezius and dark semitendinosus, but not the dark biceps femoris. Beecher et al. (1969) found that the porcine rectus femoris, a red muscle, contained a higher LDH V content (based on percentage of total activity) than the gluteus medius and longissimus dorsi. Moreover, Allen et al. (1967) histochemically demonstrated more LDH-positive fibers in the dark biceps femoris than the gluteus medius and longissimus dorsi. Therefore, it is difficult to categorize some porcine skeletal muscles as definitely aerobic or anaerobic (as in the case of the gluteus medius in the present study). There also appears to be no physiological reason or principle which would indicate that muscles with high LDH activity must contain low levels of oxidative enzymes.

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APPLYING PROTEOLYTIC ENZYMES ON SOYBEAN. 2. Effect of Aspergillopeptidase A Preparation on Removal of Flavor from Soybean Products

SUMMARY—Chemical and organoleptic tests showed that partial digestion of soybean curd and defatted soybean flour by aspergillopeptidase A preparation removed flavor compounds and related fatty materials from these soybean products. Amounts of ether-soluble compounds, total carbonyl compounds and volatile reducing substances released during incubation of soybean curd with the enzyme were higher than those released during incubation without the enzyme. Amounts of volatile flavor compounds, including n-hexanal and n-hexanol, released during the incubation of defatted soybean flour with the enzyme were also higher than those released during incubation without the enzyme. Related compounds, i.e., lipids, pigments, saponins, etc., also were released during the enzymation. The enzymic digestion product from either soybean curd or defatted soybean flour had less odor, taste and color. Stability of the enzymic digestion product from the defatted soybean flour against autoxidation during the preservation, measured by TBA-reaction and organoleptic test, was superior to that of the product treated similarly without the enzyme.

INTRODUCTION

IN THE PRECEDING paper, we reported that the objectionable flavor was effectively removed from a soybean protein preparation during its digestion by proteolytic enzyme (Fujimaki et al., 1968). According to this paper, among 12 proteolytic enzymes tested, the aspergillopeptidase A preparation (trade name Molsin) was evaluated as the most effective in removing the objectionable flavor.

The present work concerns some experiments to afford a chemical basis for interpretation of the enzymatic removal of flavor.

MATERIALS & METHODS

Soybean curd

100 g of raw soybean was dipped in 300 ml of water for 12 hr at room temperature, crushed in a mortar, mixed with 200 ml of water, boiled for 30 min, and filtered. By adding 3 g of $MgCl_2$ to the filtrate, 30 g of soybean curd was salted out on the dry basis. Composition: water, 88.7%; nitrogen, 1.04%; crude fat, 0.32%; carbohydrate, 3.70%; ash, 0.61%.

Defatted soybean flour

Ground raw soybean was defatted with sufficient volume of *n*-hexane at room temperature, and ground further into flour. Composition: water, 9.0%; nitrogen, 8.0%; crude fat, 0.7%; carbohydrate, 31.6%; ash, 6.4%; crude fiber, 2.3%.

Enzymic digestion

10 g of the soybean curd suspended in 1,000 ml of dilute HCl (pH 2.8) was digested with 0.1 g of aspergillopeptidase A preparation at 50°C for 2 hr. This enzyme preparation, trade name Molsin, was obtained from Seishin Seiyaku Co. The proteolytic activity measured for casein or a soybean protein preparation as a substrate was described by Fujimaki et al. (1968). The enzymation was stopped by neutralizing the digest with NaOH. The digest was centrifuged and the resulting supernatant submitted to chemical analyses. The precipitate was treated with tenfold weight of 50% ethanol to remove bitterness that appeared during the enzymation, and filtered. By lyophilizing the residue, the final product was obtained and submitted to sensory tests.

20 g of the defatted soybean flour was treated with 900 ml of dilute HCl (pH 1.5) at 30°C for 2 hr and centrifuged. The extract thus obtained was filled up to 1,000 ml with dilute NaOH so that the pH was adjusted to 2.8, and digested with 80 mg of the enzyme at 50°C for 2 hr. The enzymation was stopped by lyophilization. The resulting powdered digest was treated with tenfold weight of 90% ethanol to remove the flavor compounds and the related fatty materials, and filtered. The 90% ethanol extract was submitted to chemical analyses. By drying the residue under reduced pressure at room temperature, the final product was obtained and submitted to sensory tests.

Analyses of the supernatant of the soybean curd digest

A given volume of the supernatant was treated with a fivefold volume of ether. Total substances extracted with the ether were represented as crude fat. Genistein content in the crude fat was roughly estimated by measuring the optical density at 262 mμ according to the method of Horowitz et al. (1961). Phenolic compounds in the ether extract were investigated by the method of Arai et al. (1966a). Reducing substances in the crude fat were determined by potassium permanganate consumption according to Arai et al. (1967); total amounts of the ether-extractable acids were determined by the usual KOH titration. Ninhydrin-positive substances in the ether extract were determined by Kalant's method (1956); and carbonyl compounds by converting them to 2,4-dinitrophenylhydrazones and by weighing the hydrazones insoluble in 2 N HCl; anthrone-positive substances were determined by Dreywood's method (1946).

Analyses of the 90% ethanol extract of the defatted soybean flour digest

The extract, after evaporated to dryness, was treated with a 100-fold weight of ether, and the ether-soluble substances were represented as crude fat. Constituents of the 90% ethanol extractable substances, including nonpolar triglycerides, free fatty acids, phospholipids, saponins, sitosteryl glucoside, genistein and the like were investigated semiquantitatively. Nonpolar triglycerides, diglycerides and fatty acids were examined by thin-layer chromatography with silica gel G (Merck) according to Malins et al. (1960), using petroleum ether–ether–acetic acid (90:10:1). The chromatograms were made visible by spraying 5% phosphomolybdic acid in ethanol.

Phospholipids, sitosteryl glucoside and genistein were examined by thin-layer chromatography with silica gel G (Merck) according to Mangold (1961), using chloroform-methanol-water (65:25:4). The chromatograms were made visible by spraying 5% phosphomolybdic acid in ethanol, 50% H_2SO_4, 1% $FeCl_3$, 5% ninhydrin in *n*-butanol, or 7% sulfosalicylic acid–0.1% $FeCl_3$ in 70% ethanol. Phosphatidylcholine, phosphatidylinositol and phosphatidylethanolamine were identified by detecting choline, inositol and ethanolamine, respectively, after alkaline hydrolysis of the phospholipids under mild conditions with the method of Dawson (1960). Probable identification of sitosteryl glucoside and genistein was made by comparing the chromatograms with those reported by Nash et al. (1967), the latter being determined spectrometrically according to Horowitz et al. (1961). Saponins were detected also by the method of Sumiki (1929).

Analyses of volatile substances

Total amounts of volatile reducing substances released during the enzymation were measured by the method of Farber et al. (1956), partly modified as follows: Either soybean curd or defatted soybean flour was incubated with the enzyme in a round-bottomed flask with two openings under the conditions mentioned previously. Nitrogen gas was let into the flask through one opening and let out with the volatile substances through the other opening into a series of two traps containing respectively 10 ml of 0.1 N $KMnO_4$ in 1 N NaOH. The potassium permanganate consumption was measured by the usual back-titration with standardized $Na_2S_2O_3$ solution.

Otherwise, the flavor compounds swept out with nitrogen gas were trapped in a series of bottles dipped in ice bath, solid carbon dioxide–acetone bath and liquid nitrogen bath and submitted to gas chromatography under the conditions described in Figure 1. *n*-Hexanal and *n*-hexanol were identified according to the method of Arai et al. (1967), and these amounts were calculated from the corresponding peak areas calibrated by the standard solutions.

Sensory test

Odor, taste and color were evaluated by the rating-scale method. Some rheological characters were shown by simple descriptive tests.

Stability evaluation

Stability against autoxidation during preservation of the final product from the defatted soybean flour was examined. 10 g of the prod-

uct mixed with 20 g of purified sea-sand was placed in a 500-ml volume triangular flask with an air-tight stopper, and allowed to stand in a dark place at 37°C. Development of autoxidation and appearance of off-flavor at the various stages of preservation were investigated by the TBA method of Tarladgis (1964) and by the sensory test, respectively.

RESULTS

Effects of enzymation on soybean curd

Table 1 shows results of chemical and sensory evaluation of the supernatant of the soybean curd digest and the final product. Table 1 also shows amounts of volatile reducing substances released during the incubation. Significantly larger amounts of ether-extractable substances were found to be removed from the soybean curd to the supernatant by incubating with the enzyme than without the enzyme. Significantly larger amounts of

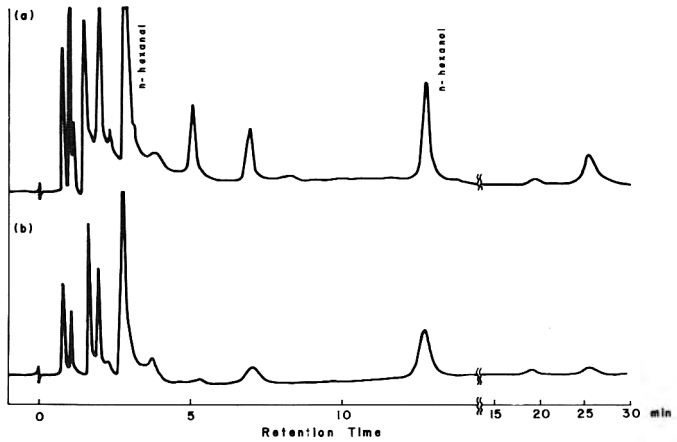

Fig. 1—Comparison of gas chromatograms of volatile compounds released during the incubation between enzymation group (a) and control group (b). Conditions: 200- by 3-mm stainless column, 10% carbowax 6000 on Diasolid L; temperature, 80°C; flow, 30 ml of N_2 per min; detection, hydrogen flame ionization.

Table 1—Chemical analyses and sensory evaluation of the supernatant, volatile substances and the final product from soybean curd.

	Enzymation group	Control group[8]
Supernatant		
Ether-extractable fraction		
Crude fat[1]	0.260	0.184
Optical density at 262 mμ	0.208	0.164
Potassium permanganate consumption[2]	5.60	4.30
Titration acidity[3]	0.63	0.50
Ninhydrin-positive substances[4]	0.081	0.040
Carbonyl compounds[5]	0.020	0.016
Phenolic acids	Trace	Trace
Anthrone-positive substances[6]	0.257	0.251
Odor	++	+
Taste	+++	+
Color	++	+
Volatile reducing substances[7]	3.25	1.55
Final product		
Odor	–	++
Taste	–	+
Color	–	–
Texture	Viscous	Granular

[1] Grams per 10 g of soybean curd on dry basis.
[2] Milliliters of 0.1 N $KMnO_4$ per 10 ml of the supernatant.
[3] Milliliters of 0.1 N KOH per 10 ml of the supernatant.
[4] The ether-extractable substances in 10 ml of the supernatant were dissolved in 13 ml of the ninhydrin reagent and the resulting color intensity measured at 570 mμ.
[5] Milligrams of 2,4-dinitrophenylhydrazones per 10 ml of the supernatant.
[6] One milliliter of the supernatant was diluted to 100 ml with water and 5 ml aliquot of it added to 10 ml of the anthrone reagent. The resulting color intensity was measured at 625 mμ.
[7] Milliliters of 0.1 N $KMnO_4$ consumed by the volatile reducing substances that released from 10 g of soybean curd on dry basis.
[8] Material was treated without enzyme under conditions similar to those of the enzymation group.

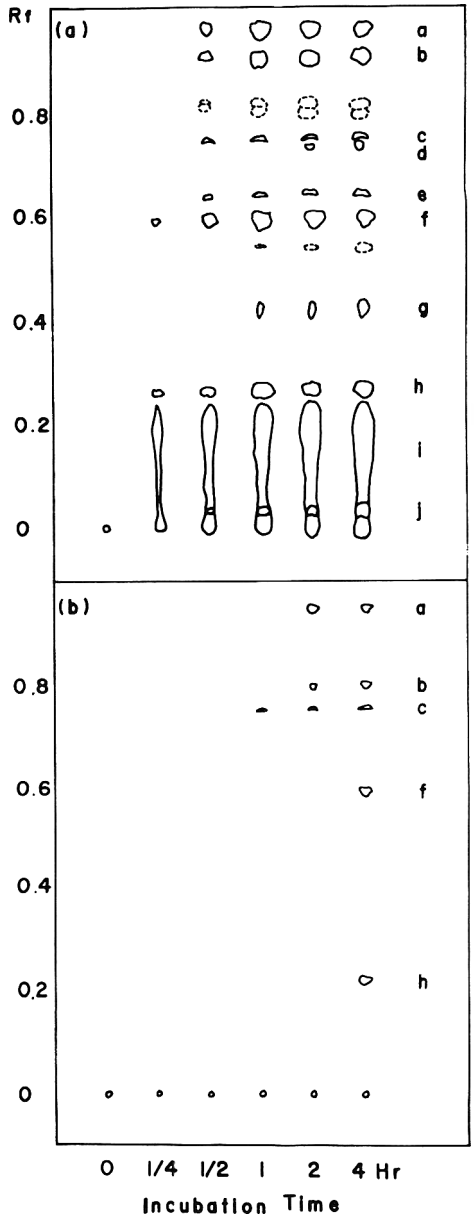

Fig. 2—Comparison of thin-layer chromatograms of fatty materials released during the incubation between enzymation group (a) and control group (b). Conditions: silica gel G 0.3 mm in thickness; solvent, chloroform-methanol-water (65:25:4); travel-length, 15 cm. Positive tests: phosphomolybdic acid, a, b, d, f, g, h and j; 50% H_2SO_4, a(brown), b(brown), c(yellow), d(purple), e(brown), f(brown), g(brown), h(brown) and j(violet); ninhydrin, f and i; sulfosalicylic acid, f, g and h; $FeCl_3$, c. Probable identity: a, nonpolar triglycerides; b, fatty acids; c, genistein; d, sitosteryl-D-glucoside; e, diglycerides; f, phosphatidyl ethanolamine; g, phosphatidyl inositol; h, phosphatidyl choline; (i, amino acids and peptides) and j, saponins.

volatile reducing substances also were found to be liberated from the soybean curd by incubating with the enzyme than without the enzyme. Sensory evaluation showed that the resulting final product had less odor, taste and color.

Effects of enzymation on defatted soybean flour

Table 2 shows results of chemical and sensory evaluation on the 90% ethanol extract of the defatted soybean flour digest, volatile substances liberated during incubation and the final product. Figure 1 shows the difference in liberation amounts of volatile substances between the enzymation group and the control group. As shown in Figure 2, a similar difference was found in liberation of lipids, i.e., triglycerides, diglycerides, fatty acids, phosphatidyl choline, phosphatidyl inositol, phosphatidyl ethanolamine and sitosteryl glucoside, etc. Sensory evaluation showed that the resulting final product had less odor, taste and color.

Enzyme amount and digestion time in relation to deodorization effect

Table 3 shows the relation between the amount of enzyme and that of *n*-hexanal or *n*-hexanol liberated during the enzymation for 2 hr; 1% amount of the enzyme per the substrate protein proved to be sufficiently effective. Figure 3 shows the behaviors of volatile reducing substances, *n*-hexanal and *n*-hexanol, during incubation of the defatted soybean flour, either with the enzyme or without the enzyme. Figure 3 also shows changes in flavor scores. In the enzymation group, the releasing amounts of volatile substances are already reaching their final values after 2 hr of incubation time; whereas, in the control group, they continue to increase linearly even after 4 hr. The sensory tests indicated that the enzymation group began to have bitter flavor after 4 hr and maltol-like flavor after 8 hr.

These results led us to a conclusion that 2-hr incubation was the most satisfactory way to prepare an enzymatically modified defatted soybean flour having little if any beany and bitter flavor.

Stability of the final product during preservation

As shown in Table 4, significant difference either in TBA-number or in flavor score was found between the enzymation group and the control group, when they were stored at 37°C. This result obviously shows that enzymation improves the preservation stability of the final product.

DISCUSSION

FATTY acids are known to be capable of binding with proteins (Boyer et al., 1946a; 1946b; Bull et al., 1967a; 1967b). Not only fatty acids but also various fatty substances in foods may possibly exist in protein-bound state. It is known that the flavor of soybean protein is not completely removable by simple distillation or extraction methods. This indicates that flavor components also are capable of binding with soybean protein. Various aliphatic flavor components of soybean have been elucidated: carbonyl compounds (Fujimaki et al., 1965), volatile fatty acids and amines (Arai et al., 1966b) and volatile alcohols (Arai et al., 1967). These flavor components are mostly removed in the process of defatting soybean, but partly remain in defatted soybean flour, presumably in the protein-bound state. Accordingly, it is reasonable that proteolytic enzyme treatment of defatted soybean flour breaks down the protein-flavor binding to facilitate the liberation of flavor components.

In the present work, defatted soybean flour was partially digested with the aspergillopeptidase A preparation, degree of proteolysis probably being about 50%. Subsequently, the digest was washed with 90% ethanol to obtain the final product having less flavor and fatty substances.

Appearance of off-flavor due to lipid oxidation may be one of the most serious problems encountered during preservation of soybean products. Most of the soybean phospholipids are difficultly extractable with *n*-hexane or similar nonpolar solvents under normal industrial conditions. Nielsen (1960) has demonstrated that phospholipids amounting to 0.5% of soybean are difficultly extract-

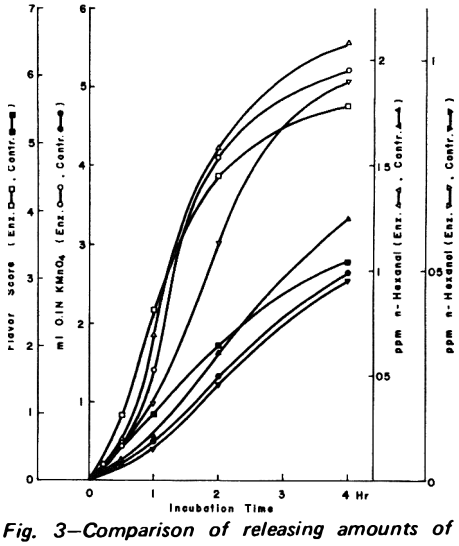

Fig. 3—Comparison of releasing amounts of volatile substances and flavor scores between enzymation group and control group at various stages of incubation. 100 g of the defatted soybean flour was used for each run. As for the enzymation group, 500 mg of the enzyme, amounting to about 1% of the protein contained in the soybean flour, was applied.

Table 2—Chemical analyses and sensory evaluation of the 90% ethanol extract, volatile substances and the final product from defatted soybean flour.

	Enzymation group	Control group
90% Ethanol extract		
Crude fat[1]	0.468	0.321
Odor	++	+
Taste	+++	+
Color	++	+
Volatile substances		
Volatile reducing substances[2]	4.10	1.30
n-Hexanal[3]	1.63	0.61
n-Hexanol[3]	0.63	0.23
Final product		
Odor	–	++
Taste	–	++
Color	–	++

[1]Grams per 100 g of defatted soybean flour.
[2]Milliliters of 0.1 N $KMnO_4$ consumed by the volatile reducing substances that released from 100 g of the soybean flour.
[3]Parts per million of the soybean flour.

Table 3—Effect of enzyme amount on releasing of n-hexanal and n-hexanol by digestion of defatted soybean flour.[1]

	Enzyme amount[2]				
	0	50	250	500[3]	1000
n-Hexanal	0.55[4]	0.75	1.38	1.63	1.62
n-Hexanol	0.23[4]	0.30	0.52	0.63	0.66

[1]The digestion time was 2 hr.
[2]Milligrams of enzyme vs. 100 g of defatted soybean flour.
[3]Amounting to about 1% of the protein contained in the soybean flour.
[4]Parts per million of the soybean flour.

able. Constituent unsaturated fatty acids of phospholipids can act as precursors of TBA-positive substances having objectionable flavor. Consequently, defatted soybean flour, when it contains phospholipids, may lack in preservation stability. Fujimaki et al. (1965) elucidated the formation of *n*-hexanal and several conjugated carbonyls during storage of defatted soybean flour, and pointed out the serious effect of residual lipids. *n*-Hexanal, along with various conjugated aldehydes, is known to be formed through autoxidation of soybean oil and the participant of off-flavor of oxidized soybean oil (Endres et al., 1962). *n*-Hexanal may be produced also through lipoxygenase-aided oxidation of lipids (Tappel et al., 1952).

As shown in Table 2 and Figure 2, crude fat and fatty substances are effectively removed from the defatted soybean flour by its digestion with the enzyme for 2 hr. The final product thus obtained seems, therefore, to contain little if any amounts of lipids and, in practice, is stable during preservation (Table 4).

Another factor relating to preservation stability may be the peptide nature of the final product. In this connection, Sato et al. (1968) reported that an enzymatic hydrolyzate of casein exhibited higher antioxidant activity than the original casein.

Chemical and physical properties of enzymatic hydrolyzates of soybean protein will be presented in another study.

Table 4—TBA-numbers and flavor scores of the final product from defatted soybean flour at various stages of the preservation.

	Preservation time[1]				
	0	7	14	21	28
TBA-number[2]					
Enzymation group	0.75	0.80	0.86	0.92	0.99
Control group	1.00	2.75	4.40	6.10	7.40
Flavor score					
Enzymation group	–	–	–	–	–
Control group	–	+	++	+++	+++

[1] Days at 37°C.

[2] Represented as μmoles 10^{-2} of malonaldehyde per 100 of sample.

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APPLYING PROTEOLYTIC ENZYMES ON SOYBEAN. 3. Diffusable Bitter Peptides and Free Amino Acids in Peptic Hydrolyzate of Soybean Protein

SUMMARY—Isolation and characterization of 7 sorts of diffusable bitter peptides in peptic hydrolyzate of soybean protein were presented. One liter of dilute HCl solution containing 25 g of soybean cold-insoluble protein and 250 mg of pepsin was incubated at 37°C for 24 hr. The resulting peptic hydrolyzate was dialyzed against water and the diffusable fraction was, after being concentrated at 40°C, investigated by gel permeation chromatography using Sephadex G-10, ion-exchange chromatography of Dowex 50 (w)-X2 (pyridinium) and thin-layer chromatography with silica gel G. Bitterness of the diffusable fraction was attributable to free amino acids having bitter flavor, such as isoleucine, leucine, phenylalanine and valine; also to peptides such as H · Gly-Leu · OH, H · Leu-Phe · OH, H · Ser-Lys-Gly-Leu · OH, H · Leu-Lys · OH, H · Phe-(Ile, Leu$_2$)-Gln-Gly-Val · OH, H · Arg-Leu-Leu · OH and H · Arg-Leu · OH. It was characteristic that almost all diffusable bitter peptides investigated here had leucine at the termini. Through carboxypeptidase A treatment on these peptides, the bitterness decreased considerably. Furthermore, applying carboxypeptidase A or Nagarse, a bacterial neutral proteinase, to the diffusable fraction of the peptic hydrolyzate was also effective in removing the bitterness somewhat.

INTRODUCTION

SOYBEAN has a peculiar and unfavorable flavor. This flavor can not be completely removed during the defatting process but still exists even in soybean protein preparations. The authors found that the unfavorable flavor was mostly removed by treating soybean protein preparation with proteolytic enzyme (Fujimaki et al., 1968). In many cases, however, the enzymic hydrolyzate of soybean protein was accompanied by a bitter flavor which would inhibit positive utilization of the product for foodstuff. It has been known that bitter flavor produced during enzymic hydrolysis of protein is due to some types of peptides. Raadsveld (1953) reported first that the bitterness of cheese resulted from a peptide. Carr et al. (1956) isolated a bitter octapeptide from tryptic hydrolyzate of casein. Ichikawa et al. (1959) isolated a bitter nonadecapeptide from casein hydrolyzed with a neutral proteinase of *Bacillus subtilis*. Gordon et al. (1965) isolated a bitter octapeptide from reconstituted nonfat milk treated with *Streptococcus cremoris*. Harwalkar et al. (1965) reported that a bitter peptide isolated from Cheddar cheese was composed of all sorts of amino acids found in milk protein. However, relation between structures of bitter peptides and their bitterness has apparently not been fully elucidated.

The present work deals with bitter substances, especially with bitter peptides, in the diffusable fraction of peptic hydrolyzate of soybean cold-insoluble protein.

METHODS

Material

Ground raw soybean was defatted with *n*-hexane at room temperature, from which the cold-insoluble protein (CIP) was prepared according to the method of Briggs et al. (1953). CIP was dialyzed using a cellophane tube against sufficient volume of dilute HCl (pH 1.6) at room temperature for 48 hr, to remove amino acids and peptides per se existing in CIP. The inner solution was used as the substrate of the following peptic hydrolysis.

Peptic hydrolysis

One liter of 2.5% CIP in dilute HCl (pH 1.6) was preincubated at 37°C for 15 min, 250 mg of pepsin (Sigma Chemical Co., recrystallized, 1:60,000) added and then incubated at 37°C for 24 hr. The enzymation was stopped by neutralizing the incubation mixture with NaOH.

Estimation of degree of hydrolysis

Degree of hydrolysis was measured at appropriate intervals of incubation time, using the ninhydrin method (Kalant, 1956) and the biuret method (Gornall et al., 1949).

Sensory test on bitterness

Two methods were used: a simple rating-scale method and the phenylthiourea comparison method. In the latter case, 1 ml of 0.1 mM aqueous solution of a bitter peptide was compared with 1 ml of a standard aqueous phenylthiourea solution for bitterness. Phenylthiourea solutions ranging from 10^{-1}% to 10^{-6}% were used.

Determination of free amino acids in peptic hydrolyzate

A given amount of the peptic hydrolyzate of CIP was treated with trichloroacetic acid (TCA), and free amino acids in the TCA-soluble fraction were determined with the aid of the Hitachi model KLA-3B amino acid analyzer.

Fractionation of bitter peptides

One liter of the peptic hydrolyzate of CIP was dialyzed, using a cellophane tube, against 4 liters of water at 4°C for 72 hr and the diffusate evaporated at 40°C under reduced pressure. The diffusable fraction thus obtained was submitted to gel permeation chromatography using Sephadex G-10 (2.5 by 95 cm). Elution was with water at room temperature, the flow rate being approximately 1 ml per minute. The bitter fraction was evaporated at 40°C under reduced pressure and submitted to ion exchange chromatography using Dowex 50 (w)-X2 (3 by 42 cm) according to the method of Guidotti et al. (1962). Beforehand, the ion exchanger was equilibrated with the starting buffer (Buffer-A). A small volume of the bitter fraction was applied on the column and developed stepwise with Buffer-A, B, C, D and E at 40°C under the flow rate of 1 ml per minute where the compositions of the buffer solutions were as follows: A, 1.4% pyridine + 1.5% acetic acid, pH 4.7; B, 3.2% pyridine + 3.7% acetic acid, pH 4.6; C, 8.6% pyridine + 3.0% acetic acid, pH 5.4; D, 18.0% pyridine + 6.0% acetic acid, pH 5.4; E, 16.0% pyridine, pH 8.75. A part of each fraction was treated with ninhydrin reagent and the resulting color measured by the method of Kalant (1956). The ninhydrin-positive fractions were examined for their bitterness. Both ninhydrin-positive and bitter fractions were submitted to thin-layer chromatography. Silica gel G (Merck) was used as the absorbent and *n*-butanol–acetic acid–water (4:1:2) as the solvent. Chromatography was carried out in the usual manner, and then each thin-layer plate made visible in part by splaying 1% ninhydrin in *n*-butanol and heating at 100°C for 5 min. According to the resulting guide chromatograms, zones to be ninhydrin-positive were separately extracted with hot water. Each extract was concentrated to 1 ml and examined for its bitterness. Through these procedures, the following seven bitter peptides were isolated: peptides A-1, A-2, C-1, C-2, D-1, D-2 and D-3. These peptides were chromatographically pure, as were their DNP-derivatives.

Constituent amino acid composition

Each bitter peptide (0.1 mg) was hydrolyzed with 6 N HCl (1 ml) either at 110°C for 20 hr or 105°C for 16 hr, and liberated amino acids were determined with the amino acid analyzer. Constituent tryptophan was not detectable in each peptide from surveying its ultraviolet spectrum. Presence of constituent cystein, cystine and methionine in each peptide was denied, since its hydrolyzate contained neither cysteic acid nor methionine sulfoxide.

N-Terminal amino acid

The DNP method proposed by Sanger et al. (1953a) was used. Bitter peptide (0.2 μmole) was dissolved in 1% trimethylamine (0.1 ml) and adjusted accurately to pH 9.0 with 0.1 N NaOH. To the solution was added 0.2 ml of 5% ethanol solution of 1-fluoro-2, 4-dinitrobenzene and the mixture allowed to stand for 3 hr in a dark place at room temperature. The DNP-peptide was hydrolyzed with azeotropic HCl at 105°C for 16 hr and the liberated DNP-amino acids identified by two-dimensional paper chromatography by the method of Biserte et al. (1951). Further identification of DNP-amino acids was attained by submitting the acid hydrolyzate of DNP-peptide to the amino acid analyzer and confirming the disappearance of peaks on the chromatogram, owing to the 2, 4-dinitrophenylation.

Table 1—Increase in the degree of hydrolysis and the accompanying increase in bitterness during peptic hydrolysis of soybean cold-insoluble protein.

Incubation time (hr)	Degree of hydrolysis: Free amino acid[1]	Degree of hydrolysis: TCA-soluble peptide[2]	Intensity of bitterness[3]
0	0	0	0
0.25	0.242	1.63	1.0
0.5	0.350	2.55	1.3
1	0.428	2.98	2.0
1.5	0.485	3.15	2.5
3	0.550	3.30	3.3
6	0.631	3.36	4.3
12	0.727	3.50	4.7
24	0.882	3.61	5.0

[1]Determined by ninhydrin method and represented as mmole leucine per milliliter of 3.3%-TCA-soluble fraction.

[2]Determined by biuret method and represented as milligrams ovalbumin per milliliter of 3.3%-TCA-soluble fraction.

[3]5: Very strong, 4: strong, 3: medium, 2: weak, 1: very weak.

Table 2—Free amino acids contents in peptic hydrolyzate of soybean cold-insoluble protein at the various stages of the hydrolysis.

Amino Acid	Amino Acid content[1]: 1 hr	12 hr	24 hr	Intensity of bitterness[2]
Phe	0.110	0.61	1.08	3
Glu	0.030	0.20	0.26	0
Tyr	0.026	0.16	0.25	0
Leu	0.031	0.16	0.24	2
Ile	0.010	0.06	0.08	4
Val	0.006	0.05	0.07	1
Asp	0.002	0.04	0.06	0

[1]Grams amino acid per 100 g of the substrate on dry basis.

[2]4: Very strong, 3: strong, 2: weak, 1: very weak, 0: not bitter.

Table 3—Bitterness comparison between bitter peptides and the constituent amino acid mixtures.

Bitter peptide[1]	Bitterness[2]	Amino Acid mixture[3]	Bitterness[2]
A-1	10^{-3} – 3×10^{-3}	Gly + Leu	10^{-6} – 3×10^{-5}
A-2	5×10^{-2} – 10^{-1}	Leu + Phe	5×10^{-5} – 5×10^{-4}
C-1	7×10^{-2} – 2×10^{-1}	Gly + Leu + Lys + Ser	4×10^{-5} – 7×10^{-5}
C-2	10^{-3} – 10^{-2}	Leu + Lys	5×10^{-4} – 8×10^{-4}
D-1	10^{-2} – 3×10^{-2}	Gln + Gly + Ile + 2Leu + Phe + Val	7×10^{-4} – 10^{-3}
D-2	3×10^{-3} – 4×10^{-3}	Arg + 2Leu	2×10^{-6} – 6×10^{-6}
D-3	6×10^{-3} – 2×10^{-2}	Arg + Leu	10^{-6} – 5×10^{-6}

[1]0.1 mM aqueous solution of the peptide was examined.

[2]Percentage concentration of phenylthiourea revealing the bitterness equal to that of the peptide or the amino acid mixture.

[3]L-Amino acids were used. A mixture of 0.1 mM of each amino acid was examined.

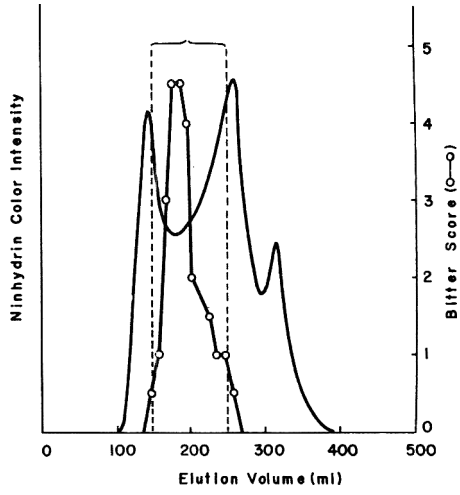

Fig. 1—Fractionation of bitter components by gel permeation chromatography of Sephadex G-10. A fraction from 150 to 250 ml, put in a bracket, was submitted to ion-exchange chromatography.

C-Terminal amino acid

Carboxypeptidase A (CPase A) was applied to determine C-terminal amino acids. Crystalline CPase A of bovine pancreas (Sigma Chemical Co.), having the acitivity of 70 μmole hippuryl-L-phenylalanine/min/mg enzyme (at 25°C), was used. The enzyme solution was prepared by the method of Fraenkel-Conrat et al. (1955). Approximately 0.04 μmole of the bitter peptide was dissolved in 0.1 ml of 0.1% $NaHCO_3$ and adjusted to pH 7.7 with 0.1 N NaOH. The peptide solution was preincubated at 25°C for 5 min and 1 ml of the enzyme solution, containing 14.4 μg of CPase A, added. The resulting mixture was incubated at 25°C and amino acids liberated at this time were analyzed, using the amino acid analyzer.

Applying carboxypeptidase A and a bacterial neutral proteinase on the bitter diffusate

100 ml of the diffusable fraction was adjusted to pH 7.7 with $NaHCO_3$ and preincubated at 37°C for 15 min. 5 mg of CPase A dissolved in a small volume of water was added to the solution. The mixture was incubated at 37°C and the decrease in bitterness measured at appropriate intervals of incubation time. A similar experiment was also carried out using a bacterial neutral proteinase, Negarse (Nagase Sangyo Co., lyophilized and crystallized, 1,500,000 P.U.N. in vial). In this case, incubation was performed at pH 7.0, other conditions being similar to those in the CPase A. Degree of hydrolysis was represented in each case as a total amount of amino acids liberated during incubation.

RESULTS

Appearance of bitterness during peptic hydrolysis of CIP

As shown in Table 1, bitterness appeared at the early stage of the peptic hydrolysis, increased gradually with hydrolysis time, and reached maximum intensity after 24 hr. The degree of hydrolysis by the ninhydrin and biuret methods is shown in Table 1.

Determination of free amino acids

Amounts of free amino acids in the diffusable fraction of CIP hydrolyzate are shown in Table 2. Seven free amino acids were detected in this fraction. Among those, isoleucine, phenylalanine, leucine and valine were found to be bitter.

Isolation of bitter peptides

Gel permeation chromatography of the diffusable fraction of CIP hydrolyzate showed a pattern as in Figure 1. Ion-exchange chromatography of the bitter fraction obtained above showed a pattern as in Figure 2. Each bitter fraction separated by ion-exchange chromatography was separated further by thin-layer chromatography as shown in Figure 3.

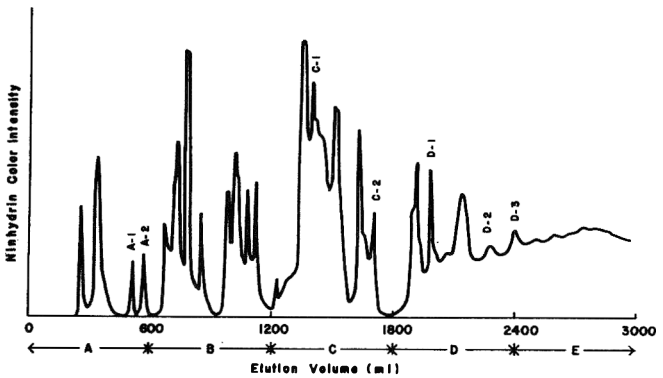

Fig. 2—Fractionation of bitter peptides by ion-exchange chromatography of Dowex 50 (w)-X2 (pyridinium). A—E mean the buffer solutions used (compositions described in the text).

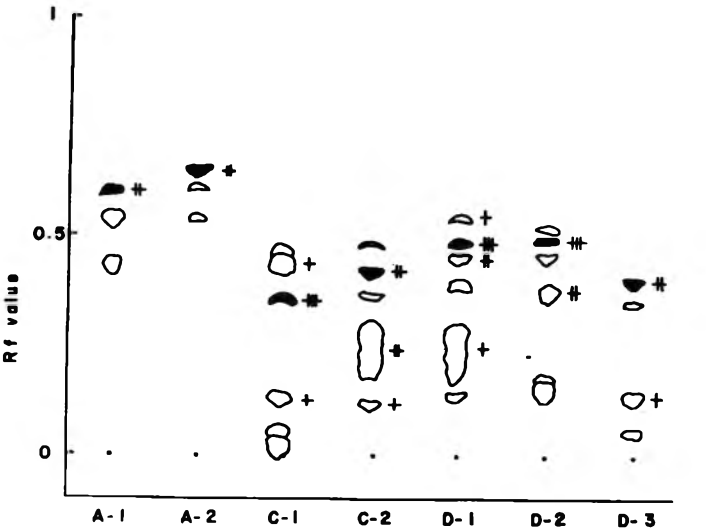

Fig. 3—Fractionation of bitter peptides by thin-layer chromatography of silica gel G. The spots marked in black were isolated. +++: Very bitter, ++: bitter, +: slightly bitter.

Structures of bitter peptides separated

Peptide-A-1. Acid hydrolysis of peptide-A-1 produced equal molar ratios of glycine and leucine. Acid hydrolysis of DNP-peptide-A-1 produced DNP-glycine. This acid hydrolyzate did not contain free glycine. These results show that the structure of peptide-A-1 is H · Gly-Leu · OH.

Peptide-A-2. Acid hydrolysis of peptide-A-2 produced equal molar ratios of leucine and phenylalanine. Acid hydrolysis of DNP-peptide-A-2 produced DNP-leucine. This hydrolyzate did not contain free leucine. These results show that the structure of peptide-A-2 is H · Leu-Phe · OH.

Peptide-C-1. Either acid hydrolysis of peptide-C-1 or exhaustive hydrolysis of this peptide with Nagarse produced equal molar ratios of glycine, leucine, lysine and serine. Acid hydrolysis of DNP-peptide-C-1 produced DNP-serine and ε-*N*-DNP-lysine. This hydrolyzate did not contain free serine, free lysine, di-DNP-lysine and α-*N*-DNP-lysine. Hydrolysis of this peptide with CPase A liberated glycine and leucine in the order shown in Figure 4(a). These results show that the structure of peptide-C-1 is H · Ser-Lys-Gly-Leu · OH. Serine-β-*O* seems to not be in the bound state, since free serine is liberated by Nagarse hydrolysis of peptide-C-1. Lysine-ε-*N* also seems not to be in the bound state, since ε-*N*-DNP-lysine is detected in the acid hydrolyzate of DNP-peptide-C-1.

Peptide-C-2. Acid hydrolysis of peptide-C-2 produced equal molar ratios of leucine and lysine. Acid hydrolysis of DNP-peptide-C-2 produced DNP-leucine and ε-*N*-DNP-lysine. This hydrolyzate did not contain any free amino acid, di-DNP-lysine and α-*N*-DNP-lysine. These results show that the structure of peptide-C-2 is H · Leu-Lys · OH. Lysine-ε-*N* seems not to be in the bound state.

Peptide-D-1. Acid hydrolysis of peptide-D-1 produced 1 mole of glutamic acid, glycine, isoleucine, phenylalanine and valine and 2 moles of leucine. Acid hydrolysis of DNP-peptide-D-1 produced DNP-phenylalanine. The hydrolyzate did not contain free phenylalanine. CPase A hydrolysis of peptide-D-1 liberated glutamine, glycine and valine in the order shown in Figure 4(b). These results show that the structure of peptide-D-1 is H·Phe-(Ile, Leu_2)-Gln-Gly-Val·OH.

Peptide-D-2. Acid hydrolysis of peptide-D-2 produced 1 mole of arginine and 2 moles of leucine. Acid hydrolysis of DNP-peptide-D-2 produced DNP-arginine. This hydrolyzate did not contain free arginine. These results show that the structure of peptide-D-2 is H·Arg-Leu-Leu·OH.

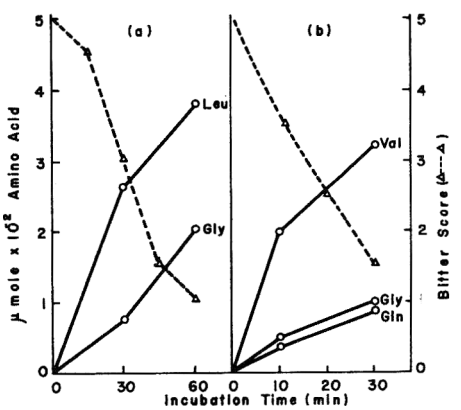

Fig. 4—Liberation of amino acids and the accompanied decrease of bitterness during the hydrolysis of peptide-C-1 (a) and peptide-D-1 (b) by carboxypeptidase A.

Peptide-D-3. Acid hydrolysis of peptide-D-3 produced equal molar ratios of arginine and leucine. Acid hydrolysis of DNP-peptide-D-3 produced DNP-arginine. This hydrolyzate did not contain free arginine. These results show that the structure of peptide-D-3 is H·Arg-Leu·OH.

Intensity of bitterness. Table 3 shows intensity of bitterness of each bitter peptide and that of an admixture of its constituent amino acids.

Debittering effects of CPase A and Nagarse. As shown in Figure 5, bitterness of the diffusable fraction of CIP hydrolyzate was lessened during incubation either with CPase A or Nagarse.

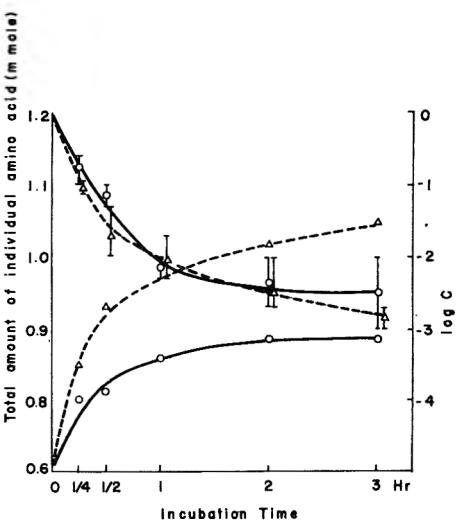

Fig. 5—Liberation of amino acids and the accompanied decrease in bitterness during the hydrolysis of diffusable fraction of peptic hydrolyzate by carboxypeptidase A (o—o) and Nagarse (Δ--Δ). Total free amino acid amount was represented as mmole leucine. C means %-concentration of phenylthiourea.

DISCUSSION

DIALYSIS and the gel filtration study indicate that the bitterness in the peptic hydrolyzate of CIP is mainly ascribed to low-molecular-weight peptides, presumably less than 1,500 in molecular weight. These peptides are considered to be difficultly hydrolyzable further by pepsin, since pepsin is an endopeptidase. As shown in Table 1, the bitterness increases gradually with incubation time, reaches maximum intensity after 24 hr, and seems to be almost unchanged after 24 hr. This fact probably means that most of the bitter peptides accumulates in the peptic hydrolyzate.

Countless sorts of peptides are possibly produced by the peptic hydrolysis under the described conditions. Other bitter peptides than elucidated in the present work also exist in the peptic hydrolyzate (Fig. 3). Almost all bitter peptides, so far as elucidated here, were found to bear leucine at the termini.

Summarizing substrate specificity studies of pepsin using synthetic substrates (Bergmann et al., 1941), oxidized insulin (Sanger et al., 1951; 1953b), corticotropin (Shepherd et al., 1956), oxidized RNase (Bailey et al., 1956), TMV protein (Anderer et al., 1960; Tsugita et al., 1960) and human hemoglobin α-chain (Konigsberg et al., 1962), it is concluded that peptide bonds involving aromatic amino acids, i.e., phenylalanine and tyrosine, are easily hydrolyzed to produce free phenylalanine, tyrosine and peptides bearing phenylalanine or tyrosine at the termini, and peptide bonds involving hydrophobic amino acids, i.e., leucine, valine, alanine and isoleucine, are also hydrolyzed to produce peptides bearing leucine, valine, alanine or isoleucine at the termini. The substrate specificity of pepsin also shows that peptic peptides frequently bear leucine, valine, phenylalanine and alanine, and scarcely ever aspartic acid, asparagine, glutamic acid, glutamine, serine or glycine at the N-termini. One example concerning N-terminal arginine has been reported by Anderer et al. (1960). The substrate specificity of pepsin further shows that peptic peptides frequently bear leucine, phenylalanine and alanine, and scarcely ever glutamic acid, glutamine, etc., at the C-termini. One example concerning C-terminal lysine has been reported by Anderer et al. (1960). Examples have apparently not been reported concerning hydrolysis of -Gly-Leu-, -Leu-Phe-, -Ser-Lys-, -Lys-Gly-, -Leu-Lys- and -Arg-Leu-. It may be possible that -Leu-Leu- is hydrolyzable.

Based on this information, it can be considered that peptides A-1, A-2, C-1, C-2 and D-3 are final products, peptide-D-2 is hydrolyzed further to produce peptide-D-3 and peptide-D-1 is evidently an intermediate product.

The substrate specificity of pepsin may afford a basis for interpretation of quantity and quality of free amino acids in the peptic hydrolyzate of CIP (Table 2). Larger amounts of glutamic acid in this hydrolyzate may rather be attributable to its high content in soybean protein.

As shown in Figure 4(a), hydrolysis of peptide-C-1 with CPase A was effective in lessening the bitterness. After 30 min, 65% of constituent leucine and 20% of constituent glycine were liberated and the bitterness decreased from 5 points to 3 points. After 1 hr, 95% of constituent leucine and 50% of constituent glycine were liberated and the bitterness decreased from 5 points to 1 point. After 2 hr, when the original peptide was almost completely hydrolyzed, no bitterness was perceptible. A similar result was obtained also in the case of CPase A treatment of peptide-D-1 (Fig. 4b).

Table 3 shows there is a remarkable difference in bitterness between bitter peptide and an admixture of its constituent amino acids. Accordingly, if bitter peptides are exhaustively hydrolyzed, the resulting hydrolyzate must be far less bitter.

As a practical approach, experiments were carried out aimed at lessening the bitterness of peptic hydrolyzate of soybean CIP. As shown in Figure 5, at the initial stage of incubation, the bitterness of the hydrolyzate was equivalent to that of 1% aqueous phenylthiourea (log C = 0; C, %-concentration of phenylthiourea). When this hydrolyzate was incubated with either CPase A or Nagarse, its bitterness decreased remarkably within 1 hr. As a result of the incubation for 3 hr, this bitterness was no more than that of $10^{-2}-10^{-3}$% phenylthiourea.

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CORRELATION BETWEEN GAS-CHROMATOGRAPHIC PATTERNS AND FLAVOR EVALUATION OF CHEMICAL MIXTURES AND OF COLA BEVERAGES

SUMMARY—Two methods of discriminant analysis, Chi-square tests, "t" tests and analysis of variance were applied to gas-liquid chromatographic (GLC) data to compare GLC measurements of food volatiles with organoleptic evaluation of flavor. Model systems composed of ethyl butyrate, ethyl heptylate and benzaldehyde were used for one set of trials. A second system consisted of blends of Pepsi-Cola® and Coca-Cola®. The stepwise discriminant analysis (SDA) procedure described by Powers et al. (1968) was only moderately successful in classifying the mixtures wnen applied to the GLC data for the model systems, but the cola blends could be classified readily. From GLC data, cola blends differing from each other by only 7–10% could be distinguisheo. A new discriminant analysis program, designated MUDAID by its originator (Bargmann, 1967), is more useful for predictive purposes. The analysis yields the correlation coefficients for each peak area with sampie differences and the weighting factors. Discriminant equations can thus be written. By substitution of measurement values for unknowns, their identity may be predicted. Organoleptic subthreshold additive effects could not be demonstrated with binary mixtures of flavor substances, but judge-compound interaction effects were observed.

INTRODUCTION

THE IDEA of using gas-liquid chromatography (GLC) for evaluation of the flavor of food is nearly as old as GLC itself. Powers (1968) pointed out that flavor evaluation by GLC has not attained the role it might have because suitable methods of correlating complex GLC data with sensory evaluation have been lacking. Powers et al. (1968) showed that stepwise discriminant analysis (SDA) could be used to classify foods for flavor from GLC data.

The present study was undertaken to learn 1) whether assessment of differences by the SDA method could be done at closer intervals than by organoleptic evaluation; 2) whether alternate methods of using GLC data efficiently for flavor evaluation could be found; and 3) whether some subthreshold additive effects, as questioned by Keith et al. (1968), result from sensitivity of particular judges to specific compounds or are truly additive. This has a bearing on correlation, since GLC is a separation process, whereas organoleptic assessment of flavor is an integrative process.

REVIEW OF LITERATURE

SJÖSTRÖM (1967), Baker et al. (1967), Powers et al. (1968) and Powers (1968) have reviewed much of the literature reporting comparisons between GLC measurements and sensory evaluation of flavor.

For some foods, simple correlation methods are suitable because one or a few components are closely correlated with flavor quality (Powers, 1968). Hammond et al. (1964), Arnold et al. (1966) and Badings et al. (1968) have each described a flavor defect in milk or cheese resulting from a single compound. Powers et al. (1968) cited other examples of simple correlation effects.

More often, the true flavor or off-flavor of a food must be attributed to several compounds. Aurand et al. (1965) found that the flavor of pickles is due to a blend of volatile compounds rather than the presence or absence of a single component. Buttery et al. (1967) reported that American-grown hops of different varieties could be distinguished by the relative percentages of certain components. MacLeod et al. (1966) stated that chromatograms of lemon oils from throughout the world were very similar and that differences were primarily quantitative rather than qualitative. Wong (1963) observed that GLC differences between fresh and decomposed cream were primarily quantitative. Sevenants et al. (1966) concluded that typical peach aroma was probably an integrated response to a wide spectrum of compounds and, in fact, none of the individual compounds was at all peachlike in aroma.

Miller (1966), Stephens (1966), Keith (1967), Buttery et al. (1967), de Becze et al. (1967), Hawkes et al. (1967) and Powers et al. (1968) have each attempted to compare, upon some mathematical or statistical basis, flavor differences with chromatographic differences. Henis et al. (1966), O'Brien (1967), Dravnieks et al. (1967) and MacGee (1968) have carried on similar studies to identify from GLC data different species or strains of microorganisms or of other biological entities.

EXPERIMENTAL

GLC vs. organoleptic comparisons

Two types of trials were used to compare GLC and organoleptic evaluation. One consisted of a model system and the other of blends of Pepsi-Cola® and Coca-Cola®.

Ethyl butyrate, ethyl heptylate and benzaldehyde were dissolved in double-distilled water at concentrations of 2.272, 1.664 and 5.125 mg/liter to form a control solution. Two of the compounds were then held at these levels and the third compound reduced in concentration until the resulting mixture could be distinguished from the control solution by a six-member taste panel. The same triangular method of sensory evaluation was used by Keith et al. (1968).

For the GLC analysis, 500-ml portions of the solutions were extracted in a distillation-extraction apparatus (Likens et al., 1964). Approximately 40 ml of pentane was used as the solvent, and the solutions were extracted for 2 hr. The solvent flask was then stored at 10°F for not less than 2 hr to freeze out dissolved or emulsified water. The pentane was decanted and then concentrated in a Kuderna-Danish evaporator partially submerged in a water bath at 37°C. The pentane extract was reduced to 60–70 μliters. The concentrated sample was held in an ice bath while GLC runs were being made; 5-μliter portions were used for chromatographic injection.

The chromatographic packing consisted of 10% Triton X-305 (alkyl aryl polyether alcohol) coated on to 60-80-mesh Diatoport S. The columns were 20 ft, 1/4-in. stainless steel, and a dual-column gas chromatograph (F & M Model 810) was used. The programming sequence consisted of a 5-min post-injection interval at 160°C, followed by programming at 6°/minute to 210°C. The injection port temperature was 295°C; the detector temperature was 275°C, and the nitrogen, hydrogen and oxygen flow rates 30, 63 and 150 ml/minute, respectively.

The areas of the various peaks were measured with a planimeter and each area converted to the percentage it represented of the total for the areas of all peaks (Powers et al., 1968).

Cola-beverage trials

Blends of Pepsi-Cola and Coca-Cola were prepared in ratios of 67:33; 60:40; 50:50; 40:60 and 33:67. Using the triangular procedure, the panel was then asked to select the odd sample in comparisons involving Pepsi-Cola, Coca-Cola or different blend ratios. The panel was also asked to place 4 solutions in order. They were 100, 67, 50 and 33% of each cola beverage.

The various blends were extracted as described for the model system, except 450 ml of the cola solutions were extracted, ethyl ether was used as the solvent instead of pentane and the extraction period was 1 instead of 2 hr. One further difference was that 0.1 μliter of γ-undecalactone was injected into the concentrated extract just before gas-chromatographic analysis, to provide a reference compound for retention times and area. For statistical purposes, the measured area of each peak, the area transformed to percentage and the ratio of the area to the area of the reference compound were thus available.

Table 1—Minimum change[1] in 1 compound necessary to enable solutions to be distinguished organoleptically.

Compound	Solution A (Control)	Solution detectably different B	C	D
Ethyl butyrate	2.272	1.717	2.272	2.272
Ethyl heptylate	1.664	1.664	0.422	1.664
Benzaldehyde	5.125	5.125	5.125	1.294

[1]Concentrations are in milligrams/liter.

Table 2—Differentiation[1] of cola beverages by organoleptic evaluation for flavor.

Cola beverages and blends						
100% Pepsi-Cola	67P:33C	60P:40C	50P:50C	40P:60C	33P:67C	100% Coca-Cola

[1]Any 2 treatments not underlined by the same line are significantly different.

Statistical methods

The same stepwise discriminant analysis procedure (Sampson, 1967) as used by Powers et al. (1968) was applied to the data. They observed some evidence of subthreshold additive effects, but after the panel was already disbanded they wondered whether the subthreshold additive effects observed resulted from the sensitivity of a particular judge for a particular compound, rather than 2 or more compounds, each below their levels of detectability, augmenting each other so as to make their presence detectable.

For these trials, a 12-member panel was used and the compounds were ethyl acetoacetate, benzaldehyde, 2-methyl butyric acid, isovaleric aldehyde, amyl butyrate and γ-undecalactone. The threshold levels reported by Keith et al. (1968) were used as a starting point. By increasing or decreasing the concentration, the threshold level for the panel was found. This, in effect, was a pooled Chi-square test. In conformity with Keith et al. (1968), the 0.05 level of significance was used. Each panelist was also tested individually to establish his threshold level for each compound. Binary mixtures of these 6 compounds were then prepared for sensory evaluation with each compound at 50% of the threshold level established by the panel. The theoretical threshold was, thus, 100% of the panel threshold.

To learn whether significant results came from judge-compound interactions or were truly additive, the panel was split into 3 groups. Judges whose threshold was neither greater nor less than that of the panel were put into Group A. The most sensitive judges were put into Group B.

Judges in Group A were given solutions with each compound at 50% of their (and the panel's) threshold levels. Judges in Group B were given solutions with the two compounds at 75 or 67% of the judge's respective threshold levels. For each judge, the sum naturally exceeded 100% of his theoretical level, but the sum was set to approximately 100% of the panel's threshold level.

Since there were 6 compounds and a judge might be sensitive for 1 compound but not for another, a judge sometimes was in Group A, Group B or temporarily eliminated according to the particular binary combination under study and his sensitivity to these compounds.

RESULTS & DISCUSSION

Organoleptic evaluation

The model system was used in the hope that a simple system would facilitate the development of means of correlating sensory and objective evaluation. This turned out not to be so. Rather sizable differences had to exist in the concentrations of the 3 chemicals before the change became organoleptically detectable. Recorded (Table 1) are the minimal changes necessary for the panel to detect a significant difference as one compound at a time was decreased in concentration. For ethyl butyrate, ethyl heptylate and benzaldehyde the respective decreases had to be 24.5, 74.6 and 74.7% before the panel could tell the difference against the background of the 2 other chemicals. Though only 3 solutions were tested at a time, the harsh chemical flavor of some combinations over-rode organoleptic differences.

The panel proved itself to be moderately sensitive in detecting differences among the cola solutions. It could detect differences when the blends varied by 17% but not when they varied by 10% or less. Results are listed in Table 2.

SDA analysis

Of 195 chromatograms available from the same solutions as listed on Table 1, 13% of the solutions were erroneously classified by the SDA procedure from the GLC data. The SDA procedure applied to the data for the cola solutions was remarkably effective. Compositional differences to within 7% could be resolved (see Table 3).

Table 3 is a classification based upon using the percentage peak areas of peaks 4, 5 and 6 as denominators. The procedure followed was the same as that of Powers et al. (1968). First, the peak areas were transformed to percentage peak areas. Then all possible simple ratios were formed using each percentage peak area as the denominator. The SDA program was written to handle only 80 variables at a time. As there were 24 peaks for the cola solutions, the data were subdivided into groups consisting of 3 peaks as denominators. The number of steps required to yield correct classification by each of the 8 groups is shown in Table 4. Shown also is the number of steps required when the 9 most effective ratios from each of the 8 subgroups were combined.

That a composite of the 72 most effective ratios from each of the 8 subgroups required more steps than most of the subgroups needs explanation. Stepwise discriminant analysis depends upon the intercorrelations between variables and the degree of dispersion of each variable. In generating 72 ratios with 9 different denominators instead of 3, heterogeneity apparently was increased.

One question arises: Rather than form ratios, can discrimination be effected from the peak areas themselves? This could not quite be done for the cola solutions. 69 out of 70 chromatograms were correctly classified as to blend, but 1 chromatogram for a 33:67 blend was incorrectly classified as a 40:60 blend. Powers et al. (1968) did not try to classify their coffee chromatograms from percentage peak heights. Their data were subjected to the SDA procedure except the 33 percentage peak heights were used instead of ratios. 1 peak area alone was sufficient to classify the coffee. This demonstrates that for some applications there is no need of forming ratios from the peak areas before the SDA procedure is applied. In trials to be reported, Milutinovic et al. (1970) were able to discriminate among samples without resort to ratios. In this study, formation of ratios was necessary for the model system and the cola beverages, if one sets a standard of 100% success. Actually, successful classification of 69 out of 70 chromatograms without resort to the formation of ratios is entirely acceptable. Upon a probability basis, one should not expect every chromatogram out of 70 to be always classified correctly.

Concerning ratios, a recent patent (Mahlmann et al., 1969) is pertinent. The patent points out that the process leads to flavorful soluble coffee and the flavor is defined as having an unique GLC spectrum. The spectrum is given in terms of ratios among volatiles.

Noncomputer methods

1 limitation of the SDA program is that a computer is required. Thought was given to the possibility of utilizing all the data from relatively complex chromatograms without having to resort to high-speed computing. 3 different methods of hand calculation were tried: 1) analysis of variance combined with Duncan's multiple range test; 2) a pooled Chi-square test

and 3) a group "t" test. For the last-named procedure, the significance of the number of significant "t" tests was estimated from an upper and lower binomial-confidence-limits table (Mainland et al., 1956).

Analysis of variance and Duncan's multiple range tests were moderately effective for the model solutions. One has to make a decision from the multiple range test for each peak area separately. There is risk of error in each decision and these risks are cumulative. Actually, for only 3 peaks this was no real problem. Table 5 shows Duncan's multiple range tests for 8 of the 24 peak areas of the cola trials. One can see that if one had measurement values for an unknown, one could probably decide to which group the sample belonged.

Chi-square analysis also could be used. This is unorthodox, for the Chi-square test is normally used for "expected" values, generally discontinuous, such as expected number of progenies in genetical experiments. By Chi-square tests of one group against another, just about the same peaks were picked as being critical to discrimination as by the analysis of variance. When group "t" tests were used for the same purpose, there was good agreement, too, as to the peaks most useful in distinguishing among the 7 cola treatments. Young (1968) correctly identified 5 out of 7 unknown blends by using the Chi-square procedure.

The analysis of variance indicated there was significant interaction between peak areas and treatment. Some peaks were useful to separate groups at one end of the blend series, but not at the other end (see Table 5). Curvilinearity of most of the regression lines accounted for much of the interaction.

Multiple discrimination

The techniques of regression and stepwise discrimination are primarily concerned with the problem of classifying individual chromatograms in terms of their similarities to the groups of chromatograms for each treatment in the present study. Since a new discriminant function is established for each pair of observed compounds, it would not be easy to generalize such results to a class of, say, all cola beverages. For this latter problem, a multiple discriminant analysis on peak areas as response variables seems more appropriate. The MUDAID program (Bargmann, 1967) performs such an analysis in one of its phases. Each variable, i.e. peak area, is studied by an irregular 2-way classification-design analysis. Adjusted mean values for replications and treatments are calculated as well as F values for replication, treatment and interaction effects. This univariate pass for each peak area is used primarily to detect experimental outliers or recording errors, which invariably result in significant interaction effects.

A second run is then made on the edited data. The F values for treatments for each peak are valuable indicators of the degree of discrimination due to each peak. A multiple discriminant function is then calculated (largest eigen vector of a matrix product) which is, in fact, a linear combination of the area under each peak. It is an artificial variable or score given to each compound, with the weights chosen in such a way that the distance between treatments is maximized. The program then proceeds to calculate correlations between each variable (peak area) and the best discriminant function. This is thus a measure of proximity of each peak area to the best discriminator. If some peak areas show low correlation, they obviously do not contribute to the over-all discrimination between treatments.

The variables with low correlation are eliminated and a second or third run is made on the remaining peak areas. Finally, only those peaks having the highest F values for treatment effects and the highest correlations with the over-all best discriminator are retained.

The discriminant function for 9 of the 24 variables of the cola data was:

$$Z = 0.673\ x_4 + 14.7\ x_7 - 9.68\ x_8 + 2.56\ x_{10} + 0.748\ x_{11} + 9.4\ x_{12} - 1.02\ x_{13} + 24.6\ x_{15} - 10.6\ x_{21}$$

The respective correlation values, in the order of peaks just listed were −0.194, 0.257, 0.136, 0.289, 0.176, 0.294, −0.187, 0.104 and −0.156. Peak 12 was

Table 3–Classification of cola beverages from ratios of percentage peak areas involving peaks 4, 5 and 6 as denominators.

	Classification						
Solution	100%	67P:33C	60P:40C	50P:50C	40P:60C	33P:67C	100%
100% Pepsi-Cola	9						
67P:33C		10					
60P:40C			10				
50P:50C				9			
40P:60C					12		
33P:67C						11	
100% Coca-Cola							9

Table 4–Number of steps required to classify chromatograms of cola solutions.

Peaks used as denominators	No. steps to successful classification
1, 2, 3	24
4, 5, 6	19
7, 8, 9	24
10, 11, 12	39
13, 14, 15	19
16, 17, 18	24
19, 20, 21	29
22, 23, 24	34
Composite[1]	39

[1]The composite consisted of the 9 most efficient ratios from each of the 8 trials.

Table 5–Peaks selected by analysis of variance and Duncan's multi-range test as being useful to distinguish among cola solutions.[1]

Peak							
2	100P	40:60	50:50	67:33	33:67	60:40	100C
4	100P	67:33	40:60	60:40	50:50	33:67	100P
7	100C	40:60	33:67	50:50	60:40	67:33	100P
10	100C	33:67	60:40	50:50	40:60	67:33	100P
11	100C	33:67	40:60	60:40	50:50	67:33	100P
12	40:60	50:50	100C	60:40	33:67	67:33	100P
13	100P	67:33	60:40	50:50	40:60	33:67	100C
21	100P	67:33	60:40	50:50	40:60	33:67	100C

[1]The first figure in each ratio designates the percentage of Pepsi-Cola.

most highly correlated with treatment change. The degree of dispersion enters into the weighing values as well as correlation. Peaks with the highest correlation to the best discriminating equation are likely to be involved in flavor. If they are not flavor substances themselves, they affect flavor in such subtle ways as by subthreshold additive effects, reaction with other components or through dilution of those compounds which are odorous.

Table 6 lists the 9 variables chosen and the Z values calculated for each cola treatment. To predict unknowns, one merely substitutes in measurement values for each of the variables and calculates the Z value. From this value one can estimate to which group the unknown belongs.

Comparison of Tables 5 and 6 shows that nearly the same peaks were selected as being important to discrimination by the analysis of variance procedure as by the discriminant analysis method. As already pointed out, the Chi-square and group "t" tests were in agreement with the analysis of variance procedure. Both tables also show the virtual impossibility of discriminating between the 50:50, 40:60 and 33:67 solutions on the basis of chromatograms. This illustrates the overly optimistic results obtained by internal validity checks. Note that, in the pairwise discrimination, these 3 solutions were almost always correctly classified. The single multiple discriminant function which permits application to unknown substances is more indicative of the quality of discrimination between future chromatograms.

Table 6—Values[1] of components of the discriminant function for cola mixtures.

Peak No.	100P	67:33	60:40	50:50	40:60	33:67	100C
4	-0.064	-0.055	-0.053	-0.052	-0.055	-0.046	-0.037
7	0.021	-0.044	0.060	0.076	0.100	0.081	0.113
8	-0.028	-0.015	-0.016	-0.013	-0.010	-0.014	-0.001
10	0.204	0.244	0.258	0.256	0.254	0.266	0.304
11	0.142	0.160	0.174	0.170	0.177	0.178	0.198
12	0.001	0.001	-0.047	0.066	0.085	0.042	0.062
13	-0.055	-0.048	0.047	-0.041	-0.039	-0.035	-0.023
15	0.020	0.027	0.022	0.022	0.020	0.014	0.002
21	-0.034	-0.030	-0.029	-0.020	-0.014	-0.013	-0.010
Z Value	0.207	0.328	0.415	0.464	0.518	0.473	0.608

[1] Each value is the product of peak area times weighing factor.
[2] The first figure in each blend ratio designates the percentage of Pepsi-Cola.

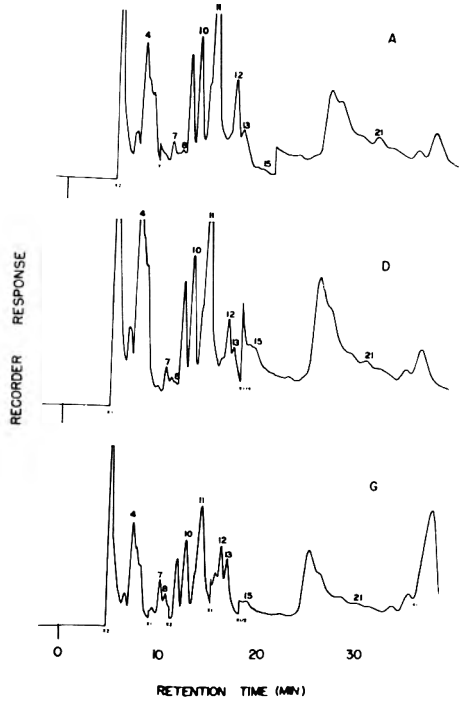

Fig. 1—Chromatograms of cola solutions. A = Pepsi-Cola, G = Coca-Cola; D = a 50/50 blend of each cola beverage. The last peak on chromatogram G is the reference compound, γ-undecalactone. The peaks, which are numbered, are the same as those used in the discriminant equation. Before calculation, areas were adjusted to compensate for attenuation changes.

Limitations and problems

Failure of the model system to be as amenable to SDA analysis as the cola solutions needs to be explained. 3 problems were observed: Use of the percentage area transformation was inappropriate for the model system since it contained only 3 variables. Whenever one compound was reduced in concentration—and thus in peak area—the areas of the 2 other compounds were automatically increased upon the percentage basis. With our method of analysis, this produced an error component. A symmetrical complementation design (Beyer, 1961) would have permitted a more sophisticated analysis to be performed.

When the transformation was not made, then error as a result of extraction, column and instrument variation was more obvious. This represented a second type of error. Analysis of variance demonstrated that when only 3 compounds were present, the percentage efficiency of extraction changed as concentration changed. No attempt was made to check extraction efficiency of the cola beverages because the original concentrations naturally were not obtainable. From observation of the variance for each of the 24 peaks, distillation efficiency of each component of the 24-component system was more stable than in the 3-component system. Even from the extremes of Pepsi-Cola and Coca-Cola the differences in volatile components were less drastic than for the model systems. Figure 1 shows chromatograms for Pepsi-Cola, Coca-Cola and a 50:50 blend of each.

A third source of error was differential volatilization from the extraction concentrate. If 5 or 6 injections were made from the same extract, the first peaks gradually decreased in size, suggesting that the more volatile substances were escaping from the solution. The concentrate was kept in small sealed vials, at 10°F when not in use, in ice water between injections. As cumulative sample withdrawal became appreciable, apparently new liquid:headspace equilibria were established. Again, in the 3-component system this was more serious than in the 24-component extract.

Organoleptic subthreshold additive effects

Threshold values for the panel and the most sensitive members of the panel are shown in Table 7. Among the 30 possible combinations of the 6 compounds listed

Table 7—Threshold values for compounds used in subthreshold additive effect study.

Compound	Threshold concentration[1]	Threshold concn of most sensitive panel member
2-Methyl butyric acid	1.27	1.02
Ethyl acetoacetate	5.20	2.08
Benzaldehyde	2.35	1.41
Isovaleric aldehyde	0.28	0.17
Amyl butyrate	1.29	0.77
Gamma-Undecalactone	0.15	0.12

[1] Concentrations are in parts per million.

in Table 7, only 5 combinations could be used for Group A with the limitation that each judge's sensitivity equal the panel average. 6 combinations were suitable for the Group B trials. None of the 5 combinations for the Group A trials showed a statistically significant subthreshold additive effect. Judge-compound interactions were detected. 1 panel member, for example, could consistently pick out the amyl butyrate + ethyl acetoacetate mixture, and another member could detect the benzaldehyde + amyl butyrate mixture. The same thing was true for the Group B trials. No combination showed a statistically significant subthreshold additive effect when judge-compound interaction was removed.

The trials were not intended to disprove that subthreshold additive effects exist. Logically, they must exist. The trials affirmed the reservation of Keith et al. (1968) that precautions need to be taken to separate judge-compound interaction in attempting to demonstrate subthreshold additive effects. Whether flavor differences are detected through a subthreshold additive effect or the sensitivity of a particular individual for a particular compound, there are other factors which complicate organoleptic-objective correlations more seriously.

To cite only a few, no extraction procedure removes the volatiles 100%. At whatever levels of efficiency they are extracted, the ratio among volatiles is upset. At the GLC stage, the chromatographic pattern is determined by the column and the operating conditions. These and many other factors mean that correlation is restricted to particular conditions of sample treatment and analysis. As pointed out by de Becze et al. (1967) no single column or operating condition is satisfactory for all components. Upon adding to 100° proof ethanol pure components shown to be in Bourbon, de Becze et al. reported that there was no resemblance in either odor or taste between the synthetic mixture and Bourbon. These investigators used 4 columns to separate several types of alcoholic beverages. Notwithstanding lack of compatibility between human sensors and GLC detectors, objective analysis of GLC data has much to commend it. Though our panel could generally select the odd cola solution from the 2 identical samples, it invariably failed in attempts to line up correctly 67:33, 50:50 and 33:67 blends, whether Pepsi-Cola or Coca-Cola was the anchor beverage at one end of the series. This could be done readily by either of the discriminant analysis procedures tried.

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COMPARISON OF FLAVOR AND VOLATILES OF TOMATO PRODUCTS AND OF PEANUTS

SUMMARY—The volatiles of canned tomatoes acidified with citric and with malic acid differed significantly from those of control, nonacidified tomatoes and from each other. Tomatoes treated with sugar and acid also differed significantly in volatiles. Blends of tomato juice prepared from various proportions of canned juice and juice reconstituted from tomato powder differed significantly in flavor. Through the application of discriminant analysis (DA) to gas-liquid chromatographic (GLC) data, blends could usually be predicted correctly from the discriminant equations. Peanuts roasted for 12, 16, 20 and 24 min were similarly evaluated organoleptically and gas chromatographically. For the 3 products above, one or a few peaks were generally not sufficient to characterize the sample. When 10–15 peak areas were used to make the discrimination, each of the 3 products could be catagorized readily according to treatment.

INTRODUCTION

THE WORK reported here had two objectives. One was to ascertain whether nonvolatile acids used as acidulants affect the volatiles of canned tomatoes. The second is part of a long-range program: to seek efficient methods of evaluating gas-liquid chromatograms so as to facilitate correlation of GLC data with sensory evaluation of flavor.

For many years, the chief acidulants in the food industry were acetic, citric, lactic and phosphoric acids. Each of these differs in its taste qualities. Today, acids closer in taste to each other, such as malic and citric, are available at competitive prices. The question then arises: Do nonvolatile acids affect taste only, or do they induce changes in volatiles? If the kind and amount of volatiles differ, and some are flavor substances, this might be important in selecting an acidulant for a particular purpose. Furthermore, though probably not applicable here, formation of new compounds is entirely different from a toxicological point of view than judging the parent compound alone for suitability in foods.

Both citric and malic acids are used commercially as acidulants for canned tomatoes. Pray et al. (1966) compared adipic, citric, fumaric, malic and succinic acids as acidulants for canned tomatoes. They observed that palatability varied according to the acid and that flavor was most important in determining over-all acceptability of the tomatoes, followed by texture and then color. Supran et al. (1966) showed by partial regression analysis that flavor was more important to acceptability than texture or color in trials involving acidification of canned pimientos. Powers (1967) found no clear-cut preference for control tomatoes or tomatoes acidified with malic or citric acids. Leonard et al. (1960) made trials with citric acid, and Lamb et al. (1962) studied acidification of canned tomatoes with citric and hexamic acids. Valdés et al. (1956) and Pangborn et al. (1964) have studied interrelations between or among salt, sugar and acids.

Tomato volatiles have been studied by Spenser et al. (1954); Hein et al. (1963); Pyne et al. (1965); Miers (1966); Wong et al. (1966); Johnson et al. (1968); Yu et al. (1967; 1968a; 1968b); Guadagni et al. (1968) and Nelson et al. (1969).

Efforts to use GLC to complement organoleptic evaluation of foods have intensified in the past few years. Hawkes et al. (1967); Powers et al. (1968); Powers (1968); Hoskins (1968) and Young et al. (1970) applied various statistical procedures to evaluate chromatograms. Young et al. (1969) cited applications to substances other than food. Most recently, Biggers et al. (1969) modified the procedure described by Powers et al. (1968) in that statistical analysis was applied to the sums of peak ratios to form "quality indices."

MATERIALS & METHODS

Materials

Two sets of canned tomatoes were used. One had been previously evaluated organoleptically by Powers (1967). The tomatoes were packed in a commercial cannery in Indiana. NaCl was used as seasoning, calcium salts as firming agents, and 10 grains of either malic or citric acid were added as the acidulant per No. 303 can. The Standard of Identity for canned tomatoes permits sugar to be added only if acid is added and then only in a quantity reasonably necessary to compensate for any tartness resulting from the added acid. The various ingredients above were combined in tablet form. When sugar was added, 10 grains of it were also incorporated in the tablets.

To ascertain the effects of extensive heating, a second set of canned tomatoes was prepared in the laboratory. Green-wrap tomatoes were ripened in the laboratory, the same substances were added (except the calcium salts were omitted), the prepared tomatoes were packed in pint jars and then processed 2 hr at 100° C.

To provide further tests of the statistical method to be described below, blends of canned tomato juice and juice reconstituted from dried tomatoes were prepared. The canned and the reconstituted juices were blended in respective ratios of 95:5, 92.5:7.5, 90:10, 85:15, 80:20, 60:40, 40:60, 20:80, 15:85 and 10:90.

The juice blends changed progressively in flavor as the blend ratio changed. To apply the statistical method to a food which peaked in flavor, then declined, shelled peanuts were roasted at 177°C for 12, 16, 20 and 24 min. The resulting optimally, under- and over-roasted peanuts provided a third food for testing.

Methods

For the GLC analysis, 1 lb of tomatoes was extracted for 2 hr in the extraction assembly described by Likens et al. (1964). Ethyl ether was the solvent. The ether extract was held 4 hr at −12°C to freeze out water (Young et al., 1970), the ether was decanted and then reduced in volume to 60–70 μliters in a Kuderna-Danish evaporator (Gunther et al., 1951). The lower part of the evaporator was submerged in water at 42°C. After final evaporation, 0.05 μliter γ-undecalactone was added to the extract to provide a reference compound for retention times and peak areas. The reference compound also helped establish the volume of the concentrate.

GLC analysis was generally carried on immediately after final concentration and addition of the γ-undecalactone. If not, the samples were kept at −12°C. Between GLC injections, the vials containing the tomato concentrate were kept in ice-water.

The tomato juices were handled in the same fashion except that 170 g of NaCl was added to the 1-lb sample to increase vapor pressure during extraction. The weight of peanuts used for extraction was 100 g. They were covered with 200 ml of saturated brine during distillation.

GLC methods

For the canned tomatoes and the tomato juice, the GLC operating conditions were the same as those described by Young et al. (1970). For the peanuts, a single-column chromatograph with a flame detector was used. The column was 5-ft-long, ¼-in. stainless steel tubing packed with 0.05% SE 52 on 140–170-mesh glass beads. Injection was at 60°C with a 4-min delay, followed by programming at 10°/min to 250°C. The nitrogen, hydrogen and oxygen flow rates were 25, 30 and 20 ml/min.

Areas of the peaks were measured with a K & E compensating Polar planimeter. The peak areas were analyzed as: a) actual area measured, b) each peak area converted to percent area based on the total area of the chromatogram (Powers et al., 1968), c) an arc-sine transformation of the percent values, and d) the ratio of each peak area to the reference area (Young et

Table 1—Peaks of control, citric acid and malic acid–treated tomatoes which differed significantly, area expressed on reference-area basis.

Peak No.	Area[1] Control	Citric Acid	Malic Acid
3	1.809	2.406	2.156
4	.308	.345	.434
6	.237	.120	.711
8	.688	2.418	2.284
9	.144	.208	1.424
10	.360	.610	.966
11	.082	.129	.193
13	.067	.102	.146
14	.182	.188	.413
16	.065	.103	.240
20	.068	.098	.091

[1] Values underscored by the same line do not differ significantly.

Table 2—Peaks of citric acid, citric acid–sugar, malic acid, and malic acid–sugar-treated tomatoes which enabled acid-treated tomatoes to be differentiated from the corresponding sugar-acid treatment, area expressed as reference-area basis.

Peak No.	Area[1] Citric Acid	Citric Acid–sugar	Malic Acid	Malic Acid–sugar
1	1.956	1.386	2.005	1.422
3	2.406	1.604	2.156	1.017
4	.345	.415	0.434	0.424
6	.120	.794	.711	.992
8	2.418	1.188	2.284	1.990
9	.208	.881	1.424	1.157
11	.129	.208	.193	.224
13	.102	.085	.146	.070
14	.188	.350	.413	.326
16	.103	.140	.240	.127
20	.098	.040	.091	.036

[1] Within each pair, values underscored by the same line did not differ significantly; those not underscored did differ significantly. Comparisons should be made only within pairs.

al., 1970). The arc-sine transformation was used, as it is often an appropriate one to "normalize" the distribution of percentage values.

Statistical methods

A slightly modified version of the MUDAID program of Appelbaum et al. (1967) was used for analysis of variance and discriminant analysis. The most recent version permits variables to be reduced in number with greater ease than did the original version. For the canned tomatoes, 23 peaks were considered; for the tomato juice, 25 and for the peanuts, 47. Initially, all the peak areas were considered. Those areas which contributed little to discrimination were then discarded and a second analysis was carried on with the reduced set of variables. The selection of variables (peak areas) is made on the basis of correlation of peak area with the best linear discriminant function among variables (see Bargmann, 1968).

For the 47-variable peanut data, a different approach was taken. The MUDAID program is designed for only 30 variables. Two preliminary analyses were made using peaks 1–30 and 18–47. The most discriminatory values from each set were combined into a new set of 25–30 variables. A second analysis was then made. From this analysis, the number of variables was reduced to 10–15 for the final equations.

Organoleptic evaluation

The flavor of the products was evaluated by 6-member panels. For the canned tomatoes, the triangular method was used to detect differences. The juice blends were similarly evaluated for differences, but the juices were also scored separately for palatability. The samples were evaluated hedonically, using a 5-point scale. The peanuts were also scored hedonically, except that a 9-point scale was used. 14 comparisons were made for the sensory examination of the peanuts. 6–18 trials were made for the canned-tomato difference tests; 12–21 for the tomato-juice difference tests and 9–27 trials for the tomato-juice palatability comparisons.

RESULTS

ADDITION of 10 grains of citric or malic acid per pound of tomatoes caused the distribution of volatiles to differ from those of the control tomatoes. Furthermore, tomatoes treated with citric and malic acids differed significantly from each other. This is shown in Table 1. 6 peaks distinguished the control tomatoes from those treated with citric acid. 11 peak areas distinguished malic acid–treated tomatoes from the control and 8 peak areas differentiated citric from malic acid–treated tomatoes. That citric and malic acids might react differently is not surprising in view of differences in reactivity (Morse, 1929; Gardner, 1966).

Table 2 shows that the citric acid and the citric acid–sugar tomatoes differed in volatiles as did the malic acid and the malic acid–sugar tomatoes.

Tomatoes processed 2 hr did not show as great differences as did those given the standard commercial process. This was somewhat surprising, as it was thought that tomatoes heated longer would show greater differences, if any were to exist. The role played by the sugar cannot be explained. Approximately 0.14% sugar was added. Tomatoes already contain about 5% sugar; the added sugar should thus be almost inconsequential. Glasstone et al. (1925); McKeown et al. (1927); Jennings (1965) and Wientjes (1968) have studied the influence of nonvolatiles on volatiles.

The fact that the acids produced a difference is easier to explain. The 10 grains of acid lowered the pH approximately 0.2 unit. In hydrogen ions, this is an appreciable change. Pectin could be hydrolyzed more readily, for example, or the ionization of volatile acids could be affected.

The taste panel was not able to distinguish between the citric and malic acid treatments of either the laboratory or commercially canned tomatoes, although the volatiles did differ significantly. No attempt was made to identify the volatiles as this is a major project in itself. Some of the volatiles were nonodorous, but probably the chief reason the panel was not able to distinguish between the samples was that most of the volatile changes produced by citric acid were also produced by malic acid. Statistical differentiation rested more on quantitative than on qualitative differences.

The panel could tell the acid and sugar-acid treatments apart. There was not a significant preference for either type of treatment. As to the latter point, this agrees with Powers' observation (1967) and is probably a result of the small amount of sugar added.

Juice blends

The canned-reconstituted tomato juice blends were set up specifically to test correlation of objective with sensory methods. Organoleptic evaluation itself has an error in it according to the sensitivity and reliability of the judges. Rather than using organoleptic scores as the primary standard, both the sensory scores and the GLC values were studied with reference to the blend ratios, as they were mathematically more precise.

The taste panel could not differentiate among the canned-reconstituted blends when the blend ratios differed by increments of less than 10. The 90:10 blend could be distinguished from the 100% canned juice, but the 95:5 and the 92.5:7.5 blends could not be separated from either the 100% canned juice or the 90:10 blend. This was true for the 85:15 blend also. The panel could not consistently separate it from the 90:10 or the 80:20 blends, though the last-mentioned two blends could be separated. When the blends were scored hedonically for prefer-

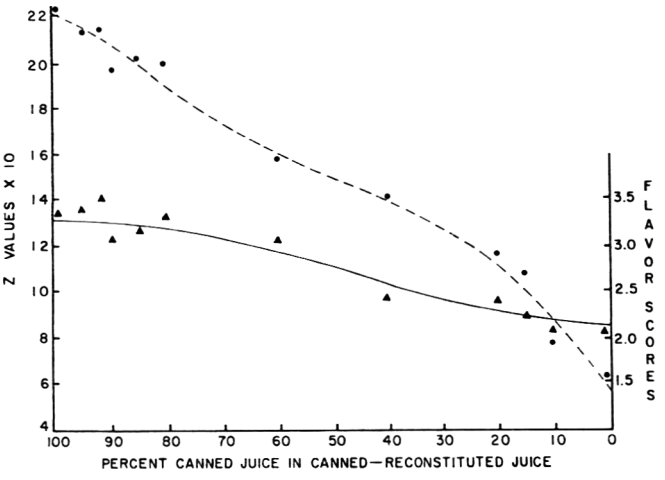

Fig. 1—The solid line shows the regression of flavor scores with change in ratio of canned to reconstituted tomato juice. The broken line depicts the change in Z values based upon the discriminant equation utilizing 25 percentage peak areas.

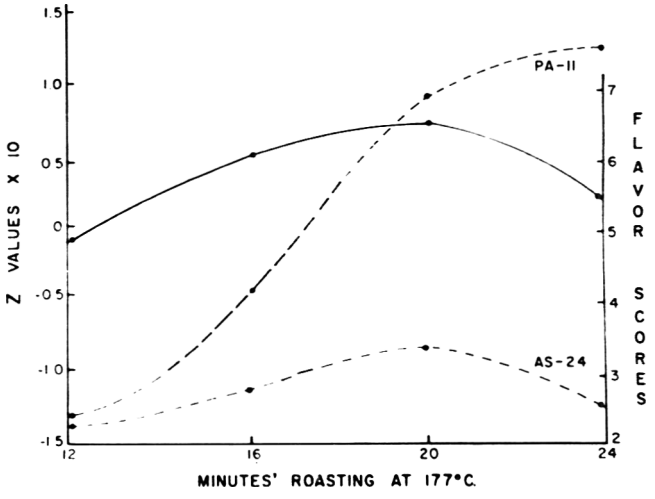

Fig. 2—The solid line shows the relation of flavor scores to roasting time. Curve AS-24 is a plot of the Z values based upon the 14 arc-sine transformations most closely correlated with flavor. Curve PA-11 illustrates a plot which might be used to provide information about scores near the maximum, so as to distinguish under- from over-roasted peanuts.

ence, the difference between the blends had to be much greater, for significance to result. Usually a 40% difference in blend ratio was necessary. The solid line in Figure 1 shows a plot of the preference scores for flavor. Statistically, a linear or curvilinear line fit the data almost equally, but the sigmoidal form is shown, as it is more logical. The canned tomato juice and the reconstituted juice differed in flavor, but not greatly. Unless an added substance is exceptionally potent, the first few increments of addition are likely to be below the sensitivity of the panel. Similarly, beyond some certain point the added substance may so dominate the flavor that further additions are without effect on scores. This results in a sigmoidal curve, or plateaux at each end until the increment of change exceeds the panel's sensitivity.

Figure 1 also shows a plot of the Z values calculated from the discriminant equation based on the percent area for each of the 25 peaks of the tomato juice blends. Discriminant equations were also calculated for the measured area, percent, arc-sine transformation and reference area. Discriminant equations were likewise calculated with reduced sets of variables—generally 10–15 variables—to yield equations easier to work with and to discard peak areas contributing little to discrimination. Table 3 illustrates the success encountered in predicting unknowns based on the discriminant equations. Predictions utilizing all the variables generally came within 10% of the correct mixture. One mixture was predicted to be a 40/60 blend, when it was a 20/80 blend. Based on sets of peak areas reduced to those most closely correlated with treatment change, the predictions were better. The tomato blends could be resolved at intervals at least as close as by taste testing and generally closer. Young et al. (1970) observed the same thing for the cola beverages they worked with. They endeavored to have their panel line up the cola beverages according to blend ratio; the task was entirely beyond the panel. The same thing was true here. By objective means, unknowns almost invariably could be ranked in order of blend ratio. This could not be done organoleptically. In that respect, the objective method is superior.

Peanut trials

Figure 2 shows the panel scores for the peanuts roasted 12, 16, 20 and 24 min. The peanuts roasted 16 min did not differ significantly from those roasted 20 or 24 min, but those roasted 20 min did differ significantly from those roasted 12 or 24 min.

Two plots of Z values are shown in Figure 2. When arc-sine values based on the 24 most discriminating variables were used, the plot correlated well with the flavor scores. Compare curve AS-24 and the flavor-score curve of Figure 2. In carrying out computations involving different ways of expressing area or in reducing the number of variables, some regression curves showed little similarity with the plot for flavor scores. One of these is shown in Figure 2 as curve PA-11. The divergence from the flavor-score pattern can be put to advantage. Peanuts might be scored the same but for the two different reasons of being under- or over-roasted. As Z values of plot PA-11 change progressively with roasting time, they could be used to resolve the ambiguity of identical scores on each side of the optimum, or to set the optimum roasting time more precisely than can be done with curve AS-24 alone. Figure 3 shows chromatograms for the 4 roasting times.

DISCUSSION

THROUGHOUT the short history of gas chromatography, gas chromatographers have been looking for unique compounds correlated with flavor change. Being unique, such compounds obviously are rare. Results with the peanuts suggest that unique combinations of GLC data may exist for particular purposes. Making the calculations is not an especially difficult task. The MUDAID program on an IBM 360 computer yields a complete analysis in 50–75 sec. Reduced to 10–15 variables, computation takes about 7 sec. One can thus afford to make several calculations to ascertain the best discriminant equation for correlation with flavor scores over the complete range of samples expected, to calculate a new discriminant equation for a portion of the organoleptic range so as to give maximum correlation with a more limited range or to seek plots which provide information complementing taste testing. Curve PA-11, for example, might be used to hold production just as close to the optimal roasting time as possible. While attainment of uniformity is highly desirable, quality-control rejection levels often have to be set somewhat wider than intended goals. Curve AS-24 is more suited for this. Whatever tolerance would be acceptable in flavor scores, either side of the optimum could be readily related to the Z values of curve AS-24, since the 2 curves are nearly identical.

Powers et al. (1968) started to study the methodology of analyzing gas chromatograms, because they felt that much information was being overlooked for

Table 3–Predictions of tomato juice blends based on GLC peaks.

	Unknowns[1]						
	Based on 25 peaks			Based on reduced varieties[2]			
Identity	Measured area	% Area	Ref area	Measured area-12	% Area-11	Arc-sine area-10	Ref area-10
100% CJ	X	X	X	X	X	X	X
90/10	X	95/5	85/15	X	X	X	X
80/20	85/15	85/15	X	X	X	X	X
60/40	X	X	X	X	X	X	X
40/60	X	X	X	X	X	X	X
20/80	10/90	40/60	X	X	X	X	X
10/90	15/85	X	X	20/80	20/80	15/85	X
100% RJ	X	X	10/90	X	X	X	X

[1] An X indicates the unknown was correctly identified; if a sample was incorrectly identified, the erroneous blend ratio is then listed.

[2] The number following each type of area shows the number of peak areas to which the set was reduced.

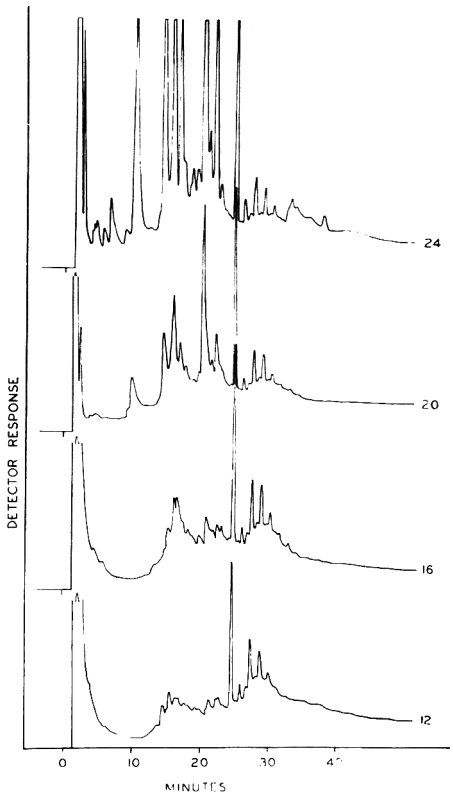

Fig. 3–Typical chromatograms for peanuts roasted various length of time.

want of suitable methods of interpreting gas chromatograms. Their stepwise method permitted separation of relevant information, but the calculation procedure is cumbersome. The DA procedure used here is simpler. This study established that peak areas themselves are adequate to catagorize samples and that resort to ratios of peak areas is not always necessary. If they are needed for some particular application, calculation by the DA procedure could still be employed. Powers et al. (1968) originally thought that most of the GLC data might need to be used to effect discrimination. Generally, some combination of reduced variables correlates as well with flavor as do all the variables. However, statistical examination of all the data is necessary to select the peaks most highly correlated with flavor differences.

Objection may be raised that the DA procedure is a blind application, as the identity of the compounds is not taken into account. Knowledge of their identity is desirable but not absolutely necessary for some applications. Wide use has been made of Tenderometer values in purchasing or quality control for more than 30 years, but even today no one claims that all relations between mechanical shearing and tactile or kinesthetic response are known. When the DA procedure shows peaks to be highly correlated with flavor, this is good reason to get on with the task of identifying the compounds if that has not previously been done. Once the peaks have been identified, even wiser use of the GLC data would then become possible.

In this study, correlation was not always obtained. The panel had no preference for and could not tell the citric acid–treated from the malic acid–treated tomatoes, but the packs were statistically different by GLC analysis. Two reasons have already been suggested: a) that some of the volatiles were nonodorous, and b) that the differences were primarily quantitative rather than qualitative. The net effect may have been less than the panel was capable of detecting. In any event, even with statistically significant correlation, erroneous decisions will be arrived at in a certain number of instances. By GLC analysis, correlations of volatiles with flavor, if they exist, may be discerned, but that is all. Even as to extraction, as Pyne et al. (1965) pointed out, no method is so mild as not to upset the balance of volatiles. For this, and for many other chemical and physiologic reasons, GLC analysis is not flavor analysis.

Though discriminant analysis applied to GLC data does not measure flavor itself, information about flavor can be acquired and expressed in mathematical, easily manageable forms. In turn, the equations may be used to control quality, to detect adulteration or to add insight to organoleptic response.

Discriminant analysis need not be confined to changes in volatiles. The variables could include other factors such as sugar content, moisture, shear press values or any other quantifiable attribute, and the procedure described could be used for industrial processes other than those of the food industry.

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EFFECT OF FREEZING RATE AND FREEZE DRYING ON THE SOLUBLE PROTEINS OF MUSCLE. 1. Chicken Muscle

SUMMARY—Freezing treatment, immersion in liquid nitrogen and air freezing at −10°C had a significant influence on the soluble proteins of frozen and freeze-dried chicken muscle. Greater protein solubility occurred in muscle frozen at −10°C than in muscle frozen by immersion in liquid nitrogen. This was observed in both raw and cooked muscle tissue. The effect of freeze-drying on protein solubility was not the same for raw and cooked muscle. Total protein solubility increased when cooked muscle was freeze dried. There was no protein solubility difference between frozen and freeze-dried raw muscle.

INTRODUCTION

SEVERAL processes are associated with freeze dehydration which may influence the physical and chemical characteristics of muscle tissue. A primary factor which must be considered is the freezing treatment.

The effect of freezing on muscle proteins has been studied extensively but considerable disagreement exists in the literature. Connell (1962) observed that freezing does not affect the extractability of proteins from cod muscle. Pavlovskii et al. (1966) demonstrated that freezing and subsequent defrosting of light and dark chicken muscle increased the extractability of the sarcoplasmic proteins. However, Reay (1933) reported that freezing denatures haddock muscle proteins, especially the globulin and albumin fractions.

The effect of freezing rate is also nonconclusive. Minimal damage to cellular components has been correlated with rapid freezing rates (Snow, 1950; Deatherage et al., 1960; Khan, 1966; Khan et al., 1967). Conversely, Love et al. (1958) concluded that freezing rate had no relative effect on the soluble proteins when fish muscle was thawed immediately after freezing. Rapid freezing at −195°C, as opposed to slow freezing, did not have a significant effect on cod muscle proteins (Love, 1967).

MacKenzie et al. (1967) attempted to isolate the effects of freezing from those of freeze drying. They reported that freeze drying did not reduce the extractability of proteins, provided the specimen chamber is maintained at −10°C throughout the drying process. Partmann et al. (1963) reported that the extractability of myofibrillar proteins from freeze-dried carp muscle was similar to samples frozen and thawed immediately. Similar observations have been made for the globular muscle proteins (Hamm et al., 1960). However, deleterious effects have been attributed to freeze drying (Hunt et al., 1958; Hamdy et al., 1959; Aitken et al., 1962; Suden et al., 1964).

The effect of freezing rate on the proteins of freeze-dried muscle has not been thoroughly investigated. The need for additional research, as pointed out by Burke et al. (1964), is apparent. The purpose of this investigation was to determine the effect of freezing rate and the subsequent effect of freeze drying on the extractable proteins of chicken muscle.

MATERIALS & METHODS

MUSCLE samples were obtained from mature White Leghorn hens. The investigation was divided into 2 experiments: Experiment I, a study of raw chicken muscle; Experiment II, investigation of cooked chicken muscle. In each experiment muscle proteins were extracted from muscle in the frozen and freeze-dried state.

Experiment I

The pectoralis major muscle was obtained 24 hr post-mortem from 20 White Leghorn hens. The muscles were diced into 1-cm cubes. Samples were frozen using 2 treatments, immersion in liquid nitrogen and air freezing at −10°C.

Approximately 60 g of diced meat were placed on a fine mesh screen. The sample was immersed for 45 sec in a Dewar-type flask containing liquid nitrogen.

A single depth of diced muscle tissue was placed on trays and frozen on a shelf of an upright freezer maintained at −10°C. The samples were frozen in approximately 2 hr.

Experiment II

60 chicken carcasses were cooked in water 24 hr post-mortem, to an internal breast temperature of 85°C. The temperature of the water varied between 94.4 and 97.8°C. The internal temperature was recorded on a Honeywell 24-point recorder at 3-min intervals. After the cooking treatment, the carcasses were chilled for 24 hr at 1–4°C. The pectoralis major and pectoralis minor muscles were removed and diced into 1-cm cubes. The diced meat was thoroughly mixed before freezing. Approximately 115 g of diced meat were immersed in liquid nitrogen for 30 sec. A single depth of diced muscle was placed on trays and frozen at −10°C in an upright freezer. Freezing time was approximately 2 hr.

Freeze drying

Samples were freeze dried in a Stokes Model 21 freeze dryer. A platen temperature of 51.7°C was used throughout the drying cycle. No heat was applied until the chamber pressure measured 250 μ. Approximately 300 g (1.76 lb/sq ft) of muscle tissue were dried during each cycle. The drying cycle times were 13 hr for raw muscle and 12 hr for cooked muscle samples.

Extraction systems

2 fractions, the total soluble proteins and the sarcoplasmic proteins, were obtained from frozen and freeze-dried samples. The total soluble proteins were extracted using 0.049 M $Na_2HPO_4 \cdot 7\ H_2O$, 0.0045 M $NaH_2PO_4 \cdot H_2O$ and KCl to give an ionic strength of 1 (Khan, 1962). The pH was 7.3. The sarcoplasmic proteins were extracted with 0.03 M potassium phosphate buffer at a pH of 7.4 (Helander, 1957).

Sample preparation

Approximately 8 g of each freeze-dried muscle sample were pulverized in a Lourdes Model VM mixer. Aliquots of each sample were placed in 50-ml centrifuge tubes. The sample weight of the raw freeze-dried tissue was about 0.5 g. About 0.75 g of freeze-dried cooked muscle was added to each centrifuge tube.

About 60 g of each frozen muscle sample were homogenized into a fine powder using a Waring Blendor equipped with a glass blender jar. The blender jar had been previously chilled to −20°C. The Blendor was operated in a chest-type deep-freeze unit. Extreme caution was exercised to prevent the muscle powder from thawing. The sample weight of the raw frozen tissue was 2 g. About 3 g were used for the frozen cooked tissue.

Extraction procedure

Proteins were extracted at 4°C. Triplicate samples were extracted from cooked muscle. The first solvent addition was 20 ml. Glass stirring rods were placed in each tube and the tubes continuously agitated by mechanical means for 2 hr. The samples were centrifuged for 15 min at 1,670 × g. The residue was re-extracted twice with 10 ml of solvent. The raw samples were extracted in duplicate. The first, second and third solvent quantities were all 25 ml.

Protein determination

A modification of the biuret method was used for protein determinations (Gornall et al., 1949). Protein concentration was determined from a standard bovine albumin solution. Absorbancy was measured on a Bausch and Lomb Spectronic 20 colorimeter at a wavelength of 540 mμ. The myofibrillar protein content was determined by difference. Results were expressed as milligrams per gram dry tissue.

Nonprecipitated protein was determined after the proteins were precipitated with 30% trichloroacetic acid. The biuret reagent was also

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Table 1—Treatment means for frozen and freeze-dried raw muscle (mg/g dry tissue).

	Frozen		Freeze dried	
	N_2	−10°C	N_2	−10°C
Total soluble	501.35	578.15	533.15	554.50
Sarcoplasmic	339.80	347.70	327.15	355.40
Myofibrillar	161.55	230.45	206.00	199.10

used for the nonprecipitated protein analysis.

Statistical analysis

The data were subjected to an analysis of variance to determine the significance of treatment effects (Steel et al., 1960). The experimental design was a split-plot design.

Table 2—Analysis of variance of raw chicken muscle protein solubility, sarcoplasmic and total soluble protein.

Source	d.f.	MS	F Value
Bird (B)	19	5438.24	3.66**
Freezing treatment (T)	1	90139.14	60.19**
Error a	19	1497.52	
Muscle state (F)	1	57.72	<1.00
T × F	1	6261.82	2.99
Error b	38	2090.17	
Extraction system (P)	1	3178027.85	1659.20**
T × P	1	18990.82	9.90**
Error c	38	1915.40	
F × P	1	852.72	<1.00
T × F × P	1	28960.05	20.87**
Error d	38	1387.03	

**P < .01.

RESULTS

Experiment I

The treatment means for total soluble, sarcoplasmic and myofibrillar proteins are included in Table 1. The analysis of variance for the sarcoplasmic and total soluble proteins is presented in Table 2. The analysis of variance for myofibrillar protein solubility is presented in Table 3.

There was a significant difference in protein solubility among the individual chickens. This difference was attributed to the sarcoplasmic proteins, since no difference existed between birds when data for the myofibrillar fraction were analyzed by a separate analysis of variance.

Freezing treatment had a significant effect on protein solubility. Total protein solubility was greater when chicken muscle was frozen at −10°C than when frozen by immersion in liquid nitrogen.

There was a significant interaction between freezing treatment and extraction method (Table 2). Extraction method refers to the 2 fractions extracted, the total protein fraction and the sarcoplasmic fraction. This interaction is illustrated in Figure 1. The magnitude of difference between liquid nitrogen freezing and freezing at −10°C increased in the total soluble fraction which contained both the myofibrillar and sarcoplasmic proteins. This increase, therefore, must be attributed to the myofibrillar proteins. This was confirmed by the statistical analyses for the myofibrillar data (Table 3).

There was no significant difference between frozen and freeze-dried muscle protein solubility. With the myofibrillar fraction there was a significant interaction between freezing treatment and muscle state. Muscle state refers to the physical state of the muscle tissue, i.e., frozen and freeze-dried. This interaction is demonstrated in Figure 2. Myofibrillar solubility of frozen muscle was greater at the slower freezing rate. This was reversed in freeze-dried muscle. Myofibrillar solubility of liquid nitrogen frozen muscle was enhanced by freeze drying. However, the solubility of myofibrillar proteins decreased when muscle frozen at −10°C was freeze dried. Conversely, the solubility of the sarcoplasmic proteins increased slightly when muscle frozen at −10°C was freeze dried, but decreased in muscle frozen in liquid nitrogen.

There was a significant difference between extraction methods or extraction systems. The difference between the total soluble and the sarcoplasmic protein extracts was expected, since the total protein fraction not only included the sarcoplasmic proteins but also the myofibrillar proteins.

Experiment II

The extraction systems for total soluble proteins (Khan, 1962) and for sarcoplasmic proteins (Helander, 1957) were used on the cooked meat. After heat denaturation, the proteins extracted by each of the systems may not be the same fractions as extracted from raw muscle but in terminology the terms total soluble, sarcoplasmic and myofibrillar proteins are applied, as the same extraction systems were employed for both raw and cooked muscle. The means for the total soluble, sarcoplasmic and myofibrillar proteins extracted from frozen and freeze-dried cooked muscle are presented in Table 4.

Cooking muscle tissue to an internal temperature of 85°C had a pronounced effect on protein solubility. The soluble protein content of cooked muscle was less than 1/10th that of raw muscle, which indicates that protein denaturation is almost complete at 85°C.

Freezing treatment had a significant effect on protein solubility (Table 5). The soluble sarcoplasmic protein content, especially in freeze-dried muscle, was greater in muscle frozen at −10°C. The effect of freezing treatment on the soluble myofibrillar proteins was not significant.

Table 3—Analysis of variance of myofibrillar solubility from raw chicken muscle.

Source	d.f.	MS	F Value
Bird (B)	19	2393.72	1.68
Freezing treatment (T)	1	19021.19	13.29**
Error a	19	1431.62	
Muscle state (F)	1	846.24	<1.00
T × F	1	28998.30	20.93**
Error b	38	1385.33	

**P < .01.

Table 4—Treatment means for frozen and freeze-dried cooked muscle (mg/g dry tissue).

	Frozen		Freeze-dried	
	N_2	−10°C	N_2	−10°C
Total soluble	35.37	35.37	41.75	58.00
Sarcoplasmic	25.87	28.12	26.25	38.75
Myofibrillar	9.50	7.25	16.50	19.25

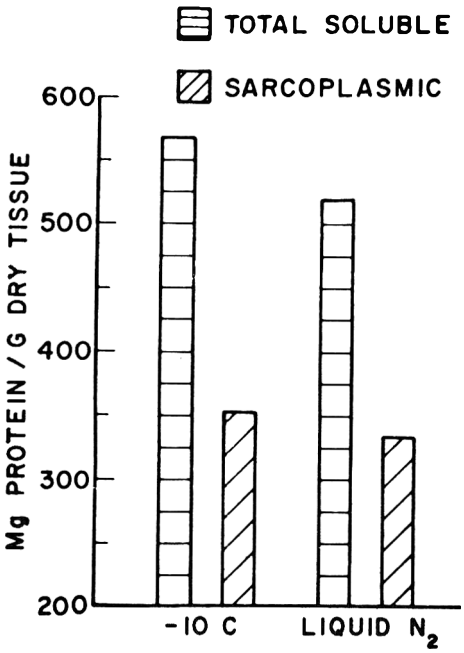

Fig. 1–Interaction of freezing treatment and extraction system in raw chicken muscle.

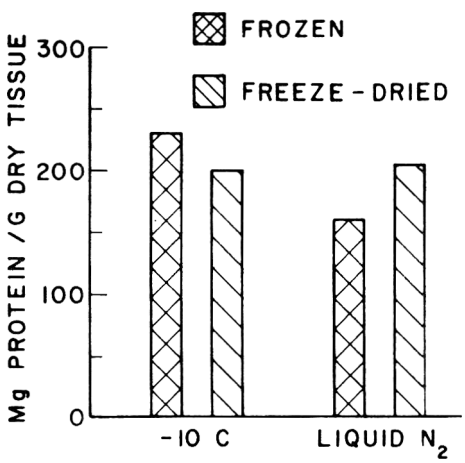

Fig. 2–Interaction of freezing treatment and muscle state of myofibrillar proteins in raw chicken muscle.

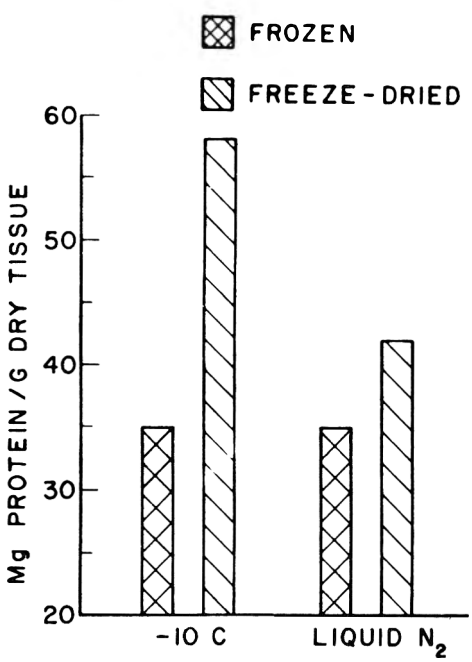

Fig. 3–Interaction of freezing treatment and muscle state in total soluble proteins of cooked chicken muscle.

The difference between frozen and freeze-dried muscle protein solubility was significant (Table 5). Protein solubility increased when cooked muscle was freeze dried. The difference between frozen and freeze-dried muscle was significant for the myofibrillar proteins (Table 6). The latter observation clarifies the significant interaction which occurred between muscle state and the extraction method (Table 5). The difference between frozen and freeze-dried muscle tissue was more pronounced in the total soluble protein fraction which contained the myofibrillar proteins.

The interaction between freezing treatment and muscle state was significant (Table 5). This interaction is illustrated in Figure 3. The difference between freezing in liquid nitrogen and at −10°C was greater in freeze-dried muscle samples. A larger increase in solubility occurred in freeze-dried muscle initially frozen at −10°C than in that frozen by immersion in liquid nitrogen.

The nonprecipitated protein content of raw and cooked chicken muscle was similar. The average for raw muscle was 18.79 mg per gram of dry tissue and 15.7 mg per gram for cooked tissue. No differences were observed between freezing treatment, muscle state or extraction system with respect to nonprecipitated protein.

DISCUSSION

PROTEIN solubility was affected by the freezing treatment. Greater solubility was observed in muscle tissue frozen at −10°C. This occurred in both raw and cooked muscle tissue. With cooked muscle the sarcoplasmic proteins, but not the myofibrillar proteins, were influenced by the freezing rate. The extractability of the sarcoplasmic proteins was also greater than the extractability of myofibrillar proteins in cooked muscle.

The total soluble protein content of raw muscle was greater when frozen at −10°C. This difference existed in both frozen and freeze-dried tissue. These observations are not consistent with all reports in the literature (Snow, 1950; Deatherage et al., 1960; Khan, 1966; Khan et al., 1967). Other reports indicate that freezing rate has no effect on protein solubility (Love, 1958; 1967). Love (1958) found that protein solubility of fish muscle was maximum at very rapid rates of freezing and also at slower freezing rates. A minimum point of solubility existed at an intermediate freezing rate.

In other studies with turkey and porcine muscles, the authors found that muscle from different species did not

Table 5–Analysis of variance for cooked chicken muscle protein solubility, sarcoplasmic and total soluble protein.

Source	d.f.	MS	F Value
Batch (B)	7	46.67	1.27
Freezing treatment (T)	1	2858.56	77.95**
Error a	7	36.67	
Muscle state (F)	1	4755.48	51.39**
T × F	1	2068.50	22.35**
Error b	14	92.53	
Extraction system (P)	1	7929.48	419.33**
T × P	1	4.22	<1.00
Error c	14	18.91	
F × P	1	989.90	23.22**
T × F × P	14	108.63	2.55
Error d	14	42.64	

**P < .01.

Table 6–Analysis of variance for myofibrillar solubility of cooked chicken muscle.

Source	d.f.	MS	F Value
Bird (B)	7	6.46	<1.00
Freezing treatment (T)	1	8.38	<1.00
Error a	7	13.39	
Muscle state (F)	1	599.01	29.38**
T × F	1	53.33	2.62
Error b	14	20.39	

**P < .01.

respond identically to freezing rates (Huber et al., 1970; McSweeney et al., 1968). This species difference may account for some of the conflicting reports in the literature regarding the effect of freezing rate on muscle tissue. A species difference has also been reported by Partmann (1963). He reported that the extractability of structural proteins was less in rosefish than in cod muscle at storage temperatures below −20°C. The response of the sarcoplasmic and myofibrillar proteins to freezing treatment was not the same in frozen and freeze-dried muscle. This was demonstrated by the significant interactions which occurred between freezing rate and frozen and freeze-dried muscle.

When raw muscle was freeze dried, the solubility of myofibrillar proteins increased in muscle samples initially frozen in liquid nitrogen. The solubility of myofibrillar proteins in freeze-dried muscle frozen at −10°C decreased when compared with frozen muscle. Therefore, when the entire freeze-drying procedure is considered, the myofibrillar proteins are enhanced by liquid nitrogen freezing. In cooked muscle the solubility of the sarcoplasmic and myofibrillar proteins increased more when muscle frozen at −10°C was freeze dried.

The effect of freeze drying on protein solubility was not the same for raw and cooked muscle. The difference between frozen and freeze-dried muscle was significant for cooked muscle, but not for raw muscle. Total protein solubility of cooked muscle significantly increased when freeze dried. Hamdy et al. (1959) also found that protein solubility increased when cooked meat was freeze dried. They suggested that the sarcolemma was altered, which allowed peptides and nucleotides to diffuse during extraction. Additional evidence by Pool (1967) shows that connective tissue tenacity was decreased by cooking and freeze drying. Both of these treatments were associated with freeze-dried cooked muscle.

It has also been reported that calcium and magnesium increased in water extracts of freeze-dried muscle (Hamdy et al., 1959; Hamm et al., 1960). Cleavage of metallic cross linkages between protein chains apparently occurred. This would be a basis for the increased solubility of freeze-dried muscle.

Hamdy et al. (1959) also suggested that freeze drying may reverse the solubility of some proteins. Under certain conditions denaturation of proteins may be reversible. Initial observations of this phenomenon were reported by Anson et al. (1931) for hemoglobin and other proteins.

Cooking chicken muscle to an internal temperature of 85°C decreased protein solubility. The solubility of cooked tissue was less than 1/10th that of raw tissue. This was consistent with the results of Khan et al. (1965), who observed a similar decrease in protein solubility when chicken muscle was cooked to an internal temperature of 85°C.

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EFFECT OF FREEZING RATE AND FREEZE DRYING ON THE SOLUBLE PROTEINS OF MUSCLE. 2. Turkey Muscle

SUMMARY—Effect of freezing rate on the soluble proteins of frozen and freeze-dried turkey breast muscle was investigated. Both raw and cooked muscles were frozen and freeze dried. Samples were frozen in liquid nitrogen and in a plate freezer at −10°C. The total soluble proteins and the sarcoplasmic proteins were extracted from frozen and freeze-dried muscle. Greater solubility was observed at the slower freezing rate (−10°C). Freeze-dried raw muscle had greater solubility than the frozen control. No differences were observed between frozen and freeze-dried cooked muscle.

INTRODUCTION

THE EFFECT of freezing rate on the proteins of turkey muscle has not been as thoroughly investigated as muscle from other species. A need for additional research is evident since the response of muscle from different species to freezing may not be identical. Partmann (1963) reported that the extractability of structural proteins was less in rosefish than in cod muscle at storage temperatures below −20°C. In a recent investigation Huber et al. (in press) reported that the sarcoplasmic and myofibrillar protein content of chicken muscle was greater when muscle was frozen at −10°C than when frozen in liquid nitrogen. McSweeney et al. (1968) observed that the myofibrillar solubility of porcine muscle was greater in muscle frozen in liquid nitrogen than at −10°C. Conversely, the sarcoplasmic proteins of porcine muscle were more soluble in muscle frozen at −10°C.

Deleterious effects on protein solubility have been attributed to freeze drying (Hunt et al., 1958; Hamdy et al., 1959; Aitken et al., 1962; Suden et al., 1964). However, evidence for the adverse effects of freeze drying on protein solubility has not been conclusive for all types of muscle (Cole et al., 1960; Partmann et al., 1963). The globular proteins, in particular, do not appear to be affected by the freeze-drying process (Hamm et al., 1960).

The effect of freeze drying on cooked muscle proteins has not been clearly defined. Hamdy et al. (1959) reported that freeze drying cooked muscle increased the soluble protein concentration. Huber et al. (in press) also observed that freeze drying cooked chicken muscle increased the concentration of the soluble proteins. Additional investigation of this effect in other species would be meaningful. This is not only true for cooked muscle but raw muscle as well. Therefore, the purpose of this investigation was to determine the effect of freezing rate and the subsequent effect of freeze drying on the extractable proteins of turkey muscle in the raw and cooked state.

MATERIALS & METHODS

LARGE White tom turkeys approximately 24 wk of age were used in this investigation. Two different experiments were conducted: Experiment I was a study of raw turkey muscle; cooked turkey muscle was investigated in Experiment II.

Experiment I

The pectoralis major was obtained 24 hr postmortem from 24 tom turkeys. The muscles were diced into 1-cm cubes and frozen immediately. Two freezing methods were employed: a) immersion in liquid nitrogen and b) plate freezing at −10°C.

Liquid nitrogen freezing. Approximately 60 g diced meat were placed on a fine-mesh screen. The sample was immersed for 45 sec in a Dewar-type flask containing liquid nitrogen.

Plate freezing. A single layer of diced cubes was placed on a shelf of an upright freezer maintained at −10°C. The samples were frozen within a 2-hr period.

Experiment II

12 turkeys were cooked 24 hr post-mortem in water to an internal breast temperature of 85°C. Temperature of the water varied between 94.4 and 97.8°C. A thermocouple was placed into the breast of each specimen and the internal temperature recorded on a Honeywell 24-point recorder at 3-min intervals. After the cooking treatment, the carcasses were chilled for 24 hr at 1–4°C. The pectoralis major and pectoralis minor muscles were removed and diced into 1-cm cubes and thoroughly mixed before freezing. As with the raw muscle samples, two freezing methods were employed.

Liquid nitrogen freezing. Approximately 115 g diced meat were immersed in liquid nitrogen for 30 sec.

Plate freezing. A single layer of diced cubes was placed on trays and frozen at −10°C.

Freeze drying. Samples were freeze dried in a Stokes Model 21 freeze dryer. A platen temperature of 51.7°C was used. No heat was applied until the chamber pressure measured 250 μ. Approximately 800 g (1.76 lb/sq ft) of muscle tissue were freeze dried during each cycle. Liquid nitrogen and plate-frozen samples were freeze dried simultaneously.

Extraction systems. Two fractions, the total soluble proteins and the sarcoplasmic proteins, were obtained from frozen and freeze-dried samples. The total soluble proteins were extracted using 0.049 M $Na_2HPO_4 \cdot 7\ H_2O$, 0.0045 $Na\ H_2PO_4 \cdot H_2O$ and KCl to give an ionic strength of 1.0 (Khan, 1962). The pH was 7.3. The sarcoplasmic proteins were extracted with 0.03 M potassium phosphate buffer at pH 7.4 (Helander, 1957).

Sample preparation. About 60 g of each frozen muscle sample were homogenized into a fine powder using a Waring Blendor equipped with a glass Blendor jar which had been previously chilled to −20°C. The Blendor was operated in a chest-type deep freeze unit. Extreme caution was exercised to prevent the muscle powder from thawing since if thawing occurred, especially during the early stages of grinding, particles would adhere to the side of the Blendor. A very fine, homogeneous powder was prepared when the samples were not permitted to thaw. Grinding time was approximately 30 sec. The sample weight of the raw frozen tissue was 2.0 g. About 3.0 g of frozen cooked tissue was used.

Approximately 8 g of each freeze-dried muscle sample was pulverized in a Lourdes Model VM mixer for 30 sec. Aliquots of each sample were placed in 50-ml centrifuge tubes. The sample weight of raw freeze-dried tissue was about 0.5 g. About 0.75 g of freeze-dried cooked muscle was added to each centrifuge tube.

Extraction procedure. Proteins were extracted at 4°C. Triplicate samples were extracted from cooked muscle both frozen and freeze-dried. The first solvent addition was 20 ml. Glass stirring rods were placed in each tube and the tubes agitated gently for 2 hr. The samples were centrifuged for 15 min at 1,670 × g. The residue was re-extracted twice with 10 ml of solvent.

The raw samples, frozen and freeze dried, were extracted in duplicate. The first, second and third solvent quantities were 25 ml each.

Protein determination. A modification of the biuret method was used for protein determination (Gornall et al., 1949). Absorbancy was measured on a Bausch and Lomb Spectronic 20 Colorimeter at a wavelength of 540 mμ. Concentration was determined from a standard bovine albumin solution. The myofibrillar protein content was determined by difference between the two extraction systems. Results were expressed as milligrams per gram dry tissue.

The nonprecipitated protein content was determined after the proteins were precipitated with 30% trichloroacetic acid. The biuret reagent was also used for the nonprecipitated protein analyses.

Storage of freeze-dried turkey muscle. Freeze-dried raw muscle samples from 8 turkeys were stored in glass jars at a temperature of 21.1 ± 1°C. Total and sarcoplasmic proteins were extracted after 2 and 4 months.

Statistical analysis. The data were subjected to an analysis of variance to determine the significance of treatment effects (Steel et al., 1960).

Table 1—Treatment means for frozen and freeze-dried raw muscle.

	Frozen		Freeze-Dried	
	N_2	−10°C	N_2	-10°C
Total soluble	406	428	454	444
Sarcoplasmic	278	290	298	316
Myofibrillar	128	138	156	128

[1] Milligrams protein per gram dry tissue.

Table 2—Analysis of variance of raw turkey muscle protein solubility.

Source	d.f.	M.S.	F value
Bird (B)	19	4,729.83	1.91
Freezing treatment (T)	1	8,537.35	3.46*
Error a	19	2,470.81	
Muscle state (F)	1	61,604.45	37.70***
T × F	1	3,506.35	2.15
Error b	38	1,634.08	
Extraction Method (P)	1	1,508,630.48	1,083.36***
T × P	1	1,577.98	1.13
Error c	38	1,392.55	
F × P	1	1,621.44	1.16
T × F × P	1	7,233.69	5.19**
Error d	38	1,394.49	

*Significant at the α .10 level.
**Significant at the α .05 level.
***Significant at the α .01 level.

Table 3—Analysis of variance of myofibrillar solubility from raw turkey muscle.

Source	d.f.	M.S.	F value
Bird (B)	19	1,579.40	1.30
Freezing treatment (T)	1	1,521.38	1.25
Error a	19	1,216.99	
Muscle state (F)	1	1,672.35	1.21
T × F	1	7,112.67	5.13**
Error b	38	1,385.92	

**Significant at the α .05 level.

Table 4—Treatment means for frozen and freeze-dried cooked muscle.

	Frozen		Freeze-dried	
	N_2	−10°C	N_2	−10°C
Total soluble	24	29	24	31
Sarcoplasmic	21	23	20	23
Myofibrillar	3	6	4	8

*Milligrams protein per gram dry tissue.

RESULTS

TREATMENT means for the frozen and freeze-dried muscle extractions are included in Table 1. Only the total soluble and the sarcoplasmic proteins were extracted from raw turkey muscle. The myofibrillar proteins were calculated by determining the difference between the two extraction systems. Analyses of variance for these data are included in Tables 2 and 3.

Freezing treatment had an effect on the soluble proteins of raw turkey muscle ($P < .10$). The sarcoplasmic proteins were more soluble in muscle frozen at −10°C. The myofibrillar proteins were not influenced by the rate of freezing.

When the myofibrillar fraction was analyzed independently a significant interaction occurred between freezing treatment and muscle state (Fig. 1). The significant interaction between freezing treatment and muscle state implies that the differences between responses of muscle state vary with the freezing treatment. Alternately, the differences among responses to freezing treatment vary with the muscle state. (Muscle state refers to frozen and freeze-dried muscle tissue.) This interaction is demonstrated in Figure 1 and was significant ($P < .05$). Myofibrillar solubility of frozen muscle was greater when turkey muscle was frozen at −10°C. This was not the case with freeze-dried muscle. Greater myofibrillar solubility occurred in freeze-dried muscle frozen in liquid nitrogen.

There was a difference ($P < .01$) between frozen and freeze-dried muscle protein solubility (Table 2). The solubility of freeze-dried muscle was greater than that of frozen muscle. This difference was attributed to the sarcoplasmic proteins since there was no difference between freeze-dried and frozen muscle samples when the solubility of the myofibrillar proteins was compared (Table 3).

The difference between extraction methods (total soluble vs. sarcoplasmic) was expected. This was anticipated since the total soluble extracts contained not only the sarcoplasmic but also the myofibrillar proteins. It should be pointed out, however, that the sarcoplasmic proteins comprised the major portion (approximately 2/3) of the total soluble proteins. There was also a significant interaction between freezing treatment, muscle state and extraction method (Table 2). This second-order interaction can probably be explained by referring to Table 3. When the myofibrillar fraction was analyzed independently a significant interaction occurred between muscle state and freezing treatment. Therefore, only the myofibrillar fraction would be involved in the T × F × P interaction.

The average nonprecipitated protein content of raw turkey muscle was 10.59 mg per gram of dry tissue. No differences were observed between extraction methods, freezing treatments or frozen and freeze-dried muscle.

Experiment II

The extraction systems for total soluble proteins (Khan, 1962) and for sarcoplasmic proteins (Helander, 1957) were used on the cooked meat. After heat denaturation the proteins extracted by each of the systems may not be the same fractions as extracted from raw muscle, but for uniformity in terminology the terms total, sarcoplasmic and myofibrillar proteins are applied.

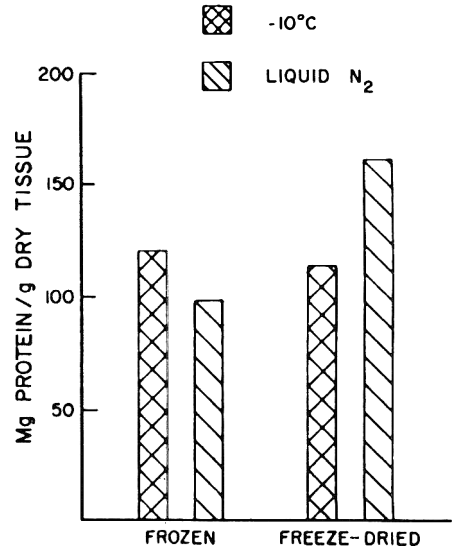

Fig. 1—Interaction of freezing treatment and muscle state of myofibrillar proteins in raw turkey muscle.

Table 5–Analysis of variance for cooked turkey muscle protein solubility.

Source	d.f.	M.S.	F value
Batch	7	117.91	7.36**
Freezing treatment (T)	1	843.99	52.68***
Error a	7	16.02	
Muscle state (F)	1	5.27	<1.00
T × F	1	29.87	<1.00
Error b	14	117.31	
Extraction method (P)	1	1,472.37	91.79***
T × P	1	84.65	5.08**
Error c	14	16.04	
F × P	1	2.75	<1.00
T × F × P	1	1.24	<1.00
Error d	14	21.08	

**Significant at the α .05 level.
***Significant at the α .01 level.

Table 6–Analysis of variance for myofibrillar solubility of cooked turkey muscle.

Source	d.f.	M.S.	F value
Bird (B)	7	7.16	<1.00
Freezing treatment	1	51.79	4.11*
Error a	7	12.61	
Muscle state	1	1.08	<1.00
T × F	1	1.51	<1.00
Error b	14	13.50	

*Significant at the α .10 level.

Table 7–Treatment means for stored freeze-dried turkey muscle.

	Initial		2 Months		4 Months	
	N_2	−10°C	N_2	−10°C	N_2	−10°C
Total soluble	445.32	427.90	454.20	472.99	427.17	438.27
Sarcoplasmic	295.19	319.38	303.11	324.37	289.43	311.54
Myofibrillar	150.13	108.52	151.09	148.62	137.74	126.73

[1] Milligrams protein per gram dry tissue.

Heating turkey muscle to an internal temperature of 85°C significantly decreased protein solubility. Compared with raw muscle, percentage decrease was greater than 93%. The sarcoplasmic proteins represented a major fraction of the total soluble proteins. The difference between extraction methods was significant. Treatment means for cooked turkey muscle are included in Table 4.

Cooked muscle, like raw muscle, was influenced by the freezing treatment. This difference was significant ($P < .01$), Table 5. The difference between freezing treatments was also significant for the myofibrillar proteins (Table 6). Greater solubility occurred in muscle samples frozen at the slower freezing rate.

In contrast to raw turkey muscle there was no solubility difference between frozen muscle and muscle which was freeze dried.

A significant interaction occurred between freezing treatment and extraction method (Table 5). The magnitude of difference between liquid nitrogen freezing and freezing at −10°C increased in the total soluble fraction (Fig. 2). This provides additional evidence that the myofibrillar proteins were affected by the freezing treatment.

The average nonprecipitated protein content of cooked muscle was 8.56 mg per gram of dry tissue. This was slightly lower than the value obtained from raw muscle.

Effect of storage on protein solubility. Treatment means for the raw turkey muscle freeze-dried and subsequently stored are included in Table 7.

Analysis of variance for stored freeze-dried muscle is presented in Table 8. Storage for 4 months had no significant effect on protein solubility. The difference between freezing treatment was significant ($P < .05$). The sarcoplasmic proteins were more soluble when muscle tissue was frozen at −10°C. Greater myofibrillar solubility occurred in muscle frozen in liquid nitrogen. This was consistent with results shown in Table 1 for nonstored muscle samples.

Table 8–Analysis of variance of stored freeze-dried turkey muscle.

Source	d.f.	M.S.	F value
Birds (B)	7	4,799.89	1.35
Storage (S)	2	7,457.14	1.73
S_l	1	11,352.79	2.64
S_q	1	3,561.49	<1.00
Error a	14	4,307.33	
Freezing treatment (T)	1	8,545.87	6.99**
S × T	2	275.01	<1.00
S_l	1	.00013	<1.00
S_q	1	550.01	<1.00
Error b	21	1,221.19	
Extraction method (P)	1	902,726.79	87.86***
S × P	2	97.87	<1.00
S_l	1	87.83	<1.00
S_q	1	10.04	<1.00
Error c	21	759.96	
T × P	1	4,043.42	6.01**
S × T × P	2	93.83	<1.00
Error d	21	672.42	

**Significant at the α .05 level.
***Significant at the α .01 level.

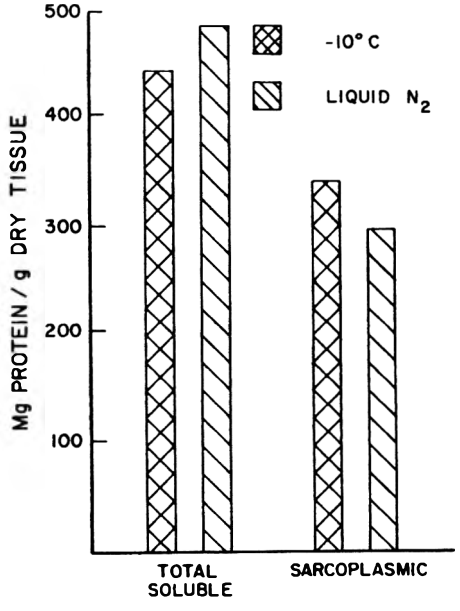

Fig. 2–Interaction of freezing rate and extraction method in stored freeze-dried turkey muscle.

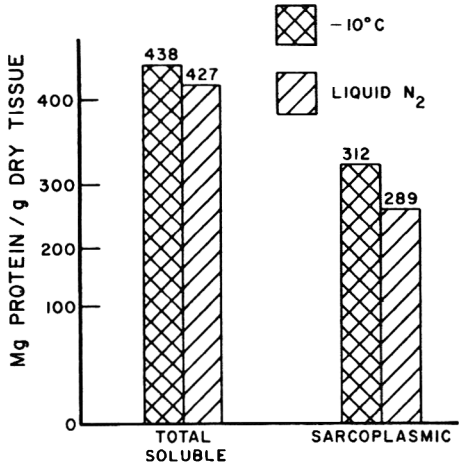

Fig. 3–Interaction of freezing rate and extraction method in stored freeze-dried turkey muscle.

A significant interaction occurred between freezing treatment and extraction method (Fig. 3). The difference between the freezing treatments increased in the sarcoplasmic fraction. The sarcoplasmic proteins were more soluble in muscle frozen at the slower rate, whereas the myofibrillar proteins had greater solubility in muscle frozen at the faster rate.

The nonprecipitated protein content remained fairly constant during the 4-month storage period. Initially it was 10.59 mg per gram of dry tissue and after 4 months it was 10.47.

DISCUSSION

FREEZING treatment had a significant effect on the protein solubility of raw turkey muscle. Only the sarcoplasmic proteins, however, were influenced by the rate of freezing. These proteins were more soluble, in muscle frozen at −10°C. Similar results have been obtained with chicken and porcine muscle (Huber et al. (in press); McSweeney et al. (1968).

Freezing treatment did not affect the myofibrillar solubility of raw turkey muscle. These results do not agree with other investigations. McSweeney et al. (1968) found that the myofibrillar proteins of porcine muscle were more soluble when frozen in liquid nitrogen than at −10°C. Conversely, Huber et al. (in press) reported that the myofibrillar proteins of chicken muscle were more soluble when frozen at −10°C than were muscle samples frozen in liquid nitrogen.

An explanation for these conflicting results may be a species difference. The effect of different freezing temperatures on protein solubility has been reported by Partmann (1963). Differences in the electrophoretic patterns of fish, beef and pork proteins have been reported (Connell, 1953; Thompson, 1960; Payne, 1963).

Age is another factor which may account for some of the solubility differences of muscle proteins (Koenig et al., 1949; Connell, 1953). Brandt et al. (1951) observed that the total protein α-globulin and γ-globulin content of chicken muscle increased with age.

Changes occurring in postmortem muscle may not be identical for all species (de Fremery et al., 1960; Weinberg et al., 1960; Sayre et al., 1963; Goll, 1964; and McIntosh, 1967). Variation among the species exists in rigor mortis development and the period of time required for maximum protein extractability.

The solubility of the myofibrillar proteins in frozen muscle was greater when samples were frozen at −10°C. The reverse was true for freeze-dried muscle. Greater myofibrillar solubility occurred in freeze-dried muscle frozen in liquid nitrogen. Therefore, when the entire process of freeze dehydration is considered, myofibrillar solubility may be enhanced with a rapid freezing rate.

It was observed that freeze-dried muscle had greater solubility than frozen muscle. The sarcoplasmic proteins were responsible for this difference. Other researchers have also reported that freeze drying did not decrease the solubility of the sarcoplasmic or globular proteins (Connell, 1962; Hamm et al., 1960).

Heat had a pronounced effect on the protein solubility of turkey muscle. The presence of soluble protein in cooked extracts was demonstrated by precipitate formation when TCA was added. The solubility of cooked tissue was less than 1/10 that of raw tissue. This is consistent with the findings of Khan et al. (1965), who observed a similar decrease in protein solubility when chicken muscle was cooked to an internal temperature of 85°C.

Protein solubility of cooked muscle was also influenced by the freezing rate. Solubility was greater in cooked muscle frozen at the slower rate.

No differences in protein solubility existed between frozen and freeze-dried cooked turkey muscle. These results are not consistent with other reports. Hamdy et al. (1959) reported that freeze dehydration of cooked meat increased the concentration of the soluble protein fractions. Freeze-dried cooked chicken muscle had greater solubility than frozen muscle, Huber et al. (in press).

Storage of freeze-dried turkey muscle for 4 months had no effect on protein solubility. These results agree with those of Reiger et al. (1956) for similar storage conditions.

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ANTHOCYANIN PIGMENTS IN RED TART CHERRIES

*SUMMARY—The anthocyanin pigments of red tart cherries (*Prunus ceracus *L., var. Montmorency) were extracted with 0.1% methanolic HCl and partially purified by adsorption on cation-exchange resin, then separated into 7 bands by paper chromatography. The anthocyanins were identified by their R_f values, aglycones, sugar moieties, partial acid hydrolysis and spectral properties. The major pigment was a branched triglycoside, cyanidin 3-glucosylrhamnosylglucoside, followed by cyanidin 3-rutinoside. Small amounts of cyanidin, peonidin, cyanidin 3-glucoside, peonidin 3-rutinoside and cherries. It is the first time that a branched triglycoside has been reported for Montmorency cherries.*

INTRODUCTION

OUR STUDY grew out of a program initiated to determine the quantitative and qualitative changes that take place after harvest in the pigments of fruits for the purpose of maintenance of color quality.

Anthocyanin pigments give red tart cherries their attractive and appealing bright-red color. Only 2 pigments in the skin of sour cherries have been reported by Li et al. (1956), cyanidin 3-rhamnoglucoside and cyanidin 3-diglucoside, obtained by purification on a silicic acid column. Dekazos (1966) obtained chromatograms of anthocyanin pigments from the skin of red tart cherries showing 7 pigments. Recently, cyanidin 3-monoglucoside obtained by purification on a silicic acid column was reported by Schaller et al. (1968) for Montmorency cherries. The identification of additional anthocyanin pigments in the skin of red tart cherries is of scientific interest and should provide better understanding as to the chemical components contributing to their color. This should also yield valuable information applicable to future research with this fruit.

The present work describes the extraction, purification and identification of anthocyanin pigments in red tart cherries. They were identified by chromatographic, spectral and chemical properties.

MATERIALS & METHODS

MATURE Montmorency cherries were harvested from an orchard of the C.H. Musselman Company, Biglerville, Pennsylvania. The cherries were transported to the laboratory in a container with ice-water. They were washed, stemmed and peeled with a scalpel; adhering flesh was removed from the skin. The clean skins were frozen immediately in liquid nitrogen and stored in polyethylene bags at −15°F until analyzed for identification of the anthocyanin pigments.

Authentic pigments

A generous supply of pure cyanidin 3-rutinoside (Cn-3RG) from Professor J.B. Harborne was acknowledged. Cyanidin and cyanidin 3-glucoside (Cn-3G) were provided by Dr. Sam Asen. Peonidin 3-glucoside (Pn-3G) and peonidin 3-rutinoside (Pn-3RG) were isolated from Bing cherries (Lynn et al., 1964). Cyanidin aglycone was purchased from Calbiochem, Los Angeles, California. Peonidin aglycone was obtained from acid hydrolysis of peonidin 3,5-diglucoside received from K & K Laboratories, Inc., Plainview, New Jersey.

Extraction and partial purification of anthocyanins

50 g of mature (overripe) skin of frozen red tart cherries were extracted at 32°F by macerating for 90 sec in a chilled Waring Blendor with 100 ml of cold methanol containing 0.1% HCl (v/v). The macerate was filtered and the residue was re-extracted with the same solvent until all the anthocyanins were removed. The combined extracts were centrifuged and filtered through Celite. All centrifugations were at 10,000 × g for 5 min in a refrigerated International centrifuge at 32°F. Then the filtrate was mixed with AG 50W X 4 cation-exchange resin (100–200 mesh, Calbiochem) in the hydrogen form, with sufficient resin to adsorb most of the pigments.

After 2 hr at 32°F the solution was filtered and the resin washed several times with distilled water. This separated the pigments from the free sugars. Then the resin was transferred to a 4- by 50-cm column and washed with pure methanol to remove organic residue. The pigments were eluted from the resin by successive extractions with 0.1, 0.5 and 1% HCl in methanol (Smith et al., 1965). The eluates were combined, filtered and then concentrated in a rotary evaporator under vacuum in a nitrogen atmosphere at 40°C. The concentrate was taken up in 0.01% methanolic HCl and stored at −10°F under a nitrogen atmosphere.

Skin of fresh red tart cherries was also used. The extraction of the pigment was prepared as above except that the filtrate was not passed through the ion exchange resin. The extracts were stored for 3 days at −20°C, having settlement of some particles. An aliquot of the supernatant was concentrated and streaked on filter paper.

Chromatographic methods

All separations and purifications were made using Whatman No. 3 or 3MM paper, and No. 1 paper was used for obtaining R_f and R_g data. In all cases descending chromatography was used and performed in the dark at about 20°C. The solvent systems used for anthocyanins, aglycones and sugars are presented in Table 1.

Chromatographic separation and purifications

Chromatograms were prepared by streaking the solution containing the anthocyanins in a band approximately 1 cm wide across the narrow width of thick filter paper. The papers were developed for 16–18 hr in HOAc-HCl solvent. 7 well-defined bands were obtained. When the papers were almost dry, they were cut into 7 bands. Each band was cut and eluted with 0.1% HCl in methanol by siphonage. The separated anthocyanins were filtered and further purified by rechromatographing with BAW (1-butanol–acetic acid–water; 4:1:5, v/v) solvent for 24 hr, 1% HCl, and finally 15% HOAc.

Chromatograms developed in the HOAc-HCl solvent as above were cut vertically into strips 4 cm wide and pigment band densities measured photodensitometrically.

Paper chromatography of anthocyanins

The purified pigments were chromatographed on Whatman No. 1 papers in a descending direction with BAW, BuHCl (n-butanol-2 N

Table 1—Solvent systems used in chromatography.

Abbreviations	Composition	Proportions (v/v)	Layer used	Solvent used for	Time (hr)
BAW[1]	1-Butanol-acetic acid-H_2O	4:1:5	Upper	Anthocyanins	18, 24
				Sugars	36
BuHCl[2]	1-Butanol-2 N HCl	1:1	Upper	Anthocyanins	24
1% HCl	Water-conc HCl	97:3	Miscible	Anthocyanins	4
HOAc-HCl	Acetic Acid-H_2O-12 N HCl	15:82:3	Miscible	Anthocyanins	16
Forestal	Acetic Acid-con HCl-H_2O	30:3:10	Miscible	Anthocyanidins	14
Formic	Formic Acid-con HCl-H_2O	5:2:3	Miscible	Anthocyanidins	6
Et-HOAc-W	Ethylacetate-acetic acid-H_2O	3:1:3	Upper	Sugars	15
Bu-Py-W	1-Butanol-pyridine-H_2O	6:3:1	Miscible	Sugars	36
15% HOAc	Acetic Acid-H_2O	15:85	Miscible	Anthocyanins	5

[1] Used with 1–2 hr of mixing.

[2] Paper equilibrated 24 hr, after spotting and before running, in a tank containing the lower phase of BuHCl.

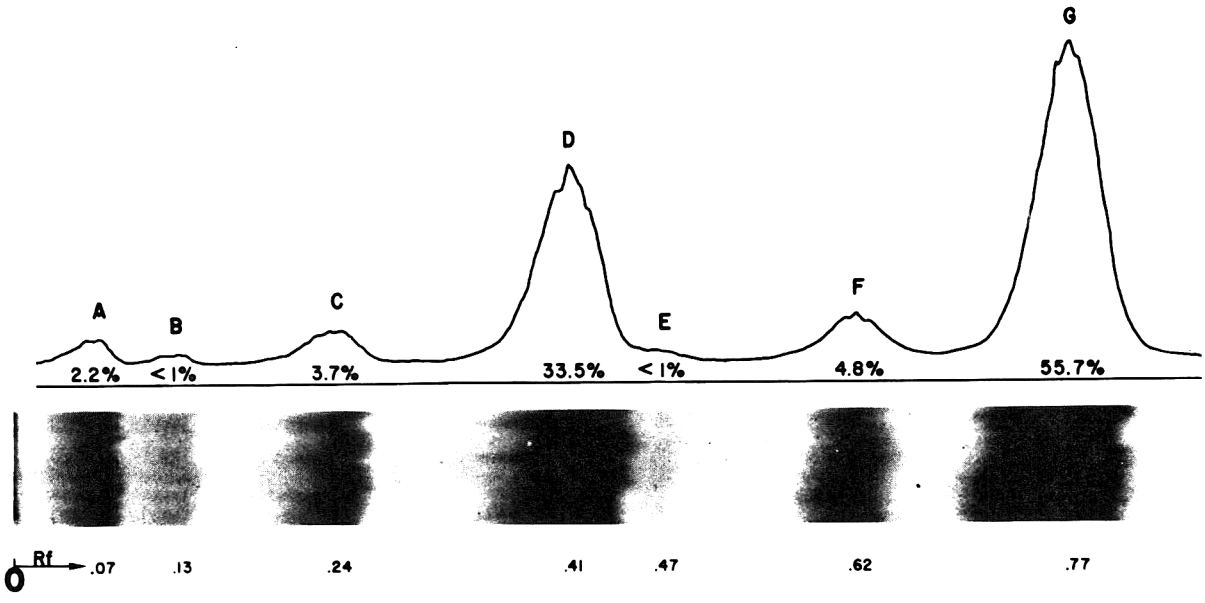

Fig. 1—Paper chromatogram and photodensitometric measurement of the relative intensity of anthocyanin pigments from the skin of red tart cherries.

HCl; 1:1, v/v), 1% HCl or HOAc-HCl solvent systems. The average R_f value of each pigment from 2 or more papers was reported. Pure pigments were also co-chromatographed in the above 4 solvents. The papers were examined for fluorescence under long-wave ultraviolet radiation using a Chromato-Vue ultraviolet cabinet. The chromatograms developed with BAW were sprayed with $AlCl_3$ solution in absolute ethanol.

Identification of sugar moieties of anthocyanins

A 3-ml portion of each purified concentrated anthocyanin was refluxed for 1 hr with 3 ml of 1 N HCl in a boiling-water bath in a 12-ml centrifuge tube fitted with a cold-finger condenser. The hydrolysate was cooled and mixed with sufficient AG 50W X 4 cation-exchange resin in the hydrogen form and AG 1 X 4 anion-exchange resin in the acetate form to remove the anthocyanidins and HCl, then filtered (Lynn et al., 1964). The filtrate was spotted on Whatman No. 1 chromatography papers along with authentic samples of 0.5% each of glucose, galactose, rhamnose, arabinose and xylose. The chromatograms, irrigated in a descending direction with BAW, Bu-Py-W (1-butanol-pyridine-H_2O; 6:3:1, v/v) and Et-HO Ac-W (ethylacetate-acetic acid-H_2O; 3:1:3, v/v), were allowed to run off for the determination of R_g values. After development, the papers were dried, dipped in aniline hydrogen phthalate reagent (0.93 g aniline, 1.66 g phthalic acid and 100 ml water-saturated n-butanol) and heated for 5 min in an oven at 105°C (Partridge, 1949). The spots were clearly visible under UV or visible light.

Table 2—R_f values of anthocyanins in red tart cherries.

Pigment	Identification[1]	Solvent system				Appearance		Color change
		BAW	BuHCl	1% HCl	HOAc-HCl	Visible	UV	$AlCl_3$
		R_f						
A	Cn	0.59	0.68	0.01	0.08	Light pink	Weak fluorescence	+
B	Pn	0.70	0.70	0.03	0.13	Light pink	Dull	–
C	Cn-3G	0.38	0.24	0.08	0.24	Light pink	Dull	+
D	Cn-3RG	0.36	0.23	0.19	0.43	Dark pink	Dull	+
E	Pn-3RG	0.37	0.24	0.21	0.48	Light pink	Dull	–
F	Cn-3GG	0.33	0.21	0.34	0.60	Light pink	Dull	+
G	Branched Cn-3GRG	0.26	0.10	0.60	0.75	Dark pink	Dull	+
Authentic markers								
	Cyanidin	0.59	0.66	0.01	0.09			
	Peonidin	0.69	0.70	0.03	0.12			
	Cyanidin 3-glucose	0.38	0.24	0.08	0.26			
	Peonidin 3-glucose	0.39	0.28	0.09	0.32			
	Cyanidin 3-rutinoside	0.37	0.24	0.19	0.42			
	peonidin 3-rutinoside	0.38	0.25	0.21	0.49			
Reported by Harborne (1967)								
	Cyanidin							
	3-Sophoroside	0.33	0.22	0.34	0.61			
	3-Gentiobioside	0.20	0.10	0.14	0.46			
	3-(2^G-Glucosylrutinoside	0.26	0.11	0.61	0.73			

[1] Notation: Cn = Cyanidin, Pn = peonidin, G = glucose, R = rhamnose.

Identification of the anthocyanidins

The aglycone from acid hydrolysis of the anthocyanin in 1 N HCl was adsorbed on AG 50W X 4 cation-exchange resin, washed several times with distilled water and methanol and then eluted with methanol containing 0.1% HCl. The eluate was concentrated to a small volume in a flash evaporator and spotted on Whatman No. 1 paper with authentic markers. The chromatograms were developed in Forestal and formic acid solutions. R_f values were measured shortly after the papers were dry.

Partial acid hydrolysis

Partial acid hydrolysis of individual anthocyanins was accomplished by the method of Abe et al. (1956), to study the types of glycoside linkage in anthocyanins by paper chromatography. 3 ml of each of the purified concentrated pigments E and G were refluxed with 3 ml of 1 N HCl in a boiling-water bath. Starting with the onset of boiling, samples were removed from the reaction mixture at intervals of 0, 5, 10, 20, 30, 40, 50 and 60 min. Each sample was placed immediately into a vial and cooled in ice-water. The samples were spotted in sequence on Whatman No. 1 papers and developed with BAW, BuHCl, 1% HCl and HOAc-HCl solutions. All the chromatograms of partial hydrolysis products were observed under UV light.

Absorption spectra

The absorption spectra of the pigments were measured in 0.01% methanolic HCl and in the range of 240–700 mμ with a Cary recording spectrophotometer, Model 14, using 1-cm cuvettes, against a blank solution of the same solvent. The $AlCl_3$ shifts were measured 1 min after adding 3 drops of a solution of the salt in absolute ethanol (5% v/v) to the cuvette.

RESULTS & DISCUSSION

Separation and chromatographic properties

Anthocyanin identification is largely dependent on effective separation and purification of the pigments. The partially purified pigments by cation-exchange resin were separated by descending paper chromatography with HOAc-HCl as solvent. This yielded chromatograms with 7 bands (Fig. 1). The slowest-moving band was designated Pigment A, then B, etc. The solvent systems BAW, 1% HCl, 15% HOAc were used for further purification of the individual anthocyanins. The purity of the isolated pigments was checked by the characteristic absorption spectra.

Paper chromatography of the purified and separated pigment bands was carried out with both aqueous and alcoholic solvents. The purified pigments were chromatographed on paper No. 1. Table 2 presents the R_f values of individual anthocyanin pigments in 4 solvent systems: BAW, BuHCl, 1% HCl and HOAc-HCl. There was an increase in R_f value for each increase of a sugar molecule in the pigment when aqueous solvents were used. When alcoholic solvents were used, this was reversed.

The chromatograms were examined under visible and ultraviolet lights. Pigment A exhibited weak pink fluorescence under ultraviolet light. Pigments A, C, D, F and G changed from red to blue when sprayed with aluminum chloride solution, indicating the presence of ortho-dihydroxy anthocyanins. No change in color was observed for pigments B and E. The test enabled a broad differentiation between the derivatives of the cyanidin, delphinidin and petunidin group, which showed a positive color change, and those of the pelargonidin, peonidin and malvidin group, which showed no color change (Harborne, 1958a).

Table 3–Products of acid hydrolysis of red tart cherry anthocyanins.

Band	Anthocyanidins		Sugars			Identification[1]	
	Forestal	Formic	BAW	Bu-Py-W	Et-HOAc-W	Aglycone	Sugar
	R_f		R_g (glucose = 1)				
A	0.49	0.23	–	–	–	Cn	–
B	0.60	0.30	–	–	–	Pn	–
C	0.48	0.24	1.00	1.00	1.00	Cn	G
D	0.48	0.24	1.00	0.98	1.00	Cn	G
			1.69	2.33	2.10		R
E	0.63	0.33	1.00	0.99	1.00	Pn	G
			1.71	2.32	2.10		R
F	0.49	0.23	1.00	1.00	1.00	Cn	G
G	0.48	0.24	1.00	1.00	1.00	Cn	G
			1.70	2.34	2.10		R
Standards							
Cyanidin	0.49	0.23					
Peonidin	0.60	0.30					
Petunidin[2]	0.46	0.20					
Glucose			1.00	1.00	1.00		
Galactose			0.91	0.80	0.89		
Rhamnose			1.70	2.34	2.10		
Arabinose			1.16	1.32	1.31		
Xylose			1.28	1.52	1.49		

[1] Notations: Cn = Cyanidin, Pn = peonidin, G = glucose, R = rhamnose.
[2] Obtained from hydrolyzed pigment of petunias.

R_f values suggested that pigment A was cyanidin and pigment B was peonidin. Pigment C was a monoglycoside of cyanidin; pigments D, E and F were diglycosides of cyanidin, peonidin and cyanidin, respectively; pigment G was a triglycoside of cyanidin. The R_f value of pigment C was found to be in agreement with previous workers (Schaller et al., 1968).

The relative intensities of the various bands, measured photodensitometrically, are presented in Figure 1. Bands D and G represent the major pigments of red tart cherries.

The aglycone

The aglycones obtained by acid hydrolysis of anthocyanins A, B, C, D, E, F and G were co-chromatographed with known anthocyanidins. The results are presented in Table 3. The R_f values from Forestal and formic solvents indicated that pigments A, C, D, F and G contained cyanidin and B and E contained peonidin. The color reactions of the intact pigments (Table 2) also supported the above conclusion.

Sugar moiety

The sugars in each purified anthocyanin were determined by paper chromatography of the products after hydrolysis of the pigments with 1 N HCl. Table 3 shows the R_g (glucose = 1) values of sugars from acid hydrolysis of the anthocyanins and those of authentic sugars in BAW, Bu-Py-W and Et-HOAc-W as solvent systems. On the basis of R_g values and the color response with Partridge's reagent, pigments A and B had no sugar attached, pigments C and F contained glucose and pigments D, E and G contained glucose and rhamnose. However, the R_f values for pigments F and G (Table 2) suggested that pigment F is a diglucoside and pigment G is a triglucoside. When hydrolyzed, pigment F yielded only glucose; thus, 2 molecules of glucose must be connected and both attached to the aglycone.

The spot for glucose in pigment G was much denser than the spot for rhamnose, as judged by visual examination. Visual comparisons were facilitated by having the authentic sugars spotted from 1 solution (containing all 5 sugars) run as standards. These data indicated that pigment G contained 2 molecules of glucose and 1 of rhamnose.

Partial acid hydrolysis

Determining the number of intermediates obtainable through partial acid hydrolysis, the nature, position of attachment and the number of sugar molecules

Table 4—Paper chromatography of controlled acid-hydrolysis products of pigments E and G isolated from red tart cherries.

Pigment	Solvent system			
	BAW	BuHCl	1% HCl	HOAc-HCl
	R_f			
E				
Pn-3RG	0.37	0.24	0.20	0.49
Pn 3-glucoside	0.39	0.29	0.09	0.32
Pn	0.71	0.69	0.03	0.13
G				
Cn-3 GRG	0.25	0.10	0.61	0.75
Cn 3-diglucoside	0.32	0.21	0.34	0.62
Cn 3-rutinoside	0.35	0.20	0.18	0.42
Cn 3-glucoside	0.37	0.22	0.08	0.25
Cn	0.59	0.68	0.01	0.08

Table 5—Spectral data of anthocyanins in red tart cherries.

Pigment[1]		λ_{max} (mμ)	OD_{440}/OD_{max} (as %)	$AlCl_3$ shift	Absorption for acylation[2]
A	Cn	535	20	+	No
B	Pn	532	26	–	No
C	Cn-3G	525	24	+	No
D	Cn-3RG	525	23	+	No
E	Pn-3RG	524	26	–	No
F	Cn-3GG	526	25	+	No
G	Branched Cn-3GRG	530	23	+	No

[1] Notation: Cn = Cyanidin, Pn = peonidin, G = glucose, R = rhamnose.
[2] Absorption at 312, 326, 328 mμ.

present in the molecule can quickly determine the anthocyanin structure on a microscale by paper chromatographic methods.

The controlled acid hydrolysis method was used to determine the structure of pigments E and G isolated from red tart cherries because these were the 2 complex pigments of specific interest. The results are given in Table 4. Pigment E gave 1 intermediate identified as peonidin 3-glucoside by comparison with authentic pigment. The pigment must be peonidin 3-rhamnoglucoside. This was substantiated by comparison with the authentic pigment.

Pigment G yielded, as intermediates of partial acid hydrolysis, cyanidin 3-diglucoside, cyanidin 3-rhamnosylglucoside and cyanidin 3-glucoside. This pigment is proposed to be a branched triglycoside, cyanidin 3-glucosylrhamnosylglucoside (Cn-3GRG), as commonly found in Rubus, Ribes and Prunus fruits and reported as cyanidin 3-(2^G-glucosylrutinoside) by Harborne et al. (1964). If this pigment was a linear triglycoside, as found in asparagus spears (Francis, 1967), then one would expect to find cyanidin 3-rhamnosylglucoside and cyanidin 3-glucoside as the only intermediate pigments. The intermediates which were found were identified by comparison with authentic pigments and reported R_f values.

Spectral properties

The spectrophotometric data of the pigments in 0.01% methanolic HCl are presented in Table 5. The OD_{440}/OD_{max} values (as percent) obtained from spectrophotometric analysis of all the pigments agree with those in the literature (Harborne, 1958a). The ratio of optical density at 440 to optical density at the maximum is useful in distinguishing anthocyanins substituted at the 3- and 3,5-positions (Harborne, 1958b). The corresponding 3,5-diglycosides have ratios of the order of 12; the 3-glycosides have ratios of the order of 23. Thus, the ratios OD_{440}/OD_{max} in the present work indicated that all 7 pigments had the 5-position free.

Addition of aluminum chloride to the sample caused a shift in the maximum absorption peak of pigments A, C, D, F and G, but no shift was observed when the same reagent was added to pigments B and E. This spectral shift is characteristic of pigments with free orthodihydroxyl groups. A positive $AlCl_3$ shift for A, C, D, F and G and a negative shift for B and E confirmed that the former group contained cyanidin as aglycone and the latter group had peonidin as aglycone.

In the ultraviolet region of the spectrum only 1 peak was revealed by the absorption curves. 2 peaks would have been evident if acyl groups were present, because of the superposition of the acyl group absorbance. Absence of an absorption peak in the 308–328 mμ range indicated that the pigments were not acylated.

The cumulative evidence from chromatographic, spectrophotometric and chemical methods of analysis indicates that red tart cherries contain 7 anthocyanins. Chromatograms of extracts (not passed through the ion exchange resin) from the skin of fresh red tart cherries yielded an equivalent number of pigments. This demonstrated that the ion exchange method does not alter the original individual anthocyanin composition of the sample. The differences in the number of pigments present and those reported by other researchers could be due to real differences in the composition of the fruit or to the experimental techniques.

The first 2 pigments of this study were found to be cyanidin and peonidin. The existence of the anthocyanidin in fresh unpurified pigment extract has been confirmed in other studies. Since it has been said that anthocyanidins are unlikely to occur naturally in plant tissues in the free state, chromatograms of extracts were run within minutes of extracting tissue (skin of overripe, fresh red tart cherries) with ice-cold methanol containing 0.1% conc HCl (Harborne, 1967) at 32°F to minimize hydrolysis. Paper chromatography, using HOAc-HCl as solvent, showed that the 2 anthocyanidins were noticeable. Special effort was expended to minimize hydrolysis; however, it might possibly still occur.

The pigments, cyanidin 3-rhamnosylglucoside and cyanidin 3-diglucoside, reported by Li et al. (1956), correspond to pigments D and F and indicate they are cyanidin 3-rutinoside (Cn-3RG) and cyanidin 3-sophoroside (Cn-3GG), respectively.

Comparison with an authentic marker substantiated the identification of pigment D as cyanidin 3-rutinoside. Pigment F has been referred to as cyanidin 3-diglucoside or cyanidin 3-gentiobioside. In this report it is identified as cyanidin 3-sophoroside. Values for this pigment agree with those reported by Harborne (1967).

The predominant pigment G was identified as a branched triglycoside, cyanidin 3-glucosylrhamnosylglucoside (Cn-3GRG). This compound is noteworthy since it is the major pigment of Montmorency cherries. Harborne et al. (1964) reported that this triglycoside is present in 7 other varieties of sour cherries examined but is uniformly absent from sweet cherries. Recently, Oldén et al. (1968) reported finding the triglycoside cyanidin 3-glucosyl-rutinoside in their chemotaxonomical studies of 10 cultivars of sour cherries.

The data show that in red tart cherries the branched triglycoside, cyanidin 3-glucosylrhamnosylglucoside, occurs with the related diglycosides, rutinoside and sophoroside.

It is of interest to note the presence of peonidin 3-rhamnosylglucoside (pigment E) which is peonidin 3-rutinoside (Pn-3RG), since an anthocyanin with peonidin as aglycone has not previously been isolated from sour cherries.

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Mention of specific instruments, trade names or manufacturers does not imply endorsement over others not mentioned.

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QUANTITATIVE DETERMINATION OF ANTHOCYANIN PIGMENTS DURING THE MATURATION AND RIPENING OF RED TART CHERRIES

SUMMARY—The anthocyanins of red tart cherries (Prunus cerasus *L. var. Montmorency), at different maturity levels, were extracted with 1% methanolic HCl and the total anthocyanin content of each stage calculated with the aid of a molar-extinction coefficient at 530 mμ. The pigment solution for each stage of maturity was purified by adsorption on AG 50W X 4 cation-exchange resin, concentrated and separated by descending chromatography. The relative intensities of the 7 well-defined pigments were measured photodensitometrically. The individual pigment content was calculated from the total anthocyanin content and the ratio of individual anthocyanin for each stage of maturity. The stage of maturity of the red tart cherry is an important factor influencing the total and individual anthocyanin content in cherries. This work also helps to give a greater insight into the development of anthocyanins in the maturing red tart cherry.*

INTRODUCTION

RED TART cherries (*Prunus cerasus* L. var. Montmorency) are grown as a commercial crop mainly in the 5 Great Lakes states. 95% of the crop is sold for processing into canned and frozen cherries. The stability of color in red tart cherry products is highly desirable.

Anthocyanin pigments give cherries and cherry products their attractive and appealing bright-red color. The anthocyanin content of fruit in general depends on factors such as species, variety, environmental conditions and physiological state of the plant and fruit, size, application of chemicals, etc. (Blank, 1958; Ribereau-Gayon, 1959; Shawa et al., 1968). It has been reported that reddening of cherries depends on the temperature and that light has no direct action on this phenomenon (Obaton, 1923). The copper spray treatment on Montmorency cherries generally resulted in more color (Langer et al., 1949; Marchall, 1954; Bedford et al., 1962). Furthermore, the color of red tart cherries showed increased darkening as the harvests were delayed until 1,000 heat units were accumulated (Lawver et al., 1965). Variation in the anthocyanin structure has a definite effect on the chemical stability of these pigments (Lamort, 1958; Horubala, 1964; Robinson et al., 1966). Thus, the quantitative estimation of anthocyanins would aid in developing cultural, handling, processing and storage practices which would result in a high degree of color stability of red tart cherry products.

The study presented here grew out of a program initiated to determine the quantitative changes that take place in the pigments of fruits for maintaining color stability during processing and storage.

The present work describes the quantitative analysis of the total and individual anthocyanins in red tart cherries at different maturity levels.

MATERIALS & METHODS

MONTMORENCY cherries were harvested from an orchard of the C.H. Musselman Company, Biglerville, Pennsylvania. They were cut carefully, with their stems attached, from a number of similar trees selected for uniform size. A distribution of maturities was established by periodic harvesting (12- to 15-day intervals) of cherries selected on the basis of visual skin color development. 4 stages of maturity were selected: very immature (yellow with spot of light-red); immature (light-red color); partially mature (red color); and mature (bright-red). These cherries were placed in a container with ice-water and transported to the laboratory. Cherry samples of the various stages of maturity were washed, stemmed, pitted and then frozen immediately in liquid nitrogen and stored in polyethylene bags at −15°F for quantitative analysis of anthocyanins.

Determination of total anthocyanins during maturation

The anthocyanins were extracted from 50 g of pitted cherries for each stage of maturity. Pigment was extracted from the skin and flesh by macerating each sample separately for 90 sec at 32°F in a chilled Waring Blendor with 100 ml of cold 1% HCl in methanol. The macerate was filtered through Whatman No. 1 filter paper in a Büchner funnel under mild vacuum. The residue was re-extracted with the same solvent. Soaking and extracting the residue 4–5 times removed all the anthocyanins. The combined extracts were centrifuged and filtered through Celite. All centrifugations were at 10,000 × g for 5 min in a refrigerated International centrifuge at 32°F.

Each of the 4 pigment solutions was diluted with 1% methanolic HCl to give the same total volume (410 ml each) for the absorption determinations. The absorption spectra of these pigment extracts were measured in the range of 350–700 mμ with a Cary recording spectrophotometer, Model 14, using 1-cm cuvettes, against a blank solution of 1% methanolic HCl. All the optical density values were expressed on the same basis: the density of the 410-m solution diluted 1:4. The maximum absorption peak of these extracts in the visible region of the spectrum was 530 mμ (λ_{max} = 530 mμ). Calculations of anthocyanin content were based on the molar-extinction coefficient for Idaein ($C_{21}H_{21}O_{11} \cdot 2.5\ H_2O$) at 530 mμ ($E = 3.43 \times 10^4$), the peak absorption of Idaein in 1% methanolic HCl (Siegelman et al., 1958).

Determination of individual anthocyanins during maturation

After determination of the total anthocyanins, the pigment solution for each stage of maturity was mixed with AG 50W X 4 cation-exchange resin (100-200 mesh, Calbiochem, Los Angeles, California 90054) in the hydrogen form. This resin is strongly acidic and was previously washed with deionized, distilled water until the pH rose to approximately 6.0. While the resin was soaking in the water, occasional

Table 1—Mean concentration of individual anthocyanins[1] (calculated as Idaein) in mg per 50 g of pitted red tart cherries at various stages of maturity.

Pigment	Stages of maturity (harvest dates)			
	a(6/15)	b(6/27)	c(7/11)	d(7/25)
A Cyanidin	0.12 ± .01[2]	0.26 ± .01	0.99 ± .02	1.53 ± .02
B Peonidin	0.04 ± .01	0.30 ± .01	0.68 ± .01	0.70 ± .01
C Cyanidin 3-glucoside	0.16 ± .01	1.00 ± .03	2.41 ± .03	4.08 ± .04
D Cyanidin 3-rutinoside	0.28 ± .02	1.09 ± .03	3.94 ± .03	5.80 ± .04
E Peonidin 3-rutinoside	0.07 ± .01	0.21 ± .01	0.58 ± .01	0.66 ± .01
F Cyanidin 3-sophoroside	0.11 ± .01	0.94 ± .02	2.12 ± .03	3.54 ± .03
G Branched cyanidin 3-glucosyl-rhamnosyl-glucoside	0.21 ± .02	1.33 ± .02	3.81 ± .03	5.50 ± .04

[1] Each value is an average from 10 chromatographic applications.
[2] Standard error of the mean.

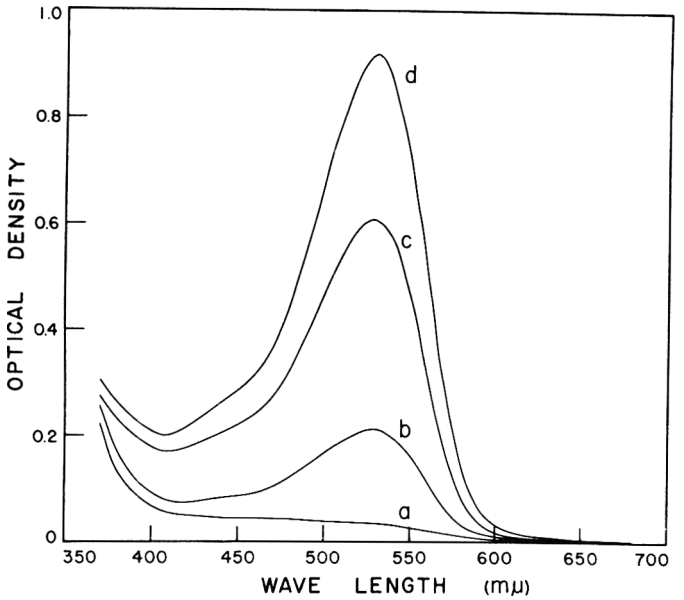

Fig. 1—The absorption spectra of anthocyanin pigments from pitted red tart cherries in MeOH (1% HCl) at 4 stages of maturity: a) very immature, b) immature, c) partially mature, d) mature.

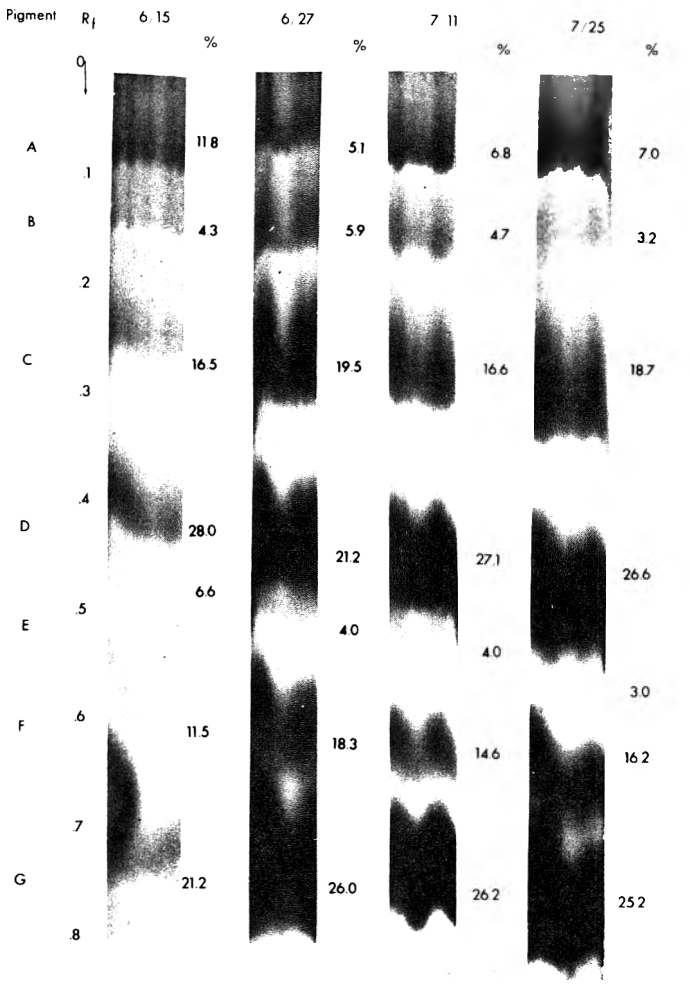

Fig. 2—Paper chromatograms and photodensitometric measurements of the relative intensity of anthocyanin pigments from pitted red tart cherries at 4 stages of maturity: very immature, immature, partially mature, mature. Dates indicate days of harvest.

decanting of the supernatant removed the undersize particles. A sufficient amount of this resin was added to adsorb most of the pigments, and the solution was white. After 2 hr at 32°F the solution was filtered through 2 layers of Whatman No. 2 paper in a large Büchner funnel and the resin was washed several times with distilled water. This separated the pigments from the free sugars. Then the resin was transferred to a 4- by 50-cm column and washed with pure methanol to remove organic residue. The pigments were eluted from the resin by successive extractions with 0.1, 0.5, and 1% HCl in methanol (Smith et al., 1965). The eluates were combined, filtered and concentrated almost to dryness in a Buchi rotary evaporator under vacuum in a nitrogen atmosphere at 40°C (104°F). The concentrated pigments were then redissolved in 5 ml of 0.01% HCl in methanol and the mixture stored at −10°F under a nitrogen atmosphere.

Separation of individual pigments by paper chromatography and their photodensitometric measurement

Chromatograms were prepared for each stage of maturity by streaking the pigment solution in a band approximately 1 cm wide across the narrow width of Whatman No. 3MM paper (20- by 46-cm). The papers were irrigated in a chromatographic jar by descending method with HOAc-HCl (acetic acid:water:conc HCl; 15:82:3, v/v) solvent for 7 hr at about 68°F. 7 pigments were obtained. The bands were lettered in increasing distance from the origin—A, B, C, etc.

After drying at room temperature in a fume hood, the developed chromatograms were stored in the dark until pigment densities were measured. The papers were cut vertically into strips 4 cm wide and pigment band densities measured in the Beckman Spinco Analytrol, Model RB, using interference filters to pass light at 550 mμ which gave the greatest response. The instrument was operated with the 4-mm slit width, and equipped with the B-2 balancing cam. Using this cam, the Spinco Analytrol, Model RB (a photoelectric densitometer), will record a linear plot of the optical densities of the strip being scanned. The chart synchronous motor with the scanning speed of 1.96 inches/minute gave the best resolution. A Servo-type integrating scanner was used to measure the area under the density curves. To assure comparable results, each strip was steamed briefly and fumed with conc HCl vapors before examination in the Analytrol (Albach et al., 1959; Akiyoshi et al., 1963). The technique of measuring on the papers the quantity of pigment in the various bands separated chromatographically gives results with moderate accuracy. However, the photodensitometric method was favored over elution and spectrophotometric measurement of individual pigment concentration because it was simple and rapid.

Individual anthocyanin content calculation in absolute quantities

The individual pigment content in milligrams was calculated from the total anthocyanin content in milligrams and the ratio of individual anthocyanins for each stage of maturity. The ratio of individual anthocyanins was obtained by taking the densitometric peak area for each pigment and expressing it as a percentage of the total densitometric peak area for all pigments of a given stage of maturity.

RESULTS & DISCUSSION

Quantitative determination of total anthocyanins during maturation

To study the development of the pigments during maturation of the fruit, the absorption spectra were determined of the methanolic HCl extracts of the pigment from fruits at 4 different stages of maturity. The absorption spectra, with absorption peaks at 530 mμ, are given in Figure 1. The total pigment of the red tart cherries was estimated by measurement of the optical density at 530 mμ. The pigment content was calculated in absolute quantities with the aid of the molar-extinction coefficient. The total pigment for the 4 maturity levels analyzed increased from 0.99–5.13 to

14.54—21.82 mg/50 g of pitted red tart cherries. The total pigment content at the mature stage was approximately 0.05% of the fresh weight. Additional work, with skin alone, showed that the total pigment was 0.4% of the fresh skin.

Quantitative determination of individual anthocyanins during maturation

Work done on anthocyanins has been mostly qualitative rather than quantitative analysis. The data available on quantitative determination of the amounts of each pigment in fruits are few. In this research, 7 well-defined colored bands were observed when purified on AG 50W X 4 cation-exchange resin in the hydrogen form and concentrated pigment extract of red tart cherries, at each of the 4 stages of maturity, was paper-chromatographed descendingly using the HOAc-HCl solvent. The anthocyanin pigments A, B, C, D, E, F and G were identified in an earlier paper (Dekazos, 1969) as cyanidin, peonidin, cyanidin 3-glucoside, cyanidin 3-rutinoside, peonidin 3-rutinoside, cyanidin-3-sophoroside and a branched triglycoside, cyanidin 3-glucosylrhamnosyl-glucoside, respectively. The relative intensities of the various bands, measured photodensitometrically, at the aforementioned stages of maturity, are shown in Figure 2. Bands C, D, F and G are major pigments of the sour cherry. The relative concentrations (based on 10 determinations) of individual anthocyanins in milligrams at the 4 stages of maturity are listed in Table 1.

Results show that all the individual contents are increased during the maturation process but at different rates. Pigments B and E show almost no change during the harvest period. It is clear that the stage of maturity of the red tart cherry is an important factor influencing the total and individual anthocyanin content in the cherry. Environmental conditions, cultural practices and the growing area may also have an effect on anthocyanin synthesis. An earlier histological study has shown that the pigment is primarily located in the skin of the sour cherry (Dekazos, 1966).

The above procedure for quantitative estimation of anthocyanins is presently in use to investigate the effect of the growth retardant, Alar (succinamic acid 2,2-dimethylhydrazide), on the pigment ratios of red tart cherries.

The work here helps to give a greater insight into the development of anthocyanins in the maturing red tart cherry. Harborne (1958) has correlated bright fluorescence in anthocyanins with the presence of sugar on the No. 5 carbon. In the present work, lack of fluorescence, under long-wave ultraviolet radiation, indicates that all 7 pigments had the 5-position free. For the chromatograms of the 4 stages of maturity, Bands A, C, D, F and G produced a blue coloration when sprayed with 5% aluminum chloride solution in absolute ethanol. No color change was observed for pigments B and E. The reagent caused a positive change in color for anthocyanins with 2 hydroxyl groups at ortho position in the B ring (cyanidin, delphinidin and petunidin) and no change in color for anthocyanins containing only 1 phenolic group (peonidin, pelargonidin and malvidin) (Harborne, 1958). From these observations, chromatographic comparisons and comparison with earlier work (Dekazos, 1969) under the same conditions it may be concluded that the 7 pigments are all present at each of the 4 stages of maturity and that the concentration of each pigment increases as ripening progresses.

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EFFECT OF SMOKING PROCESS ON SOLUBILITY AND ELECTROPHORETIC BEHAVIOR OF MEAT PROTEINS

SUMMARY—The solubility characteristics and starch gel electrophoretic properties of untreated, heated, and heated and smoked pork longissimus dorsi muscle samples were investigated. The percentages of the low ionic strength fraction, the sarcoplasmic and the soluble fibrillar protein nitrogen fractions decreased in the heated and heated and smoked samples when compared to the untreated samples. The myofibrillar protein nitrogen fraction increased in the heated samples and decreased in the heated and smoked samples. The stroma fraction from the untreated to the heated state remained almost constant, but increased considerably in the heated and smoked samples. Electrophoretic studies of the sarcoplasmic fraction indicated numerous changes in the heated and heated and smoked samples. The electropherograms of the heated and smoked samples from the Weber-Edsall and meat-urea extracts showed definite changes in protein components. These studies indicated that smoke definitely caused changes in protein solubility and the electrophoretic behavior of meat proteins.

INTRODUCTION

A GREAT DEAL of biochemical research on muscle proteins in the raw or native state has been carried out. However, little investigation on the chemical changes of muscle proteins during the heating process has been made. Hamm (1966) stated that the most drastic changes in meat during heating are those that involve the muscle proteins. Proteins of muscle can be separated by solubility into various fractions; major alterations occur in the solubility of these fractions during heating (Bol'shakov et al., 1968; Hamm et al., 1960; Paul et al., 1966; Usborne et al., 1968). Electrophoretic and chromatographic studies of the sarcoplasmic fraction and meat-urea extracts have also demonstrated numerous changes in the protein components of the heated sample (Grau et al., 1963; Kakō, 1968a). There have been infrequent investigations of the effect that the smoking process has upon meat proteins. Kihara (1962) demonstrated that protein solubility decreased during the smoking of chicken and pork muscles. Electrophoretic and chromatographic analysis of water and meat-urea extracts showed that the smoking process caused alterations in the protein patterns (Kihara, 1962; Kakō, 1968b).

The objectives of this research were a) to study the changes taking place in the various protein nitrogen components of untreated, heated, and heated and smoked porcine muscle obtained under 2 smokehouse conditions and b) to investigate the electrophoretic properties of certain fractions during heating and heating and smoking.

EXPERIMENTAL

Materials

Pork loin samples from the right and left longissimus dorsi muscles of 150-160-lb hog carcasses were used in this study. Each loin section (15 per treatment) was sliced 1.1 cm thick; the slices were divided randomly into 3 equal groups: untreated, heated, and heated and smoked samples. The slices to be heated or heated and smoked were treated similarly in the smokehouse with the exception of smoke being added to the latter samples. The samples were in the smokehouse for 2.25 hr for either treatment and 2 different temperatures were used. To obtain a heated and smoked sample, the smokehouse was operated at 60°C (140°F) and 45% R.H., resulting in an internal meat temperature of 58.8°C (138°F). A cold smoked sample was obtained when the smokehouse was operated at 32.2°C (90°F) and 45% R.H., which resulted in an internal sample temperature of 32.0°C (89.6°F). After smoking, separable fat was removed and the samples ground through a 1-cm plate, then through a 2-mm plate.

Protein fractionation

The fractionation procedure was adapted from that used by Hegarty et al. (1963) and Weiner (1967). The muscle samples were fractionated into a low ionic strength fraction, a sarcoplasmic and a nonprotein nitrogen fraction, a myofibrillar protein nitrogen fraction, a soluble and a denatured fibrillar protein nitrogen fraction and a stroma nitrogen fraction. Total nitrogen content of the tissue was determined by the micro-Kjeldahl procedure (American Instrument Co., 1961). Protein nitrogen in the extracted solutions was determined by the same procedure and expressed as percentage of the total nitrogen.

Preparation of extracts for electrophoresis

Samples for electrophoretic studies were obtained at the same time as the samples for protein fractionation studies. The samples subjected to a smokehouse condition of 60°C (140°F) and 45% R.H. were used.

A water extract containing the sarcoplasmic proteins was prepared for starch gel electrophoresis using the method of Scopes (1964). The pH adjustment step was abandoned since no apparent improvement was observed in the protein patterns.

A Weber-Edsall extract containing the myofibrillar proteins was prepared for disc gel electrophoresis utilizing the method of Rampton (1969). The samples were extracted in 12 vol of a pH 7.6 buffer (0.25 M sucrose, 1 mM EDTA, 0.05 M Tris) and then gently agitated for 30 min on a magnetic stirrer. The meat slurry was centrifuged in a Sorvall Model RC2-B centrifuge at 15,000 × g for 15 min. The supernatant was discarded and the residue resuspended in 12 vol sucrose, EDTA, Tris buffer, stirred and centrifuged as described. The supernatant was again discarded and the residue suspended in 6 vol Weber-Edsall solution (0.6 M KCl, 0.04 M $KHCO_3$, .01 M K_2CO_3, pH 9.2) (Perry, 1953) and gently mixed with a magnetic stirrer for 24 hr. The mixture was centrifuged 1 hr at 25,000 × g. The supernatant was designated as the Weber-Edsall extract and dialyzed 12 hr against 15 vol 8 M urea before being placed on the disc gels.

A meat-urea extract was prepared using a 7.7 M urea-containing 0.055 M Tris-HCl buffer (pH 8.6; Kakō, 1968a) and this extract subjected to disc gel electrophoresis.

Electrophoresis

Horizontal starch gel electrophoresis was carried out in a discontinuous buffer system as described by MacRae et al. (1965) but with the modification that pH of the gel buffer was increased to 8.5. For detection of separated protein, the gels were stained for 1 min in a solution of 1% Amido Black 10B and 0.5% nigrosine in methanol:acetic acid:water (5:1:4 v/v). The unbound dye was removed by washing the gel in several changes of solvent.

The disc gel electrophoresis technique as described by Davis (1964) was used with Cyanogum (E. C. Apparatus Co.) replacing acrylamide and N,N′-methylenebisacrylamide. A 6.5% running gel and a 5% spacer gel were used and the concentration of urea in each gel was 7 M. During electrophoresis and destaining, a current of 2 ma per tube was applied. The gels were stained 20 min in a solution of 0.67% Amido Black 10B; after destaining, the gels were stored in a 7% acetic acid solution.

RESULTS & DISCUSSION

Protein fractionation

The averages of the nitrogen composition for the different protein fractions from untreated, heated, and heated and smoked pork samples subjected to two smokehouse conditions are presented in Tables 1 and 2. The total nitrogen content was uniform for all sample treatments.

An appreciable change was observed in the solubility of the low ionic strength fraction; these changes were more noticeable in samples subjected to increased temperature and heating and smoking. Paul et al. (1966) observed similar results with cooked rabbit muscle as did Kihara (1962) with smoked chicken and pork muscle. This low ionic strength fraction was divided into a nonprotein nitrogen (NPN) fraction and a sarcoplasmic pro-

tein fraction to determine where the changes occurred. The NPN values were uniform throughout for the 60°C samples (Table 2), whereas noticeable decreases occurred in the sarcoplasmic protein nitrogen fractions of the heated and heated and smoked samples with a greater decrease of solubility in the latter samples. Thus, it appeared that the low ionic strength fraction of the heated and heated and smoked samples subjected to a smokehouse temperature of 60°C consisted primarily of NPN. Outside of the significant decrease in the NPN fraction of the heated sample for which no explanation is available, a similar trend of results was obtained with the samples heated and heated and smoked at 32.2°C (Table 1).

There was a definite increase in the amount of myofibrillar protein nitrogen extracted from the heated samples and a definite decrease in this fraction from the heated and smoked samples, with these changes being more noticeable at 60°C. Similar results were obtained by Usborne et al. (1968) with heated samples and by Kihara (1962) with heated and smoked samples. The soluble fibrillar protein fraction was examined in the samples heated and heated and smoked at 60°C (Table 2) and a significant decrease in solubility was obtained with both samples. The loss in solubility observed in both the sarcoplasmic and soluble fibrillar protein nitrogen fractions of the heated smoked samples was, therefore, not entirely due to heating; the smoke ingredients were also involved in this decrease in solubility.

A significant increase ($P < .01$) was observed in the stroma (insoluble) nitrogen fraction of the heated and smoked samples, with this increase being greater at 60°C (Table 2). Although this fraction contained the connective tissue proteins, the increase observed was attributed to the insolubilization of some of the other protein constituents.

In general, heating alone caused definite changes in the solubilities of the various nitrogen fractions obtained, which were more noticeable at 60°C than at the lower temperature of 32.2°C. In the heated and smoked samples (Table 2), noticeable changes were observed in the amounts of nitrogen containing compounds extracted in either of the salt buffers, i.e., the low ionic strength fraction and the soluble fibrillar fraction, and in the myofibrillar and stroma fractions. The results indicated that smoke ingredients, in addition to heat, caused additional changes in the solubility of meat protein components. However, the changes in the solubility of the various protein nitrogen fractions of the heated and smoked samples were not entirely due to smoke ingredients as indicated by Kihara (1962). The results obtained with the cold (32.2°C) smoked samples (Table 1) supply further proof of the action of smoke on meat protein constituents.

Table 1—Distribution of nitrogen in various protein fractions of untreated, heated, and heated and smoked pork samples.[1,2]

Variables	State of muscle		
	Untreated	Heated	Heated-smoked
% Total nitrogen[3]	14.70	14.83	14.52
Protein nitrogen solution extracted at low ionic strength[4]	29.88	24.63	21.34**
Sarcoplasmic protein nitrogen[4]	17.16	13.51	8.88**
Nonprotein nitrogen[4]	12.72	11.12*	12.46
Myofibrillar protein nitrogen[4]	52.88	61.57	46.66**
Stroma protein nitrogen[4]	17.24	13.82	32.00**

*P < 0.05, **P < 0.01.
[1] Smokehouse condition: 32.2°C (90°F), 45% R.H.
[2] The means underlined do not differ significantly.
[3] Of muscle on a dry, fat-free basis.
[4] Calculated as percent of total nitrogen.

Electrophoretic studies

The starch gel electrophoretic patterns of sarcoplasmic proteins of pork muscle samples extracted with water are shown schematically in Figure 1. Distinguishable differences were present in the protein patterns of the untreated and treated samples. Protein bands were either totally absent or decreased in stainability, and these changes were greater in the heated and smoked sample than in the heated sample. The cationic proteins were more thermostable than the anionic components, in agreement with Lee et al. (1966). Using free-boundary electrophoresis Kihara (1962) observed the disappearance and decrease of peaks in the water-soluble proteins of smoked chicken muscle.

The Weber-Edsall extract contains the majority of the myofibrillar protein fraction and the electrophoretic behavior of this extract from untreated, heated, and heated and smoked pork samples is shown in Figure 2. The electropherograms of the untreated and heated samples were very similar. Only small differences were observed in the color intensity of the 6 fastest-moving bands; however, the color intensities of the 10 to 12 slower-moving bands were more distinct in the heated sample. With the exception of the fastest-moving bands, changes were observed in all bands of the heated and smoked sample, with the majority of the bands disappearing. These results reflect changes in the solubilization of the various components of the myofibrillar protein fraction.

It was suggested by Kakō (1968a) that a 7.7 M urea-containing 0.055 M Tris-HCl buffer (pH 8.6) is the most suitable solvent to solubilize meat proteins before and after heat coagulation. Thus, this extraction method of solubilizing meat proteins was explored and the disc gel electrophoretic patterns of the meat-urea extracts are presented in Figure 3. The

Table 2—Distribution of nitrogen in various protein fractions of untreated, heated, and heated and smoked pork samples.[1,2]

Variables	State of muscle		
	Untreated	Heated	Heated-smoked
% Total nitrogen[3]	14.82	14.74	14.49
Protein nitrogen solution extracted at low ionic strength[4]	30.17	17.48	13.92**
Sarcoplasmic protein nitrogen[4]	18.09	5.59	1.99**
Nonprotein nitrogen[4]	11.89	12.08	11.93
Soluble fibrillar protein nitrogen[4]	7.99	5.76	4.32**
Myofibrillar protein nitrogen[4]	47.57	60.33	35.79**
Stroma protein nitrogen[4] (insoluble in any solution used)	22.26	22.19	50.32**

**P < 0.01.
[1] Smokehouse condition: 60°C (140°F), 45% R.H.
[2] The means underlined by the same line do not differ significantly.
[3] Of muscle on a dry, fat-free basis.
[4] Calculated as percent of total nitrogen.

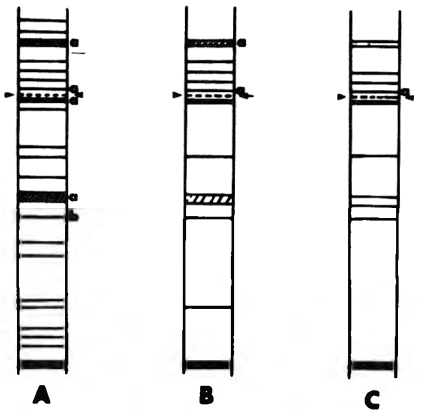

Fig. 1—A comparisor. of protein patterns of the water-soluble extract of pork loin samples. The arrows indicate point of sample application. N.B: In Figures 1, 2, 3 A = untreated sample, B = heated sample, C = heated and smoked sample.

mobility of the 16 to 18 bands was similar for all extracts; however, the color intensity of the protein pattern of the heated and smoked sample was considerably decreased and some bands disappeared. With all 3 samples, there appeared to be a large amount of protein which did not migrate into the gel, indicative of denaturation during the extraction process.

Results obtained from the electrophoretic studies of the water extracts, Weber-Edsall extracts and meat-urea extracts substantiated the results obtained with the protein fractionation studies. Obvious changes were shown in the electrophoretic patterns of the heated and smoked samples which indicated that smoke caused changes in the electrophoretic behavior of meat proteins.

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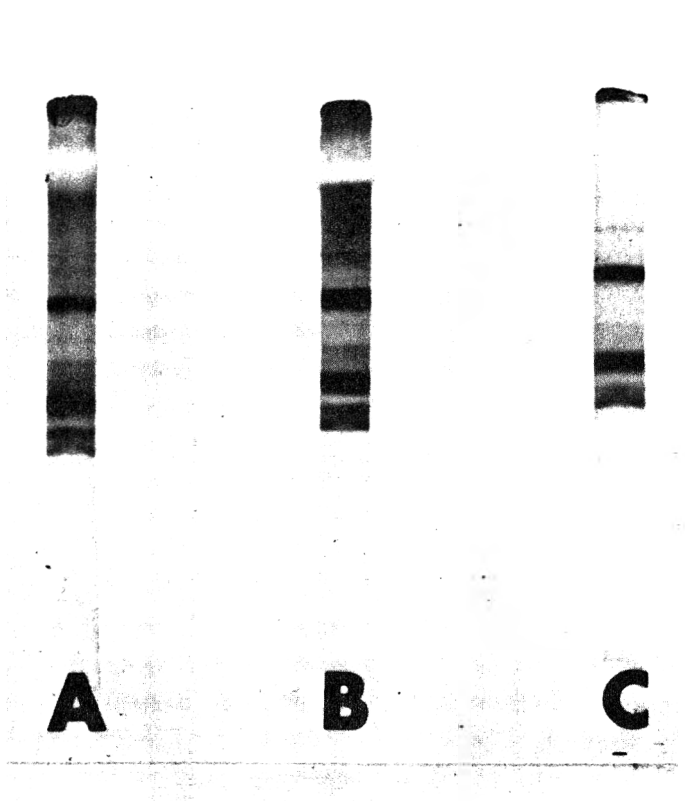

Fig. 2—A comparison of the protein patterns of the Weber-Edsall extracts of pork loin samples.

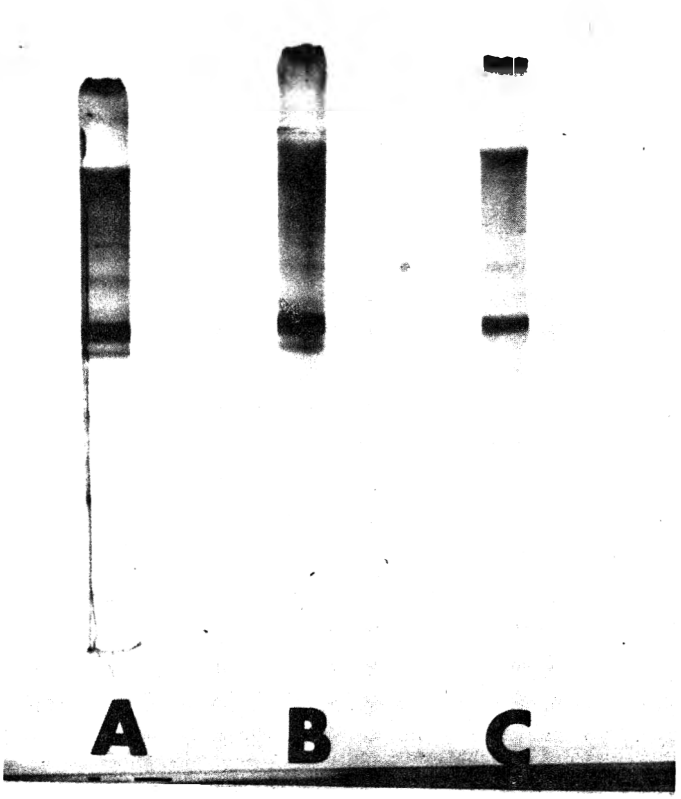

Fig. 3—Disc gel electropherograms of meat-urea extracts of pork loin samples.

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CHANGES IN VARIOUS PROTEIN PROPERTIES OF PORK MUSCLE DURING THE SMOKING PROCESS

SUMMARY—Changes in the pH, free sulfhydryl groups, amino nitrogen content and total free amino acids of untreated, heated and heated and smoked pork longissimus dorsi muscle samples were investigated. This study demonstrated that heating and heating and smoking caused changes in the pH, free sulfhydryl and amino nitrogen content of pork samples. An interesting observation was the increase in the myofibrillar protein nitrogen fraction, pH and free sulfhydryl groups of the heated samples, and the decrease of these values in the heated and smoked samples. Results of this study indicated that smoke constituents react with the functional groups of meat proteins.

INTRODUCTION

IT HAS BEEN demonstrated that changes occur in the functional groups of meat proteins during heating. The pH of muscle tissue of various meats and poultry increased during heating (Hamm et al., 1960; Kauffman et al., 1964; Paul et al., 1966) more rapidly and to a higher level with increased temperature. Using cured hams, Cohen (1966) and Karmas et al. (1964) observed a similar change in pH upon heating. Hamm et al. (1965) observed the release of free sulfhydryl groups during the heat denaturation of meat and Bautista et al. (1961) observed a decrease in the amino nitrogen content upon heating beef muscle. Studying the total free amino acid content of raw and cooked pork, Krol (1966) and Usborne et al. (1968) observed no significant difference due to heating. The effect of the smoking process upon the functional groups of proteins has been examined by several workers. Kakō (1968) and Krylova et al. (1962) observed a decrease in pH of smoked meat. Krylova et al. (1960) indicated that the chemical activity of functional groups (amino, hydroxyl and sulfhydryl) increased as proteins became denatured during the smoking process. Phenols and polyphenols react with sulfhydryl groups and carbonyls with amino groups (Krylova et al., 1962), and interactions occur between various phenol components and individual amino acids (Kurko, 1967).

Although it has been demonstrated that heating as well as the smoking process affects protein functional groups, it was the objective of this study to determine whether smoke per se had any additional effect besides that exerted by heat during the smoking process.

EXPERIMENTAL

THE SAMPLES used in this study were prepared similarly to those reported by Randall et al. (1970). The samples for pH measurements were prepared by homogenizing 10 g of the meat sample in 100 ml of distilled water for 1 min. All pH measurements were performed with a Corning Model 12, expanding-scale pH meter.

The Sørensen method as outlined in A.O.A.C. (1965) was used to determine the amino nitrogen content. A determination of the total ninhydrin positive material was used as an estimate of the total free amino acids in the sample and is an adaptation of that used by McCain et al. (1968). The samples were prepared by the method of Tallan et al. (1954).

Ellman's reagent (1959), 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), a water-soluble disulfide for the determination of free sulfhydryl groups, was adapted for use. The reagent was prepared by adding 39.6 mg DTNB to 10 ml 95% ethanol. A 2.5-g meat sample was homogenized with 25 ml of 8 M urea for 1 min. The slurry was centrifuged at 25,000 × g for 10 min at 0°C. For analysis, this supernatant was diluted 1 to 5 with phosphate buffer (μ = 0.1, pH 8.0) and filtered through Whatman No. 2 filter paper. The colorimetric procedure was carried out as described by Ellman (1959).

The myofibrillar protein nitrogen fraction was obtained as outlined by Randall et al. (1970).

RESULTS & DISCUSSION

DATA on the changes of protein properties of untreated and treated pork loin samples are given in Table 1. The pH of the muscle tissue increased with heating and decreased with heating and smoking. A slight increase in pH values with heating was observed by Cohen (1966), Hamm et al. (1960), Kauffman et al. (1964) and Paul et al. (1966) with ham, beef, pork and rabbit muscles, respectively. Hamm (1966) has suggested that the pH changes occurring during heating of meat may be caused by charge changes or hydrogen bonding, or both, within the myofibrillar proteins. A decrease of pH on smoking meats at 20°C (Krylova et al., 1962) and at 25°C (Kakō, 1968) has been observed. The changes observed in the heated and smoked samples were probably caused by the penetration of smoke components, such as organic acids, into the meat.

Appreciable differences in the free sulfhydryl groups of untreated, heated and heated and smoked pork samples were observed (Table 1). The significant increase of sulfhydryl groups of heated pork samples was in agreement with an earlier study by Hamm et al. (1965). These workers observed a steady increase within the temperature range of 30 to 70°C and attributed this increase of free sulfhydryl groups to the unfolding of peptide chains, especially those of actomyosin. With the smokehouse conditions utilized in this study, a loss of 24% of the free sulfhydryl groups occurred in the heated and smoked pork samples. Krylova et al. (1962) observed a 60% decrease in the free sulfhydryl groups of smoked beef. The decrease observed was probably due to the formation of complexes between smoke components and free sulfhydryl groups, since Krylova et al. (1962) noted that the phenolic fraction of smoke exerted the greatest effect upon these groups.

Table 1—Effect of heating and heating and smoking on protein properties of pork samples.[1,2]

Variables	State of muscle		
	Untreated	Heated	Heated-smoked
pH	5.31	5.48	4.95**
Free sulfhydryl groups (μmoles/g protein)	91.87	120.37	69.81**
Amino nitrogen (mg/g protein)	9.05**	7.06	6.57
Ninhydrin positive material (μmoles/g protein)	526.67	559.67	540.30
Ninhydrin positive material (μmoles/g material)	179.90*	259.70	229.90

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

[1]Smokehouse condition: 60°C (140°F), 45%R.H., 2.25 hr.

[2]Means underlined by the same line do not differ significantly.

There was an appreciable change in the amino nitrogen content of the heated and heated and smoked pork samples (Table 1), with the majority of the decrease being due to heat effects. Upon heating beef longissimus dorsi muscle to 65°C, Bautista et al. (1961) observed a similar decrease. Kihara (1962) obtained a slight increase in the amino nitrogen content (mg/g meat) of smoked poultry and pork as did Kido et al. (1967) with smoked herring, but this increase was probably a reflection of the weight lost during smoking rather than an actual increase in the amino nitrogen content.

McCain et al. (1968) stated that the determination of the total ninhydrin-positive material may be used as an estimation of the total free amino acids in the sample. By this determination, no appreciable changes were observed between any of the samples (Table 1) when calculated on a protein basis. Usborne et al. (1968) observed a noticeable increase of total free amino acids during heating of pork. de Abreu et al. (1962) had a similar increase during smoke drying of sausages and Kido et al. (1967) found a 50% increase of total extractable amino acids of smoked herring based upon the wet weight of the sample. By calculating the ninhydrin-positive material on a wet weight basis (Table 1), a significant increase ($P < .05$) was observed between the treated and untreated samples.

It appeared that a relationship existed between the pH, free sulfhydryl groups and the myofibrillar protein nitrogen fraction (Table 2). An increase was observed in all 3 values from the heated pork samples, whereas a decrease in the values was obtained from the heated and smoked samples. The increases observed in the heated samples were in agreement with the studies of Hamm (1966), who was of the opinion that changes in pH and free sulfhydryl groups were related to the unfolding of the peptide chains of myofibrillar proteins. The decreases observed in the heated and smoked samples were probably due to smoke constituent interactions with the various reactive groups within the proteins.

Table 2—A comparison of the pH, free sulfhydryl groups and the myofibrillar protein nitrogen content of untreated, heated and heated and smoked pork samples.[1]

State of muscle	pH	Variables: Sulfhydryl groups (μmoles/g protein)	Variables: Myofibrillar content (% of total nitrogen)
Heated	5.48	120.37	60.33
Untreated	5.31	91.87	47.57
Heated-smoked	4.95	69.81	35.79

[1]Smokehouse conditions: 60°C (140°F), 45% R.H., 2.25 hr.

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A Research Note

EFFECT OF SMOKE UPON ACID PHOSPHATASE ACTIVITY OF SMOKED MEAT

INTRODUCTION

IN THIS COUNTRY and in some European laboratories, coagulation tests are being used to determine the maximum temperature attained in heat processing of hams and picnics. However, these tests present difficulties in interpretations and often give false results (Lind, 1965b; Olsman, 1968).

It has been suggested (Lind, 1965a; 1965b; Gantner et al., 1968; Olsman, 1968) that acid phosphatase activity may be used as a criterion for the heat treatment of hams and picnics. Although salt content (Lind, 1965b), polyphosphates (Körmendy et al., 1967) and pH (Körmendy et al. 1960) have been shown to affect the phosphatase activity, the effect that smoke exerts upon acid phosphatase activity is apparently unknown.

EXPERIMENTAL

Materials

Pork loin samples from the longissimus dorsi muscle were used in this study. The samples were deboned, most of the external fat removed, and sliced on a meat slicer to 1.1-cm thickness. The slices were divided randomly into 3 equal groups to obtain control, heated, and heated and smoked samples. The slices to be heated or heated and smoked were treated similarly in the smokehouse with the exception of smoke being added to the latter samples. The smokehouse temperature was 60°C and the relative humidity was 45%, resulting in an internal temperature of the meat of 58.8°C after 2.25 hr of treatment.

Acid phosphatase activity. 2 methods were used to determine the acid phosphatase activity of pork samples. The Andersch et al. (1947) method for the determination of serum acid phosphatase was adapted for determination of acid phosphatase activity in meat. A 10-g sample of meat was homogenized in 20 ml of 0.05 M phosphate buffer (pH 7.6) for 1 min. The resulting slurry was centrifuged at 25,000 × g for 30 min. The supernatant from the fresh sample was diluted 1 to 5 for analysis; the supernatant from the heated and smoked samples was not diluted. The remainder of the procedure was unchanged from the original method. A second method (Lind, 1965a), used in Europe as an indicator of the heat processing of hams, was also employed to determine acid phosphatase activity.

Nitrogen analysis

Nitrogen was determined by the micro-Kjeldahl method as described by the American Instrument Company (1961).

RESULTS & DISCUSSION

RESULTS obtained by 2 methods of determining acid phosphatase activity are given in Table 1. With either method, there was a significant decrease in the acid phosphatase activity of the heated and the heated and smoked samples. Although heat caused a definite decrease in acid phosphatase activity, and this decrease may be related to heating temperatures (Lind, 1965b; Olsman, 1968), it would appear from the above results that if smoke accompanies heating, the effect that smoke exerts upon acid phosphatase activity would also have to be taken into consideration if this test were used to measure heat treatment of smoked meats.

Table 1—Effect of heating and heating and smoking on the acid phosphatase activity of pork samples.[1]

	State of muscle		
	Untreated	Heated	Heated-smoked
nm moles Substrate hydrolyzed/min/mg N (Andersch et al., 1947)	7.12	0.56	0.24**
μmoles Phenol/g sample (Lind, 1965a)	7.75	1.19	0.39**

**$P < 0.01$.

[1]Smokehouse condition: 60°C (140°F), 45% R.H.

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A Research Note

RADIO RESISTANCE OF BYSSOCHLAMYS FULVA ASCOSPORES AS SHOWN BY STORAGE TESTS

INTRODUCTION

THE ASCOMYCETE *Byssochlamys fulva* was first detected and described in England (Hirst et al., 1932; Olliver et al., 1934). It is known as an important factor of spoilage in the fruit industry (Senser et al., 1965). Since its spores enter the factories together with the fruits coming from the plantations, it is very difficult to get this organism under control. It can grow in heat pasteurized fruit juices because its ascospores are very heat resistant (Hull, 1933; 1934). The temperatures used in the various methods of pasteurization which normally are sufficient to destroy most of the moulds, fail completely to eliminate the ascospores of this organism. Temperatures in the range of 75–80°C even favor the germination and growth of ascospores. In these investigations, fresh apple juice, grape juice and commercial orange juice were used.

Studies on the radio resistance of moulds as shown in storage tests have the advantage over the conventional plating techniques, in that methods, applied in industry, may be reduced to a laboratory scale, thus allowing a more realistic study on the reactions of moulds and microorganisms in general. The reaction of moulds may, therefore, be studied in the medium where the infection plays such an important role in industry. However, this method does not allow quantitative determinations of their sensitivity. Only those dose ranges are taken into consideration in which no germination of spores occurs after a certain period of storage at optimal growth temperatures. Formation of mycelium, visible to the eye, indicates acute infection. Another important factor is the concentration of the spores in the fruit juices. In carrying out laboratory tests with the plate-counting method it is necessary, owing to the rapid growth of the moulds, to keep the concentration of spores within certain limits because the proliferation on the surface would make evaluation impossible. With storage tests, the concentration of the spores may be considerably increased.

MATERIAL & METHODS

THE STRAIN of *Byssochlamys fulva* used for our investigations was supplied by the Agriculture Collection Northern Utilization Research and Development Division (NRRL), with the designation A-3849. For the preparation of a single cell suspension see Partsch, 1969. To activate the spores, they were heated for 10 min at 75°C. Apple juices and grape juices (Sylvaner and Grünveltliner) of the Weinbauschule Klosterneuburg, and commercial orange juice ("Jaffa Gold Brand", unsweetened, pure, nature, produced by Citrus and Canned Products Association, Tel Aviv, Israel) were used. The grape and orange juices had to be filtered for the separation of floating particles. 11-ml flasks, closed with a rubber stopper and an aluminum cap, served as storage containers. The quantity of juice was 10 ml per trial.

To meet the conditions of industry as far as possible, samples were pasteurized in an ultrathermostat for 10 min at 72°C. The flasks were inoculated with 0.1 ml of the prepared spore suspension, using sterile, plastic syringes (Johnson). The concentration of the spore suspension was approximately 10^5 per milliliter. Irradiation was carried out in a 12,000 C_i Co^{60} Gamma-cell, at a dose rate of 660,000 rad/hr. To eliminate the possibility of too high an error factor in the homogeneity of the source, no more than 10 flasks at a time were irradiated. In the two main trials 100 flasks per juice were treated. The irradiation doses applied were 100, 120, 140, 150, 160, 180, 200, 220 and 240 krad. The irradiation temperature was 24°C. After irradiation the flasks were incubated at 37°C and examined daily for formation of mycelium and those in which growth of mycelium could be detected were removed. The tests were run for a minimum of 3 months (Fig. 1).

RESULTS & DISCUSSION

IN ADDITION to storage tests, survival curves of ascospores on apple juice–agar, Sabouraud-agar and potato dextrose–agar have been made. The LD_{90}-value was 35 krad for these experiments. Comparing this value with the results of the storage tests it becomes obvious that with a significantly higher dose there are still samples showing growth of mycelium. This difference is probably due to the modified experimental conditions.

Mean values were calculated from the results of the 2 main tests and, as seen in Figure 1, the microorganisms in different fruit juices showed different reactions. In the nonirradiated controls, growth of mycelium was perceptible in all cases by the second day. This growth was just as regular on the 5th day, at an irradiation dose of 100 krads. At the next higher dose (120 krads), some differences between apple and grape juice, on the one hand, and orange juice on the other began to show. There was no difference between the 2 varieties of grape juice. At a dose rate of 140 krads and above, growth of mycelium could be observed in all

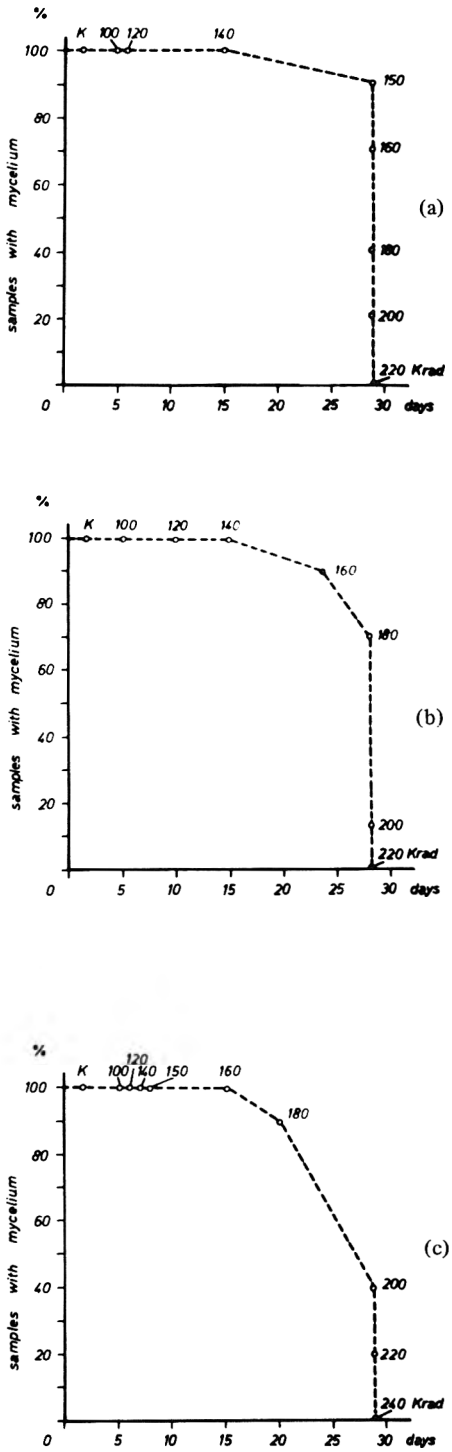

Fig. 1a–Effect of irradiation on germination and growth of ascospores of Byssochlamys fulva in apple juice. 1 b–In grape juice. 1c–In orange juice.

samples of orange juice after 1 wk, whereas in apple and grape juice only after 2 wk. At 150 krads, differences could be noticed also between grape juice and apple juice. While all samples of grape juice still showed spore germination, in some samples of apple juice, even after an extended period of storage, no growth occurred. At 200 krads the number of infections in apple juice decreased still further and above 200 krads they disappeared completely. Grape juice showed a similar tendency, but the decrease in germination was less apparent. Orange juice continued to show mycelial development even at doses above 200 krads. At 240 krads no germination could be observed.

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ANTIGENICITY OF SALT-SOLUBLE BEEF MUSCLE PROTEINS HELD FROM FRESHNESS TO SPOILAGE AT LOW TEMPERATURES

SUMMARY—Ground semitendinosus beef muscle was allowed to undergo normal spoilage at 7°C for 30 days. Changes in the antigenicity of the muscle proteins were determined periodically by injecting rabbits with 3% NaCl extracts of beef from freshness to spoilage. According to the serological methods employed, the salt-soluble muscle proteins were highly antigenic in all meat extracts. Although the anti-putrid and anti-fresh extract antibodies cross reacted they were, nevertheless, serologically distinct, in that the former always yielded a greater amount of precipitate and a larger number of precipitating bands than did the latter, when the two were tested against their homologous antigens. This study revealed that spoiled meat extracts contained at least 2 new antigenic species not demonstrable in fresh meat extracts, in addition to the antigens serologically common to both extracts. These findings indicate a lack of complete breakdown of salt-soluble beef proteins by the spoilage flora.

INTRODUCTION

PREVIOUS reports from this laboratory have shown that an increased hydration capacity is the most demonstrable change that occurs in meats such as beef and poultry as they undergo microbial spoilage at refrigerator temperatures (Jay et al., 1964; Jay, 1964; 1966a). It has also been shown that spoilage of this type occurs in the absence of detectable breakdown of primary beef muscle proteins, and that the spoilage flora is generally devoid of strong proteolytic activities at the temperatures of spoilage (Jay, 1966b; 1967). The low-temperature spoilage flora of fish has also been shown to be generally lacking in proteolytic activities (Lerke et al., 1965). While post-mortem aseptic autolysis of muscle proteins was reported as early as 1917 by Hoagland et al. and claimed by various investigators since (Zender et al., 1958; Soloviev et al., 1964), recent findings by several investigators (Bodwell et al., 1964; Martins et al., 1968) on the lack of capacity of meat cathepsins to significantly degrade muscle proteins raise doubts about the significance of aseptic autolysis of primary meat proteins during aging and spoilage. To shed more light on the nature of the changes, if any, that occur in muscle proteins as they undergo microbial spoilage, a study was undertaken to determine the effect of microbial numbers and microbial activities on the antigenicity of muscle proteins allowed to undergo spoilage at refrigerator temperatures. Results of this study constitute the basis of this report.

MATERIALS & METHODS

Preparation of beef extracts

25 g of freshly ground semitendinosus beef muscle were extracted with 100 ml of 3% (wt/vol) NaCl solution at pH 7.0 by homogenizing for 2 min at 30°C in a single-speed Waring Blendor. The slurry was centrifuged at room temperature at 3,000 rpm for 15 min and the supernatant filter-sterilized employing 0.8-μ Gelman membranes. This extract was then aseptically dispensed into glass vials in 1-ml amounts and kept frozen at −15°C without preservatives. In spoiled beef, it was necessary to use 140 ml of NaCl solution per 25 g of beef to obtain any supernatant. Before use, fresh and spoiled meat supernatants were adjusted for protein content by dilution.

Immunization procedures

1-year-old New Zealand male rabbits weighing approximately 3 kg and not previously im-

Table 1—Summary of immunization schedule employed.

Days of inoculations:	1, 3, 5, 7, 9 and 11 via marginal ear vein.
Size of inocula employed:	1 ml each containing 5 mg protein.
Animals used:	White New Zealand male rabbits, 12 months of age.
Time of final bleeding:	On 18th day, or 7 days from last injection, via cardiac route.
No. of animals yielding positive antisera:	3 of 4.

munized were injected with 6 ml of salt-soluble proteins via the marginal ear vein. Each rabbit received 6, 1-ml injections spaced by 1-day intervals and was bled via cardiac puncture 5–7 days after the last injection. With the exception of route of injection, this is essentially the schedule of Fox et al. (1966), which they employed to immunize rabbits with myosins from canine skeletal and cardiac muscles. The sera were harvested after clot formation, cleared of erythrocytes by centrifugation and stored at −15°C without preservative.

Serologic methods

The capillary precipitin assay of Swift et al. (1943) was 1 of the 2 methods used. It consisted of mixing equal volumes of reactants into capillary tubes approximately 1.0 mm in diameter and 90 mm in length. The tube was then inverted several times to permit complete mixing of reactants and inserted into a plasticine block. The tubes were incubated at 37°C for 30 min followed by incubation at 6°C for 16 hr. Readings were recorded according to the amount of visible precipitate formed in the tubes at this time.

The micro double-diffusion assay was carried out basically according to the procedures described by Crowle (1961). Briefly, microscopic slides precoated with 0.1% purified agar containing 0.02% sodium azide were overlaid with a 1–2-mm layer of sterile agar containing the following ingredients/liter: 6.98 g sodium barbital, 6 g sodium chloride, 0.02 g sodium azide, 10 g Noble's agar and 2.7 ml of 1 N HCl. The pH of this preparation was 7.4 and its ionic strength (μ) was 0.15. Plexiglass templates with funnel-shaped wells were placed over this agar layer immediately after solidification (the 4 peripheral wells were spaced about 5 mm apart around the center well). The wells were then filled with reactants by use of 0.25-ml syringes and 27-gauge needles. In all experiments, antibodies were placed in the central well while antigens were placed in the peripheral wells. These slides were incubated in humid atmospheres at room temperature for 3–6 days. Following elution with large volumes of phosphate-buffered saline at pH 7.4 and μ of 0.15, the slides were stained by use of a double protein stain as proposed by Crowle (1961). Precipitating bands were observed with an ordinary 3× magnifying glass and recorded.

Preparation of meat samples and their quality evaluation

Ground beef in 25-g quantities was wrapped in aluminum foil and allowed to undergo spoilage at 7°C for 30 days. The microbial quality of this meat was measured by the extract-release volume (ERV) method as previously described (Jay, 1964). By this method, beef having an ERV value of less than 25 is taken to be spoiled, whereas values above 25 denote beef of good microbial quality.

Table 2–Serologic reactivity of antibodies made to salt extracts of fresh and spoiled beef employing capillary precipitin assay.

	ERV of beef from which extracts were made					
Antibodies	45	17	11	9	0	3% NaCl
Anti-ERV 45	2+	2+	1+	1+	±	–
Anti-ERV 0	4+	5+	6+	5+	7+	–
Normal rabbit serum	–	–	–	–	–	–
Saline	–	–	–	–	–	–

+ = Intensity of reaction; – = no reaction.

RESULTS & DISCUSSION

THE IMMUNIZATION schedule used in this study is presented in Table 1. 3 of the 4 animals yielded positive sera within 18 days after receiving approximately 30 mg of beef protein. Protein content was estimated by 280/260 ratios using a Beckman DB-G spectrophotometer. Since high titers of antisera were not sought, a brief and simple schedule of this type was found to be advantageous; that is, requiring less than 3 wk, single route of injection, and lacking adjuvants.

The relationship between ERV and bacterial numbers in ground beef undergoing natural spoilage is presented in Figure 1. In succeeding tables, beef with ERV values of around 5 to 25 was designated as being spoiled, whereas that with ERV values of 0 was designated as being putrid.

Table 2 presents results obtained by capillary precipitin assay when an antibody to fresh meat extracts (ERV of 45) and an antibody to extracts of putrid meat (ERV of 0) were reacted against an extract from fresh meat and extracts from meats of varying stages of spoilage. Although the homologous systems formed the greatest amounts of precipitate, these two antibodies cross reacted. That the amount of precipitate formed by anti-ERV 45 antibodies was decreasing when it reacted against extracts of declining ERV values suggests strongly that the small amount of precipitate (±) between anti-ERV 45 antibodies and ERV 0 antigens was not the result of a false positive reaction but instead reflected a lower degree of sensitivity. The reactivity pattern of anti-ERV 0 antibodies was comparable to that of anti-ERV 45 antibodies, with the exception that the former's titer was higher than the latter's. This pattern is further illustrated in Table 3 where antibodies were tested by the precipitin assay method, and in Table 4 by the double diffusion assay for extracts of meats with ERV values from 48 to 0. From the data (Table 4), it appears that fresh beef may be differentiated from spoiled beef by use of an anti-spoiled meat antibody in the immunodiffusion assay. From a large number of observations, anti-ERV 0 antibody preparations form at least 2 precipitating bands with any extracts of fresh meat but 4 or more bands with any extract of salt-soluble

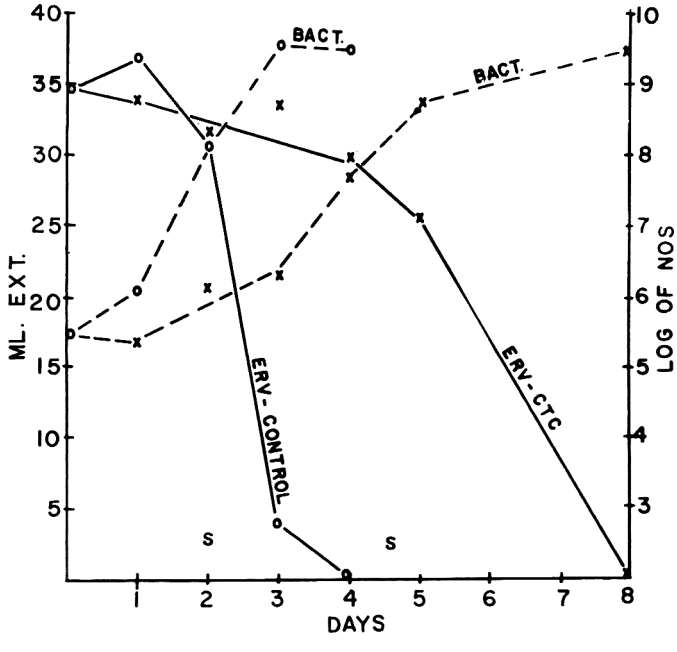

Fig. 1–Relationship between ERV and bacterial numbers on all-lean ground beef round held and extracted at 7°C with distilled water (-o- = control; -x- = chlortetracycline-treated). From Jay, 1964.

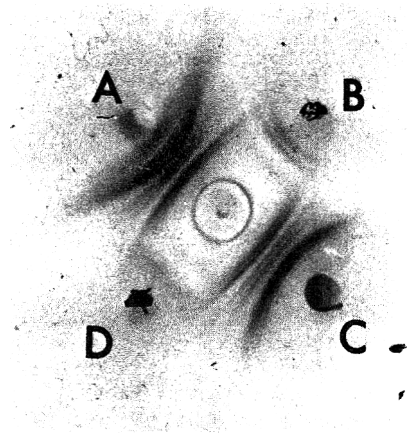

Fig. 2—Photograph of double-diffusion assay slide with anti-ERV 0 antibodies in center well, extracts from ERV 0 beef in wells A and C and extracts of ERV 48 beef in wells B and D.

Table 3—Capillary precipitin assay of antigen-antibody reactions employing fresh and putrid beef extracts.

	ERV of beef from which salt extracts were made			
Antibodies employed	48	45	0 (No. 1)	0 (No. 2)
Anti-ERV 48	2+	2+	1+	±
Anti-ERV 45	3+	2+	1+	1+
Anti-ERV 0 (No. 1)	5+	4+	7+	8+
Anti-ERV 0 (No. 2)	5+	5+	6+	7+
Normal rabbit serum	–	–	–	–

Table 4—Extracts from beef of varying degrees of freshness tested by double-diffusion assay against antibodies made to fresh and spoiled beef.

	ERV of beef from which extracts were made				
Antibodies employed	48	45	33	17	0
Anti-ERV 48	++	++	*	++	++
Anti-ERV 0	++	++	++	++++	++++
Normal rabbit serum	–	–	–	–	–

+ = No. of precipitating bands; – = no reaction; *no data.

proteins having an ERV value below 25 (see also Fig. 2).

Data presented in Table 5 reveal that meat-spoilage pseudomonads and their cell-free filtrates did not form precipitating bands when reacted with antibodies to fresh and spoiled beef. In this assay, both salt extracts and cell-free filtrates of pseudomonads were tested by the capillary precipitin assay, whereas pseudomonads suspended in saline were tested against salt-soluble protein antibodies by the slide agglutination method. The genus *Pseudomonas* is the most important group of bacteria involved in the low-temperature spoilage of beef (Kirsch et al., 1952; Brown et al., 1958; Ayres, 1960). Before injection into rabbits, bacterial cells were removed from the salt-soluble protein extracts by filtration, as previously stated.

Table 5—Activity of fresh and spoiled meat antisera against Pseudomonas sp. cells and cell-free filtrates.

	Meat extracts[1]		Pseudomonas preparations	
Antibodies employed	Fresh	Spoiled	Cells only[2]	Cell-free filtrates
Anti-fresh	2+	+	–	–
Anti-spoiled	5+	10+	–	–
Normal rabbit serum	–	–	–	–
Saline	–	–	–	–

[1] Assayed by capillary precipitin method.
[2] Saline suspensions tested by slide agglutination.

By either of the assay methods in this study, putrid meats were found to be antigenic, indicating that complete protein breakdown was not produced by the spoilage flora. Both assay methods disclosed a cross reactivity between the two types of antibodies, anti-ERV 48 and anti-ERV 0. While the capillary precipitin assay revealed only a gradual loss in the amount of the original serologically precipitable proteins as beef underwent spoilage, the double diffusion method showed that new antigenic species appeared in the extracts from spoiled and putrid meats. Since these antigenic species were not characterized in the present study, the precise way in which the new antigens came about is unknown. While not yet substantiated by direct experimental data, the following is one of several possible explanations for cross reactivity between anti-ERV 45 antibodies and ERV 0 antigens.

If it is assumed that the putrid meat contained, in addition to its spoiled proteins, a small quantity of the original unchanged proteins of the fresh meat, the entire reactivity pattern of these serologic systems becomes more meaningful. Thus, anti-ERV 48 antibodies would be expected to react, as they do, with ERV 0 extracts because the putrid meat extract contained original antigens to which anti-ERV 48 antibodies were made. Likewise, when putrid meat extract was injected into rabbits, the antibodies produced were directed toward both the original and spoiled meat proteins, allowing anti-ERV 0 to react not only with its homologous antigens but with the antigens of the fresh meat extracts as well.

The reason for the greater number of antibody species when antibody to putrid meat was reacted with extracts from fresh and putrid meats could be related to the unfolding of protein molecules that leads directly to increased hydration capacity. In putrid beef, where a greater amount of unfolding exists, it is not inconceivable that these unfolded proteins elicit the production of antibodies with more reactive potential. While this degree of reactive potential apparently does not exist in fresh beef proteins where the protein molecules are tightly coiled, antibodies made to putrid beef apparently consist of some species made to antigenic sites on both folded and unfolded proteins. If it is assumed that the antigenic sites of the tightly coiled, folded proteins of fresh beef also exist in reactive form in the more loosely arranged spoiled and putrid beef proteins, this would account for the greater number of precipitating bands when anti-putrid antibodies are employed against extracts of beef in varying stages of spoilage. Attempts to better understand the nature of this phenomenon are in progress.

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LYSOSOMAL-TYPE ENZYMES IN BEEF LONGISSIMUS DORSI MUSCLE

SUMMARY—Biochemical evidence for the presence of lysosomes in bovine longissimus dorsi muscle has been presented. Comparisons of enzymatic activities among lysosomal fractions from rat liver, bovine liver and bovine l. dorsi muscle indicate that there are specific as well as organic differences with respect to the activities of some of the enzymes.

INTRODUCTION

THE PRESENCE of lysosomes in bovine muscles is of interest since cathepsin, one of the hydrolases present in lysosomes, may be a factor in the tenderization of postmortem bovine muscle.

Recently, Buchanan et al. (1967) and Canonico et al. (1969) presented evidence for the presence of lysosomes in the rat hindleg. Parrish et al. (1967) showed that cathepsin from bovine diaphragm muscle was lysosomal; more recently, the comprehensive work by Stagni et al. (1968) showed that lysosomes are present in the rat and bovine skeletal muscles.

Since muscles from the same animal vary in tenderness, there is still a need to study the lysosomes from specific muscles. Such a study may help clarify the relationship of cathepsin to tenderness. Also, there are specific and organic differences in the distribution of lysosomal enzymes (Shibko et al. 1963; Stagni et al., 1968). The relative distribution of the enzymes in the various bovine muscles is of interest.

EXPERIMENTAL

Sampling

Samples of beef longissimus dorsi muscle and liver were obtained from 36-month-old Black Angus steers about 3 hr after slaughter and were chilled immediately.

Adult male Wistar rats were decapitated and their livers excised and used immediately.

Enzyme extraction

The muscle was trimmed of fat and cut into small pieces. About half of a 150-g sample of these pieces was mixed with 200 ml of cold 0.175 M KCl in 0.25 M sucrose solution, then blended with an Omni-Mixer for 10 sec. This process was repeated with the remaining half of the sample. The pooled homogenate was centrifuged for 10 min at 650 × g in a Sorvall RC-2 centrifuge. After filtering the supernatant through 3 layers of cheese cloth, the residue was suspended in 500 ml of the solvent, homogenized in portions for 1 min with a motor-driven teflon-pestle homogenizer and then centrifuged for 10 min at 650 × g. This supernatant was combined with the first and centrifuged again for 10 min at 650 × g. The resultant supernatant was centrifuged for 10 min at 20,200 × g (SS-34 rotor). The brownish pellet was washed twice with 70 ml of the solvent, each time centrifuging for 10 min at 20,200 × g. After decanting the solvent, the residue was mixed with about 10 ml of 0.01 M ethylenediaminetetraacetic acid (EDTA) in 0.25 M sucrose, poured into a teflon-pestle homogenizer and uniformly suspended by 6 gentle strokes of the pestle. Just before use, appropriate portions of this suspension were taken and the enzymes activated by the various techniques.

Activation

A 0.1% (W/V) suspension of Triton x-100 and freeze-thaw techniques were used to activate the enzymes.

Enzymatic assay

Acid phosphatase activity was determined with p-nitrophenylphosphate and β-glycerophosphate as substrates. With p-nitrophenylphosphate, 2.5 ml of the incubation mixture contained 25 μM of the substrate, 1.0 ml of 0.15 M acetate buffer (pH 5.2), enough 1 M sucrose solution to give a final sucrose concentration of 0.25 M and 0.4 ml of the enzyme suspension. After incubating the reaction mixture for 10 min at 37°C, 2.5 ml of cold 10% trichloroacetic acid was added and after standing for 5 min at 5°C, the preparation was centrifuged. 3 ml of the supernatant was treated with 1.0 ml of 1 N NaOH and then with 3.0 ml of alkaline glycine buffer (pH 10.5) (Fishman, 1963). The yellow color of the p-nitrophenol was read at 410 mμ. The Beckman DU spectrophotometer was used for all spectrophotometric assays.

Essentially the method of Gianetto et al. (1955) was used when β-glycerophosphate was the substrate; however, the concentration of the substrate was reduced by half, to lower the blank phosphate reading. Also, a modified Fiske-Subbarow method was used to analyze for inorganic phosphate (Clark, 1964).

For the assay of β-glucuronidase, the incubation mixture of Gianetto et al. (1955) was used and for assay of β-galactosidase the reaction mixture of Sellinger et al. (1960). Both mixtures were incubated at 37°C for 60 min, after which 2.0 ml of alkaline glycine buffer (pH 10.5) was added. After standing for 10 min the suspension was centrifuged for 10 min at 35,000 × g. The absorption of the red phenolphthalein color was read at 550 mμ; that of the yellowish o-nitrophenol color at 420 mμ.

Cathepsin was assayed by the method of Sliwinski et al. (1959) except that the incubation period was 1 hr.

Protein analysis

Protein concentration was determined by a modified biuret method (Gornall et al., 1949) using bovine albumin as standard.

RESULTS & DISCUSSION

TABLE 1 indicates that bovine muscle β-galactosidase, β-glucuronidase and cathepsin are activated by Triton x-100 and freeze-thaw treatments. However, no acid phosphatase activation is indicated. The reason for the failure to obtain activation is not clear, although permeability of the particulate membrane to β-glycerophosphate can not be excluded as a factor. Appelmans et al. (1955) reported that the rat liver lysosomal membrane may be permeable to β-glycerophosphate. To determine whether the substrate or its hydrolytic product, glycerol, had a labilizing effect on the particulate matter, β-glycerophosphate was incubated with

Table 1—Activation of bovine muscle, liver and rat liver lysosomal-type enzymes by physical and chemical means.

Tissue (No. of animals)	Substrate	Treatment[1]		
		Freeze-thaw	Triton x-100	None
Bovine muscle (6)	β-Glycerophosphate	6.68[2] a	6.69a	6.88a
	β-Galactoside	.60a	.67a	.29b
	β-Glucuronide	.33a	.33a	.07b
	Hemoglobin	2.96a	3.12a	1.37b
Bovine liver (3)	β-Glycerophosphate	83.76a	81.37a	61.17b
	β-Galactoside	12.31a	8.69b	8.60b
	β-Glucuronide	2.33a	1.73a	.44a
Rat liver (3)	β-Glycerophosphate	206.38a	219.19a	29.76b
	β-Galactoside	14.83a	15.33a	5.65b
	β-Glucuronide	6.54a	7.00a	.67b

[1] Means in the same row, if followed by the same letter, are not significantly different as determined by Duncan's multiple range test (5% level).

[2] Specific activities expressed as nM substrate hydrolyzed per minute per milligram protein.

phenolphthalein glucuronide. The results indicated that the activity of the acid phosphatase, although quite high, was not enhanced by Triton x-100 nor by freezing and thawing; the β-glucuronidase activity was enhanced by both treatments, which indicates that neither β-glycerophosphate nor glycerol has a lytic effect.

Activation also was absent when p-nitrophenylphosphate was the substrate for the acid phosphatase. Again, there is no definite explanation for the lack of activation. Buchanan et al. (1967) postulated that p-nitrophenylphosphate, the substrate, or p-nitrophenol, the hydrolytic product, is a labilizing agent, since detergent activation was absent when rat skeletal muscle acid phosphatase was assayed with this substrate but activation was present when β-glycerophosphate was the substrate.

Apparently, the response towards various activating conditions differs when bovine-liver β-galactosidase is used (see Table 1). There was little or no activation when Triton x-100 was used, but freezing and thawing affected activation. Shibko et al. (1963) found inhibition with Triton x-100. The reason for the inhibitory effect is not clear. It is clear that the Triton x-100 ruptures the lysosomal membrane since the other enzymes are activated; the effect of the detergent on the substrate is probably not a factor since the reaction proceeds without inhibition when rat-liver enzyme is used. Neither freeze-thaw nor Triton x-100 treatments activated β-glucuronidase.

Results for rat liver in Table 1 are similar to those reported by others (Duve et al., 1955; Gianetto et al., 1955; Sawant et al., 1964).

Table 1, in summary, provides some evidence that β-galactosidase, β-glucuronidase and cathepsin are lysosomal. Also, the data confirm the report by others (Shibko et al., 1963; Stagni et al., 1968) that the distribution and behavior of lysosomal enzymes vary among species as well as among organs.

Parallel activations of some of the rat-liver enzymes were used by Gianetto et al. (1955) as evidence that these enzymes are enclosed within a common membrane. Figure 1 shows a typical activation curve using data from a single animal. Analysis of variance showed no significant differences among the 3 substrates at each point for each of the 6 animals, but there were significant variations within a substrate group for a given point. The parallel activation curves provide further evidence that 3 of the enzymes, reported to be mostly lysosomal in origin, are activated simultaneously and thus have a common origin.

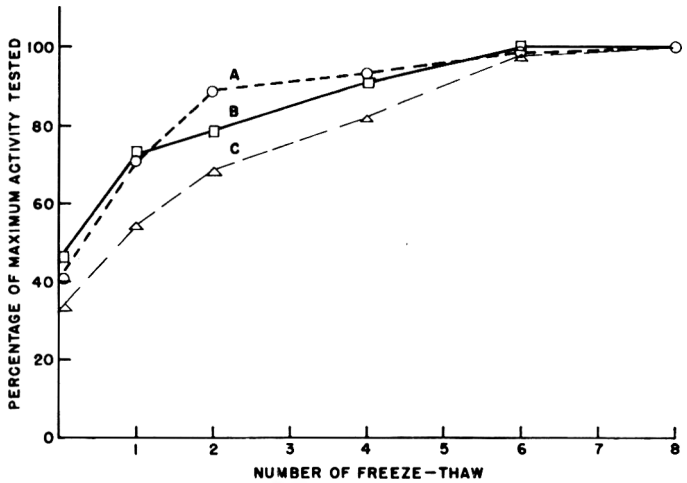

Fig. 1—Simultaneous activation of bovine l. dorsi β-galactosidase (A), cathepsin (B) and β-glucuronidase (C) by freezings and thawings. Ordinate is expressed as increase in specific activity as percentage of maximum activity tested.

The evidence presented herein gives strong indication that lysosomes are present in the bovine l. dorsi muscle. The relative magnitude of the enzymatic activities may serve as a guide in investigating other bovine muscles commonly used as food. Certainly for future work some of the comprehensive approaches and techniques of Romeo et al. (1966) and Stagni et al. (1968) should be considered.

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EFFECT OF POST-MORTEM AGING ON ISOLATION OF INTRAMUSCULAR CONNECTIVE TISSUE

SUMMARY—The yield of bovine and porcine intramuscular connective tissue (IMCT) was quantitated at various time intervals post-mortem. The pH of the muscle tissue was followed through the aging period, and the heat absorbed during hydrothermal shrinkage (ΔHs) and the temperature of the transition (Ts) determined on the IMCT. The yield of bovine IMCT at 72 hr post-mortem was 50% lower than that at 0 hr, whereas 34% lower yields were found for the porcine tissue ($P < 0.01$). Loss in the yield of IMCT was paralleled by the drop in pH post-mortem. Recovery of IMCT reached a minimum in the pH range 5.43–5.53. No marked differences in ΔHs or Ts were observed in any of the samples studied.

INTRODUCTION

IT HAS long been recognized that the post-mortem changes occurring in muscle tissue are intimately related to meat tenderness. The biochemistry of these changes has been extensively investigated in relation to the sarcoplasmic and myofibrillar proteins (Briskey et al., 1966). The nature of the alterations occurring in the connective tissue proteins, however, has received little emphasis. Work based on the nitrogen content of solubilized hydrolysates produced by autoclaving has indicated that the collagen content remains unchanged during the aging period (Hershberger et al., 1951; Wierbicki et al., 1955). However, McClain et al. (1965), reported a decrease in salt-soluble collagen and a concurrent increase in acid-soluble collagen in steer, calf and lamb fore-shank muscle aged 7 days. Winegarden et al. (1952) found a slight but consistent decrease in shear force in connective tissue aged for 35 days.

During investigations involving the development of a method for isolating the intramuscular connective tissues (McClain, 1969a), it was noted that the yield of this tissue appeared to decrease as the time post-mortem increased. This investigation was designed to study these changes.

MATERIALS & METHODS

THE RIGHT and left longissimus dorsi muscles were removed from 5 choice grade Angus steers (450–500 kg) and 3 cross-bred barrows (90–100 kg). The muscles were removed within 30–45 min after exsanguination, dissected free of adhering fat and connective tissue and placed in a 4°C cooler. Samples were removed at various time periods post-mortem, frozen on dry ice and stored at −40°C.

The method utilized for isolating the intramuscular connective tissues has been described previously (McClain, 1969a). The only deviation from this procedure was the elimination of dry ice in the blending of samples stored at −40°C. The intramuscular connective tissues (IMCT) isolated in the above manner were weighed and the yield expressed as a percent of the wet weight of muscle tissue.

Aliquots of the IMCT were removed for determination of the heat absorbed during thermal shrinkage (ΔHs) and the thermal shrinkage temperature (Ts). The DuPont 900 Differential Scanning Calorimeter (DSC) was utilized for these studies. The T scale was set at 10°C/in., the ΔT setting at 0.2°C/in. and the heating rate was 10°C/min for all DSC determinations. Samples were allowed to thaw and equilibrate in physiological saline for 30 min before determining the ΔHs. 3 to 10 mg of IMCT and 3–4 μliters of 0.9% saline were utilized for the DSC determinations. Aliquots were also removed at this time for hydroxyproline determinations (Woessner, 1961) and the ΔHs values were expressed in terms of grams of collagen in the sample, assuming a hydroxyproline content of 13%.

The pH of the samples at the various time periods post-mortem was determined on 2 g of muscle powder blended with 25 ml of 0.005 M sodium iodoacetate.

RESULTS

RESULTS of the post-mortem study with bovine l. dorsi muscle are shown in Table 1. The initial yield of intramuscular connective tissue was 0.61% of the wet weight of muscle tissue. On a dry weight basis this is equivalent to approximately 1.0% collagen. This value compares reasonably well with those reported by Carmichael et al. (1967) who found values of approximately 1.2–2.2% collagen in bovine l. dorsi muscle. Thus it appears that the isolation method utilized was effective in recovering most, if not all, the connective tissue from the muscles.

The data from Table 1 also reveal that the yield of bovine IMCT at 24 hr had fallen to a value approximately 50% lower than at 0 hr. These differences were significantly different at the 1% level. The yield at 72 hr was comparable to that at 24 hr.

Similar data for porcine l. dorsi muscles at 0 and 72 hr post-mortem are shown in Table 2. The value of 0.44% IMCT agrees reasonably well with the value of 0.68% total connective tissue for porcine l. dorsi reported by Kauffman et al. (1964). The yield of porcine IMCT at 72 hr was approximately 34% less than that at 0 hr ($P < 0.01$). Therefore, post-mortem changes in porcine IMCT were similar to those observed for the bovine, albeit to a lesser extent.

The pH data shown in Tables 1 and 2 indicate that the decrease in recovery of IMCT was accompanied by the drop in pH post-mortem. This finding is substantiated by the data shown in Figure 1, showing plots of yield and pH versus time post-mortem (0, 4, 8, 12, 24, 72 and 168 hr). The yield of IMCT reached a min-

Table 1—Post-mortem pH values[1] from porcine longissimus dorsi muscles and yield[2] of intramuscular connective tissue.

Animal No.	IMCT yield, Post-mortem time (hr) 0	24	72	pH, Post-mortem time (hr) 0	24	72
1	0.85	0.33	0.44	6.20	5.50	5.42
2	0.62	0.32	0.39	6.27	5.41	5.45
3	0.38	0.15	0.18	5.58	5.58	5.47
4	0.55	0.31	0.17	6.51	5.55	5.39
5	0.66	0.40	0.40	6.51	5.62	5.43
Mean	0.61	0.30**	0.31*	6.21	5.53**	5.43**
Std. dev.	0.17	0.09	0.13	0.38	0.08	0.03

*Means significantly different from 0 hr ($P < 0.05$).
**Means significantly different from 0 hr (< 0.01).
[1]Values determined on the muscle powder.
[2]Expressed as a percent of the wet weight of muscle tissue.

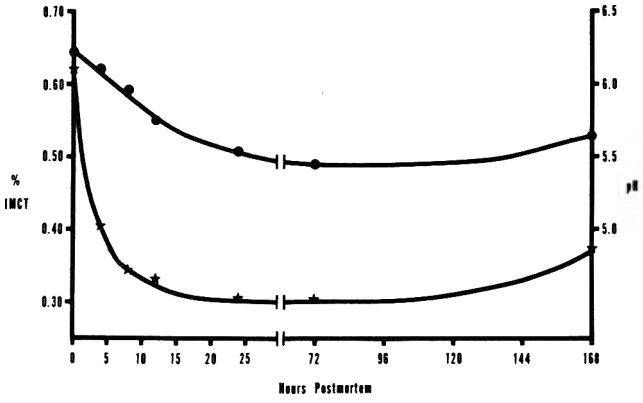

Fig. 1—Relationship between yield of intramuscular connective tissue, time post-mortem[1] and pH values from bovine l. dorsi muscles.

[1] 0 hr = 30–45 min post-mortem.

** = Yield of IMCT. • = pH post-mortem.*

Table 2—Post-mortem pH values[1] from porcine longissimus dorsi muscles and yield[2] of intramuscular connective tissue.

Animal No.	IMCT yield Post-mortem time (hr) 0	IMCT yield Post-mortem time (hr) 72	pH Post-mortem time (hr) 0	pH Post-mortem time (hr) 72
1	0.39	0.31	6.48	5.51
2	0.43	0.26	6.67	5.59
3	0.49	0.29	6.53	5.62
Mean	0.44	0.29**	6.56	5.57**
Std. dev.	0.05	0.03	0.09	0.06

**Mean significantly different from 0 hr ($P < 0.01$).

[1] Values determined on the muscle powder.

[2] Expressed as a percent of the wet weight of muscle tissue.

Table 3—ΔHs[1] and Ts[2] values for intramuscular connective tissue from bovine longissimus dorsi muscles.

Animal No.	Post-mortem time (hr) 0 Ts	0 ΔHs	24 Ts	24 ΔHs	72 Ts	72 ΔHs
1	61.5	6.27	60.0	7.42	60.0	5.87
2	64.5	10.11	63.5	10.14	63.0	12.50
3	63.0	12.79	63.0	15.94	63.0	12.78
4	63.0	10.48	57.0	17.71	62.0	11.63
5	64.0	11.84	63.0	9.62	–	–
6	62.0	9.80	62.0	9.22	–	–
Mean	63.0	10.22	61.4	11.68	62.0	10.70
Std. dev.	1.1	2.24	2.5	4.13	1.4	3.25

[1] Heat absorbed during thermal shrinkage in cal/g.

[2] Thermal shrinkage temperature in °C.

imum at a pH of approximately 5.4, attained within 24–72 hr post-mortem. The relationship of yield to pH is further brought out by noting the data obtained for animal No. 3 (Table 1). This animal inexplicably had an extremely low initial pH value and, consequently, a low yield of IMCT. The slight increase in pH and connective tissue yield at 169 hr post-mortem was a consistent observation (Fig. 1).

The ΔHs and Ts values found for the 0, 24 and 72 hr post-mortem IMCT are shown in Table 3. No significant differences were evident in these parameters, indicating that the secondary and tertiary structure of those tissues which are removed has not been greatly altered (McClain et al., 1968; 1969b).

DISCUSSION

THIS INVESTIGATION has served to emphasize that the intramuscular connective tissues are also changed in some way during the post-mortem aging process, as are the sarcoplasmic and myofibrillar constituents. That the decrease in yield of IMCT actually represents a loss in connective tissue is considered doubtful. Rather, it is more likely that the IMCT becomes more closely associated with the myofibrillar proteins, causing them to be less easily separated during the isolation procedure. Alternatively, it is entirely possible that the connective tissue proteins are altered by the drop in pH, rendering them more easily fragmented and thus resulting in a diminished yield or recovery.

That the ΔHs and Ts values did not change significantly with post-mortem aging indicates that the connective tissues isolated have not been greatly altered.

The nature of the changes occurring in the intramuscular connective tissues with post-mortem aging and the causes of these changes are areas of investigation which should contribute significantly to understanding the nature of meat tenderness.

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SOME POST-MORTEM CHANGES IN PORCINE MUSCLE HELD AT 25°C

SUMMARY—Eight market-weight gilts were used in this study. The animals were slaughtered and a section of the longissimus dorsi muscle extending from the last lumbar vertebra anterior to the 6th thoracic vertebra excised and sliced into chops for samples. Each chop was held for a different period post-mortem (30, 60, 120, 180, 240, 300, 360 or 480 min). Shear force, percent kinkiness, pH, press fluid values and fiber diameter were used to indicate the changes. Differences in shear force due to holding times were highly significant for the right. Shear force values became smaller as pH declined. Fiber diameter and percent kinkiness exhibited a direct relationship; as one decreased so did the other. Shear values decreased with a decrease in fiber diameter and percent kinkiness. Press fluid was not significantly affected by the holding periods. The interaction found was attributed to the varied muscle tension caused by the method used to suspend the carcass during exsanguination.

INTRODUCTION

COMMERCIAL interest in pre-chill processed pork has led to the need for more information concerned with the effect of this process on muscle held at room temperature. Such information could help to determine handling methods for a commercial operation in the event of a line breakdown or similar disorder that would cause the hot pork to remain without refrigeration for extended periods of time.

Variation in the rate and severity of post-mortem change has been reported to have an effect on the subsequent quality of pork muscle. It has generally been considered that the onset of rigor at high temperatures or low pH values is associated with soft, pale and watery pork less tender than normal tissue. Sayre et al. (1964) concluded that shear values were higher for muscles in which the onset of rigor occurred at pH values below 6.0 than for those in which rigor commenced at higher pH values. Lewis (1959) found that stress, which lowered initial glycogen and resulted in a higher muscle pH, was associated with increased tenderness of certain muscles. DeFremery et al. (1960) found that every treatment which resulted in a more rapid loss of ATP, more rapid loss of glycogen or a more rapid decline of pH decreased tenderness. Busch et al. (1967) reported that shear force increased as pH declined at 2°C, but that shear values became progressively smaller as pH declined at 37°C.

Condition of the muscle fiber also has been indicated to have an effect on tenderness. Locker (1960) reported that relaxed muscles were more tender than contracted ones. Tuma et al. (1962) stated that after correcting for age, fiber diameter was a poor indicator of tenderness. Carpenter et al. (1963) indicated that with an increase in maximum fiber diameter there was a decrease in desirability as judged by a taste panel. Hiner et al. (1953) revealed that the order of 9 muscles ranked on fiber diameter was essentially the same as those same muscles ranked on tenderness. Herring et al. (1965) reported that muscles allowed to shorten on the carcass had larger fiber diameters, shorter sarcomeres and were less tender than muscles under strain on the carcass. Gillis et al. (1968) stated that with an increase in degree of rigor (percent kinkiness) there was a corresponding increase in shear force. These workers further stated that with a decrease in fiber diameter, there was also a decrease in shear force.

The objective of this study was to determine the effect of holding hot pork muscle at room temperature (25°C) for extended periods of time on pH, press fluid, shear force, fiber diameter and percent kinkiness.

EXPERIMENTAL

EIGHT MARKET-weight Hampshire gilts were used in this study. The animals were stunned, shackled by one leg, suspended from the ceiling and bled in the normal manner. Immediately upon death (5 min) of the animal, a small incision was made in the skin and fat and a pH reading taken of the longissimus dorsi muscle on both sides. The carcass was then lowered to the floor, skinned, raised, eviscerated and rapidly split. A section of each longissimus dorsi muscle extending from the last lumbar vertebra anterior to the 6th thoracic vertebra was removed (about 18 in.). Each section was sliced into 8, 2-in.-thick chops, tagged according to location. Each chop was placed into a polyethylene bag, the air removed by vacuum and the bag sealed. This was done to simulate the anaerobic conditions of the muscle of an intact carcass. The chops were assigned to the treatments so as to use each number chop at each time period and were held at room temperature

Table 1A—Analysis of variance for pH, shear force, fiber diameter, fiber kinkiness and press fluid of porcine longissimus dorsi muscle.

	pH		Shear force		Press fluid	
	R	L	R	L	R	L
Source	M.S.		M.S.		M.S.	
Animal	0.16**	0.16**	79.94**	32.60**	3.50**	4.26**
Location	0.02	0.06**	11.35	7.97	0.67	0.63
Treatment	0.82**	0.68**	16.89	41.61**	0.59	0.71
Error	0.02	0.02	10.25	9.27	0.71	0.76

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

Table 1B—Analysis of variance for pH, shear force, fiber diameter, fiber kinkiness and press fluid of porcine longissimus dorsi muscle..

	Fiber diameter		Fiber kinkiness	
	R	L	R	L
Source	M.S.		M.S.	
Animal	227.55**	236.55**	272.72*	234.46
Location	27.63	23.34	156.54	68.80
Treatment	30.84	29.52	139.18	183.72
Error	28.17	22.87	93.43	109.02

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

(25°C) for 30, 60, 120, 180, 240, 300, 360 or 480 min after the animal's death.

At each analysis time the chop assigned to a treatment was removed from its bag. pH readings were taken by placing the electrode directly upon the cut surface of the chop at 3 locations and the average of the 3 used as the value for that chop. A ½-in. core was removed for press fluid and histological samples and the remainder of the chop cooked to an internal temperature of 70°C to obtain cores for shear determination. Histological samples were placed in a bottle of buffered 10% formalin solution for later observation. Three 500-mg samples were taken from the ½-in. core, each sample placed on Whatman No. 1 filter paper and pressed for 1 min at 10,000-lb pressure on a Carver press. The total ring area was measured with a compensating polar planimeter.

The histological samples were blended at a slow speed with the blades of the blender reversed, to dislodge but not break the individual fibers. The resulting suspension was placed in a 2-in.-diameter petri dish. Fifty fibers from each sample were measured using a microscope equipped with an ocular micrometer. Each fiber was measured at the widest portion of the fiber that was straight. While the fiber was under the ocular micrometer, a subjective score (0–6 depending upon the condition of fiber) was assigned to each measured fiber. The total for the 50 fibers was then divided by 300 (highest score 50 fibers could receive) to get a percentage kinkiness score.

The cooked chops were placed in a 4°C cooler overnight to allow all shear determinations to be made at the same time and temperature. Two ¾-in. cores were sheared 3 times each, which gave 6 values that were averaged and used as the shear value for that chop.

Table 2A–Effect of holding longissimus dorsi at 25°C on pH, shear force, fiber diameter, fiber kinkiness and press fluid.[1]

	pH		Shear force[2] (lb)		Press fluid	
Minutes	Side R	L	R	L	R	L
30	6.45	6.48	20.04	24.03	61.99	54.77
60	6.47	6.30	20.95	21.27	51.46	55.96
120	6.33	6.22	20.22	21.62	49.83	52.19
180	6.20	6.08	21.28	20.76	49.96	53.56
240	6.03	5.99	19.90	20.94	52.50	51.92
300	5.87	5.81	19.66	18.32	53.40	48.49
360	5.72	5.72	18.41	17.72	51.62	43.47
480	5.62	5.57	17.10	17.28	51.62	43.79

[1] Each value is an average of 8 chops.
[2] Pounds required to shear 3/4-in. core.

Table 2B–Effect of holding longissimus dorsi at 25°C on pH, shear force, fiber diameter, fiber kinkiness and press fluid.[1]

	Fiber diameter (u)		Fiber kinkiness (%)	
Minutes	R Side	L	R	L
30	81.06	80.63	61.99	54.77
60	79.91	76.59	51.46	55.96
120	81.19	75.64	49.83	52.19
180	77.75	77.96	49.96	53.56
240	79.31	78.40	52.50	51.92
300	78.59	77.54	53.40	48.49
360	77.31	76.91	51.62	43.47
480	74.44	74.19	57.62	43.79

[1] Each value is an average of 8 chops.

RESULTS & DISCUSSION

pH

A highly significant difference was found among pH values due to treatment effect (Table 1). Means for pH values indicated a constant decline with increasing time post-mortem (Fig. 1). The highest value was present immediately after death and declined to 8 hr post-mortem (Table 2). The decline was much less rapid during the last 2 hr (Fig. 1). However, it should be noted that at 480 min there was still a decline of pH; thus, the ultimate pH had not yet been reached. A highly significant difference was also revealed for the location of the chop on the left longissimus dorsi muscle, but this source of variation was found nonsignificant for the right muscle. This appeared to be due to some side-by-treatment interaction not isolated in the design.

Shear force

Differences in shear force values due to treatment were found to be highly significant for the left but nonsignificant for the right longissimus dorsi muscles (Table 1). Here again, the interaction was evident. The treatment means for shear force exhibited an interesting relationship to pH in that as pH declined, so did shear force beyond 240 min post-mortem (Table 2). Busch et al. (1967) suggested that the increase in tenderness for muscle held at higher temperatures could be due to high-temperature aging. The early high shear values in this study prompted the belief that slicing the muscle rapidly after death may have caused a decrease in tenderness. In this respect Cagle et al. (1968) substantiated that hot-sliced muscle was less tender than muscle sliced after a 24-hr chill. The increase in shear force was believed due to slicing of the muscle in the hot condition. It may be

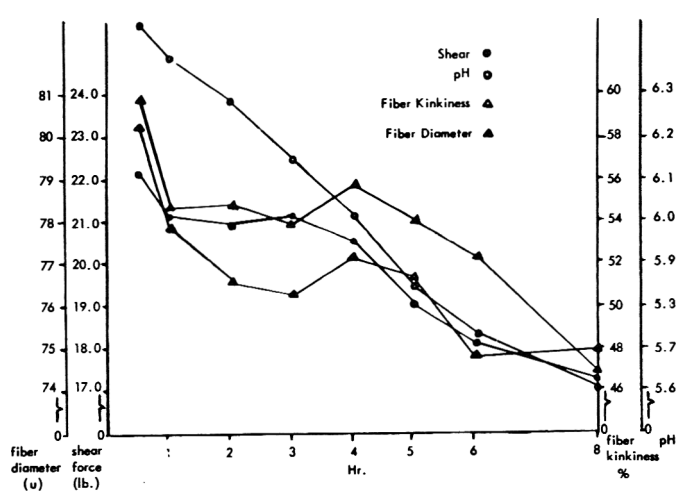

Fig. 1–Relationship of pH, shear force, fiber diameter and percent kinkiness changes in porcine muscle held at 25°C.

that the sudden release of tension caused by slicing the intact muscle while it was still in the elastic state allowed the fibers to contract. There was a slight variation present due to location effect.

Fiber diameter and percent kinkiness

Differences in fiber diameter and percent kinkiness were nonsignificant for location effect (Table 1). However, the "F" values for treatment were considerably greater, which indicated a difference may exist but the measurement was not precise enough for it to be demonstrated. Fiber diameter and percent kinkiness followed essentially the same pattern (Table 2). As fiber diameter decreased, percent kinkiness decreased approximately the same amount (Fig. 1). The early high values for fiber diameter and percent kinkiness lend further support to the theory that slicing the muscle before removal of body heat resulted in contraction of the fibers. No definite relation was apparent for shear force and percent kinkiness. The means revealed a decline of shear force as fiber diameters became smaller and percent kinkiness scores decreased.

There was no significant change in press fluid values with increasing time post-mortem (Table 1). The means showed only a slight variation and were quite erratic from one time period to the next (Table 2).

The apparent interaction of the left side was thought to be caused by the manner in which the animals were suspended during death. Upon examination of the slaughter procedure, it was discovered that the animals were shackled by the left leg and suspended from the ceiling by that leg during exsanguination. A physical strain may have been induced in the muscles of the left side during death. No apparent differences in pH between left and right sides were noted, thus indicating no difference in the rate of biochemical change between the left and right sides. This could cause some of the fibers in the muscles of the left side to contract, which would account for the decreased tenderness of the longissimus dorsi muscles of the left side as found in this study.

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CAROTENOIDS IN 3 STAGES OF RIPENING OF MANGO

SUMMARY—The distribution of carotenoids, both qualitative and quantitative, during 3 stages of ripening of mango has been studied using chromatographic, spectroscopic and chemical methods. There was an increase in content as well as in number of carotenoids during ripening. The present study showed there were 15, 14 and 17 different carotenoids in the unripe, partially ripe and fully ripe mangoes, respectively. Even though phytofluene (39.26%) was the major carotenoid in the partially ripe mango, β-carotene constituted the major carotenoid in the unripe (37.47%) and fully ripe mango (50.64%). cis-β-Carotene was present only in the fully ripe mango. Only the unripe mango contained ξ-carotene, whereas γ-carotene was present in all the 3 stages of ripening. The major xanthophyll present in the unripe mango was mutatoxanthin (9.44%), whereas auroxanthin constituted the major hydroxylated carotenoid of the partially ripe (5.07%) and fully ripe (10.40%) mangoes. The percent of cryptoxanthin dropped to lower levels during ripening. As ripening proceeded, lutein completely disappeared. There were significant quantities of zeaxanthin in the partially ripe and fully ripe mango. Epoxy carotenoids such as 5,6-monoepoxy-β-carotene, mutatochrome, cis-violaxanthin, luteoxanthin, mutatoxanthin and auroxanthin were observed in all 3 stages of ripening.

INTRODUCTION

SADANA et al. (1946), employing chromatographic and colorimetric methods, detected β-carotene, neo-β-carotene, xanthophyll (lutein) and 2 other pigments of hydrocarbon nature in fully ripe mangoes. They studied the changes taking place in the individual carotenoid pigments. Sadana et al. (1949) also reported that during ripening there is a steady increase in the β-carotene and xanthophyll content. Ramasarma et al. (1940) observed a steady and definite increase of the total carotene content during ripen-

Table 1—Total carotenoids in mango.

Stage	Total carotenoids present per 100 g of the fresh pulp (mg)	Total carotenoids present per 100 g of the dry pulp (mg)
Unripe	0.041	1.776
Partially ripe	3.36	15.98
Fully ripe	8.92	48.85

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Table 2–Oxycarotenoids in the 3 stages of ripening of mango.

Chromatographic adsorption analysis and identification of mono- and dihydroxy xanthophylls and their epoxides of unripe, partially ripe and fully ripe mangoes. (The different pigments were eluted according to the order given below in the respective stages.)

Concentration of % (v/v) acetone used for elution	λ max in light petroleum	Partition ratio: Hexane 95% methanol	Partition ratio: Hexane 85% methanol	Partition ratio: Hexane 75% methanol	Partition ratio: Hexane 70% methanol	Iodine isomerization test	λ max after conversion of the 5,6-epoxide to the 5,8-epoxide	Co-chromatography with authentic sample	Identification	% Total carotenoids present: Unripe	Partially ripe	Fully ripe
The first band (containing carotene hydrocarbons and their epoxides) in each stage was eluted with light petroleum containing 20% diethyl ether.												
3–5	425,448,476	86:14	100:00	–	–	*trans*	–	From Hoffmann-La Roche	Cryptoxanthin	1:27	1.41	0.40
6–8	400,425,452	77:23	90:10	–	–	–	–	Synthesized, Subbarayan et al. (1964)	Cryptoflavin	Nil	Nil	0.06
8–10	415,437,463	–	12:88	–	–	*cis*	378,397,423	–	*cis*-Violaxanthin	1.99	0.93	7.08
10–12	430,450,469	21:79	36:64	–	–	–	–	–	Unidentified-450	Nil	Nil	0.75
13–15	422,445,475	10:90	45:55	–	–	*trans*	–	From egg yolk Gillam et al. (1935)	Lutein	4.97	Nil	Nil
16–18	420,444,472	–	13:87	–	–	*trans*	400,422,448	Synthesized, Karrer et al. (1945a)	Anthraxanthin	Nil	0.95	3.00
20–25	411,437,468	–	13:87	–	–	*cis*	398,420,449	–	*cis*-Antheraxanthin	Nil	Nil	0.31
25–30	425,451,479	12:88	37:63	–	–	*trans*	–	From Gul-Mohr Jungalwala et al. (1962)	Zeaxanthin	Nil	0.33	0.29
30–35	400,420,448	7:93	22:78	–	–	–	–	From *Ranuculus acer*	Chrysanthema-xanthin	6:12	0.94	Nil
40–50	380,400,420	5:95	30:70	–	–	–	–	–	Unidentified-400	2.55	Nil	Nil
50–60	398,425,450	–	–	16:84	29:71		379,401,423	–	Luteoxanthin	3.12	1.38	1.93
60–70	400,420,448	–	–	20:80	33:67	–	–	–	Mutatoxanthin	9.44	2.85	3.73
70–80	380,400,425	–	–	–	24:76	–	–	Synthesized, Karrer et al. (1945a)	Auroxanthin	5.02	5.07	10.40

ing, with the maximum value reached at about the 6th day. Furthermore, Ramasarma et al. (1946) showed that Badami mango fruit contains 2 unidentified xanthophylls with absorption peaks at 446 and 493 mμ, and 468 and 497 mμ, respectively.

Jungalwala et al. (1963) carried out a qualitative and quantitative study of the distribution of carotenoids in fully ripe Alfonso mango fruit and reported the presence of 16 different carotenoids. Little information is available about the nature of minor carotenoids in the early stages of ripening of mango; therefore, we made a study of their distribution during ripening to determine their role in the carotenogenesis in mango.

EXPERIMENTAL

FULLY matured mangoes of the Badami variety were collected from a local farm. The pulp (after removing the skin) of the unripe, partially ripe (mangoes kept in an aerated box at room temperature for 5 days) and fully ripe (skin, deep-yellow) mangoes was used.

The extraction of carotenoids, saponification, desterolization and the chromatographic separation of the various carotenoids were carried out as mentioned earlier by Jungalwala et al. (1963).

Identification of carotenoids

Individual carotenoids were characterized by comparing their chromatographic properties, ultraviolet and visible absorption spectra with those of authentic samples. Co-chromatography with authentic samples also helped in the identification of many of the carotenoids.

The xanthophylls were identified by phase partition between hexane and aqueous methanol (95, 85, 80 and 75%–v/v) by the method of Petracek et al. (1956).

Iodine isomerization test by Zechmeister (1960) was carried out to ascertain the *cis-trans* configuration of carotenoids. The test was performed by the addition of 0.5 ml of 0.001% iodine in hexane to 10 ml of a solution of carotenoid in hexane and exposure of the mixture to light from an incandescent lamp for 5, 15 and 30 min. Before and after exposure to light, the ultraviolet and visible absorption spectra were recorded. The λ max of a *cis*-carotenoid after isomerization is shifted slightly to a longer wavelength, while that of a *trans*-carotenoid is shifted slightly to lower wavelength.

Carotenoids with epoxy groups were characterized by the modified ethanolic-HCl test of Jungalwala et al. (1962). Furthermore, epoxy carotenoids were characterized by the blue-green color produced when the dry pigment (solvent-free) and powdered dry mercuric chloride (1:5) were heated in a water bath at 100°C in a closed tube for 3–5 min. The λ max of the complex after dissolving in acetone was noted (Subbarayan et al., 1965; Yamamoto et al., 1961).

Quantitative determination

The weight of the pulp was determined after drying it in air to a constant weight at 80°C. Total carotenoids were determined by dissolving the unsaponifiable matter in a known volume of hexane and measuring the extinction at 445 mμ taking the $E^{1\%}_{1cm}$ for the mixture to

Table 3—Carotene hydrocarbons and their epoxides in the three stages of ripening of mango.

The first band containing the carotene hydrocarbons and their epoxides (in all the 3 stages) on the 1:1 MgO:Celite column was eluted with 20% diethyl ether in light petroleum. Individual pigments were separated by rechromatography of this band on 3% (w/v) water-deactivated alumina. (The compounds were eluted according to their order given below. The identification and the percentage of the total carotenoid present in each stage is also represented in the table.)

Concentration of % (v/v) ether used for elution	λ max in light petroleum	Iodine isomerization test	λ max after conversion of the 5,6-epoxide to the 5,8-epoxide	Co-chromatography with authentic sample	Identification	% Total carotenoid present		
						Unripe	Partially ripe	Fully ripe
–	276,286,294	–	–	From tomatoes Raubourn et al. (1954)	Phytoene	1.27	11.84	6.32
2	332,348,367	–	–	From tomatoes Koe et al. (1953)	Phytofluene	1.75	39.26	11.70
3	447,470	*cis*	–	–	*cis*-β-carotene	Nil	Nil	1.33
5	425,450,475	*trans*	–	From Hoffmann-LaRoche	β-Carotene	57.47	33.55	50.64
8	378,399,424	*trans*	–	From tomatoes Porter et al. (1946)	ξ-Carotene	1.67	Nil	Nil
10	431,458,488	*trans*	–	From Hoffmann-LaRoche	γ-Carotene	0.18	0.15	0.21
15	420,445,476	*trans*	402,420,454	Synthesized, Karrer et al. (1945b)	5,6-Mono-epoxy-β-carotene	1.83	0.04	0.75
20	403,428,448	–	–	Synthesized, Karrer et al. (1945b)	Mutatochrome	1.35	1.30	1.10

be that of β-carotene (2500—Goodwin, 1955).

RESULTS & DISCUSSION

Quantitative experiments

Total carotenoids per 100 g of the fresh pulp as well as the dry pulp for the 3 stages are shown in Table 1. Tables 2 and 3 give the relative amounts of the individual oxycarotenoids and carotene hydrocarbons. The $E^{1\%}_{1cm}$ of the unsaponifiable matter of the unripe, partially ripe and fully ripe mangoes were found to be 10.71, 34.68 and 77.82, respectively. The ratio of carotene hydrocarbons to oxycarotenoids was 6:4 for the unripe, 9:1 for the partially ripe and 7:3 for the fully ripe mangoes.

Table 2 represents the distribution of oxycarotenoids and Table 3 the distribution of carotene hydrocarbons in the unripe, partially ripe and fully ripe mangoes.

Unripe stage

Cryptoxanthin was the first xanthophyll band eluted from the column and showed the absorption maxima at 425, 448 and 476 mμ. It gave a partition ratio of 86:14 between hexane and 95% methanol. *cis*-Violaxanthin was present in small amounts, and on treatment with ethanolic-HCl it was converted to the 5,8,5′,8′-diepoxide. It formed a blue complex with mercuric chloride and exhibited absorption maximum at 635 mμ in acetone. Lutein (λ max 422, 445, 475 mμ) formed a distinguishable orange-red band on the Mgo:Celite column. Chrysanthemaxanthin formed a broad yellow band having λ max 400, 420, 448 mμ and formed a greenish-blue complex with mercuric chloride (λ max 680 mμ).

An unidentified carotenoid with absorption maxima at 380, 400, 420 mμ was found to be present in the unripe mangoes. This unidentified, 400-mμ carotenoid gave a partition ratio of 5:95 between hexane and 95% methanol and 30:70 between hexane and 85% methanol. With mercuric chloride, it gave a light-blue coloration with absorption maxima at 620—625 mμ. This unidentified carotenoid could be a 5,8-epoxide with an hydroxyl group.

Though luteoxanthin and mutatoxanthin formed very clear and distinguishable bands on the Mgo:Celite column, they could not be differentiated spectroscopically, but the former on treatment with ethanolic-HCl was converted to auroxanthin (5,8,5′,8′-diepoxyzeaxanthin). Mutatoxanthin and luteoxanthin formed bluish-green complex with mercuric chloride and exhibited the absorption maxima at 685—690 and 630—635 mμ, respectively. Auroxanthin formed a blue complex with λ max at 635 mμ in acetone.

The band containing the carotenoid hydrocarbons and their epoxides of the unripe mangoes were rechromatographed on 3% (w/v) water-deactivated alumina. Phytoene and phytofluene were collected as a single fraction.

A satisfactory separation between the two was achieved by rechromatography on fully activated alumina. Phytofluene exhibited a green fluorescence under the ultraviolet light. β-Carotene, the major hydrocarbon, was purified by rechromatography. ξ-Carotene (λ max 378, 399, 424 mμ) and γ-carotene (λ max 431, 458, 488 mμ) formed very faint bands on alumina column. 5,6-Monoepoxy-β-carotene was present in small amounts and on ethanolic-HCl treatment it was converted to the 5,8-epoxide (mutatochrome—λ max 403, 428, 448 mμ). Both 5,6-monoepoxy-β-carotene and mutatochrome formed mercuric chloride complexes and gave identical absorption maximum in acetone at 688 mμ.

Partially ripe stage

Antheraxanthin (λ max 420, 444, 472 mμ) formed a light-yellow band after *cis*-violaxanthin. With ethanolic-HCl, it showed a hypsochromic shift of 20 mμ to form the 5,8-epoxide. It formed a bluish-green complex with dry mercuric chloride and in acetone it showed λ max at 690 mμ. Zeaxanthin (λ max 429, 451, 479 mμ) formed a clear orange-red band after

antheraxanthin and gave a partition ratio of 12:88 between hexane and 95% methanol and of 33:67 between hexane and 85% methanol, respectively.

Except for ξ-carotene all the carotene hydrocarbons of the unripe mango pulp were present in the partially ripe mangoes.

Fully ripe stage

Cryptoflavin (5,8-monoepoxycryptoxanthin) was present in small amounts in the fully ripe mangoes. Its mercuric chloride complex in acetone showed λ max at 675 mμ, and phase partition between hexane and 95% methanol and hexane and 85% methanol gave partition ratios of 77:23 and 90:10, respectively. An identified band (λ max 430, 450, 469 mμ) was eluted after *cis*-violaxanthin. It gave negative results with ethanolic-HCl. On the basis of its partition ratios in 95 and 85% methanol, and the chromatographic behavior, one can suspect this unidentified band of being an hydroxy-substituted carotenoid. Only fully ripe mangoes contained *cis*-antheraxanthin.

Unlike the unripe and partially ripe mangoes, small amounts of *cis*-β-carotene (λ max 447, 470 mμ) were present in the fully ripe mangoes.

Table 4 gives the quantitative distribution of carotenoids in fully ripe Alfonso variety mango fruit (Jungalwala et al., 1963).

Table 4—Relative amounts of individual carotenoids in the fully ripe Alfonso mango pulp (Jungalwala et al., 1963).

Carotenoid	% Total carotenoids present
Carotene hydrocarbons	
Phytoene	3.70
Phytofluene	6.89
cis-β-Carotene	0.36
β-Carotene	59.50
γ-Carotene	0.01
Oxycarotenoids	
5,6-Monoepoxy-β-carotene	0.85
Mutatochrome	1.52
Cryptoxanthin	0.66
Violaxanthin	1.25
cis-Violaxanthin	9.02
Antheraxanthin	1.01
cis-Antheraxanthin	0.50
Zeaxanthin	0.01
Luteoxanthin	11.25
Mutatoxanthin	0.76
Auroxanthin	2.71

CONCLUSION

INVESTIGATIONS show there are 15, 14 and 16 different carotenoids in the unripe, partially ripe and fully ripe mangoes. The present study shows that the fully ripe Badami variety of mangoes contains almost the same type of carotenoids as the Alfonso variety (Table 4). It has been observed that the carotenoid content of fruits increases during ripening (banana skin maintains a constant carotenoid content during ripening, as reported by Goodwin, 1952). As in most varieties of mangoes, during ripening there is a tremendous increase of carotenoid content in the Badami variety. Only the unripe mango contained ξ-carotene. In the partially ripe mangoes the carotene hydrocarbons (particularly phytofluene, β-carotene and phytoene) constituted the major part (85%) of the total carotenoids. This observation of the preferential formation of carotene hydrocarbons in the partially ripe stage confirms the results of Ramasarma et al. (1940), that the total carotene content reached a maximum value on the 6th day of ripening and dropped to lower levels later.

The extent of epoxy carotenoids in the unripe and fully ripe mangoes is very high as compared to the partially ripe mangoes. This formation of epoxy carotenoids may be taking place by a mechanism by which lutein (as ripening proceeds lutein disappears completely and cryptoxanthin and zeaxanthin appear) will be converted to cryptoxanthin or zeaxanthin which, in turn, gives rise to the epoxy carotenoids.

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EFFECTS OF PRE- AND POSTMORTEM GLYCOLYSIS ON POULTRY TENDERNESS

SUMMARY—To study the effects of glycolysis on tenderness of poultry breast meat, premortem and postmortem glycolysis in muscle tissue was controlled either by epinephrine administration at suitable intervals of time before slaughtering or by allowing the birds to struggle freely just before and during slaughtering. Premortem glycolysis, occurring as a result of death struggle or epinephrine administration 1–2 hr before slaughtering, lowered the pH of the meat at the time of death and caused toughness. Minimization of postmortem glycolysis by epinephrine administration more than 5 hr before slaughtering increased the ultimate pH of meat and the tenderness. The results indicate that a pH value above 6.2 just after slaughtering, and an ultimate pH near 5.7, are desirable for maintaining quality of poultry breast meat, and that these pH values are maintained by minimum premortem and maximum postmortem glycolysis.

INTRODUCTION

ONE OF THE major problems in the meat industry as well as in meat research is the wide variation in the tenderness of meat between animals. The variability arising from the effects of age, strain and sex, environmental and nutritional conditions to which the animals were exposed during growth and the effects of slaughtering and processing conditions can be minimized by selecting animals of equal age, similar breeding, environmental and nutritional conditions and by slaughtering and processing under identical conditions. This is especially applicable to chickens. Even with the best available controls, however, shear force values for poultry meat have varied by 50% (Stadelman, 1967).

Fasting, muscular activity and emotional stress at the time of slaughter, and thoughtless or rough handling of animals in the immediate preslaughter period have been mentioned as affecting the postmortem glycolysis and the quality of meat (Briskey, 1964; Lawrie, 1966; Rose, 1963). Conditions such as fasting, muscular activity and emotional stress at the time of slaughter accelerate glycolysis, causing the accumulation of lactic acid in the muscle tissue and lowering the pH of the meat. A decrease in pH may affect quality by changing the solubility of proteins and by affecting those enzymes which remain active in the tissue after the death of the animal. The influence of lactic acid content of muscle tissue at the time of death on meat tenderness was therefore studied. In these tests the extent of glycolysis was controlled by injecting epinephrine or by slaughtering birds with varied preslaughter (voluntary) and postslaughter (involuntary) struggle.

EXPERIMENTAL

TESTS were made with pectoralis major muscles from 60 male chickens (Ottawa meat control strain, eviscerated weight 2-2.5 kg) hatched and raised in the laboratory under similar environmental and nutritional conditions. All birds were slaughtered by cutting the jugular vein and carotid arteries, bled, scalded for 2 min at 53–54°C, plucked by hand, eviscerated and aged in drained crushed ice. During aging the temperature of the breast muscles was measured with thermocouples.

Three main test conditions were used: One group of birds was slaughtered under conditions designed to minimize voluntary as well as involuntary struggling. These birds were kept in a small cage for 2–3 hr to allow them to rest and were then restrained in metal funnels (with manual restraint of the legs) during slaughtering and bleeding. A second group of birds was hung by the legs as in commercial practice, and allowed to struggle freely just before and after slaughtering. The third group of birds was administered an intramuscular dose of 4 mg of epinephrine/kg body weight, allowed to rest for a time (1–20 hr as desired) and then slaughtered in the same manner as the first group.

The tenderness of breast muscle was determined by shear force measurement using a Warner-Bratzler type apparatus (Black et al., 1931). Samples of pectoralis major from one half of the carcass were tested after 24 hr of aging, and samples from the other half after 48 hr. All samples for shear force measurement were obtained from the same location in the muscle. To reduce uncontrolled distortion of excised muscle samples during cooking, the samples were clamped in a special mold designed according to deFremery et al. (1960), and cooked to an internal temperature of 82–85°C as described by Khan et al. (1965). Strips 1-cm square in cross section were cut and 10 to 16 determinations made on each sample.

Pectoralis major muscles were analyzed for lactic acid content by an enzymatic method (Olson, 1962) and for glycogen content with anthrone reagent (Roe et al., 1966). pH measurements were made at suitable intervals during the 48-hr aging period.

RESULTS

THE EFFECT on glycogen and lactic acid content in the muscle caused by administration of epinephrine at various times before slaughter is shown in Figure 1. The glycogen content of the muscle tissue decreased rapidly to a minimum value during the initial 12-hr period, whereas the lactic acid content of the muscle tissue increased rapidly during the first 2 hr and decreased thereafter to a minimum value. After a period of 12 hr the effects of epinephrine wore off and both glycogen and lactic acid started to accumulate in the muscle tissue. Based on these results two delay periods between epinephrine administration and slaughtering were selected to study the effects of glycolysis on meat tenderness. A 2-hr period was selected to allow sufficient time for lactic acid to accumulate in muscle tissue, simulating conditions comparable to those in muscle from birds exhausted as a result of struggle or fear immediately before slaughter. A 5-6-hr delay was selected to allow time for the transport of lactic acid from the muscle to the liver, simulating muscle pH and

Table 1—Effects of death struggle and epinephrine injection on lactic acid content and tenderness of breast meat. (Values are averages of eight birds, range or standard deviation shown in brackets.)

Method of slaughtering or treatment before slaughtering	Lactic acid after slaughter mMoles/100 g muscle	Ultimate pH	Shear force value kg	
			24 hr Post-mortem	48 hr Post-mortem
With minimum struggle	3.8 (2.7–4.9)	5.7	1.1 (0.2)	1.0 (0.2)
With unrestricted struggle	8.6 (8.3–9.0)	5.7	2.5 (0.5)	2.1 (0.4)
Epinephrine injected 2 hr before slaughter	6.3 (4.7–8.0)	5.7	1.6 (0.3)	1.5 (0.2)
Epinephrine injected 6 hr before slaughter	3.2 (2.6–4.0)	6.5	0.8 (0.2)	0.7 (0.2)

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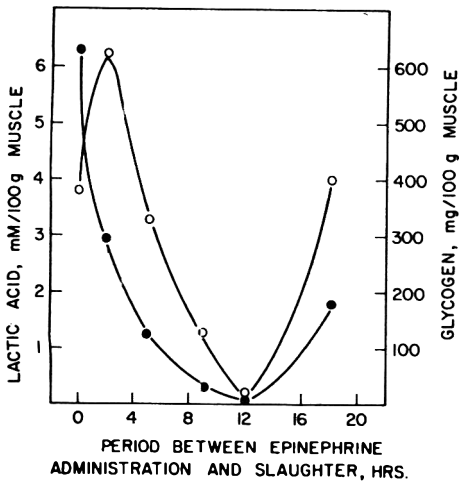

Fig. 1–Effects on lactic acid and glycogen content of breast muscle, immediately after death, of delay period between epinephrine administration and slaughter. O, lactic acid content, ●, glycogen content.

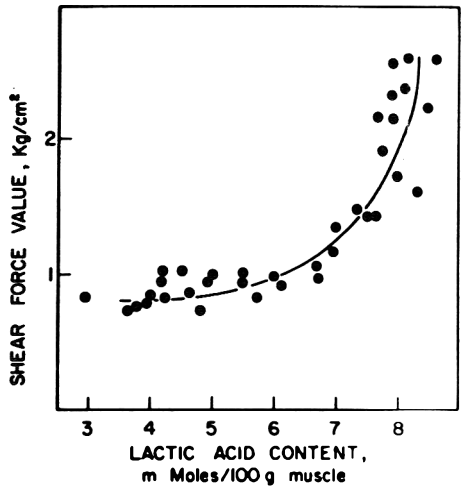

Fig. 2–Relation between lactic acid content immediately after death and shear force value after 24 hr of aging in chicken breast muscle.

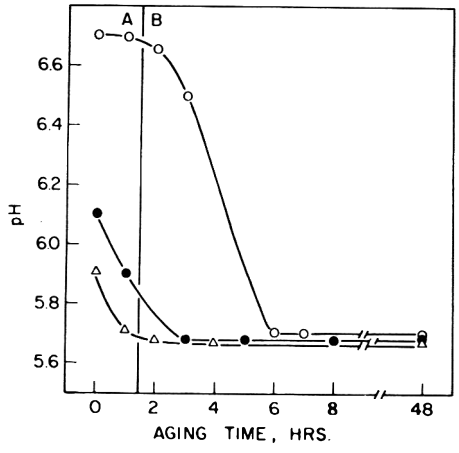

Fig. 3–Effects of aging time, struggle and epinephrine injection on pH of breast meat. O, birds killed with minimum struggle, △, birds killed with unrestricted struggle, ●, birds killed 2 hr after epinephrine administration. (A, period during which temperature dropped from 40 to 2°C; B, period during which temperature was maintained between 0 and 2°C.)

glycogen contents comparable to those found in birds starved or fatigued some time before slaughter.

Accelerated glycolysis occurring as a result of voluntary and involuntary struggle or epinephrine administration immediately before slaughtering increased the accumulation of lactic acid in the muscle tissue at the time of death and gave less tender meat (Table 1). Accelerated glycolysis followed by a resting period during which the muscle lactic acid was transported to the liver, on the other hand, raised the ultimate pH of meat and increased the tenderness. Since the effects of epinephrine and death struggle vary from bird to bird, and are difficult to reproduce, a wide variation in the lactic acid content of the samples was noted. However, the range of lactic acid and shear force values differed markedly between test conditions.

The relation between lactic acid content of the muscle tissue immediately after slaughter and its shear force value after 24 hr of aging is shown in Figure 2, where results from birds from all 3 test groups are included. Lactic acid contents up to 6-7 mMoles/100 g muscle, which corresponded to pH values of 6.1-6.2, had little effect on tenderness, but higher lactic acid contents had an adverse effect.

Ultimate pH was not affected by struggle or epinephrine injection administered less than 2 hr before slaughter, but a longer aging period was required for the pH to drop to the ultimate value when struggle was restricted (Fig. 3). In all of the tests the temperature of the carcasses dropped to about 2°C during the first 1.5 hr of aging and remained at 0–2°C thereafter. In birds killed without struggle approximately all of the pH drop occurred after the meat had cooled to 0–2°C, whereas in the other samples, the pH had dropped below 6.1 before cooling was completed. Since a low pH at temperatures near 0°C had no harmful effect in the former samples, the increased toughness in the latter samples resulting from low pH appeared to occur while the muscle was still warm.

DISCUSSION

THE RESULTS indicate that accumulation of lactic acid in muscle tissue immediately before and after slaughter causes changes in some muscle tissue components and renders the meat tough. The nature of these changes is not understood at present, but accelerated glycolysis is known to be caused by factors such as fear and muscular activity immediately before slaughter, and by death struggle that occurs when birds are hung by the legs and allowed to struggle freely. It is difficult, of course, to control the physiological state of the animal completely, but acceleration of premortem glycolysis can be minimized by careful handling of the birds immediately before slaughter and by restraining them during slaughter. Epinephrine administration more than 2 hr before slaughter, exhaustion or starvation of meat animals before slaughter has been shown to minimize postmortem glycolysis, increase the ultimate pH and the tenderness of meat (Bate-Smith et al., 1949; deFremery, 1966; deFremery et al., 1963). Since a high ultimate pH of the meat enhances microbial growth (Gibbons et al., 1950), and causes dark-cutting beef (Kidwell, 1952), postmortem glycolysis is necessary for maintaining a low pH. The present study shows that minimization of premortem glycolysis in well-fed and well-rested poultry would also ensure adequate postmortem glycolysis and increase tenderness.

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EFFECT OF TREATMENT OF PRE- AND POST-RIGOR PORCINE MUSCLES WITH LOW SODIUM CHLORIDE CONCENTRATIONS ON THE SUBSEQUENT EXTRACTABILITY OF PROTEINS

SUMMARY—Comparisons were made of the extractable salt-soluble protein content between pre- and post-rigor normal- and low-pH muscles. Pre-rigor normal-pH muscle was found to contain 69.9% more extractable salt-soluble protein than post-rigor normal-pH muscle, whereas pre-rigor low-pH muscle contained only 7.3% greater extractable salt-soluble protein than post-rigor low-pH muscle. Addition of 1, 2 and 3% sodium chloride to meat samples 24 hr before extraction of protein increased the extractable salt-soluble protein content in pre-rigor normal-pH muscle, and decreased the extractable salt-soluble protein content in post-rigor normal-pH muscle. Addition of sodium chloride decreased the extractable salt-soluble protein content in pre- and post-rigor low-pH muscle.

INTRODUCTION

MICROSCOPIC examination of sausage emulsions by Hansen (1960) revealed that a protein film enclosed fat globules to form the emulsion. Further work by Swift et al. (1961) and Trautman (1964) indicated that the protein membrane formed by salt-soluble proteins consisted of thicker layers than those formed by water-soluble proteins. The salt-soluble proteins are more effective fat emulsifiers than salt-soluble residues and water-insoluble proteins (Trautman, 1964). Hegarty et al. (1963) found that fibular proteins (actin, myosin and acto-myosin) had greater emulsifying capacities than did the sarcoplasmic proteins. However, at the pH of normal meat, 24 hr after being chilled, (pH 5.60–5.80) the sarcoplasmic proteins produced the most stable emulsions. It seems clear now that water-soluble proteins have low emulsifying capacity, but Swift et al. (1961) found that addition of sodium chloride to the meat enhanced the proteins to stabilize emulsions. One reason for this increased emulsifying capacity was considered by Swift et al. (1963), to be due to the unfolding of the protein structure, which extended its ability to enclose fat globules. Borton et al. (1968) reported that sodium chloride added to beef cheek meat approximately 20 hr before emulsion preparation did not increase the emulsifying capacity of the meat when based on the amount of oil emulsified per gram of sample, but did increase the emulsifying efficiency of the meat when based on a unit of protein. Saffle et al. (1964) used salt-soluble protein content as a measure of emulsifying capacity. They also reported that any rise in the meat pH away from the iso-electric point of the meat protein resulted in an increased amount of salt-soluble protein which could be extracted.

Purposes of this study were to 1) compare the extractable salt-soluble protein content of pre- and post-rigor muscle and 2) determine which of 3 sodium chloride levels, added to ground pre- and post-rigor porcine muscle, would give the greatest extractable salt-soluble protein content. Extractable salt-soluble protein content was used as the measure of emulsifying capacity.

EXPERIMENTAL

ELEVEN MARKET-weight hogs all with normal (pre-rigor)-pH muscle (pH 6.2 at 30 min post-mortem) and 4 market-weight hogs all with low (pre-rigor)-pH muscle (pH 5.8 at 30 min post-mortem) were used. The animals were from a similar managerial background. Slaughtering procedure was accomplished in the conventional manner, except that all animals were skinned to allow for a reduced dressing time. Pre-rigor muscle in this study is defined as that muscle analyzed within 2 hr post-mortem. Post-rigor muscle is that chilled muscle which has either been analyzed or treated approximately 26 hr post-mortem.

Sampling procedure

A 5-rib section of the loin was removed approximately 15 min post-mortem. The longissimus dorsi muscle was ground once through a 3-mm plate and thoroughly mixed. This study was conducted in 2 parts. Part I provided a comparison of the extractable salt-soluble protein content in pre- and post-rigor muscle. Part II provided a comparison of the extractable salt-soluble protein in pre- and post-rigor muscle treated with 3 sodium chloride levels.

Part I. Approximately 45 min post-mortem, duplicate 10-g pre-rigor samples were analyzed for extractable salt-soluble protein content. 24 hr later duplicated post-rigor samples were analyzed for extractable salt-soluble protein content.

Part II. Approximately 2 hr post-mortem, duplicate 10-g pre-rigor samples were treated with 0, 1, 2 and 3% sodium chloride, chilled at 2°C for 24 hr and analyzed for extractable salt-soluble protein content 24 hr later (26 hr post-mortem). Also, duplicate 10-g post-rigor samples were treated with the 3 levels of sodium chloride, chilled at 2°C for 24 hr and analyzed for extractable salt-soluble protein content (48 hr post-mortem).

The pH of the muscle was taken approximately 30 min post-mortem for the pre-rigor muscle and 24 hr post-mortem for the post-rigor muscle.

Salt-soluble protein extraction

Reference to salt-soluble protein in this paper includes any protein soluble in 3% salt-solution which includes the water-soluble proteins, water-insoluble proteins and salt-soluble proteins. The procedure used for protein extraction was similar to that of Saffle (1964). Extractions of the salt-soluble protein were accomplished using a 3% saline solution, added to muscle in a ratio of 8:1. The 10-g sample and 80 ml of extraction solution were blended 4 min in a Sorvall Omni-mixer. The homogenate was centrifuged for 10 min at 16,300 × g in a Serval refrigerated ultracentrifuge. The supernatant was decanted and again centrifuged for 10 min at 16,300 × g. The supernatant was filtered into a 200-ml volumetric flask and the fluid made to volume with distilled water. The protein solution was then thoroughly mixed. All extraction procedures were carried out at 2°C and all chemicals chilled to 2°C.

Salt-soluble protein determination

The procedure used to determine extractable salt-soluble protein content was by the biuret test (Layne, 1957).

Table 1—Extractable salt-soluble protein content in pre- and post-rigor normal- and low-pH muscle.

Muscle type	Animal No.	Pre-rigor[1]		Post-rigor[2]	
		Protein[3]	pH	Protein	pH
Normal-pH	11	114.45	6.44	67.48	5.40
Low-pH	4	72.04	5.71	67.14	5.39

[1,2]Difference ($P < .01$) between muscle condition.
[3]Expressed in milligrams of protein per gram of sample.

Table 2–Extractable salt-soluble protein content in pre- and post-rigor normal- and low-pH porcine muscle treated with 3 sodium chloride levels.

Muscle Type	Animal No.	Pre-rigor[1]				Post-rigor[2]			
		Sodium chloride							
		0[3]	1	2	3	0[4]	1	2	3
Normal-pH (mg/g)	11	70.02	72.37	78.01	78.19	68.07	67.28	66.21	63.59
Low-pH (mg/g)	4	66.76	64.73	62.9	61.25	66.14	64.92	62.31	60.17

[1]Differences ($P < .01$) among sodium chloride levels.
[2]Percent sodium chloride of sample by weight.
[3]Sodium chloride added 30 min after death and protein extracted 24 hr later.
[4]Sodium chloride added 24 hr after death and the protein extracted 48 hr after death.

Statistics

The data were analyzed by use of the F-test and by making orthogonal comparisons (Steel et al., 1960). The same analysis was run on both populations.

RESULTS & DISCUSSION

EXTRACTABLE salt-soluble protein content is expressed as milligrams of salt-soluble protein per gram of sample.

Part I. A highly significant difference was found in the extractable salt-soluble protein content between the pre- and post-rigor normal-pH muscles. This was to be expected and is in agreement with the findings of Saffle (1964), who worked with beef. The extractable salt-soluble protein content in pre-rigor normal-pH muscle was found to be 69.6% greater than that in the post-rigor muscle. The pH of the pre-rigor muscle was much farther away from the iso-electric point of the muscle proteins than the pH of the post-rigor muscle, thus allowing for greater salt-soluble protein extraction.

The difference in extractable salt-soluble protein content between pre- and post-rigor low-pH muscles also was significant. This was to be expected, since the pH of the pre-rigor muscle was higher that that of the post-rigor muscle. It should be noted that the pre-rigor low-pH muscle contained only 7.30% more extractable salt-soluble protein than the post-rigor low-pH muscle, whereas the pre-rigor normal pH muscle contained 69.6% greater extractable salt-soluble protein than the post-rigor muscle. This was anticipated, since the difference between the pH of the pre- and post-rigor low-pH muscles was much less than the difference between the pH of the pre- and post-rigor normal-pH muscles (Table 1). These data indicate that pre-rigor normal- and low-pH meat will possess greater emulsifying ability than post-rigor normal- and low-pH meat.

Part II. Differences in the extractable salt-soluble protein content among the 3 sodium chloride levels were found to be highly significant. A test of linearity proved highly significant, showing that as the added amount of sodium chloride increased, so did the amount of extractable salt-soluble protein. The test for the quadratic effect was found to be nonsignificant. The 1% sodium chloride treatment increased the amount of extractable salt-soluble protein 3.35% over the zero level, whereas the 2 and 3% salt treatments increased the extractable salt-soluble protein content 11.4 and 11.7%, respectively. The post-rigor muscle proteins are tied up in the actomyosin complex and the pH of the post-rigor muscle is at the iso-electric point of the muscle proteins; thus, the proteins are in a less soluble state when the sodium chloride is added. It appeared that as more sodium chloride was added an even further decrease in solubility of the post-rigor muscle proteins occurred (Fig. 1). This indicated that less salt-soluble protein was available in the sodium chloride–treated meat than in the nontreated meat. These results indicated that pre-rigor normal-pH meat with 3% sodium chloride added 24 hr before emulsion preparation has a greater emulsifying ability than normal chilled meat with no added sodium chloride. They also indicated that addition of sodium chloride to chilled post-rigor normal-pH meat decreased the emulsifying ability of the meat.

Significant differences were found among the sodium chloride treatments in extractable salt-soluble protein content in pre-rigor low-pH muscle (Table 2). The test for linearity was found to be highly

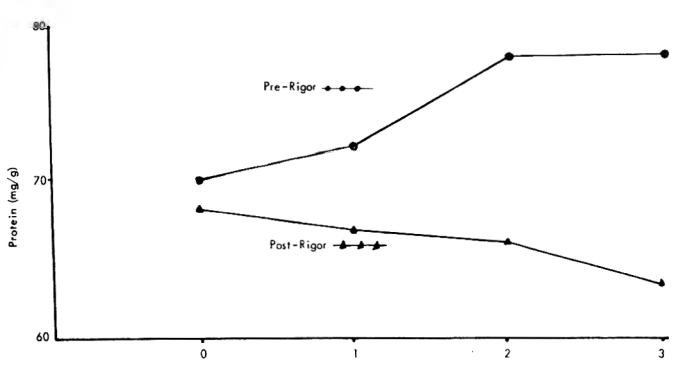

Fig. 1–Extractable salt-soluble protein content in pre- and post-rigor normal-pH porcine muscle treated with levels of sodium chloride. Means at the zero level are not significantly different.

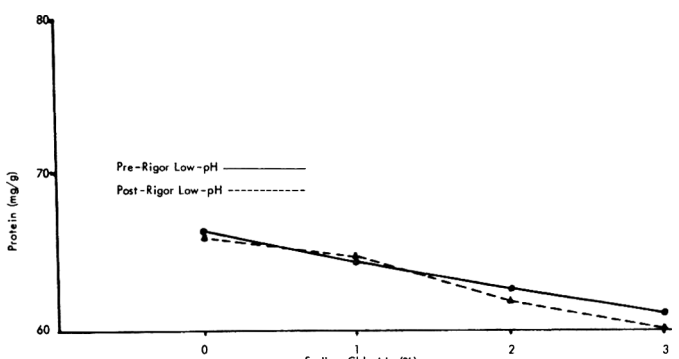

Fig. 2–Extractable salt-soluble protein content in pre- and post-rigor low-pH porcine muscles treated with levels of sodium chloride.

significant, indicating that as the added amount of sodium chloride increased, the amount of salt-soluble protein which could be extracted decreased. The 1, 2 and 3% sodium chloride treatments contained 3.14, 6.07 and 9.00% less extractable salt-soluble protein than did the 0% level, respectively. One explanation for these results is that the pH of the pre-rigor low-pH muscle was very low, nearing the iso-electric point of the muscle proteins; thus, the proteins were in a less soluble state, approximating that of the proteins of the post-rigor normal-pH muscle. As the amount of added sodium chloride was increased, the solubility of the proteins was decreased to an even greater extent. The test for the quadratic effect was found to be nonsignificant. Approximately the same results were found for the post-rigor low-pH muscle at the 3 sodium chloride levels (Table 2 and Fig. 2). These data indicated that to obtain the greatest emulsifying ability from pre- and post-rigor low-pH meat, no sodium chloride should be added to the meat before emulsion preparation.

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INFLUENCE OF SLICING WARM PORCINE MUSCLE ON FIBER DIAMETER, KINKINESS AND SHEAR FORCE

SUMMARY—Ten market-weight Hampshire pigs were used to test 2 methods of fabricating hot-processed pork loins. Longissimus dorsi muscle sliced before removal of body heat had significantly higher shear values than the corresponding muscle sliced after a 24-hr chill. This difference was believed to be due to the contraction of the muscle fibers caused by slicing the muscle soon after death. Fiber diameter and percent kinkiness were also significantly affected by method of slicing, but the larger fiber diameter and greater percent kinkiness were found for the cold-sliced muscle. An interaction between side and treatment was attributed to the manner in which the animal was suspended during death.

INTRODUCTION

CONSUMER demand for a high-quality, reasonably priced, ready-to-eat or ready-to-cook product has led to many recent changes in the meat-processing field. One such process drawing considerable attention in recent years is hot-processing of pork. It has been substantiated (Mandigo et al., 1966; 1967; Moore et al., 1966; and Barbe et al., 1967) that the hot-processing method yielded an acceptable product when chilled or cured in the wholesale cut form.

Locker (1960) found that different muscles of the carcass entered rigor mortis at widely different states of contraction. He postulated that the final state of the muscle, in respect to contraction, may be dependent upon the strain imposed on it in the hanging carcass. He further stated that the state of contraction could be modified by cutting or excising the muscle. Reddy et al. (1967) discovered that pre-rigor excised bovine longissimus dorsi muscle exhibited a higher value for percentage rigor (percent kinkiness) than the post-rigor excised muscle from the opposite side. Herring et al. (1965) reported that vertical suspension of the carcass permitted the longissimus dorsi to shorten. They also found larger fiber diameters and higher shear values for muscles that shortened on the carcass. Muscles under strain increased in length, had smaller fiber diameters and lower shear values than those that shortened. Gillis et al. (1968) revealed that induced tension up to the 1,000-g pull had a highly significant effect on fiber diameter. Also, fewer rigor kinks and decreased shear values were revealed for the 1,000-g pull sample over the unrestricted sample.

The objective of this study was to determine the effect of slicing hot pork loins into retail chops before any chilling. This would eliminate the initial chill and allow the finished product to chill faster, thus adding to the economy. Mechanical shear force, fiber diameter and percent kinkiness were used as test criteria for the warm-sliced vs. the cold-sliced samples.

EXPERIMENTAL

TEN market-weight Hampshire pigs of similar feeding and genetic background were obtained from the Oklahoma Agricultural Experiment Station herd for this experiment. The animals were stunned with an electrical tool, shackled by one leg, raised from the floor, bled, skinned and dressed in the conventional manner. Immediately after being dressed (15 min) the carcasses were split; a section of the longissimus dorsi extending from the last lumbar vertebra to the 6th thoracic vertebra was removed from both sides. One of the sections from each carcass was then sliced into 6, 2-in.-thick chops. Each chop was tagged and placed on a flat tray with the corresponding intact muscle section. Muscles from the right and left sides were alternately hot-sliced. The difference in suspension time was only during the sticking and bleeding period. The samples were then placed into a −29°C freezer for approximately 30 min to allow them to chill rapidly. After chilling, the samples were placed in a 4°C cooler for 24 hr at which time the analysis was started. As near as possible to 24 hr after the animal was killed the chilled intact muscle sections were sliced into chops identical to the hot-sliced sections. A sample for histological work was removed from the center of each chop and placed in a bottle of buffered 10% formalin solution for observation at a later date. The remainder of the chop was then placed in a deep-fat fryer with oil preheated to 135°C and cooked to an internal temperature of 68°C. It was then placed in a 2-in. pan, covered, and the temperature allowed to equilibrate to 4°C overnight. Shear determinations were made the following day. Two ¼-in. cores were removed from the cooked cooled chop. Each core was sheared 3

Table 1–Influence of slicing high-temperature porcine muscle on shear force, fiber diameter and fiber kinkiness.[1]

		F-Value		
Source	df	Shear force F	Fiber diameter F	Fiber kinkiness F
Total	19			
Right side				
X	1	13.79**	3.52	0.22
Left side				
Animal	8	3.28	8.98**	0.48
Side	1	9.14*	0.35	0.84
Hot vs. cold	1	19.01**	7.17**	6.47*
Error	8			

*P .05.
**P .01.

[1]High-temperature refers to slicing the longissimus dorsi muscle within 15 min after death. 3/4-in. cores of cooked muscle were used in the Warner-Bratzler shear machine.

Table 2–Mean for 3 properties of hot- vs. cold-sliced porcine longissimus dorsi muscle.

		Hot	Cold
Shear value	(kg)	12.33	9.77
Fiber diameter[1]	(μ)	73.6	76.4
Kinkiness	(%)	36.9	39.6

[1]Measured using an ocular micrometer.

times with the Warner-Bratzler shear device. This gave a total of 6 shear determinations which were averaged to give the value used for that chop. The histological samples were analyzed within 1 wk of slaughter. Fiber diameter and percentage kinkiness were observed using a binocular light microscope equipped with an ocular micrometer. Twenty-five fibers were measured at their widest point and averaged for each sample. Kinkiness was a subjective value assigned to a fiber by using a set of photographic guides. A value of 0 to 6 was assigned depending upon the condition of the fiber. The total for the sample was then divided by 150, the highest possible value 25 fibers could receive to give a percentage kinkiness figure for the sample.

DISCUSSION

FIBER diameter was variable and associated closely with animal difference. The interaction of side by treatment was also found to be significant ($P < .01$) for shear force; whereas, treatment effect was revealed to be highly significant for shear force and fiber diameter, but less influential on ($P < .05$) percent kinkiness.

Means for all 3 characteristics reveal a rather large difference in shear force in favor of the cold-sliced muscle. However, cold-treated muscle possessed the largest fiber diameters and the greatest percent fiber kinkiness. This appeared contrary to previous reports which stated that large fibers are associated with the less tender muscle. It may be that the state of contraction of the fiber has considerable effect on the fiber size–tenderness relationship. Locker (1960) reported that contracted muscles were less tender than relaxed ones. It could be that muscles with larger fibers are not any less tender than muscles with smaller fibers provided they are in the same state of contraction. Muscles with fibers that are large because they are contracted may be less tender than muscles with smaller fibers that are relaxed.

There appeared to be an interaction between side and treatment for shear force. This apparent interaction was also reported by Cagle et al. (1968) in a rigor mortis study in which they found a significant difference in the left side due to treatment effect, but none in the right side. Upon examination of the slaughter procedure, it was discovered that the animals were shackled by the left leg and suspended from the ceiling by that leg until death occurred from bleeding. The possibility existed that a physical strain may have been induced in the muscles of the left side due to the manner in which the animal was suspended during the death struggle. This could result in the decreased tenderness of the muscles of the left side as found in this study. The similarity of the cold sides could result because a muscle can contract only a limited amount. The right muscle contracted more due to rigor mortis than did the muscle of the left side, which was already partially contracted during the suspension by the left leg. Both sides reached about the same level of contraction, however, before being sliced cold 24 hr after death of the animal.

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EFFECT OF PSYCHROTOLERANT BACTERIA ON THE AMINO ACID CONTENT OF CHICKEN SKIN

SUMMARY—Changes induced by type-cultures of Achromobacter, *nonpigmented* Pseudomonas, *pigmented* Pseudomonas *and a mixture of all three types in the amino acid content of fresh chicken skin were studied during storage at 5°C for up to 21 days. The* Achromobacter *and nonpigmented* Pseudomonas *cultures reduced the amount of all amino acids to below detectable levels during the early log phase of growth and produced no detectable change subsequently. The pigmented* Pseudomonas *caused no appreciable change initially but produced a marked increase in the amount of most amino acids during the late log phase, after off-odor had developed; an increase in free and total extractable proline and hydroxyproline indicated that collagenous proteins were attacked. The mixed inoculum gave intermediate results, producing comparatively little change in the total free amino acid content throughout the incubation period. Results indicate that the pigmented pseudomonads are the most proteolytic of the common types of psychrotolerant spoilage bacteria and that they possess collagenase.*

INTRODUCTION

PREVIOUS studies (Adamčič et al., 1969) on bacteria-induced biochemical changes in chicken skin showed that psychrotolerant spoilage organisms (achromobacteria and pseudomonads) utilize low-molecular-weight nitrogenous compounds during the log phase of growth and later replenish these compounds through degradation of proteins during the late log or stationary phases. To understand these biochemical changes more clearly, further study was made to determine the effect of these organisms on the amino acid content of chicken skin and to determine whether collagen is attacked. This paper reports the results of this study, part of a larger project dealing with biochemical and quality changes that occur in stored poultry meat. The skin is emphasized in the microbial studies because it is the tissue that is attacked most readily by psychrotolerant bacteria (Lochhead et al., 1935; Ziegler et al., 1954).

The work included qualitative and quantitative chromatographic analyses of the free amino acids of skin inoculated with pure strains of *Achromobacter* and pigmented and nonpigmented *Pseudomonas*. Quantitative chromatographic analysis of the total extractable amino acid content (free amino acids plus amino acids from extractable proteins and peptides) of skin inoculated with pigmented *Pseudomonas* was also included. In addition, chemical analyses for changes in both the free and total proline and hydroxyproline content of inoculated skin were made to determine collagen degradation. Nearly all of the proline and hydroxyproline content of skin tissue is contained in collagen (Harkness et al., 1954; Rothman, 1954).

MATERIALS & METHODS

PROCEDURES for obtaining, preparing and inoculating the skin have been described previously (Adamčič et al., 1969). Briefly, fresh skin from 9-week-old male chickens (Ottawa Meat Control strain) was defatted, frozen in liquid nitrogen, ground, irradiated (500,000 rads, sufficient to kill the psychrotolerant bacteria present), inoculated (200,000 cells/g), dispensed in 6-g samples into Petri dishes and incubated aerobically at 5°C for up to 21 days. The inoculum was composed of pure cultures of psychrotolerant bacteria isolated from the skin of commercially processed poultry (Clark et al., 1969). In most tests, a single strain each of pigmented *Pseudomonas* (strain 1), nonpigmented *Pseudomonas* (strain 47) and *Achromobacter* (strain 89) and a mixed inoculum composed of 10 strains of *Achromobacter*, 5 strains of nonpigmented *Pseudomonas* and 2 strains of pigmented *Pseudomonas* were used. In one experiment 10 strains of pigmented *Pseudomonas* were tested individually for collagenolytic activity. Twenty-four-hour-old cells grown on SM agar (Difco) at 20°C and prepared as described previously by Clark (1968) were used to prepare the inoculum in all tests. Control samples consisting of irradiated but uninoculated skin were included in all experiments.

Samples were analyzed at regular intervals for changes in the free or total extractable amino acid content by paper and column chromatography, and for changes in the free or total extractable proline and hydroxyproline content by chemical analysis. To extract the amino acids, 5 g of skin was ground with 20 ml of water in a Sorvall Omni-mixer for 2 min (50-ml-capacity cup, 5000 rpm), and the resultant slurry filtered (Whatman No. 1). For chromatographic analysis of the free amino

Table 1—Effect of bacteria on the free amino acids in chicken skin (incubation 7 days at 5°C).

Compound	Amount, μg/g skin				
	Control	Ps. 1	Ps. 47	Achrom. 89	Mixed inoculum
(Taurine)	(120.2)	(46.3)	(81.3)	(92.6)	(63.8)
Hydroxyproline	32.8	11.8	0	0	6.6
Aspartic Acid	70.5	93.2	2.6	2.6	69.2
Threonine	32.1	40.5	0	0	47.6
Serine	89.3	35.7	2.1	1.1	92.5
Glutamic Acid	244.2	245.7	2.9	2.9	101.52
Proline	57.6	56.4	0	0	33.4
Glycine	84.1	81.8	1.5	1.5	69.1
Alanine	80.2	121.2	0	0	84.6
Valine	30.5	86.7	0	0	64.4
Half-cystine	15.4	0	0	0	1.9
Methionine	16.4	53.7	0	0	38.8
Isoleucine	19.7	68.2	0	0	51.2
Leucine	44.6	149.5	0	0	112.8
Tyrosine	43.5	61.6	0	0	56.2
Phenylalanine	28.1	72.7	0	0	54.5
Ornithine	–	129.5	0	0	99.1
Lysine	52.6	149.1	0	0	90.6
Histidine	15.5	27.9	0	0	31.0
Arginine	67.9	13.9	0	0	5.2
Totals (taurine excluded)	1,025.0	1,499.1	9.1	8.1	1,110.2

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acids, the filtrate was treated with 2 volumes of absolute ethanol for about 16 hr at 5°C to precipitate proteins, then filtered. The filtrate was evaporated to dryness under vacuum and the residue dissolved in 2 ml of water.

Part of this concentrated solution was used directly for paper chromatographic analysis (method of Hanes et al., 1961), and the remainder further purified by treatment with an equal amount of 10% TCA for quantitative analysis on a Technicon automatic amino acid analyzer. The analyzer was equipped with a 140- by 0.6-cm column of chromobead type B resin. Aliquots of 0.05 ml of the filtered TCA-treated solution were analyzed in a 22-hr elution procedure. For chromatographic analysis of the total extractable amino acid content, a 5-ml sample of the original filtered water extract was hydrolyzed with 5 ml of conc HCl in a sealed tube at 110°C for 24 hr. The hydrolysate was neutralized with Na_2CO_3, made up to volume and used directly for quantitative chromatographic analysis. Chemical analyses for free proline and hydroxyproline were also made on the original filtrate after treatment with TCA (final concentration 5%) to remove protein material. Total proline and hydroxyproline were determined on the hydrolyzed filtrate. Both amino acids were determined spectrophotometrically, proline by the method of Troll et al. (1955), and hydroxyproline by the method of Neuman et al. (1950) as modified by Leach (1960).

RESULTS

IN INITIAL tests, results of paper chromatographic analysis showed that only *Achromobacter* 89 and *Pseudomonas* 47 had a marked effect on the free amino acid content of the skin. At the beginning of the log growth phase (after 2 days of incubation), these bacteria rapidly utilized all the free amino acids, with leucine, phenylalanine, alanine, aspartic acid and glutamic acid being attacked first; near the end of the growth period (4 days of incubation), only the amine, taurine, remained (identified by eluting the spot and testing the eluate on the amino acid analyzer). No further changes were noted for the remainder of the 21-day incubation period, showing that these organisms do not utilize taurine nor replenish any of the original amino acids by protein breakdown. *Pseudomonas* 1 and the mixed inoculum gave noticeable changes in the density of the spots for some of the amino acids but none of the acids completely disappeared, and except for a noticeable reduction between the 2nd and 4th day of incubation, the total amino acid content did not appear to change during the 21 days. Figure 1 shows the chromatograms for all cultures after 4 days of incubation when the cell count was about 10^8 and off-odor was produced.

Results of subsequent quantitative analyses with the amino acid analyzer confirmed that *Achromobacter* 89 and *Pseudomonas* 47 reduce the level of all free amino acids in skin to near or below detectable limits during the log phase of growth and do not readily utilize taurine

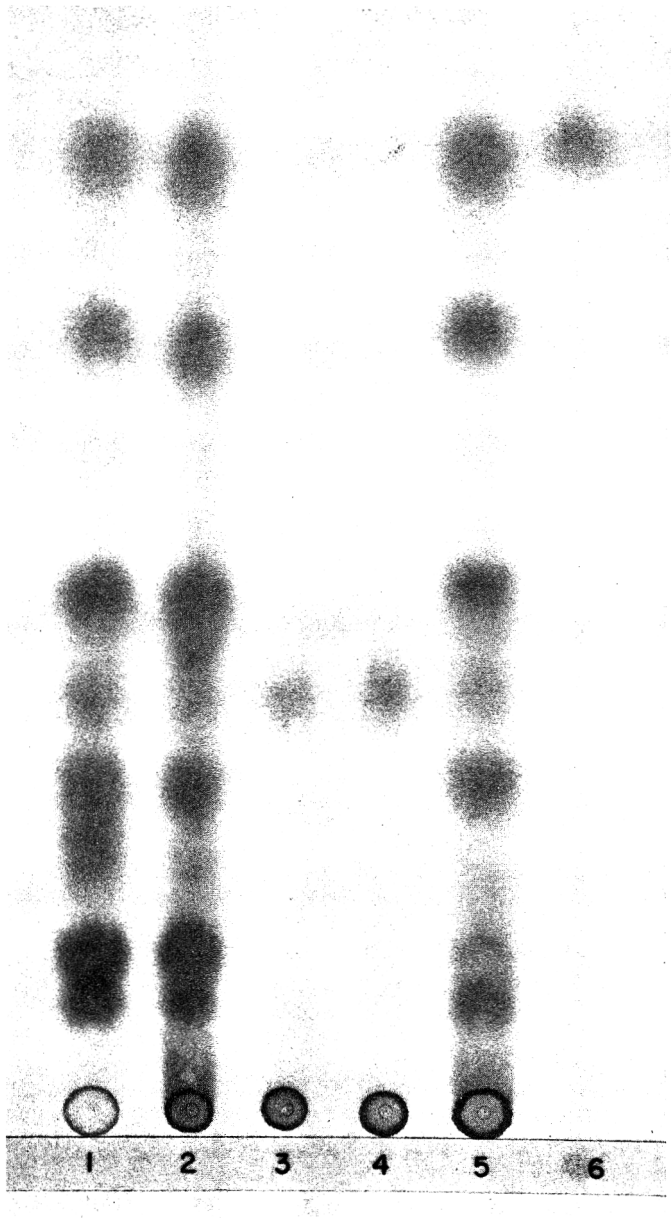

Fig. 1—Chromatograms showing effect of bacteria on amino acids in chicken skin after 4 days of incubation at 5°C. Legend: 1, incubated control; 2, Pseudomonas 1; 3, Pseudomonas 47; 4, Achromobacter 89; 5, mixed inoculum; 6, leucine (standard). (Single spot in 3 and 4 is taurine.)

(Table 1). *Pseudomonas* 1 increased the concentration of most amino acids, giving about a 30% increase in the total content. This organism appears to possess an arginase, since it produced ornithine (not found in the control) and gave a marked reduction in arginine. The mixed inoculum affected the concentration of most of the amino acids, but the total amount was about the same as in the uninoculated skin.

Results of chemical analyses showed that, of the 3 type cultures tested, only *Pseudomonas* 1 increased the free and total extractable proline (Fig. 2) and hydroxyproline (Fig. 3) content. In every case the increase did not occur until after about 5 days of incubation (end of log phase; count about 10^9/g skin) and was greatest after about 10 days (2- to 3.5-fold increase over original values). The maximum increase in free proline was about 4.5 times that for free hydroxyproline, roughly proportional to the relative amounts of each acid in the hydrolyzed extract of uninoculated skin. Consistent with earlier chromatographic tests, *Pseudomonas* 47 and *Achromobacter* 89 markedly reduced the concentration of free proline and hydroxyproline during the log phase (2-5 days of incubation), but produced little change in

Table 2—Effect of Pseudomonas 1 on the total extractable amino acid content of chicken skin (incubation 10 days at 5°C).

Compound	Amount, μg/g skin	
	Control	Pseudomonas 1
Taurine	150	115
Hydroxyproline	147	278
Aspartic Acid	538	1,496
Threonine	243	624
Serine	311	710
Glutamic Acid	1,012	2,278
Proline	424	1,059
Glycine	598	1,297
Alanine	428	955
Valine	309	881
Half-cystine	87	269
Methionine	95	304
Isoleucine	231	624
Leucine	367	976
Tyrosine	192	449
Phenylalanine	258	581
Hydroxylysine	6	39
Ornithine	0	423
Lysine	474	1,017
Histidine	143	267
Arginine	376	558
Totals (taurine excluded)	6,239	15,085

Table 3—Effect of strains of pigmented Pseudomonas on free and total extractable proline and hydroxyproline content of skin (incubation 10 days at 5°C).

Strain No.	Amount,[1] μg/g skin			
	Proline		Hydroxyproline	
	Free	Total	Free	Total
1	16.1	728	3.4	188
18	7.9	848	17.6	228
22	2.4	472	2.0	222
24	11.6	712	18.4	168
27	31.3	1,008	21.6	396
44	28.5	952	22.1	299

[1] Values for control subtracted.

the total extractable amounts of these compounds. The mixed inoculum gave intermediate results, showing that the type cultures studied were representative of the average of the 3 major groups of spoilage bacteria.

Additional tests, in which the total extractable amino acid content of skin was measured on the amino acid analyzer, confirmed that *Pseudomonas* 1 increases the concentration of extractable proline and hydroxyproline (Table 2). The increase after 10 days of incubation was about 2.5- and 2-fold for proline and hydroxyproline, respectively. The 6-fold increase in hydroxylysine also indicated collagen breakdown, since this amino acid is found in collagen but not in other skin proteins. Again, the results show that *Pseudomonas* 1 possesses an arginase, since ornithine was produced and arginine did not accumulate as much as the other amino acids.

Tests with 10 strains of pigmented *Pseudomonas* (all previously isolated from skin of commercially processed chickens, Clark et al.(1969)),showed that after 10 days of incubation, 6 of the 10 increased the content of free and total extractable proline and hydroxyproline. The remaining 4 cultures reduced the free proline and hydroxyproline content by 40-90% but gave no change in the total extractable content. Table 3 shows that of the collagenolytic cultures, two (strains 27 and 44) were even more active than the type culture, strain 1. It appears from these results that the majority of pigmented pseudomonads associated with commercially processed refrigerated poultry possess collagenase.

DISCUSSION

THE RESULTS indicate that pigmented pseudomonads are the most proteolytic of the 3 major groups of bacteria causing deterioration of poultry at low temperatures. Unlike the type cultures studied for *Achromobacter* and nonpigmented *Pseudomonas*, pigmented *Pseudomonas* 1 released more amino acids than it utilized during the late log and stationary phases of growth and apparently attacked collagen. The former organisms, however, grew as rapidly as *Pseudomonas* 1 and produced off-odor after the same duration of incubation (about 4 days; count 10^8/g of skin). Possibly *Pseudomonas* 47 and *Achromobacter* 89, after depleting the amino acids normally present in the skin, released these compounds through proteolysis but only in amounts needed to maintain growth. The small increase in total extractable proline (Fig. 2) and in the content of extractable nonprotein nitrogenous material during the stationary phase of growth (shown previously, Adamčič et al., 1969), shows that these organisms are slightly proteolytic. However, for all organisms studied, enough free amino acids and other low-molecular-weight nitrogenous compounds are present in skin to give an excellent rate of growth (Clark, 1968) and off-odor without the need for protein breakdown. Jay et al. (1967) reported similar results for organisms causing spoilage of ground fresh beef. Thus, the superior proteolytic activity of pigmented pseudomonads and their apparent ability to degrade collagen has little or no significance in low-temperature spoilage of poultry. All three types are normally present on the surface of spoiling poultry, with the achromobacteria (Ayres et al., 1950; Clark et al., 1969) or the nonpigmented pseudomonads (Nagel et al., 1960; Thornley et al., 1960) generally being the most numerous.

The increase in the content of free and total extractable proline and hydroxyproline caused by 6 of the 10 pigmented psuedomonads tested is strong evidence that these organisms possess collagenase

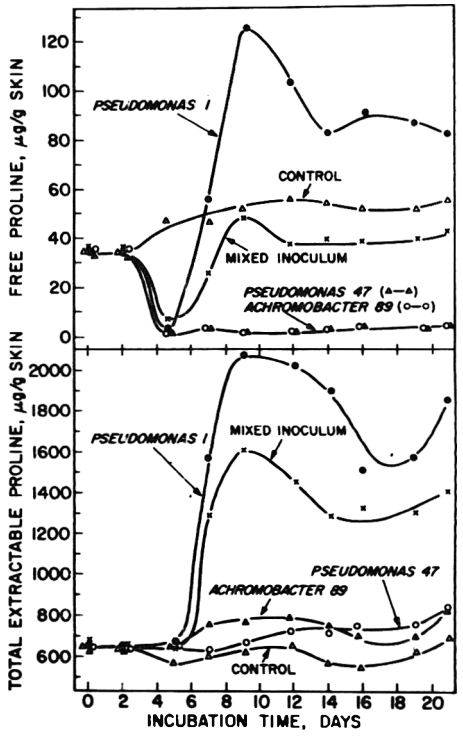

Fig. 2—Effect of bacteria on the free and total extractable proline content of chicken skin.

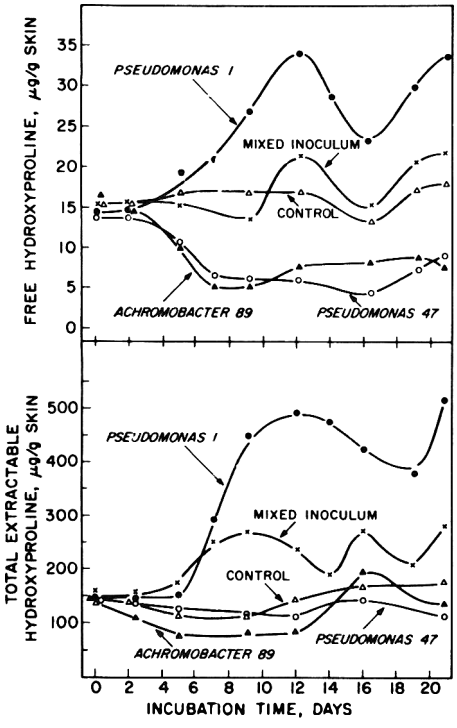

Fig. 3–Effect of bacteria on the free and total extractable hydroxyproline content of chicken skin.

and the peptidases necessary to split peptides into amino acids. The collagenase would appear to be extracellular, since it was active during the late log phase (after 4 days of incubation), at a time when little cellular autolysis would have occurred. While proteolytic enzyme systems have been described for several species of *Pseudomonas* (e.g., Camp et al., 1957; Peterson et al., 1960; Hurley et al., 1963; Li et al., 1968), the existence of a collagenase in a pseudomonad has been reported only once before: Schoellmann et al. (1966) reported finding an extracellular collagenase in *Pseudomonas aeruginosa* capable of lysing a synthetic hexapeptide which possesses an amino acid sequence susceptible to the *Clostridium histolyticum* collagenase. However, more recently, Waldvogel et al. (1969) tested 30 strains of *Pseudomonas aeruginosa* and found that none was capable of lysing reconstituted collagen. The increase in free hydroxyproline shown in Table 3 indicates that pigmented pseudomonads may possess an enzyme capable of splitting off terminal hydroxyproline residues. Such a peptidase, not heretofore described (Nordwig, 1968), would be useful in research on the structure of collagen.

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PROTEOLYTIC ACTIVITY OF CUCUMIS TRIGONUS ROXB. EXTRACTION, ACTIVITY, CHARACTERISTICS

SUMMARY—The proteolytic activity of the fruit of Cucumis trigonus *Roxb. casein as substrate was studied and the active principle isolated. Optimum conditions for maximum yield, effect of various factors on the activity and kinetics of the enzyme-substrate complex were investigated. The isoelectric point of the enzyme is pH 3.4. Analytical test on the enzyme isolate for thiol groups gave negative results, but the enzyme behaves in a manner indicative of the presence of —SH groups. The enzyme system possesses strong meat-tenderizing but apparently no milk-clotting properties. Certain fillers and activators are proposed for an effective meat-tenderizing mixture based on the powdered enzyme extract. An arbitrary name "Cucumin" is suggested for this newly discovered proteolytic factor.*

INTRODUCTION

THE FRUIT of *Cucumis trigonus* Roxb. is oval-shaped (3—4 cm in length) with dark-green strips of dots running longitudinally. When yellow-ripe the fruit tastes sweet and is used as an appetizer. It bears no latex. Cucumis is grown throughout most of West Pakistan and is used as a meat tenderizer. For this purpose, the dried, coarsely ground fruit called "Kachri" is spread over the pieces of meat at room temperature.

The authors are not acquainted with any prior work on the proteolytic activity of the Cucumis. High protease activity in plants has been associated with the presence of latex (Pirie, 1955) and commercial plant proteases come from latex-bearing plants. However, leaf extracts of several nonlatex-bearing plants contain proteases (Kemble, 1956; Brady, 1960).

Among the proteolytic enzymes of plants, papain, ficin and bromelain have been most extensively studied (Stern, 1955) for their chemistry and technology. Investigations along similar lines on the enzymes of Cucumis have been undertaken and the proteolytically active principle isolated is arbitrarily named "Cucumin."

EXPERIMENTAL

THE JUST-mature yellowish-green fruits were collected in early June from the plains of West Pakistan. Whole fruits were fractionated by first thoroughly washing and then cutting the fruit into 2 halves. Seeds were separated by hand squeezing. The peels were either assayed fresh or dried at 40°C and ground in a disc mill. The powdered peel was stored at room temperature in dry Kilner jars, whereas the fresh material was stored frozen at −25°C.

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Extraction of protease activity (Cucumin)

Extractions were made in a 10% sodium chloride solution at well-defined pH levels maintained with the help of decinormal sodium hydroxide or hydrochloric acid. The tissues were comminuted in a Waring Blendor with 1:10 w/v of the extracting medium, kept for 1 hr at 25°C with constant stirring and the homogenate thus obtained filtered through cheese cloth. Alternatively, a phosphate buffer (pH 7.0) was employed as an extractant, and the yield compared with the extracts obtained with the salt. The milk-clotting ability (Whitaker, 1958) and meat-tenderizing activity (Gottschall et al., 1942) of the enzyme isolate were examined.

Estimation of the proteolytic activity

Activity of the proteolytic extract was measured by the release of nonprotein nitrogen (Greenburg, 1955) from 1.0% (w/v) buffered casein solution maintained at pH 5.0 when the enzyme extract (1:10 w/v) was added to the casein solution in 1:10 v/v ratio. Controls were run with the enzyme solution boiled for 2 min. This was considered sufficient to inactivate the enzyme in view of the sharp drop in activity above 80°C (as seen in Fig. 5). NPN (amino nitrogen) was determined with the micro-Kjeldahl method and also by way of confirmation, with the formol titration. The samples were run in triplicate, the reported value being the mean figure.

The unit of protease activity is defined as the amount needed to give an increase of 1 mg in NPN over that of the control, when the enzyme was incubated with 1.0% w/v casein solution for 60 min at 40°C and pH 5.0.

Isoelectric point of Cucumin

The enzyme extract obtained in 10% sodium chloride solution at pH 7.0 was examined for the isoelectric point by recording the activity of the supernatant and the precipitate, if any, at several points in the pH range 1—10. The phases were separated by centrifugation at 2000 g for 20 min.

Test for thiol group

This was carried out according to the method described by Stern (1955) over the sediment obtained from active enzyme extracts by precipitation with hydrochloric acid at pH 3.5. The precipitate was separated by centrifuging, redissolving, re-precipitating and finally drying under vacuo. The dried material thus obtained was also subjected to activity assay by the method described above after mixing with 0.1% by weight of glutathione.

Cucumin in powder form

1 kg of the dried powder peel was added to 10 liters of 10% sodium chloride solution with constant stirring and the pH adjusted to 7.0 by means of ammonium hydroxide. The mixture was beaten in a mixer for 1 hr and filtered through cheese cloth. The colloidal suspension thus obtained was adjusted to pH 6.0, stirred with active charcoal (3%, w/v) at 25°C for 10 min, then centrifuged to remove carbon particles together with most of the coloring matter. The clear supernatant liquid was decanted and stirred with solid ammonium sulfate to 0.4 saturation. The solution was stirred for 1 hr at room temperature, allowed to stand for 2 hr at 5°C and the precipitate thus obtained removed by centrifugation and discarded because of its negligible activity. The saturation of ammonium sulfate in the supernatant solution was brought to 0.6. It was kept overnight at 5°C, centrifuged and the sediment dried over P_2O_5 in a vacuum desiccator and preserved. Alternately, spray drying at 60°C with a laboratory type "Anhydro" spray dryer was also carried out for preparing powdered enzyme extract.

Meat-tenderizing mixture based on Cucumin

A meat-tenderizing mixture was prepared by employing powdered pea sprouts as a source of glutathione. To obtain pea sprouts, peas were allowed to germinate at 25°C for 4 days. At the end of this period the sprouts were separated, dried at 45°C in a cabinet dryer and ground in a disc mill. The powder thus obtained was examined by the method of Barbon (1951) and its —SH contents found to be in the range of 60—70 mM/g.

RESULTS & DISCUSSION

Optimum conditions for isolation of the enzymic activity

An examination of various portions of the fruit revealed that only about 5% of

Table 1—Activity of various parts and fractions of the fruit.[1]

		Protease units/g of the material
1.	Whole fresh fruit	850
2.	Whole, dried, powdered	1,870
3.	Fresh peel	3,000
4.	Dried powdered peel	5,960
5.	Powdered Cucumin	18,000
6.	Seeds	–

[1]All values are reported on a moisture-free basis.

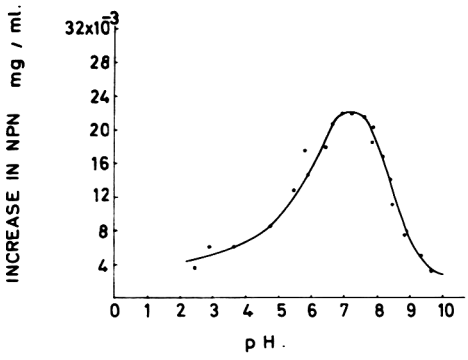

Fig. 1—Effect of pH on the extractability of the enzyme.

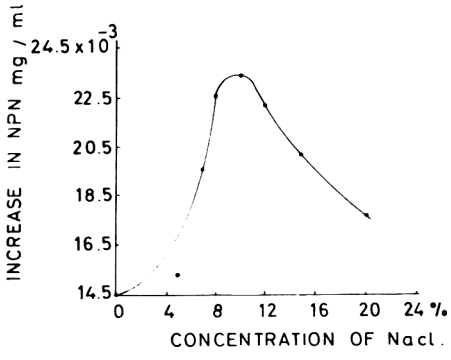

Fig. 2—Effect of concentration of sodium chloride percent of the extracting medium on the extractability of the enzyme.

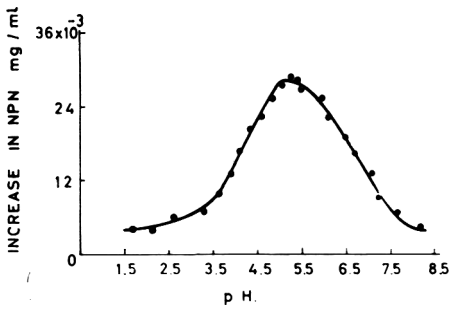

Fig. 3—Effect of pH on protease activity.

the total activity of the whole fruit is present in the endocarp, whereas the rest of the activity is concentrated in the outer cellular tissue, consisting largely of peels. Separation of the less-active fraction, including the seeds is, fortunately, easy.

Extraction of the enzyme could best be done at pH 7.0 (Fig. 1). A temperature range of 20–25°C is best suited for the extraction. A marked decrease in extractibility is observed below 5°C. As the pH rises above the stated optimum, the amount of proteinaceous nitrogen increases in solution but the enzymic activity falls rapidly. Little difference was noted in the extracts from mortar and Waring Blendor; however, it was more convenient to grind the fresh peel in a mortar before maceration in the Blendor.

Other things being equal, there was about a 50% increase in the activity of the extract obtained from the dried powdered peel over that from the fresh peel (moisture-free basis) (Table 1), probably due to the greater release of the enzymic material from the easily ruptured cell walls in the former case. Similar levels of protease activity were found in different batches of the fruits.

Maximal yields were obtained with 10% sodium chloride, maintained at pH 7.0 (Fig. 2). With phosphate buffer solutions, the activity of the isolate was 80% of the former. This contrasts with our previous observations on esterases (Hujjatullah, 1964).

The units of activity/g of the original material isolated from fresh and dry fruits and from various fractions of the fruits are shown in Table 1. The values express the units of activity that can be practically extracted, and not the actual whole activity contained in the fruit.

Effect of pH on activity

Figure 3 shows the effect of pH on the proteolytic activity of the enzymes under the conditions described. It is of interest that a plant protease should have such a low optimum pH (5.0) as is shown by the peak. The enzyme, however, is not irreversibly destroyed on either side of this optimum nor is there any indication that the proteolysis is inhibited by the substrate or split products. Maximum activity is obtained simply under the influence of the highest enzyme substrate affinity, in the vicinity of pH 5.0.

Effect of substrate concentration on activity of the enzyme

Kinetic studies revealed that activity increases in a linear relationship with concentration of the substrate until it reaches its maximum value with about 60 mg of the latter. At this optimum concentration of the substrate, the rate of hydrolysis was found to be directly proportional to the enzyme concentration. By plotting log $S_O - S_O/y$ against time, a straight line was obtained showing that the reaction obeys the first-order equation up to at least a 1-hr reaction time at pH 5.0. The slope of the curve gives the specific rate constant K, calculated to be 0.98×10^{-3} sec^{-1}. This value represents the average of K values for all susceptible but different peptide bonds present in the substrate.

The enzyme substrate intermediate

The foregoing observations give an idea of the enzyme-substrate affinity. The nature of the enzyme substrate intermediate and dissociation constant of this intermediate can be obtained by applying the classical Michaelis equation integrated

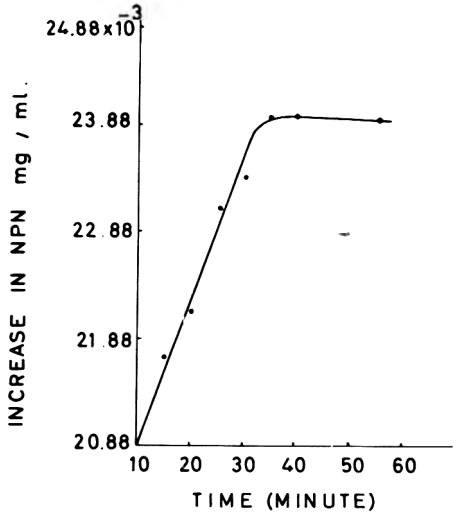

Fig. 4—Effect of time allowed for reaction on the protease activity.

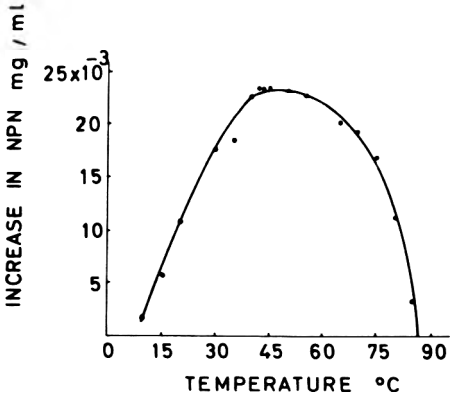

Fig. 5—Effect of temperature on protease activity.

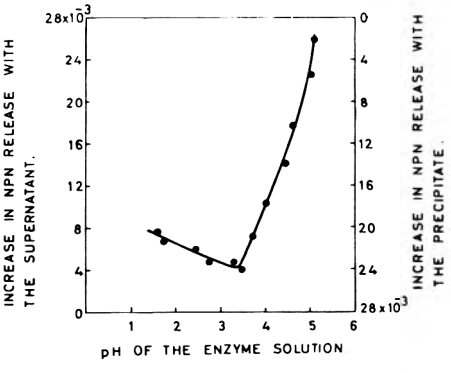

Fig. 6—Isoelectric point of the enzyme.

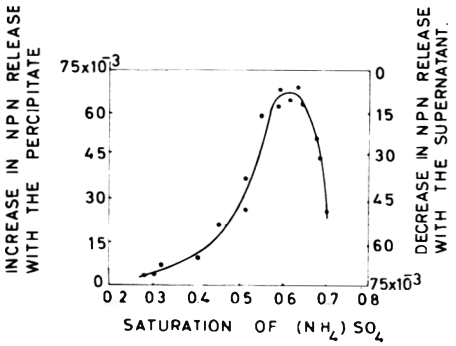

Fig. 7—Effect of salt concentration on solubility of the enzyme and maximum precipitation of the latter by salt addition.

to suit the conditions of the experiment or of the nature of enzyme-substrate mixture. Thus, the graphic method of Lineweaver and Burk (1934) based on the Michaelis Menten theory was employed to calculate the final velocity (V) and Michaelis constant (Km). The values of K_m and V obtained by the Lineweaver and Burk plot were found to be 4.8×10^{-5} g mole/liter and 5.56×10^{-7} g mole/min, respectively. However, as regards their exact significance, what has been stated in relation to the value of K, the specific rate constant, is also true for these figures.

Effect of time and temperature

The progress of proteolysis with time was studied by incubating the casein solution with 5% (w/v) of the extract at 40°C. NPN increases proportionally up to 40–45 min, beyond which further hydrolysis seems to have ceased (Fig. 4). When the incubation period exceeds 60 min, NPN virtually decreases compared to its maximum value and the time-reaction graph tends to fall. This behavior cannot be explained in terms of the available data and merits further investigation.

The rate of hydrolysis has also been determined at different temperatures. Aliquots of the enzyme and substrate solutions were incubated before mixing, so that the reactants attained the temperature at which the degree of hydrolysis was to be determined. The reaction mixture was kept for 60 min at various temperatures from 10–90°C. As the temperature rises, the reaction rate proportionally increases until it reaches the maximum at 40°C, but the enzyme retains its activity up to a temperature as high as 70°C (Fig. 5). This fact may be put to practical use, inasmuch as the stability of a proteolytic mixture based on this enzyme might well be expected to be comparatively better at ambient temperature.

Isoelectric point

The effect of pH on the solubility of the active proteases is shown in Figure 6. At pH 3.4 most of the active material is precipitated and further lowering of pH brings some of the precipitated enzymes back into solution. Simultaneous assay of the precipitate and the supernatant furnished definite information regarding the extent to which the enzyme was distributed into phases at a particular pH. The isoelectric point occurs at a pH lower than the level where most of the plant proteases precipitate out. This may only be ascribed to the individual specificity.

Thiol group

Although direct analysis of the enzyme extract for SH groups gave negative results, this could not be taken as sufficient proof for the absence of thiol contents in the enzyme system as the enzyme was not in a pure enough state. When a foreign sulfhydryl compound such as glutathione was added up to 0.1% w/v in the extract, activity doubled. This activation by glutathione is indicative of –SH groups in the proteolytic system.

Cucumin as meat tenderizer

The enzyme has no demonstrable milk-clotting activity but nevertheless tenderizes meat to a considerable extent and could serve as a base for commercial meat-tenderizing mixtures. Fractional precipitation with salt was, therefore, adopted as a tentative method for partial purification of the enzyme, to obtain it in powder form.

Of all the precipitants tried for the fractional precipitation of the enzyme, ammonium sulfate was the best. At 0.6 saturation of the salt, almost all the active enzymes are precipitated (Fig. 7). Activity rises suddenly beyond 0.5 saturation. Although the amount of the precipitate is considerable at 0.4, the activity of the sediment is negligible and it may be discarded easily. The activity of the material that settles down at 0.6 saturation is found to be 2.5 times that of the fresh extract (on a moisture-free basis), i.e., 18,000 units/g of the material. Apparently, the enzyme again goes into solution, or the precipitate assumes colloidal dimensions when the saturation of ammonium sulfate exceeds 0.6 in such a way that it cannot be separated under the conditions adopted for centrifugation. The separation has been carried out under similar conditions throughout, that is, by centrifugation at 2000 g for 20 min.

The meat tenderizers were prepared by adding components which act as promoters of the enzyme catalysis and simultaneously increase the solubility of the enzymes. Chloride ions promote the proteolytic activity and also increase the solubility. Similarly, sodium bicarbonate tends to keep the pH on the neutral side, where the proteases are most soluble.

The idea of adding a filler containing an –SH compound worked very well. The pea-sprout powder could be readily obtained and this renders the formula locally feasible. The activity of the tenderizer with powdered pea sprouts is increased considerably due to the optimum amount of glutathione in the mixture. The formula found to be the best is (in percentages): Cucumin, 30; pea-sprouts powder, 25; sodium chloride, 25; sodium bicarbonate, 15; sodium citrate, 5.

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EFFECT OF TEMPERATURE ON STABILITY OF ORANGE AROMA SOLUTION

SUMMARY—Stability of orange aroma solutions from Valencia oranges grown in Arizona, California and Florida was studied at storage temperatures of 0, 10, 20, 34 and 70°F. Samples stored in 10-ml Pyrex glass screw-capped vials lost aroma strength and fresh orange character rapidly at 70 and 34°F. Aroma strength as measured by threshold determinations remained fairly constant at 0 and 20°F for up to 1 year. Significant changes in aroma character, however, were observed within 6 months and 1 year at 20 and 10°F, respectively. At 0°F, fresh orange aroma was maintained without significant change for up to 88 weeks. The most significant change indicated by GLC headspace analysis was almost complete loss of limonene in samples held at 70°F for 9 weeks or more. Aroma strength and character during storage at 70°F were preserved by sealing in glass tubes and storing in the dark.

INTRODUCTION

TRADITIONALLY, the frozen orange juice concentrate industry has utilized fresh single-strength juice to add fresh orange aroma and taste to its concentrated products. This generally involves concentrating juice to 58–60°Brix and diluting the concentrate with 33 to 35% fresh juice, yielding a fourfold concentrate of 42–44°Brix. It would be advantageous to store and market the product at the higher concentration (50–60°Brix) if the reconstituted juice was essentially equal in quality and stability to that obtained by the standard cutback process. The savings in containers, storage and shipping costs are obvious.

Recently, practical processes have been developed for the recovery of aroma from orange juice concentration operations (Wolford et al., 1968; Bomben et al., 1966; Brent et al., 1966 and Kelley, 1965). Aroma solution obtained by the WURVAC process can satisfactorily flavor orange concentrates of 58–60°Brix (Mannheim et al., 1967) and concentrates so flavored are essentially equal to cutback concentrates in stability (Guadagni et al., 1970). The experimental evidence also indicates that it may be advantageous to store concentrate and aroma solution separately. If it is feasible or desirable to store concentrate and aroma solution separately, information on stability of the aroma solution becomes essential. This paper gives data on the effect of temperature and other storage conditions on stability.

MATERIALS & METHODS

THE AROMA solutions used in these experiments were prepared by the WURVAC process from California, Arizona and Florida Valencia oranges. The oranges were hand reamed and the juice was immediately fed to the WURVAC process, which gave a 150-fold aroma solution (1 liter of aroma solution from 150 liters of juice). 25% of the feed juice was evaporated at 180°F. The stripped juice was concentrated to 60°Brix in an agitated-film evaporator at an evaporation temperature of 120°F.

In addition to aroma solutions prepared by the WURVAC process, a sample of aroma solution made from Florida Valencia oranges by the system described by Wolford et al., 1968 (FCC-CES) was included in the storage studies.

The following samples were prepared from the above materials. Arizona Valencia juice stripped and concentrated to 60°Brix (C), and aroma solutions from: Arizona Valencia oranges (EA); California Valencia oranges (EC); Florida Valencia oranges (EFW) and Florida Valencia oranges made by the FCC-CES system (EF).

Stripped concentrate (C) canned in 6-oz cans and immediately frozen and stored at −30°F was used as a base for comparison of some of the aroma solutions. Samples of aroma solution were packaged in 10-ml Pyrex glass vials with Teflon-lined screw caps and frozen at −30°F. In addition, 1 sample of aroma solution (EC) was also sealed in small-bore Pyrex glass tubes (5 by 120 mm) under atmospheric conditions and under vacuum.

Storage

The stripped concentrate (C) was always maintained at −30°F until reconstituted with water and appropriate amounts of aroma solution to 12°Brix juice. The aroma samples in vials were stored at −30, 0, 10, 20, 34 and 70°F. The samples at −30°F served as controls. At periodic intervals, samples from the higher temperatures were compared with samples of the same code held at −30°F. The small-bore heat-sealed glass tubes were stored at 70°F under illumination from a 25-w tungsten lamp and under complete darkness.

Sensory evaluation

The threshold concentration (Tc) of all aroma solutions was determined by the procedure of Guadagni et al. (1967), except that Teflon instead of polyethylene bottles and tubes were used as containers. Briefly, the procedure consists of smelling 4 to 6 pairs of solutions in which 1 sample in each pair is odor-free water, and determining which sample in each pair contains the aroma. The order of presentation and position of blanks in each pair were completely random. 20 to 26 screened odor judges evaluated each series at 3 separate sessions. The average Tc was determined from the results of all judgments and replications.

In addition to Tc measurements, aroma solutions held at different temperatures were

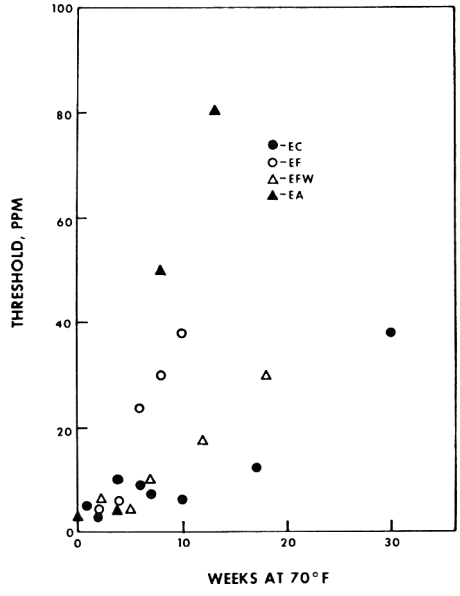

Fig. 1—Effect of storage at 70°F on threshold concentration (Tc) of different samples of 150-fold orange aroma solutions.

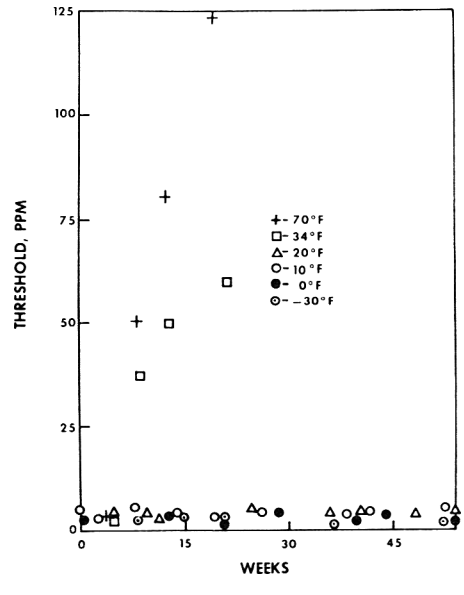

Fig. 2—Effect of temperature on threshold concentration (Tc) of 150-fold orange aroma solution (EA).

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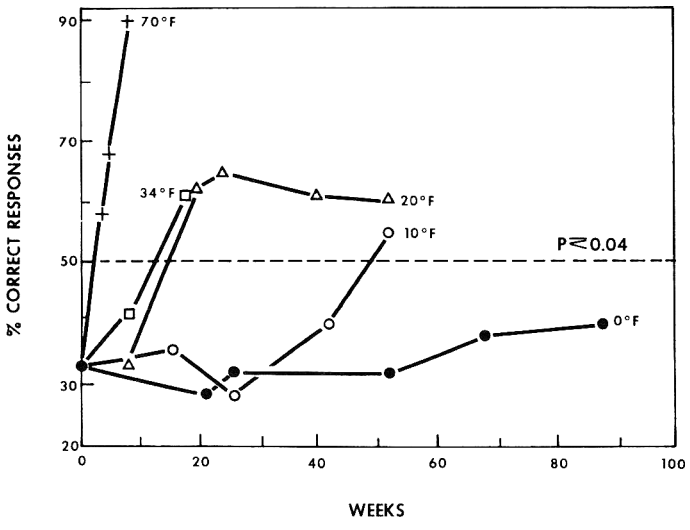

Fig. 3—Effect of time and temperature on changes in aroma character of 150-fold orange aroma solution (EA).

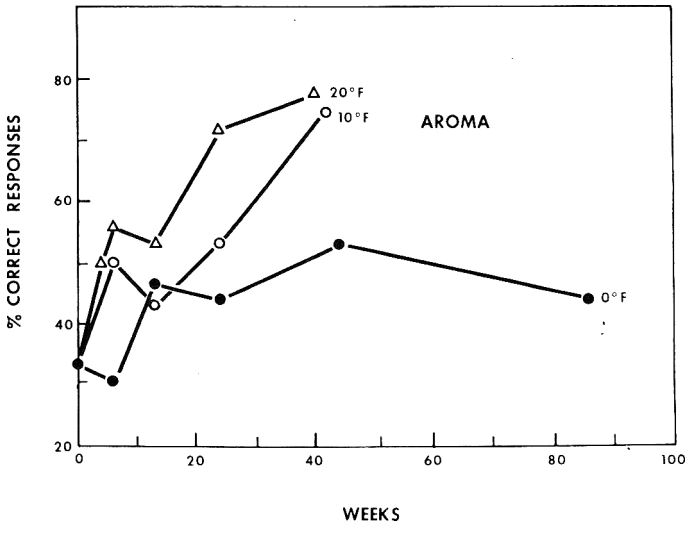

Fig. 4—Effect of temperature on aroma stability of 150-fold orange aroma solution (EA) as measured by triangle test comparison against −30°F control. Aroma solution was diluted with water and −30°F concentrate to form single-strength equivalent before conducting aroma comparisons.

diluted to single-strength equivalent (1 → 150) with triple-distilled water and compared with the control sample held at −30°F, diluted in the same manner. These comparisons were made on the basis of aroma alone by triangle test with 15 trained judges. Each comparison was replicated twice, giving 30 judgments.

Some aroma solutions at different temperatures were diluted with water and −30°F stripped concentrate (C) to give the equivalent of single-strength juice. The −30°F aroma solution was diluted in the same way and served as the control for the samples held at higher temperatures. The −30°F (C) was the base for all aroma solutions, so that the only variable affecting aroma solution was temperature alone. The diluted samples were then compared by triangle test for both aroma and taste, as described above. Details of these sensory procedures and the facilities used are described elsewhere (Mannheim et al., 1967).

Gas-liquid-chromatographic analyses of the headspace over single strength equivalent aroma solutions were made as described by Mannheim et al. (1967).

RESULTS & DISCUSSION

THE RELATION between threshold concentration (Tc) and storage in vials at 70°F is shown in Figure 1. The EA and EF samples appeared to increase in Tc value or lose aroma at the fastest rates, whereas EC and EFW samples seemed to be somewhat more stable in aroma strength. Irrespective of these minor differences, it is apparent that storage at 70°F is unsatisfactory for stability.

Figure 2 shows the effect of temperature on changes in Tc for aroma solution EA packaged in vials. Again, it is obvious that 70°F causes a rapid loss in aroma strength (Tc increase) and, while the loss is somewhat less at 34°F, the original Tc of the solution increases 20- to 30-fold in 20 weeks. At 0, 10 and 20°F, however, there was no consistent significant change in Tc values over 88 weeks. This would

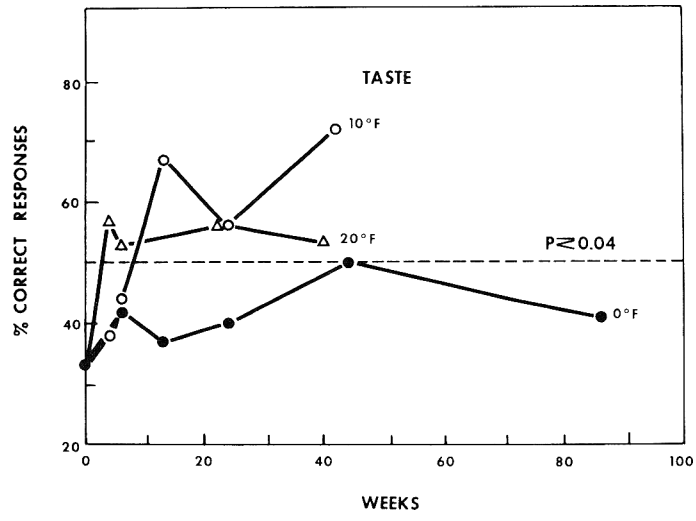

Fig. 5—Effect of temperature on taste stability of 150-fold orange aroma solution (EA) as measured by triangle test against −30°F control. Aroma solution was diluted with water and −30°F concentrate to form single-strength equivalent before conducting aroma comparisons.

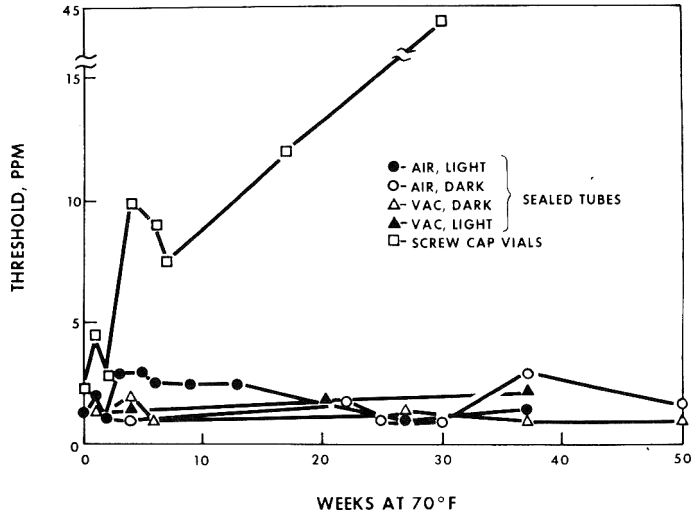

Fig. 6—Effect of sealing and storage conditions on the threshold concentration of 150-fold orange aroma solution (EC) during storage at 70°F.

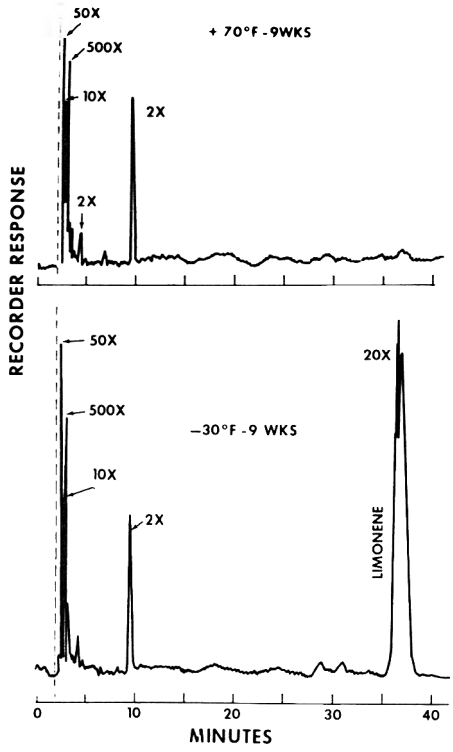

Fig. 7–GLC headspace analysis (5 ml) of 150-fold (EFW) aroma solution diluted to single-strength equivalent after 9 weeks at –30°F and 70°F. GLC conditions: column–¼ in. by 20 ft packed with Chromosorb G coated with 4% SF96-50, column temperature, 100°C. Nitrogen carrier gas at a flow rate of 4.7 cm/sec.

indicate that aroma strength or intensity does not change at these temperatures for extended periods of time, and implies that the aroma is stable at temperatures as high as 20°F. Unfortunately, aroma comparisons at single-strength equivalent show that significant differences in aroma quality occur within 20 to 52 weeks at 20 and 10°F, respectively (Fig. 3).

As indicated by the Tc measurements, changes in aroma quality occur very rapidly at 70 and 34°F. However, unlike the Tc data, at 20°F highly significant changes in aroma quality take place in 8 to 20 weeks; at 10°F, a similar change occurs between 42 and 52 weeks. It is important to note that practically all the judges who identified the odd sample in the triangle after these storage periods preferred the –30°F control. The stored samples were described as weak or possessing an off-aroma. The only temperature at which no significant changes occurred in aroma from the –30°F control was 0°F. Samples at 0°F were stable for at least 88 weeks in both aroma character and strength.

The aroma evaluation of EA samples diluted to single-strength equivalent with –30°F (C) and water is shown in Figure 4. Again, these data show that 0°F is required for reasonable aroma stability. Marked differences in aroma quality occurred in 13 to 24 weeks at 20°F, and 24 to 40 weeks at 10°F. The results at 0°F did not show any consistent significant differences over a period of 86 weeks.

Figure 5 shows results obtained when evaluation of the aroma solutions held at different temperatures was made on the basis of taste rather than aroma alone. The data are similar to those shown in Figure 4 for aroma. Storage of the aroma solutions at 10 and 20°F causes significant changes in taste of the reconstituted juice, even though the concentrate (C) was continuously held at –30°F. Again, storage at 0°F did not show any consistent significant differences over an 86-week storage period. Therefore, it is clear that even though Tc values of aroma solutions held at 10 and 20°F remain fairly constant for a year or longer, the quality or character of the aroma undergoes definite changes during this time.

Since 70°F caused very rapid changes in aroma strength and character, it was decided to study the effect of closure, atmosphere and light on aroma solutions at this temperature. Figure 6 shows the results obtained on aroma solution EC stored in sealed small-bore glass tubes. Compared with screw-capped vials, the sealed tubes prevented the drastic increases in Tc that occurred in vials held at this temperature. The sample sealed under normal atmosphere and stored under light showed a slight initial increase in Tc, but no definite trend in this direction was observed over a 50-week storage period.

Sealing the tubes under vacuum and storing in the dark appeared to be responsible for the least change in Tc value during storage for 50 weeks at 70°F. As seen before, constant Tc values indicate retention of aroma strength but do not necessarily signify retention of aroma character. Triangle test comparisons between stored and control samples indicated that tubes sealed in air and held under light developed significant differences after about 6 weeks at 70°F. The same tubes stored in the dark did not develop significant aroma differences for up to 1 year.

Tubes sealed under vacuum were not significantly changed from their controls after storage of 1 year at 70°F regardless of the presence or absence of light. Therefore, it appears that changes in aroma character are related to the combined effect of both oxygen and light. For practical purposes, packing in the absence of oxygen in hermetic containers should give added insurance against deterioration at temperatures above 0°F.

Analysis of the headspace vapors above aroma solutions diluted to single-strength equivalent did not show any consistent differences at temperatures of 0 to 20°F. Where large changes in aroma character were observed during storage at 70°F, GLC analysis showed essentially complete disappearance of limonene (Fig. 7). Since limonene is a component of the oil-soluble fraction of the aroma solution, it appears that alteration of aroma character may be associated with degradative reactions in this fraction.

Other components of the oil-soluble fraction have Tc values much lower than that of limonene. Since limonene is readily oxidized during storage at 70°F, the more odoriferous components of the oil fraction may also be oxidized to nonvolatile components or to materials of higher Tc values. The rapid rise in Tc value observed at temperatures of 34 and 70°F could be explained on this basis. However, measurement of the limonene peak is not a satisfactory indicator of aroma quality. At temperatures of 10 and 20°F, where significant sensory changes in aroma occurred, consistent significant losses in limonene were not observed.

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AVOCADO POLYPHENOLOXIDASE: PURIFICATION, AND FRACTIONATION ON SEPHADEX THIN LAYERS

SUMMARY—Polyphenoloxidase (PPO) was purified 28-fold by ammonium sulfate precipitation, dialysis and gel filtration. Enzyme constants were determined with a variety of substrates. Thin-layer gel filtration resolved the crude PPO into 5 fractions with molecular weights estimated at 14-, 28-, 56-, 112- and over 400-thousand by comparison with standard proteins. Subsequent electrophoresis at 90° to the direction of gel filtration resolved the 28 × 10^3 MW fraction into 4 to 6 components. One of these was by far the most active of all the isoenzymes toward all the substrate sprays tested. It is probably the activity of this isoenzyme which is reflected in kinetic data, even those obtained with relatively crude avocado PPO preparations.

INTRODUCTION

IN SPITE of the well-known enzymic browning potential of the avocado, this has been studied to only a limited extent. Samish (1937) and Knapp (1965) investigated this fruit's PPO (E.C.1.10.3.1). Knapp found that, of the substrates tested, the most rapidly oxidized by avocado PPO were nordihydroguaiaretic acid (NDGA), catechol and catechin. Neither worker considered the possibility that avocados might have more than one enzymic protein with PPO activity.

Recent investigators have found that instead of there being only a single phenol-oxidizing enzyme per species, multiple forms could be separated on the basis of molecular weight or electrophoretic mobility, or both. Robb et al. (1965) separated multiple forms of broad bean tyrosinase, having similar molecular weights and specificities, by starch gel electrophoresis. Constantinides et al. (1967) used polyacrylamide gel electrophoresis to separate multiple forms of PPO from mushrooms, apples and potatoes. They used D,L-3,4-dihydroxyphenylalanine (dopa) and tyrosine as substrates for detection of active PPO bands. They found that some of the forms exceeded others in resistance to heat, bisulfite and ethylenediaminetetraacetic acid and in ability to oxidize tyrosine. Sakamura et al. (1966) separated 2 eggplant PPO fractions which differed in their substrate specificities. The last 2 groups of authors did not report estimation of molecular weights.

The technique of filtration through dextran gels having controlled pore sizes offers an effective means for purification of proteins by eliminating fractions having higher or lower molecular weights than the protein of interest (Anonymous, 1966). Gel filtration on thin layers is a relatively simple method of estimating molecular weights by comparison with standard proteins (Andrews, 1964).

The objectives of the present work were to purify avocado PPO and to determine its molecular weight(s) and enzyme constants with a variety of substrates. A further objective was to determine whether the kinetic data obtained represented more than 1 enzyme fraction.

EXPERIMENTAL

ALL AVOCADOS used were West Indian–Guatemalan hybrids, grown in Florida and obtained from a local market. Enough fruit were obtained and processed at 1 time to complete all the studies described herein.

Ripe avocados, freshly peeled and cubed, were blended with acetone (1:1.5, w/v) and filtered with suction. The marc was reblended with acetone 3–4 times. After filtration and drying under vacuum, a free-flowing, creamy white oil-free powder was obtained. Typical yields were 6–10% of flesh weight. The powder was stored at −15°C for future use.

Avocado PPO was extracted from the acetone powder by blending the latter with Mac-Ilvaine buffer, pH 6.8 (1:50, w/v) and stirring mechanically for 2 days at 3–5°C. Preliminary studies have shown this to be the optimum pH for extraction and that whereas stirring longer than 2 hr increased the total activity by only a small amount, it did increase the specific activity. The low temperature of extraction was less than optimum but was necessary to avoid microbial spoilage. The extracts were centrifuged 45 min at 20°C and 2.6 × 10^4 × g, then filtered with suction through Whatman No. 1 paper. Activity values were typically 15 U/ml and 26 U/mg protein. 1 PPO unit (U) as defined by the International Union of Biochemistry (1965) is sufficient enzyme to catalyze the oxidation of 1 μM of substrate per minute. In the spectrophotometric assay used (Knapp, 1965), 1 PPO U equals 1.45 times the initial rate of absorbance increase at 420 nm, using 3.3 mM catechol as substrate and 1.3% $K_4Fe(CN)_6$ as reductant. The protein fraction precipitating between 0.5 and 0.7 saturation with $(NH_4)_2SO_4$ was resuspended in pH 6.8 buffer. The increase in specific activity was generally 2-fold, with a 50% yield. Dialysis against distilled water overnight at room temperature resulted in a specific activity 2.6 times that of the original, with an over-all yield of 35%.

After centrifugation (15 min, 0°C, 2.6 × 10^4 × g) the samples (hereafter referred to as the crude PPO) were held at 3–5°C, some for over 6 months, with little loss in activity.

Further purification was carried out by means of gel filtration through Sephadex G-150, fine, in a 2.5- by 45-cm Sephadex column (Pharmacia Fine Chemicals) carried out as described by the manufacturer (Anon., 1966). After equilibration of the column with pH 6.8 buffer, the void volume (Vo) was determined with Dextran Blue 2000. Aliquots of the crude PPO preparation were applied to the column, eluted with the buffer at a flow rate of about 25 ml per hr and collected by a fraction collector at intervals timed to give 5–10 ml per fraction. Each fraction was assayed for PPO activity and protein concentration (Lowry et al., 1959). Crystalline bovine serum albumin (Nutrition Biochemicals Corp.) was used as protein standard. The most active fractions from several runs were pooled, concentrated by $(NH_4)_2SO_4$ precipitation, redissolved in buffer, dialyzed and rechromatographed.

The most highly purified sample was used for the determination of Michaelis constants (K_m) which were calculated by least-squares analysis of S/v vs. S plots. To avoid loss of activity due to prior dilution, 1 μliter of enzyme preparation was used for each rate measurement. 10 dilution levels of each substrate were tested, with 4–6 determinations at each level. Of the substrates employed (Table 2), catechol was obtained from Fisher Chemical Co., homocatechol (4-methylcatechol) from Aldrich Chemical Co., pyrogallol from Merck and Co. and the remainder from Nutritional Biochemicals Corp. All were used without further purification.

Thin-layer gel filtration was carried out essentially as described by Andrews (1964) and Johansson et al. (1964). Sephadex G-150 (superfine) was applied in a 0.25-mm layer to 20- by 20-cm glass plates by thin-layer spreader (Brinkman Instruments, Inc.). The plates were placed at an angle of 25° below the horizontal and equilibrated overnight with the flowing pH 6.8 buffer before being spotted. Proteins of known molecular weight (see Table 2) obtained from Mann Research Laboratories, Inc. were dissolved in pH 6.8 buffer, and 12.5 μg (1.0 μliter) of each applied to the plates, except for γ-globulin, which required 25 μg for good detection. 0.25–2 μliters of PPO preparation were used. Development required about 2.5 hr. After partial drying, substrate spray of dopa, catechol or homocatechol was used to locate active enzyme. After brief oven-drying, protein spots were detected as blue areas by dipping for 30 min in 0.01% nigrosine in methanol-water-acetic acid (5:4:1), followed by a 2–3-min wash in the same solvent.

Migration rates were all expressed as distance traveled relative to bovine serum albumin (BSA) and expressed as R_{BSA}.

2-dimensional development of crude avocado PPO was carried out by thin-layer gel filtration of 2-μliter aliquots, carried out as just described, followed by electrophoresis in the

[a]Deceased, March 22, 1970.

Table 1–Typical purification of avocado PPO.

Preparation step	Specific activity PPO U/mg protein
Buffer extract from acetone powder	26
$(NH_4)_2SO_4$ precipitate (0.5–0.7 saturation)	52
Dialysate ("Crude PPO")	70–100
I. Sephadex eluate (most active fractions)	200–350
II. Sephadex eluate ("Purified PPO")	725

Table 2–K_m and V_m values for avocado PPO with various substrates.

Substrate	Purified[1]			Very crude[2]
	K_m(mM)	V_m	$V_m/2\,K_m$	K_m
NDGA[3]	0.081	1,120	6,900	.04
4-Methylcatechol	2.0	2,760	691	
Catechol	6.8	1,510	104	5.7
Chlorogenic Acid	7.0	1,220	87	2.5
Catechin	1.8	280	76	2
Pyrogallol	5.0	605	60	3.4
Hematoxylin	1.3	101	39	
Dopa	27	609	11	12
Dopamine	1.1	560	250	

[1]Purified as described in text and Table 1.

[2]Extracted with buffer directly from avocado flesh and subjected to only 1 $(NH_4)_2SO_4$ purification (0.5–0.7 saturation).

[3]Nordihydroguaiaretic Acid.

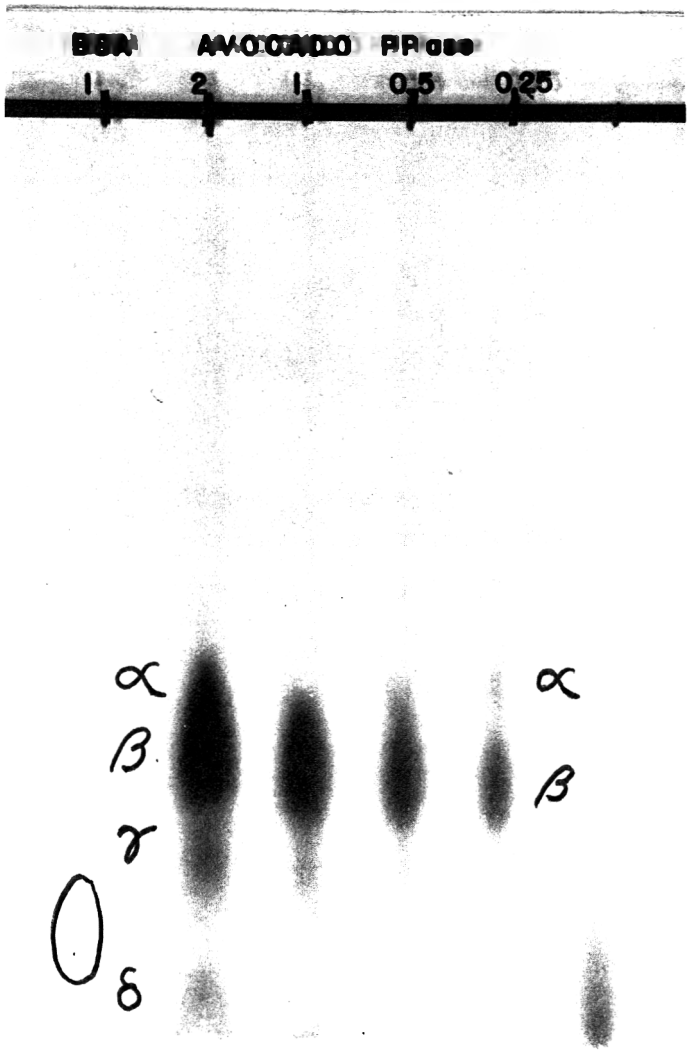

Fig. 1–Fractions of avocado PPO separated by gel filtration on Sephadex G-150(s) thin layers. Values at top indicate microliters of crude PPO preparation applied to plate. Sprayed with dopa after development.

second direction. A Gelman paper electrophoresis apparatus (No. 51170 chamber and No. 38201 power supply) was adapted for this purpose. The usual pH 6.8 buffer was employed. The potential difference across the 20-cm plate was 50 v and the current was 15-20 ma. Contact between the solvent and gel was effected with wicks of Whatman 3 MM filter paper. Running time was 24 hr. Separate plates were sprayed with dopa, 4-methylcatechol and catechol.

RESULTS & DISCUSSION

Purification

Table 1 presents typical specific activity values at the stages in the purification procedure. The increase, from 26 U/mg protein in the initial buffer extract to 725 U/mg in the second Sephadex eluate, represents approximately 28-fold purification.

When aliquots of crude PPO ranging in volume from 1 to 50 ml were applied to the Sephadex column, the resulting V_e/V_o values (elution volume of enzyme/elution volume of a compound completely excluded from the gel, i.e., Dextran Blue 2000) all fell between 1.88 and 1.98. Although the V_e/V_o values were independent of load, indicating the absence of concentration dependent association, the purification achieved (2- to 5-fold) decreased as the load volume was increased.

Michaelis constants

Results of the rate studies are shown in Table 2. Also presented for each substrate is the molecular velocity constant, $V_m/2\,K_m$, which approximates the slope of the first-order portion of the plot of v vs. S (since $K_m = S$ when $v = V_m/2$).

Table 2 also shows that these K_m values are in reasonable agreement with results obtained with much cruder avocado PPO preparations extracted directly from the fruit tissue and only purified by precipitation with $(NH_4)_2SO_4$ (0.5–0.7 saturation). The V_m values fall in the same order as previously published relative reaction rates (Knapp, 1965).

Thin-layer gel filtration

Although the measured reactivity of avocado PPO with dopa was considerably less than with catechol and 4-methylcatechol, the depth of color was much greater. Upon oxidation, dopa turned brownish-black in contrast to the lighter orange-beige of the latter compounds' products (Fig. 2). Consequently, dopa was used routinely for location of PPO fractions on thin layers.

Spraying of developed thin-layer plates with dopa revealed 4 PPO fractions of varying intensities, designated α, β, γ and δ in order of increasing migration rate and molecular weight (Fig. 1). Preliminary runs (see Fig. 2) showed that the purified PPO preparation essentially lacked the δ fraction and had a lower concentration of the γ fraction than did the crude PPO preparation. This was because the most active eluates from the Sephadex columns contained the β fraction predominantly, with smaller amounts of the other fractions. Consequently, the crude PPO preparation was used for subsequent thin-layer studies. On

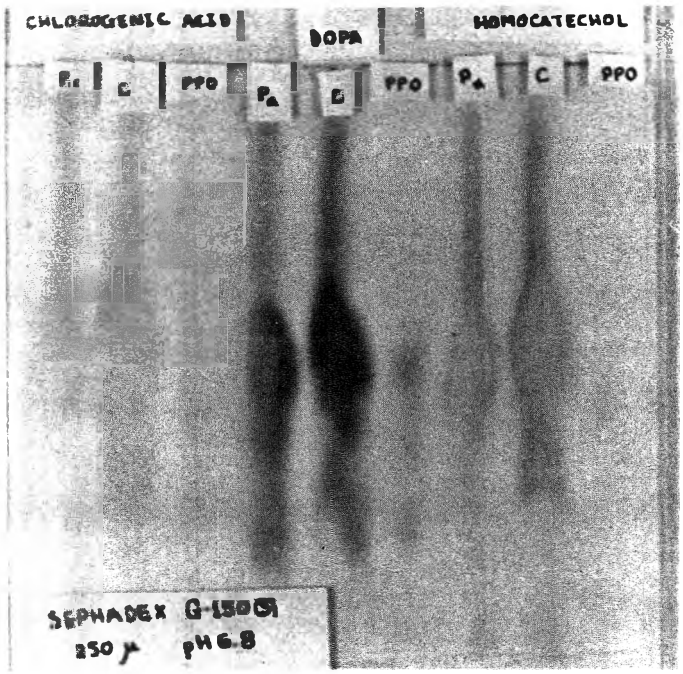

Fig. 2—Sephadex thin layer showing partial separation of purified avocado PPO (P_α), crude avocado PPO (C) and commercial mushroom PPO (Worthington Biochemical Corp.) visualized with catechol, dopa and homocatechol sprays.

plates not allowed to develop as fully as the others, a faint spot appeared considerably ahead of the δ fraction (see Fig. 2). This ϵ fraction must have been completely eluted from most of the other plates.

Table 3 lists the reference proteins, their molecular weights and R_{BSA} values based on 5 determinations. When log molecular weight is plotted against R_{BSA} values, a linear slope is obtained from cytochrome c to ovalbumin, and a 2nd, flatter slope from the latter point to gamma-globulin.

The R_{BSA} values for 4 fractions (α–δ) of crude avocado PPO and their estimated molecular weights are also presented in Table 3. The latter values were 14-, 28-, 56- and 112-thousand for fractions α, β, γ and δ, respectively. According to Andrews (1964) human gamma-globulins behave in such systems as though they had a molecular weight of 205,000. If so, the molecular weight of the δ fraction must be 130,000. The ϵ fraction had a molecular weight in excess of 4×10^5, although the exact value could not be determined because it exceeded the exclusion limit of the gel. This fraction deserves further investigation, using a dextran gel with a higher exclusion limit.

It may be noted that the molecular weights of the avocado PPO fractions apparently fall in multiples (1, 2, 4 and 8) of 14,000. Similar behavior has been reported for several enzyme systems, of which tyrosinase is the most similar in function to PPO. Nakamura et al. (1966) isolated a dimeric mushroom tyrosinase with a molecular weight of 61,000. Jolley (1966) reported ready interconversion between the monomeric, dimeric and higher polymeric forms of this enzyme. Association was favored by high enzyme concentration, while dissociation was stimulated by high ionic strength, sodium dodecyl sulfate, heat and EDTA. He believed all the monomeric units to be essentially identical.

Dilution of the crude avocado PPO (1.3 mg/ml) to 1/2 or 1/3 and concurrent increase in buffer concentration from 0.02 to 0.1 M resulted, after equilibration at 5°C, in an increase in the α fraction at the expense of the β fraction. Any changes in the relative sizes of the higher MW fractions were too small to be readily detectable. All appeared on the plates of the treated fractions, including the ϵ fraction in some cases.

Pending further investigation, nothing definitive can be said about the microheterogeneity of the monomeric (α) unit of avocado PPO. The electrophoretic separation of the β (dimeric) fraction (see below) would seem to indicate either that the subunits were not identical in composition or that they were polymerized in different ways so as to yield dimers having slightly different net charges.

Two-dimensional thin-layer gel filtration-electrophoresis

The most noticeable features of the 2-dimensional plates were the absence of the α fraction and the separation of the β (2.8×10^4 MW) fraction into several components, as shown in Figure 3. Spots β 1 and 2, on the left (anode side) were not clearly separated, and it is not certain that 2 components are present. There is somewhat less doubt about the separation of spots β 5 and 6 on the right (cathode side). Thus, instead of the 6 components indicated, there may be only 4 or 5. The largest, central component (β 4) migrated about the same distance toward the anode as did the γ and δ fractions, which remained as discrete entities.

The enzyme fractions shown in Figure 3 reacted with different substrates to differing degrees. After being sprayed with dopa, all spots eventually produced the same intensity of color (except β 6, which was much lighter).

After catechol spray, also, the β 4 spot

Table 3—Effect of molecular weight on migration of proteins on Sephadex G-150 (S) thin layers.

Protein standards	MW × 10^{-3}	R_{BSA}[1]		s.d.
Cytochrome c	12.48	0.657	±	0.016
Myoglobin	17.8	0.714	±	0.016
Chymotrypsinogen A	25.0	0.775	±	0.018
Ovalbumin	45.0	0.880	±	0.011
Bovine serum albumin	67.0	1.00	±	—
Gamma globulin (human)	160.0	1.23	±	0.004
Avocado PPO fractions				
	14.0	0.677	±	0.014
	28.0	0.801	±	0.025
	56.0	0.945	±	0.036
	112.0 (130)	1.14	±	0.006
	400			

[1] Migration rate with respect to bovine serum albumin.

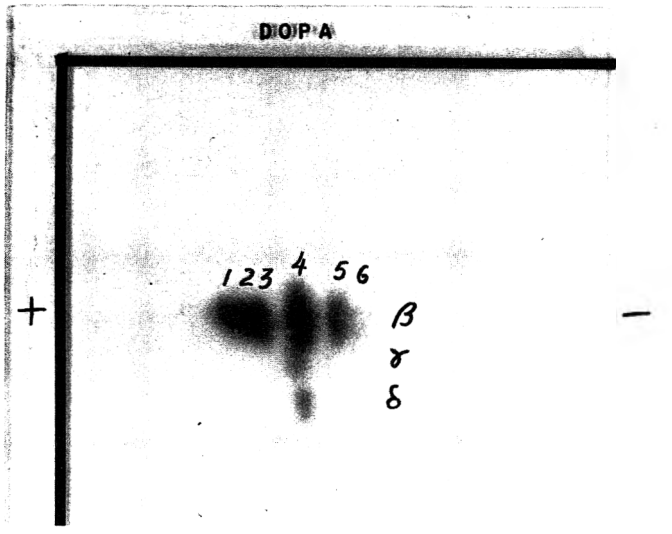

Fig. 3–Sephadex thin layer showing separation of avocado PPO fractions by gel filtration followed by electrophoresis at 90°. Sprayed with dopa after development.

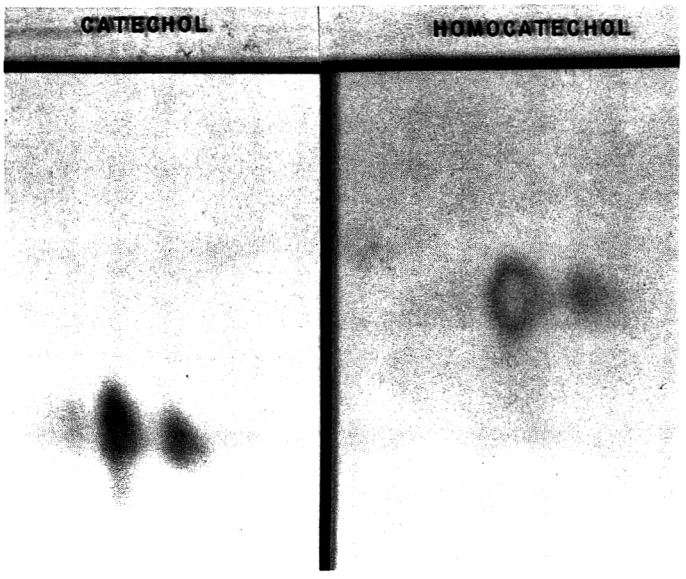

Fig. 4–Portions of 2 Sephadex thin-layer plates showing avocado PPO developed 2-dimensionally by gel filtration-electrophoresis followed by spraying with catechol (left) and homocatechol (right). The most prominent spot in each plate corresponds to the β 4 fraction indicated in Figure 3.

gave much the most intense color. The β 1–3 spots and γ and δ spots were rather weakly colored, and the β 5 spot was intermediate in intensity. The β 6 spot appeared to produce relatively more color with catechol than with dopa, so that its final intensity was equal to the dopa color of the β 1–3, γ and δ spots (Fig. 4, left).

Spraying with 4-methylcatechol produced an intense orange-tan spot at the β 4 position and a moderately intense spot (of the same color) with the β 5 fraction. Color production in the other areas was very weak. There was no ϵ spot, although one might have been expected on the particular plate shown in Figure 4 (right).

It is important to note the order in which these fractions produced color after being sprayed with the substrates. The β 4 spot began to appear within 15–20 sec, the γ and δ fractions after 15 min and the other β fractions required 30–60 min. The α fraction did not appear on any of the 2-dimensional plates. On the basis of comparative color production by dopa and nigrosine after 1-dimensional development, the α fraction must have had a low specific activity, at least after that treatment. Both α and ϵ fractions may have been totally inactivated during electrophoresis. Because color development by the β 4 fraction occurred so much sooner than with the other PPO components, it seems certain that it is the activity in the β 4 fraction measured by the spectrophotometric assay. The initial linear portion of this assay lasted only 1 min or less.

Because only dopa, catechol and 4-methylcatechol were used to spray the plates, we cannot be sure that other substrates would also have been oxidized more rapidly by the β 4 fraction than by any other PPO fraction. However, this seems a reasonable assumption and allows the conclusion that it is the activity of the β 4 fraction which is reflected in all the kinetic data obtained (Table 2).

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MAJOR VOLATILE NEUTRAL AND ACID COMPOUNDS OF HYDROLYZED SOY PROTEIN

SUMMARY—Interest in the use of hydrolyzed vegetable protein in many food products has led to the need for more basic knowledge of the types of compounds present. The present study shows that a number of aldehydes and furan-type compounds are the major compounds of significance in a neutral fraction. An acid fraction, which appeared to carry most of the odor associated with the hydrolyzed soy protein, has levulinic acid as a major component. Thirteen other acids, 2 lactones, 4 phenols plus other compounds were also isolated and identified in the acid fraction. The mechanisms for the formation of some of the compounds are discussed.

INTRODUCTION

INCREASED interest in the use of vegetable protein hydrolysates has led to a greater need for fundamental information about the formation of aroma and the stabilization of the chemical components of nutritive value during the hydrolysis process. One approach to obtaining information about aroma development is to study the volatile compounds found in the hydrolyzed material. These products, because of their volatility, are likely to influence the aroma of the hydrolyzed soybean product.

Many model systems which parallel the natural vegetable system have been investigated. Acid- and base-catalyzed fructose degradations have been studied by Shaw et al. (1967; 1968). Sugisawa (1966) separated some acids and esters formed when acidic solutions of D-glucose are heated. The volatile compounds from heated glucose have also been studied by Walter et al. (1968), Sugisawa (1966) and Heyns et al. (1966). Bailey et al. (1962), Bryce et al. (1963) and Gianturco et al. (1964; 1966) have also studied thermal effects on carbohydrates and various food products closely associated with carbohydrates. The chemistry of browning reactions occurring between amino acids and carbohydrates, in model systems and natural products, has been well reviewed by Hodge (1953), Ellis (1959) and Reynolds (1963; 1965).

Prior to the recent publication by Manley and Fagerson (1970), reports of flavor studies of hydrolyzed vegetable protein (HVP) have not been available.

However, studies on soy sauce, a chemical and microbiological hydrolysis product of the soybean, are fairly extensive. Two general reviews by Yokotsuka (1961) and Yamada (1966) offer a total view of the components isolated in soy sauce. A number of flavor studies on soybean flour before hydrolysis have been completed by Arai et al. (1966a; 1966b; 1967). They were successful in isolating many of the phenolic acids, volatile fatty acids, volatile amines and neutral volatile compounds of defatted soy flour.

The purpose of the present study was to identify the major volatile components in the neutral and acid extracts of hydrolyzed soybean flour.

EXPERIMENTAL

Soybean flour

A solvent defatted soybean flour commercially available as Textrol was obtained (Central Soya, Chicago, Ill). A typical proximate analysis showed: 50% protein, 0.6% fat, 3.0% crude fiber, 5.4% moisture and 30% carbohydrate.

The soybean flour was subjected to hydrolysis with 6 N HCl at a temperature of 110°C for 24 hr. After this reaction, the mixture was cooled and brought to a pH of 3 by addition of NaOH (50% aqueous). The material was then filtered with prewashed diatomaceous earth (Celite) to remove the humins from the crude hydrolysate. The filtered mixture was adjusted to a pH between 5 and 6 by further addition of NaOH solution. At this stage the mixture was concentrated to a dry powder by spray drying.

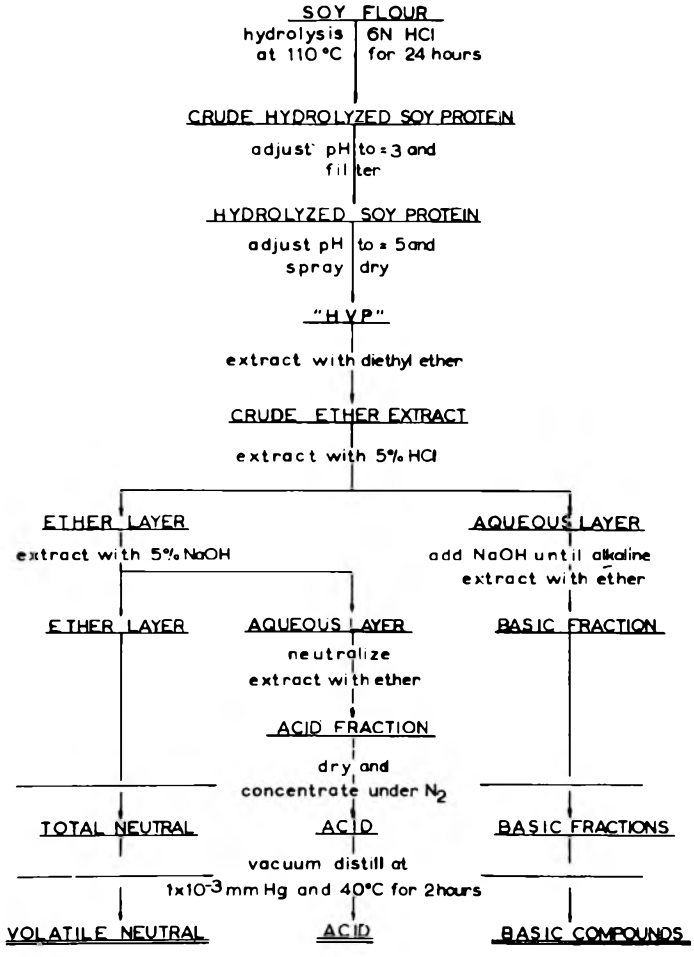

Fig 1—Isolation scheme for the volatile organic components of hydrolyzed soy protein.

[a]Present address: The Nestlé Co., New Milford, Connecticut 06776.

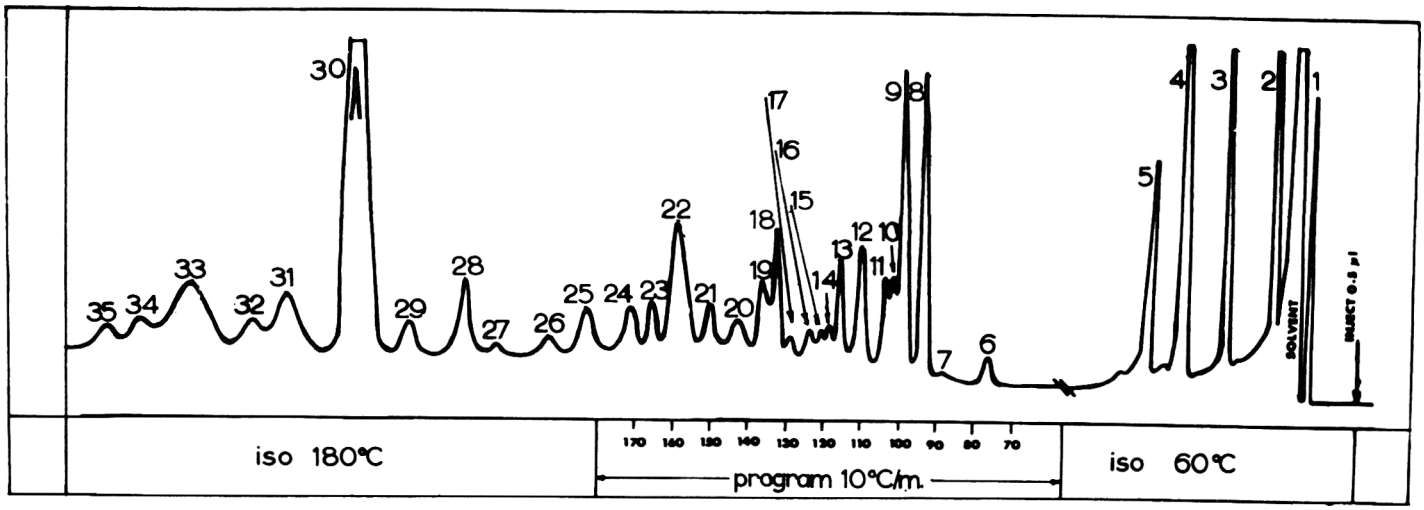

Fig. 2—Composite gas chromatogram of the volatiles of the neutral fraction of hydrolyzed soy protein.

The spray dryer inlet temperature was 180°C and the outlet temperature 110°C.

In each batch the final pH of the dried material as determined in a 2% solution was between 5 and 6, the sodium chloride concentration was approximately 40% and total solid averaged 95%.

The general scheme just outlined is similar to that used in the commercial preparation of hydrolyzed vegetable protein.

The dried material was sealed in an amber jar and stored at room temperature until needed for further analysis.

Extraction

The spray-dried HVP was exhaustively extracted with redistilled ethyl ether in a Soxhlet-type extractor. 300 g was extracted with 500 ml of the redistilled ethyl ether at a distilling temperature of 45°C. The total extraction method for all fractions is outlined in Figure 1.

A dilute ether solution of the extracted material was stored in an Erlenmeyer flask at −20°C until needed.

When required, the ether extracts of the var-

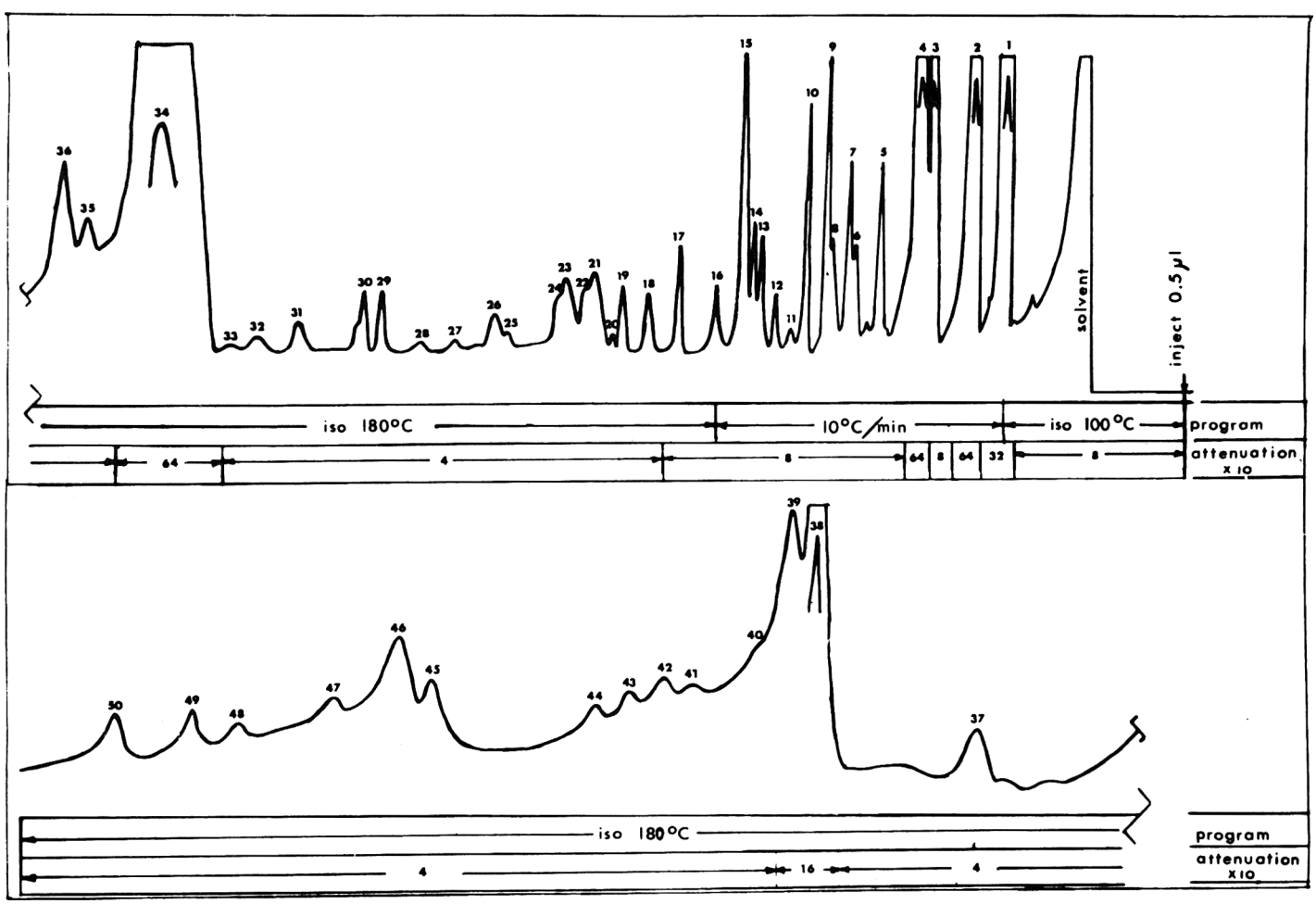

Fig. 3—Gas chromatogram of the volatiles of the acid fraction of hydrolyzed soy protein.

Table 1—Identification of components found in the neutral fraction.

Peak No.	Name of compound	Rf on GC columns: K20M	DEGS	M.S.	I.R.	Comment
1	Acetaldehyde	+	+	+		P
(s)	Furan	+	+	+		P
2	2-Butanone	+	+	+		P
3	Acrolein	+	+	+		P
4	Ethyl acetate	+	+	+		P
5	Ethanol	+	+	+		P
6	Furfural	+	+	+		P
8	Benzaldehyde	+	+	+	+	P
9	2-Furyl methyl ketone	+	+	+	+	P
10	5-Methyl furfural	+	+	+		P
12	Guaiacol	+	+	+		P
13	5-Methyl-2-acetyl furan	+	+	+		P
18	Phenyl acetaldehyde	+	+	+		P
19	Acetophenone	+	+	+		P
20	Acetyl-2-propionyl-5-furan			+		T
22	Dimethyl pyrone			+		T
25	3-Octen-4-one			+		T
29	Coumarin	+	+	+	+	P
30	Methyl coumarin			+		T

T—Tentative. P—Positive. (s)—With solvent.

ious fractions were redried and further concentrated to a small volume in a cooled centrifuge tube under nitrogen. The residue was then distilled at a temperature of 50°C and a pressure of 1 μ Hg on to a liquid N_2-cooled cold-finger. The material was removed from the cold-finger by repeated washings with diethyl ether. This material was considered to be the volatiles of the neutral and acid fractions. An analysis was carried out on the concentrate within a day of the preparation. Unused sample was rediluted and again stored at −20°C.

Table 2—Identification of components found in the acid fraction.

Peak No.	Name of compound	Rf on GC columns: DEGS	SE-30	M.S.	I.R.	Methyl ester	Comment
(s)	Formic Acid	+	+	+			P
1	Acetic Acid	+	+	+	+		P
2	Propanoic Acid	+	+	+	+		P
2	α-Angelica lactone (mixed with propanoic)	+	+	+			P
3	Butanoic Acid	+	+	+			P
3	Benzaldehyde	+		+			Trace
3	2-Furyl methyl ketone	+		+			Trace
4	Isovaleric Acid	+	+	+			P
5	Valeric Acid	+	+	+			P
7	Crotonic Acid	+	+	+			P
9	2-Methyl-2-butenoic acid			+			T
10	β-Angelica lactone	+	+	+			P
14	Isomaltol			+			P
15	Guaiacol	+	+	+	+		P
16	Ethyl phenol	+	+	+			P
17	Phenol	+	+	+	+		P
18	4-Ethyl guaiacol	+	+	+			P
19	p-Cresol	+	+	+			P
21	Maltol	+	+	+			P
23	4-Phenyl-3-buten-2-one			+			T
30	γ-Undecalactone	+		+			T
34	Levulinic Acid	+	+	+	+	+	P
35	Benzoic Acid	+	+	+		+	P
36	2-Furoic Acid	+	+	+		+	P
38	Phenyl Acetic Acid	+	+	+	+	+	P
39	N-Propyl-2-pyrrolidone			+			
42	β-Phenyl propanoic	+	+	+		+	T
46	p-Ethyl Benzoic Acid	+	+	+		+	P

T—Tentative. P—Positive. (s)—With solvent.

Mass spectrometry

For analysis, a 1-μliter sample of the concentrated ether extract was taken. Mass spectra were obtained, using a coupled gas chromatograph (Varian-Aerograph Model 1200)—mass spectrometer (Hitachi-Perkin-Elmer Model RMU-6A) unit with a capillary column, 200 ft long, 0.02 in. id and coated with a mixture of diethylene glycol succinate (DEGS) with 2% H_3PO_4. The column effluent was split with nearly equal portions being directed to the flame ionization detector and to the mass spectrometer. A Watson-Biemann (Watson et al., 1965) helium separator was used between the gas chromatograph and the mass spectrometer. The operating parameters were as follows:

Gas Chromatograph

Flow rate: Helium carrier gas at 15 ml/min

Program: 100°C for 5 min
100 to 180°C at 6°C/min

Injection block temperature: 275°C

Mass Spectrometer

Inlet temperature, 200°C. Ion source pressure, 8×10^{-6} mm. Ion source temperature, 250°C. Acceleration voltage, 2.5 kv. Target current, 100 μA. Electron multiplier voltage, 2.5 kv. Scan speed, 8 sec (m/e 12–250). Exit slit, 0.25 mm.

Infrared analysis

Trapping for infrared analysis was done by the method of Edwards et al. (1965), using a Perkin-Elmer 800 gas chromatograph with a 6-ft-long, 1/8-in. od column packed with 8% DEGS with 2% H_3PO_4 on 60/80-mesh Chromosorb W. For the neutral fraction a 12-ft-long, 1/8-in. od column packed with 15% Carbowax 20M on 80/100-mesh Chromosorb W was also used. The outlet temperature was kept at 250°C and the injection block temperature at 275°C. The column was programmed 100–180°C at 4°C/min.

The collected fractions were pressed into individual 1.5-mm KBr discs, using an ultramicro die. Infrared spectra were recorded on a Perkin-Elmer 337 infrared spectrometer (equipped with a 4 × beam condenser).

Retention data

The retention times of the known compounds and unknown sample were recorded by injection on a Perkin-Elmer 900 gas chromatograph using the following columns for the various fractions

1. A 50-ft support-coated open tubular (S.C.O.T.) column 0.02-in. inside diameter coated with Carbowax 20M (K20M).
2. A capillary column, 200 ft long, 0.02-in. id and coated with DEGS and H_3PO_4 (2%).
3. A 6-ft 1/8-in. packed column with 3% SE-30 on 80/100-mesh Chromosorb W.

RESULTS

Neutral fraction

The separation of the volatile neutral fraction of the hydrolyzed soybean protein on the K20M column is shown as a chromatogram (Fig. 2). The chromatogram is a composite of the low- and high-boiling fractions.

Identification of the compounds was based primarily on mass spectroscopy and gas chromatography retention factors. When the concentration and resolution permitted, infrared spectra also were re-

corded. The compounds identified are presented in Table 1. The peak numbers refer to those in Figure 2.

A (+) symbol in the gas chromatography column indicates retention time agreement with known compounds. A (+) symbol in the mass spectra column indicates mass spectral agreement with published or known spectra. A (+) symbol in the infrared spectra column indicates confirmation by comparison of spectrum to that of an authentic sample.

Mass spectra of 3 of the major neutral volatile constituents are shown in Figure 4 labeled as N8, N9 and N29. The mass spectral data are a presentation of the normalized major peaks of diagnostic value.

Acid fraction

Separation of the volatile acid fraction of the hydrolyzed soybean protein on the DEGS with H_3PO_4 column is shown, as a chromatogram, in Figure 3. Identified compounds are listed in Table 2, and the notation of the Table is the same as for Table 1 with the addition of the column for methyl esters.

A (+) symbol in the methyl ester column indicates that the methyl ester of the acid as prepared by the Stoffel et al. (1959) method was identified by comparison of retention times with known compounds and mass spectral data.

Mass spectra of 3 of the major volatile constituents are shown in Figure 4 labeled as A10, A15 and A34. These compounds were chosen because of their relative importance in the mechanisms to be discussed.

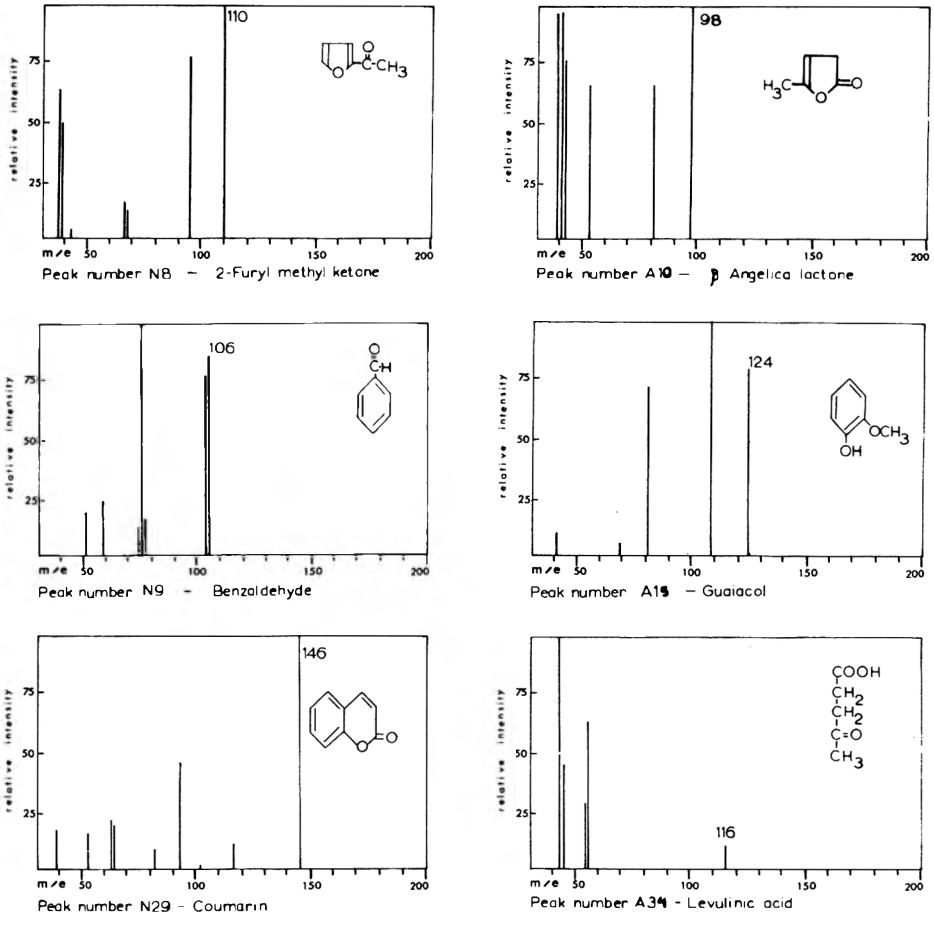

Fig. 4—Some mass spectra of volatile components isolated from the neutral (N) and acid (A) fractions of hydrolyzed soy protein.

DISCUSSION

THE MAJOR component of the acid fraction, levulinic acid, appears to indicate that the major amount of this fraction is caused by acid-catalyzed degradation of the carbohydrate in the soybean flour. Levulinic acid is a known degradation product of 5-(hydroxy methyl)-2-furaldehyde, which occurs from the degradation of glucose or fructose (Reynolds, 1963). Anet (1962; 1964) suggests that acid-catalyzed degradation of D-fructose or D-glucose occurs primarily through 1,2-enolization with loss of the 3-hydroxyl group by dehydration, and then further dehydration to give 5-(hydroxy methyl)-2-furaldehyde. The conversion to levulinic acid involves ring fission followed by a disproportionation in which the ketol group becomes reduced and the aldehydic group becomes oxidized. Figure 5 outlines the mechanism by which the levulinic acid is produced. A number of researchers have isolated the angelica lactones (the gamma lactones of the 4-hydroxy 2- or 3-pentenoic acid) from acid, base and thermal-catalyzed degradation of glucose or fructose, Walter et al. (1968); Shaw et al. (1967; 1968). Shaw (1967) notes that the work of Leonard (1956) indicates that production of the lactones may occur as an artifact during the distillation step.

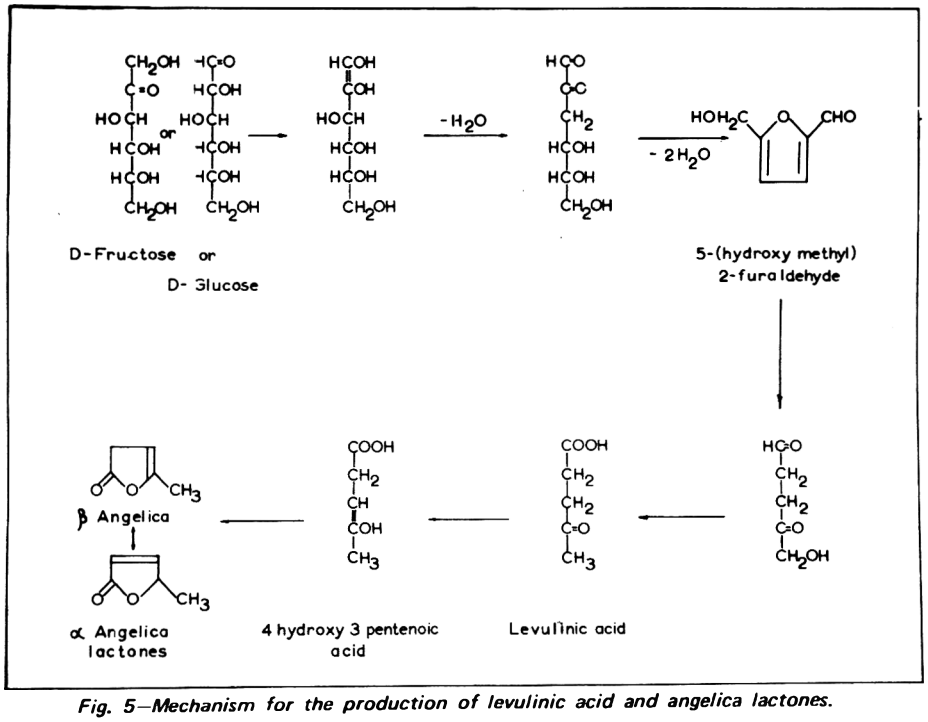

Fig. 5—Mechanism for the production of levulinic acid and angelica lactones.

However, Kuehl et al. (1950) notes that a temperature of 280°C causes a dehydration of the levulinic acid to form the α-angelica lactone and a subsequent

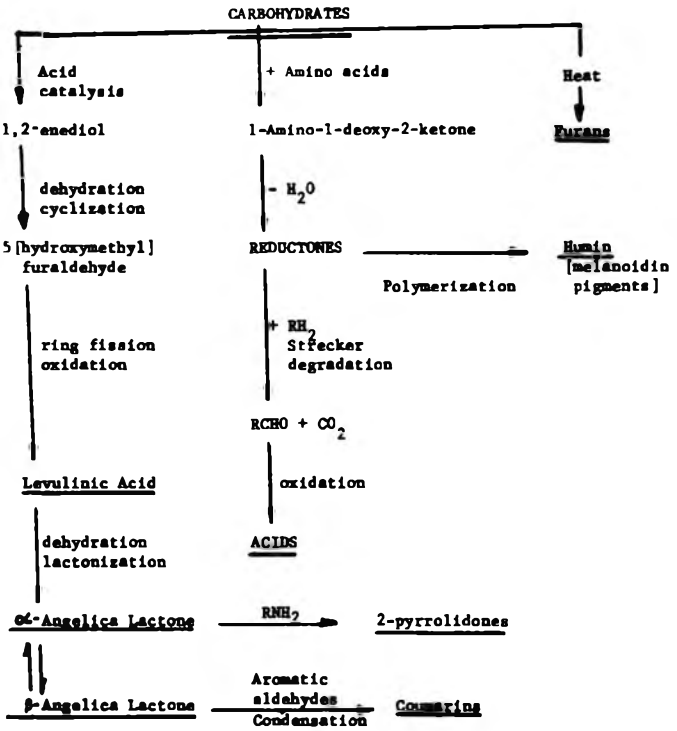

Fig. 6—Summary of some of the possible chemical reactions occurring during hydrolysis and processing of hydrolyzed soy protein.

thermal isomerization to create the β-angelica lactone. The production of the hydrolysate involves a spray-drying step where the temperature might be of an order of magnitude to cause the angelica lactone formation. Therefore, these compounds may be produced at least in part by the spray-drying process and due to an artifact during the extraction and separation.

Also of importance in the acid fraction is the presence of guaiacols and phenols. These compounds arise mainly through the degradation of the phenolic carboxylic acids. Arai et al. (1966a) have isolated and identified a number of phenolic carboxylic acids in defatted soy flour. One of the guaiacols, 4-ethyl guaiacol, has been mentioned as a possible major flavor component in soy sauce (Yokotsuka et al., 1967). Obviously, all the phenols, in general, cause an intense characteristic odor.

Maltol, found in the acid fraction, has been shown by Hodge et al. (1963) to be present in distillates of soybeans. Maltol is also assumed to arise from 4-0-substituted glucose derivatives by browning reactions with amino acids (Hodge, 1967). While browning reactions occur most rapidly in aqueous solution at alkaline pH, it is possible that appreciable browning occurs at the pH and moisture levels of the final HVP product.

Production of acids is via the Maillard type reaction of carbohydrates and amino acids followed by Strecker degradation to produce the aldehydes, which are then oxidized to the acids. It is also possible that some of the acids are being created by the degradation of the carbohydrate material alone. It is this acid fraction which appears to carry most of the notes associated with the meat-like hydrolyzed vegetable protein aroma. However, no one particular compound carries all of the associated aroma. The flavor volatiles of cooked beef have been studied by Hornstein et al. (1960), Kramlich et al. (1958), Sanderson et al. (1966) and Yueh et al. (1960). Included in the volatile compounds identified by these authors and considered to contribute to the over-all meat-like aroma are formic, acetic, propionic, iso-butyric and butyric acids.

The neutral fraction had a more caramel-type aroma. The furfural type compounds found in this fraction are likely contributors. Sugar dehydration reactions in acidic systems are known to form the furfural (Hodge, 1953). The coumarins in this fraction could possibly be caused by the condensation of hydroxy-aldehydes and aromatic aldehydes with β-angelica lactones, Marrian et al. (1946).

The identification, although not a positive structural identification, of a cyclic amide, N-propyl-2-pyrrolidone, having a molecular weight of 127 is of possible importance in HVP. Lukes (1928; 1929) has shown that some cyclic amides, such as the 2-pyrrolidones, have occurred by the condensation of α-angelica lactone with amines. Reduction of levulinic acid and its condensation with amines can also cause 2-pyrrolidone formation, Hayashi et al. (1954).

An outline of some of the possible mechanisms for the development of the compounds isolated is shown in Figure 6. Most intermediate compounds are omitted to show the over-all nature of the reactions involved.

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EFFECT OF AGING ON PALATABILITY AND SELECTED RELATED CHARACTERISTICS OF PORK LOIN

SUMMARY—Selected characteristics of 24 left pork loins representing 4 aging (chilling at 36°F) periods (1, 4, 8 and 12 days) were investigated. The loin was cut into 4 sections: a) the 4th to 8th thoracic vertebra, b) the 8th to 12th thoracic vertebra, c) the 12th thoracic to the 1st lumbar vertebra and d) the 1st to 5th lumbar vertebra. 2, 1.5-in. chops were cut from the posterior end of each section and used for measurements on the cooked meat (cooking time and losses, palatability scores, shear value, water-holding capacity, total moisture, free fatty acids and pH). The remaining portion of the sections was used for measurements on the raw meat (total moisture, free fatty acids, pH, lactic acid, TBA, ether extract and free amines). Significant increases after 12 days' aging were noted in cooking time in min/lb ($P < 0.05$), dripping cooking losses ($P < 0.05$), acid numbers for raw and cooked fat ($P < 0.01$), pH of raw ($P < 0.05$) and cooked ($P < 0.01$) muscle, TBA value of raw muscle ($P < 0.05$), tenderness score ($P < 0.05$) and free amines ($P < 0.01$). Decreases ($P < 0.05$) were noted for percentage total moisture of raw muscle and shear values. Aging pork loin for 12 days had only a slight effect on palatability. Few significant changes were noted in the pork loins until after 4 days of aging. However, free amines in raw muscle and free fatty acids of cooked meat increased ($P < 0.05$) between 1 and 4 days of aging. Generally, aging was of little benefit.

INTRODUCTION

IN GENERAL, it is believed that pork carcasses should be chilled and processed soon after slaughter. Wilson (1960) stated that fresh pork is not aged, primarily because pork fat rapidly becomes rancid when held at refrigerator temperature. He explained that most fresh pork entering retail channels is tender and does not require treatment for tenderization. He stated that some consumer complaints about fresh pork being dry and less tender than desirable suggest attention should be given to improving tenderness. Hendrix et al. (1963) also indicated that all consumers are not satisfied with pork. Gould et al. (1965) reported that aging pork loin chops from 2 to 12 days after slaughter increased tenderness as measured by the Warner-Bratzler shear.

Harrison et al. (1956) reported that pork loin from carcasses chilled (30 or 40°F) 7 days before freezing deteriorated to a greater extent during frozen storage than pork from carcasses chilled for 1 and 3 days. In general, there was little difference in acceptability of meat chilled for the latter 2 periods. Tenderness of the frozen meat did not vary significantly with time and chilling temperature or with frozen storage.

The effects of 4 aging (chilling at 36°F) periods on palatability and selected related characteristics of pork loin are reported here. Aging periods selected (1, 4, 8 and 12 days) represented the usual time between slaughter of the animal and entrance of the carcass into retail channels (1 day), and longer periods for tenderization by protein autolysis.

EXPERIMENTAL

Processing the loins

24 pigs (8 Duroc, 8 Yorkshire and 8 Hampshire) were slaughtered at a weight of approximately 220 lb. Carcasses hung for 1 day at 36°F, then left loins were cut into 4 sections from the posterior surface of a) the 4th thoracic vertebra to the 8th thoracic vertebra; b) the 8th to 12th thoracic vertebra; c) the 12th thoracic vertebra to the 1st lumbar vertebra and d) the 1st to the 5th lumbar vertebra. A fat covering of approximately ½-in. was left on the loins to provide adequate fat tissue for palatability and chemical measurements.

3 of the 4 sections were placed in Cryovac bags and aged 4, 8 or 12 days at 36°F. After aging, they were frozen (−15°F) in a blast freezer and stored in still air (−5°F) until evaluated. The 4th section was placed in a Cryovac bag, frozen (−15°F) and stored (−5°F) as the sample representing 1 day of aging. A 4 × 4 Latin square with 6 replications was followed, to age and evaluate (3–6 months after freezing) the loin sections. Each row of a square represented the left loin of 1 pig (sections A, B, C and D) and 1 evaluation period. The columns represented the position on the loin (section A, B, C or D).

At each of 24 evaluation periods the 4 sections from 1 loin were removed from the Cryovac bags and each divided into 3 pieces. 2, 1.5-in. chops were cut from the posterior end of the section and used for measurements on cooked meat. The remaining portion of the section was used for measurements on raw meat. Frozen pieces were wrapped in polyethylene laminated freezer paper and defrosted for 24 hr in a refrigerator (45°F).

Measurements on cooked meat

2 chops from each section were placed on a wire rack (4 in. high) set in a shallow pan and cooked in a rotary hearth oven (350°F) to an internal temperature of 75°C. Total cooking time (min) was noted, and cooking time (min/lb) and percentage cooking losses, based on the weight of the defrosted chops, were calculated.

The longissimus dorsi (LD) muscle from 1 cooked chop was used to measure shear value and water-holding capacity (WHC). The LD from the other cooked chop was used for palatability measurements. The muscle remaining from both chops was ground and used to measure total moisture and pH.

2 cores (½ in. diameter) were cut longitudinally through the LD at lateral and medial positions, and sheared perpendicularly to the fibers on a Warner-Bratzler shearing apparatus (25-lb dynamometer). Triplicate measurements of WHC were made on samples taken from the center of cores (selected at random) used to measure shear value. WHC was determined by the method of Miller et al. (1965), except the filter paper was dried for 2 hr at 70°C.

Percentage total moisture was ascertained by drying duplicate 10-g samples of ground LD in a C.W. Brabender Semiautomatic Moisture Tester for 1 hr at 121°C. Duplicate measurements of pH were made on homogenates of ground LD by the method of Rogers et al. (1967).

Table 1—Means for cooking time and losses.

Measurements	Aging time, days 1	4	8	12	Probability of F	LSD[1]
Cooking time (min)	63	64	62	62	ns	—
Cooking time (min/lb)	45.3	47.3	47.2	48.0	*	2.508
Cooking losses (%)						
Total	24.6	24.6	24.4	25.2	ns	—
Volatile	17.2	17.7	17.2	17.7	ns	—
Dripping	6.9	6.5	6.8	7.2	*	0.586

*$P < 0.05$.
[1]LSD, least significant difference at the 5% level; ns, not significant.

The outside fat covering on both chops from 1 piece was analyzed for free fatty acids by titrating the free fatty acids in an aliquot of fat extract in neutral alcohol. The fat extracts were prepared as described by Watts et al. (1947). Results were expressed in terms of acid number (mg KOH/g extracted fat).

An 8-member laboratory panel scored the outside covering of fat for flavor and ½-in. cubes of LD for flavor, juiciness, tenderness and texture on a 7-point scale. Flavor and texture were scored on a desirability scale and tenderness and juiciness on an intensity scale, with 7 points representing extremely desirable or intense characteristics. Panel members described any "off" flavor they detected such as acid, yeasty, moldy, oxidized or other. They also described characteristics of the muscle texture (soft, mealy or grainy, stringy, rubbery, other) when a score below 6 (desirable) was given.

Measurements on raw meat

After defrosting, the LD was ground and used to measure pH, total moisture, lactic acid, malonaldehyde (thiobarbituric acid values, TBA), ether extract and free amines. The outside fat covering was analyzed for free fatty acids as described for cooked meat.

Percentage total moisture was ascertained with duplicate 10-g samples dried at 121°C for 2 hr in a C.W. Brabender Semiautomatic Moisture Tester Lactic acid was determined by the method of Barker et al. (1941). TBA values were obtained by the distillation method described by Tarladgis et al. (1960), and percentage ether extract with a Goldfisch extraction apparatus.

Total free amines were estimated by preparing a filtrate from a slurry of muscle deproteinated with zinc hydroxide and barium sulfate. Total free amines in the filtrate were measured by the method of Yemm et al. (1955). Micromoles of ninhydrin-reactive substances were calculated from a standard curve prepared from glycine.

RESULTS & DISCUSSION

Cooking time and losses

Total cooking time and losses and volatile losses were not affected significantly by aging. However, cooking time (min/lb) and percentage dripping losses increased ($P < 0.05$) with aging. Values for both cooking time and dripping losses were highest at 12 days of aging (Table 1).

Flavor and related objective measurements

Little change in flavor of the LD muscle and fat covering was detected by the palatability panel. However, significant hydrolytic ($P < 0.01$) and oxidative ($P < 0.05$) changes occurred with aging (Table 2). Acid numbers for raw fat increased ($P < 0.05$) with each increase in aging time beyond 4 days. For cooked fat, acid numbers increased ($P < 0.05$) between 1 and 4 days and between 8 and 12 days, but not between 4 and 8 days. Harrison et al. (1956) reported that fat on carcasses chilled for 7 days had higher acid numbers for both raw and cooked fat than fat on carcasses chilled 3 and 1 day. TBA values for LD muscle aged 8 and 12 days were greater ($P < 0.05$) than those for muscle aged 1 or 4 days.

Table 2—Means for flavor and related objective measurements.

Measurements	Aging time, days 1	4	8	12	Probability of F	LSD[1]
Flavor score[2]						
Cooked muscle	5.6	5.6	5.6	5.4	ns	---
Cooked fat	5.6	5.6	5.4	5.4	ns	---
Acid number[3]						
Raw fat	11.45	13.37	17.36	20.28	**	2.227
Cooked fat	11.91	14.54	16.15	18.69	**	2.177
TBA value, raw muscle[4]	2.13	2.10	2.99	3.18	*	0.854
pH						
Raw muscle	5.54	5.56	5.58	5.59	*	0.037
Cooked muscle	5.77	5.79	5.85	5.87	**	0.036
Lactic acid, raw muscle, mg/g	9.58	10.04	10.15	10.01	ns	---
Free amines, raw muscle[5]	1,938.82	2,216.56	2,317.46	2,633.28	**	209.898

*P < 0.05.
**P < 0.01.
[1] LSD, least significant difference at the 5% level; ns, not significant.
[2] 7 (extremely desirable) to 1 (extremely undesirable "off" flavor).
[3] mg KOH/g extracted fat.
[4] mg malonaldehyde/1,000 g muscle.
[5] μmoles ninhydrin-reactive substances/g muscle.

Changes in pH of raw and cooked muscle increased ($P < 0.05$ and $P < 0.01$, respectively) with aging for 12 days (Table 2). Most glycolytic changes take place within 24 hr after slaughter; thus, most pH and lactic acid changes would have occurred during that time. Lactic acid concentration in the muscle was similar for the aging periods studied.

Changes in the protein during the aging of pork were indicated by a significant ($P < 0.05$) increase in free amines with each increase in aging period, except between 4 and 8 days. McCain et al. (1968) reported an increase in ninhydrin-positive material (NPM) (from dry-cured hams) during successive aging periods up to 8 wk. They reported a highly significant relationship ($r = 0.80^{**}$) between NPM and aged flavor. Their evidence indicated that amino acids are important as precursors of meat flavor and that, conceivably, changes occurring in free amino acids during aging were related to flavor changes during the aging period.

Tenderness, texture and juiciness

The palatability panel noted statistically significant ($P < 0.05$) changes in tenderness (Table 3). However, the small differences between mean values probably are not practical differences. Tenderness, as measured by the Warner-Bratzler shearing apparatus, also was affected significantly ($P < 0.05$) by aging.

Table 3—Means for tenderness, texture, juiciness and related objective measurements.

Measurements	Aging time, days 1	4	8	12	Probability of F	LSD[1]
Tenderness scores[2]	5.5	5.6	5.7	5.8	*	0.243
Shear (lb/½-in. core)	7.7	7.8	6.9	6.8	*	0.633
Texture score[2]	5.4	5.6	5.4	5.5	ns	---
Juiciness score[2]	5.6	5.3	5.3	5.8	ns	---
Total moisture (%)						
Raw muscle	73.28	72.94	72.49	72.60	*	0.523
Cooked muscle	63.5	63.6	63.3	63.1	ns	---
WHC, cooked muscle[3]	0.62	0.62	0.61	0.62	ns	---
Ether extract (%)	3.49	3.68	3.93	3.68	ns	---

*P < 0.05.
[1] LSD, least significant difference at the 5% level; ns, not significant.
[2] 7 (extremely tender, desirable or juicy) to 1 (extremely tough, undesirable or dry).
[3] WHC, water-holding capacity (1.0–expressible moisture index).

Measurements on cooked muscle related to moisture (juiciness score, percentage total moisture and water-holding capacity) and texture score were not affected significantly by aging. Raw muscle aged 1 day contained significantly ($P < 0.05$) more total moisture than that aged 8 or 12 days. Percentage ether extract was similar for all aging periods.

Generally, the characteristics of pork loin that affect consumer acceptance were not improved by aging longer than the period (1 day) usually provided by current commercial practice.

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URIC ACID LEVELS IN MEN FED ALGAE AND YEAST AS PROTEIN SOURCES

SUMMARY—Microorganisms grown on human or industrial waste products may be economical and nutritious food sources. Algae (Chlorella sorokiniana) *harvested from continuous culture and extracted with ethanol, and yeast (food-grade* Torulopsis utilis) *were compared with casein at 2 levels of nitrogen as the sole source of protein for men. Biological value of algal protein was superior to casein, and yeast protein was not quite as good as algae, in diets containing 25 g of protein. When 50 g of algae protein was consumed, true nitrogen digestibility was reduced from 89 to 82%. Nitrogen balances were not significantly different in groups of men fed the higher protein level, indicating that 50 g of any of these proteins met or exceeded dietary requirements. Urinary uric acid excretion was doubled when the higher level of algae was ingested; with yeast, excretion was nearly 4 times as high as the amount voided with a purine-free diet. During ingestion of these materials, renal clearance rates were not sufficient to prevent plasma uric acid concentrations from reaching abnormally high levels, comparable to those found in gout.*

INTRODUCTION

MICROORGANISMS are technologically attractive in that they offer promise of producing food without dependence on traditional agricultural methods. Some may serve a second role, as biological processors of industrial and human wastes. Algae can provide edible protein-rich cells as a by-product of sewage treatment and some yeasts of the *Torulopsis* genera flourish in residues from sugar and paper production.

Several studies have proved that the quality of these algal and yeast proteins is reasonably good (Dirr et al., 1942; Goyco et al., 1959; Kondratiev et al., 1966; Dam et al., 1965; Lee et al., 1967). However, some adverse responses do occur when large amounts of crude products are incorporated into the human diet; therefore, some food processing may be required. For example, gastrointestinal disturbances have been attributed to poor digestibility of the algal cell wall (McDowell et al., 1963) and there is evidence that utilization is improved if the dried cells are first extracted with alcohol (Kondratiev et al., 1966; Dam et al., 1965).

A major limitation to the use of microorganisms as food sources is their high nucleic acid content. Uric acid, the end-product of the purine portion of nucleic acid catabolism, is only slightly soluble at the pH of body fluids and there is some risk that salts may be deposited in the renal tract and possibly other tissues (e.g., joints) if the diet contains excessive purines. Ingestion of large quantities of yeast has been shown to produce elevated blood uric acid concentrations (Dirr et al., 1942), but no similar information is available concerning the effect of algae.

An experiment was conducted in which yeast and algae were compared with casein, a purified protein of established biological value, as the sole protein sources in a human diet. Their effect on blood uric acid and other indices of tolerance was measured.

MATERIALS & METHODS

SEVEN HEALTHY men were fed 7 test diets during sequential 9-day periods, according to the plan shown in Table 1. Three of the men received all of the diets and completed 56 days of study; the other men participated for fewer periods, totaling 27 to 45 continuous days. During the experiment, the men were confined to a metabolic unit except for brief periods of supervised activity at bi-weekly intervals. Exercise was provided by means of a motor-driven treadmill, the men being required each day to walk 30 min at 3.5 mph and to run 15 min at 5 mph, both at a 10% grade. The men were freely ambulatory but largely sedentary.

All of the men received during 1 period a protein-free (1 g N per day) but otherwise nutritionally adequate diet. Protein sources—casein, algae and yeast—were fed at 2 dietary levels, to provide 25 and 50 g of protein (N × 6.25) per day. Pure yeast ribonucleic acid (RNA) was added to the casein at the approximate level found in several microorganisms, 8 g RNA per 100 g protein. Both algae and yeast contained nucleic acid naturally.

Algae was provided by Dr. R. L. Miller, Brooks Air Force Base. The cultivated strain, *Chlorella sorckiniana*, was contaminated at a level of about 1 bacterium per 5 algal cells. Harvested cells were processed as follows: extracted with boiling ethanol (¼w/v) for 24 hr, washed with ethanol, dried; batches combined, washed 5 times with distilled water, allowed to settle, decanted, washed twice with ethanol, redried and ground. The final product was dark-

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green and gritty in texture but did not taste bitter, as unprocessed cells do. The desired protein levels were obtained by adding 31 and 64 g of dry algae to the protein-free basal diet.

The yeast, *Torulopsis utilis*, was purchased from a commercial supplier (Lake States Yeast Co., Rhinelander, Wisconsin). The 2 levels of dietary protein required consumption of 43 and 90 g of yeast per man per day, plus the basal diet.

Composition of the single-cell protein sources is given in Table 2. Nutritional adequacy of the diets was assured, despite the varying contributions from the proteins, by addition of macrominerals to a formula given as 1 meal a day. Trace minerals and vitamins were given by capsule separate from the diet (Waslien et al., 1968).

The diet was administered as 4 major meals daily with snacks mid-morning and mid-afternoon. At breakfast, protein was added to a special low-protein hot cereal; at lunch and dinner, the protein was served as flavored sauces with low-protein pastas; in the evening, it was added to a spread served with a protein-free muffin. All diets included constant amounts of apple sauce, orange-flavored beverage, carbonated beverages, decaffeinated coffee and tea. Additional calories were available, as required to maintain body weight, from protein-free cookies, candies and salt-free, milk-free margarine.

Because of the risk of urate crystallization in the urinary tract with high levels of RNA in the diet, fluid intake of at least 3 liters per day was maintained. As necessary, sodium bicarbonate tablets were prescribed to maintain urine pH above 6.5, thereby to promote solubility of salts of uric acid.

Urine was collected quantitatively every day of the experiment and feces pooled by 3-day periods. Blood samples were taken at the completion of each metabolic period and analyzed for uric acid, total protein and albumin, urea nitrogen, glucose, cholesterol and triglyceride. Urinary volume, pH and osmolality were measured every day and qualitative tests made for the presence of protein, sugar and acetone. Urinary nitrogen (Block et al., 1956), uric acid and creatinine (Technicon AutoAnalyzer) were determined daily on the last 3 days of each period. The urines from the last 3 days of each period were pooled by subject before analysis for content of phosphorus (Technicon AutoAnalyzer), sodium, potassium, calcium and magnesium (atomic absorption spectroscopy). The feces from the last 3 days of the period also were analyzed for these minerals and nitrogen, as were composite samples of the total diets.

Feces and composite diets were lyophilized prior to combustion in a ballistic bomb calorimeter to compute digestible energy value of the diets (Miller et al., 1959). Concentrations of hydrogen and methane in expired air and total volume of rectally passed flatus were recorded at intervals, as an indication of bacterial activity in the intestine (Calloway et al., 1968).

RESULTS

THERE WERE no marked physiologic disturbances due to diet in any of the subjects. Fluctuations of body weight were recorded, but these were not associated with any particular diet and were usually corrected by minor changes in caloric allowances (Table 1).

Table 1–Characteristics of subjects and their diets, caloric intake and weight change during 9-day metabolic periods.

	1301	1302	1303	1304	1305	1306	1307
Age (years)	27	22	23	30	23	26	30
Height (cm)	185	183	178	183	188	178	180
Weight (kg)	96	80	73	75	68	70	67
Creatinine (mg/kg)[1]	20.5	22.0	23.7	23.2	25.2	23.0	23.9
Period 1 Diet code[2]	50C	25C	50C	25C	–	50C	–
Intake (kcal)	2,867	2,829	2,925	2,829	–	2,874	–
Weight change[3]	–.07	–.02	+.03	–.16	–	–.06	–
2	25C	0	25C	50C	50C	0	–
	2,989	2,847	2,917	2,999	3,005	3,111	–
	–.07	–.11	–.04	–.04	–.01	–.03	–
3	0	50C	0	25A	25C	–[4]	–
	3,176	2,940	2,935	3,034	2,949	–	–
	–.07	0	–.04	+.02	–.04	–	–
4	25Y	50Y	25A	50A	25A	25C	–
	3,319	2,968	2,999	2,941	3,023	3,296	–
	0	–.03	–.02	+.03	+.02	+.02	–
5	50Y	25Y	50A	0	0	–	25A
	3,521	3,093	3,068	3,111	2,985	–	2,773
	0	+.06	+.06	–.02	+.02	–	0
6	–	25A	25Y	50Y	50A	–	0
	–	3,049	3,110	2,901	3,000	–	2,847
	–	–.02	–.04	–.06	–.01	–	–.06
7	–	50A	50Y	25Y	–	–	50A
	–	3,171	3,068	3,096	–	–	2,809
	–	+.10	+.02	+.02	–	–	+.01

[1]Creatinine in urine, average last day of each period studied, mg/kg body weight on those days.
[2]Codes 0, 25 and 50 = g protein in daily diet; sources C = casein, A = algae (*Chlorella sorokiniana*), Y = yeast (*Torulopsis utilis*). The casein contained 8 g RNA per 100 g protein.
[3]kg/day, mean for 9 days.
[4]Subject had 25 g protein diet in period 3, but from an unrelated protein source.

Fluid intake and urine volumes and concentration were uniform for all diets (Table 3). The pH was lower with yeast diets, presumably because their potassium content was lower than in the casein and algae diets (Table 4). Some men had to be given additional sodium (as $NaHCO_3$) to maintain urinary pH above 6.5 while consuming the yeast diets. These urines also showed a marked tendency to form precipitates on standing.

Fecal wet and dry weights were increased when the men ate algae and yeast (Table 3), but there were great differences between subjects in this respect.

Table 2–Composition of yeast and algae.

	Chlorella sorokiniana	*Torulopsis utilis*	Casein
g/100 g			
Nitrogen	11.35	8.01	15.50
Nucleic Acid	5.6	11.4	[1]
mg/g			
Calcium	1.19	2.45	0.78
Magnesium	0.78	1.49	0.03
Sodium	0.11	0.13	0.88
Potassium	0.12	20.0	2.12
Phosphorus	7.4	16.3	6.15
Chloride	0.031	0.26	2.64

[1]None present; for diets pure yeast RNA was added, 8 g/100 g protein.

Table 3—Fluid intake and output, urinary pH and concentration, fecal weight and intestinal gas production of men fed algae, yeast and casein

		Casein + RNA		*Chlorella sorokiniana*		*Torulopsis utilis*	
	Prot.-free	25 Prot.	50 Prot.	25 Prot.	50 Prot.	25 Prot.	50 Prot.
Fluid intake (ml/day)	3,332 ± 125[1]	3,366 ± 91	3,405 ± 80	3,515 ± 304	3,529 ± 172	3,641 ± 403	3,728 ± 515
Urine volume (g/day)	2,097 ± 504	1,824 ± 578	1,933 ± 283	2,017 ± 576	1,938 ± 564	2,155 ± 443	2,188 ± 404
Urine pH	7.0 ± 0.2	7.0 ± 0.2	6.8 ± 0.2	7.0 ± 0.2	6.9 ± 0.4	6.6 ± 0.2	6.6 ± 0.3
Urine osmolality (mOsm/liter)	226 ± 49	282 ± 75	297 ± 50	241 ± 76	257 ± 65	207 ± 50	248 ± 47
Feces weight (g/day)							
Wet	142 ± 65	125 ± 86	114 ± 61	151 ± 48	202 ± 86	206 ± 95	195 ± 104
Dry	26 ± 6.1	28 ± 7.3	25 ± 2.4	31 ± 5.4	44 ± 7.2	40 ± 9.6	39 ± 3.9
Flatus volume (ml/12 hr)[2]	393 ± 69	374 ± 46	252 ± 36	479 ± 11	446 ± 95	401 ± 54	310 ± 39
Breath hydrogen (ppm)							
At 0730	2–17[3]	3–15	4–9	3–4	2–11	5–13	2–10
Maximum[3]	4–38	6–39	5–20	5–30	4–13	5–22	3–19
Breath methane (ppm)[4]							
At 0730	3–4	3–5	3–5	2–3	2–3	3	3

[1] Mean and standard deviation.
[2] Gas egested from the rectum 1030–2230 hr. Maximum breath H_2 is average of 3 highest values recorded during this period.
[3] Range of individual values.
[4] Subject 1303 omitted. His data show marked period effects, from initial 32–17 ppm by period 3 and 3 ppm by period 5 and after.

Only one man, 1304, complained of subjective discomfort such as stuffiness and bloating with the 50-g algae protein diet, and he passed a large amount of soft stools. Dietary energy was well absorbed from all diets. About 7% of caloric intake from casein diets was recovered in the feces and 10% from algae and yeast diets.

Volumes of rectally egested flatus were somewhat increased with the algae diets but, again, there was much variation within dietary groups (Table 3). Breath hydrogen and methane patterns failed to reflect any change in microfloral activity, except in one case. Subject 1303 gradually lost his initially high level of breath methane and reached a steady low level after the 3rd metabolic period.

When the men ate the protein-free diet, they were in negative balance of all minerals except phosphorus (Table 4). With the casein diets, only calcium and magnesium balances were negative, taking the subjects as a whole, and some men were in slightly positive balance. Calcium balance was negative with the higher level of algae, but not at the lower intake. With the yeast diets, calcium, magnesium and phosphorus excretions all exceeded intake, at both protein levels.

The protein digestibility values given in Table 5 have been corrected in the conventional way for fecal endogenous nitrogen output, that is, by subtracting from the test series the fecal nitrogen found with the protein-free diet, to give "true" digestibility. On this basis, casein is seen to be 95 to 99% digestible; but, as fecal nitrogen with casein in the diet was not significantly different ($P >> .05$) from the protein-free condition, the digestibility could be regarded as 100%. Fecal nitrogen was significantly higher with algal diets than with casein and varied directly with intake. Digestibility

Table 4—Mineral balances of men fed algae, yeast and casein.

			Casein + RNA		*Chlorella sorokiniana*		*Torulopsis utilis*	
		Prot.-free	25 Prot.	50 Prot.	25 Prot.	50 Prot.	25 Prot.	50 Prot.
(g/day)								
Sodium								
	Intake	2.90	2.57	2.52	3.22	3.13	3.41 ± .27	3.47 ± .24
	Balance	−.05 ± .28[1]	.07 ± .29	.07 ± .28	.31 ± .37	.52 ± .24	.24 ± .45	.31 ± .40
Potassium								
	Intake	3.43	3.28	3.28	3.52	3.34	1.86	2.46
	Balance	−.32 ± .27	.25 ± .81	.30 ± .41	.19 ± .34	.38 ± .23	.42 ± .23	.47 ± .20
Calcium								
	Intake	0.80	0.75	0.77	0.79	0.87	0.78	0.88
	Balance	−.11 ± .16	−.14 ± .25	−.04 ± .12	.00 ± .13	−.07 ± .14	−.34 ± .19	−.24 ± .16
Magnesium								
	Intake	0.52	0.44	0.48	0.60	0.64	0.59	0.61
	Balance	−.04 ± .10	−.09 ± .15	−.03 ± .06	.08 ± .10	.07 ± .07	−.02 ± .10	−.06 ± .07
Phosphorus								
	Intake	1.57	1.60	1.67	1.47	1.40	1.41	2.00
	Balance	.12 ± .29	.27 ± .18	.25 ± .10	.16 ± .16	.30 ± .09	−.10 ± .27	−.07 ± .24

[1] Mean and standard deviation. A single entry for dietary intakes indicates there was no known deviation.

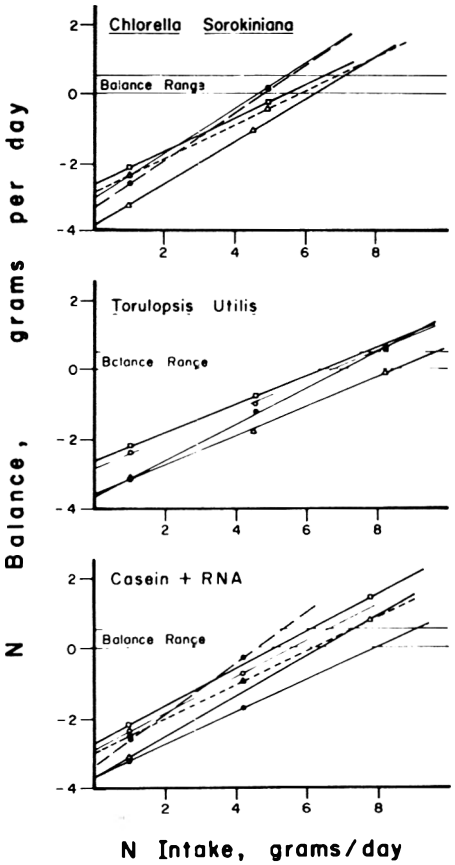

Fig. 1—Nitrogen balance as a function of nitrogen intake from casein + RNA, algae and yeast. Regression equations describing the mean slopes are where B = Nitrogen Balance, g/day and NI = Nitrogen Intake, g/day: casein + RNA B = .55 NI–3.25; algae and yeast B = .60 NI–3.20. Because B does not take into account nitrogen lost from the body surface and other miscellaneous routes, to assure adequacy B should be approximately + 0.5 g/day. When B = 0.5, then required NI is casein, 6.8; algae, 6.2; yeast, 8.3.

Table 5—Utilization of algae, yeast and casein nitrogen at 2 levels of dietary intake.

Dietary protein	Nitrogen, g/day				Nitrogen	
	Intake	Fecal	Urinary	Balance[1]	True digestibility[2] %	Biological value[3] %
Prot.-free	1.08	1.02 ± .16	2.52 ± .26	−2.61 ± .36	–	–
Casein +	4.25	1.13 ± .31	3.97 ± .40	−1.00 ± .49	95 ± 8	66 ± 4
RNA	7.84	1.08 ± .11	6.27 ± .85	+0.33 ± .88	99 ± 1	52 ± 6
Chlorella	4.83	1.58 ± .15	3.32 ± .30	−0.28 ± .41	89 ± 4	79 ± 12
sorokiniana	7.81	2.61 ± .52	5.00 ± .45	+0.05 ± .83	82 ± 6	60 ± 6
Torulopsis	4.51	1.79 ± .42	3.75 ± .13	−1.23 ± .36	83 ± 7	70 ± 5
utilis	8.20	2.04 ± .28	5.62 ± .16	.39 ± .28	87 ± 3	58 ± 6

[1] Includes a correction for blood samples taken but no correction for sweat and integumental losses. Balance = Intake − (fecal + urinary + blood). True balance is probably underestimated by 0.2–0.5 g/day if all routes of loss were accounted for.

[2] $\frac{\text{N Intake} - (\text{fecal N} - \text{fecal N at 0 protein intake})}{\text{N Intake}} \times 100.$

[3] $\frac{\text{Absorbed N} - (\text{Urinary N} - \text{urinary N at 0 protein intake})}{\text{Absorbed N}} \times 100.$

Table 6—Urinary and plasma uric acid levels of men fed nucleic acid with casein and as algae and yeast.

Diet	Nucleic Acid intake (g/day)	Uric Acid (mg) Urine per day	Uric Acid (mg) Plasma per 100 ml
Prot.-free	0	394 ± 50[1]	5.4 ± 1.0
Casein + RNA			
25 g Prot.	1.8	562 ± 47	6.9 ± 1.0
50 g Prot.	3.7	886 ± 77	8.7 ± 0.9
Chlorella sorokiniana			
25 g Prot.	1.7	605 ± 122	7.4 ± 1.1
50 g Prot.	3.6	872 ± 209	9.7 ± 1.1
Torulopsis utilis			
25 g Prot.	5.0	942 ± 65	10.2 ± 1.7
50 g Prot.	10.3	1,536 ± 65	12.6 ± 2.0

[1] Mean and standard deviation.

was 89% at the lower dietary level and 82% at the higher. Yeast-containing diets resulted in slightly more fecal nitrogen than did casein, but the difference was not statistically significant with the small number of cases and large variability within groups. At the 25-g level, true digestibility was computed to be 83% and with 50 g of yeast protein in the diet, 87%.

Comparing the lower levels of dietary nitrogen, *Chlorella* protein was significantly ($P < .05$) better in promoting nitrogen balance than either of the other sources (Table 5). This is reflected in the higher biological value computed for the algae, 79%, than for casein, 66%, and yeast, 70%. Utilization of absorbed algal nitrogen was superior to casein at the higher dietary level also, but balance was less positive (though not significantly so) because of the poorer nitrogen absorption with the larger dose of algae. Yeast protein produced about the same nitrogen balance as did casein.

Figure 1 presents the regression equations derived from nitrogen balance data at varying intake levels. The predicted total range of minimum requirements for balance in men of this population was 5.0–8.8 g casein nitrogen per day. The mean value is 6.6 g. Fewer subjects are available for the other proteins, but the mean need for algal protein appears to be 6.2 g N per day, within a range of 5.1–6.4 g per day. Yeast values are 8.3 g N per day, mean requirement for assured balance, and range, 7.0–8.8 g.

Urinary uric acid output was 394 mg per day with the protein-free, purine-free diet and plasma levels were in the normal range (Table 6). Addition of 1.8 g RNA with casein caused urinary excretion to increase to 562 mg; plasma values, though elevated, were still in the normal range. At double this intake, renal excretion rose to 886 mg per day and plasma levels reached the lower limits of the abnormal range. Responses to the algae diet were not significantly different from the reaction to the RNA–supplemented casein. Algae contributed the same amount of nucleic acid as was added to the control casein diets. Yeast used in this experiment contained 11.4% nucleic acid, which was much higher than anticipated from published values for other yeasts. Urinary excretion rose to a mean of 1,536 mg and plasma levels to 12.6 g % with 50 g of protein and 10.3 g nucleic acid present from yeast. Fortunately, none of the men experienced symptoms of gout, either during or after the feeding period.

Table 7—Blood components of men fed algae, yeast and casein.

		Casein + RNA		*Chlorella sorokiniana*		*Torulopsis utilis*	
	Prot.-free	25 Prot.	50 Prot.	25 Prot.	50 Prot.	25 Prot.	50 Prot.
Blood (mg/100 ml)							
Glucose	87 ± 4[1]	90 ± 6	93 ± 4	89 ± 4	89 ± 4	88 ± 7	89 ± 5
Urea nitrogen	5.1 ± .5	7.7 ± 1.1	10.1 ± 1.6	7.0 ± .3	9.3 ± 1.1	6.4 ± .4	8.6 ± 1.3
Total nitrogen	31 ± 1	31 ± 1	32 ± 2	31 ± 2	31 ± 1	31 ± 2	30 ± 1
Plasma (mg/100 ml)							
Protein (total)	6.4 ± 1.0	6.4 ± .4	6.6 ± .4	6.9 ± .6	6.9 ± .6	6.2 ± .2	6.4 ± .4
Albumin	4.0 ± .5	4.0 ± .2	4.1 ± .2	4.2 ± .1	4.4 ± .3	4.0 ± .4	4.2 ± .4
Creatinine	1.4 ± .2	1.2 ± .1	1.3 ± .1	1.6 ± .5	1.4 ± .2	1.4 ± .2	1.3 ± .1
Cholesterol	160 ± 24	166 ± 33	165 ± 18	154 ± 28	155 ± 33	154 ± 14	163 ± 20
Triglyceride	63 ± 20	68 ± 8	69 ± 27	85 ± 21	71 ± 17	72 ± 33	79 ± 45

[1]Mean and standard deviation.

Blood urea nitrogen varied directly with protein intake but was unaffected by protein source (Table 7). All of the men had normal blood protein, lipid and glucose levels initially and these did not vary due to either amount or source of protein in the diet.

DISCUSSION

THE HIGH biological values shown for yeast and algae in this study are good indications of their usefulness as a protein supplement for man. However, if yeast were to be used as the major source of protein at levels sufficient to maintain nitrogen equilibrium, plasma and urinary uric acid concentrations would be dangerously elevated. With algae as the major source of protein, the risk is substantially diminished, both because nucleic acid concentration is lower on a equivalent protein basis and slightly less protein is required to assure balance. Even so, extreme caution is indicated, because any normal diet to which the supplement might be added would almost certainly contain some other purine bases.

The effect of long-term consumption of such diets has yet to be demonstrated. However, in leukemia, plasma uric acid is elevated to these same levels and secondary gout is a common finding in such patients. The experience of Kondratiev et al. (1966) is illustrative of the problem. Some of their male subjects developed edema and irritation of the great toe (a prime target for gouty attacks) on being fed 150 g of dried algae per day for 14–16 days.

With algae, the adverse gastrointestinal response to large doses must also be considered. In our case, symptoms were not so severe as to impair nutrient balances significantly but indications for caution were present. Pokrovskaia et al. (1968) have reported that calcium and magnesium assimilation was depressed by continued consumption of 120 g of dry cells per day. Our balance data indicate a similar trend when the amount of algae fed was 64 g per day.

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A Research Note
DEVELOPMENT OF A MICRO-EMULSIFIER

WE REPORT herein the development of a micro-emulsifier apparatus designed to study the emulsifying role of proteins in food manufacture. Saffle (1968) has pointed out that one factor causing considerable confusion regarding the efficiency of various muscle proteins in fat emulsification has been lack of preciseness in isolation and purification of specific salt- and water-soluble proteins. Over recent years, we have been developing a program in our laboratory on the isolation and characterization of major myofibrillar and regulatory proteins of striated muscle. One objective of this program has been to evaluate the functionality of these proteins for food manufacture, while still being able to conduct a series of experiments on small quantities (about 50 mg) of isolated protein. This problem prompted the development of our apparatus which has a capacity low enough to permit the investigation of 1 or 2 mg of protein in 0.5 to 1.0 ml of aqueous phase.

Swift et al. (1961) are credited with the development of the first model system for study of emulsifying properties of meat proteins. Moss et al. (1968) developed a constant-volume apparatus from which air could be removed before test. Our apparatus enables us to remove all air and inject oil at a constant rate while keeping air excluded.

Figure 1 illustrates the micro-emulsifier, which has a mixing chamber of clear lucite with a capacity of 7 ml. The emulsification chamber is surrounded by another clear lucite chamber with an inlet at the bottom and an outlet at the top; this serves as a circulating water bath to aid in controlling temperature during operation. The chamber is fitted with a piston and the piston contains a needle-type valve. After the chamber is loaded with protein solution, the piston can be depressed to exclude air and the system is then sealed with the needle valve. A lateral inlet is designed for the addition of oil via a hypodermic syringe, and micro-knives accomplish the emulsification. The screw advance fitted to the syringe allows addition of oil under uniform rates. The entire unit is mounted on an aluminum base placed directly on a Waring Blendor for driving power. The chambers are sealed with 0 rings and held in place with screws. This design permits easy disassembly and cleaning. Our preliminary experiments revealed that the emulsion breaking point is clearly evident through the lucite chambers. Once the breaking point for given conditions is established, we can proceed to a specific point just under breaking, then stop oil addition and remove the emulsion plug. These plugs can be evaluated for physical properties (firmness, particle size) and stability.

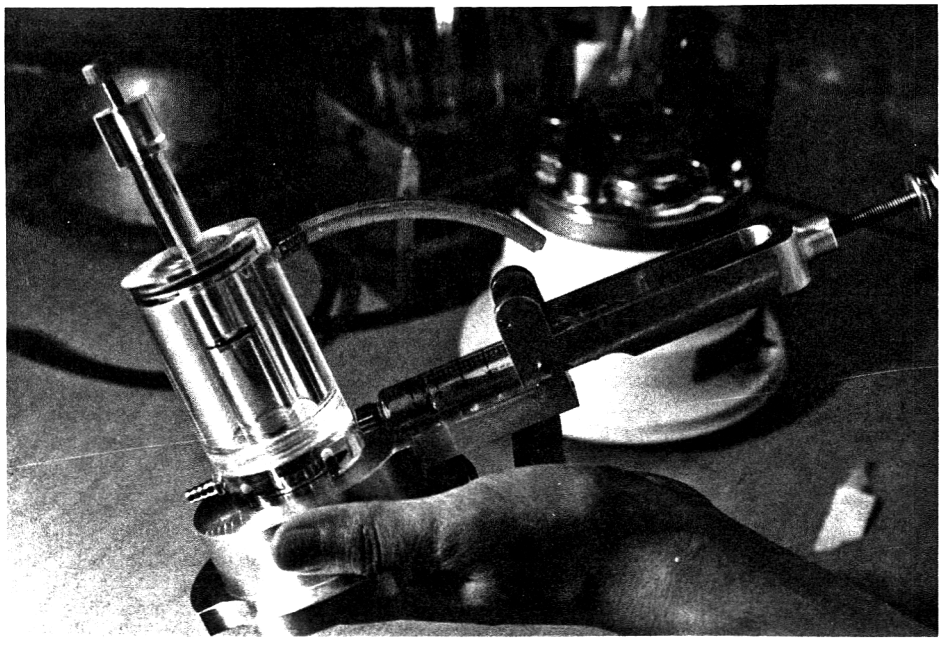

Fig. 1—Illustration of the micro-emulsifier. A complete description of the micro-emulsifier is found in the text.

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COLORIMETRIC ESTIMATION OF TOTAL ALDEHYDES IN AQUEOUS ORANGE ESSENCE USING N-HYDROXYBENZENESULFONAMIDE

SUMMARY—A spot test method for detection of aldehydes has been adapted colorimetrically for quantitative determination of total aldehydes in aqueous solutions. The final colored complex has an absorption maximum at 510–525 mμ. The technique is simple, and the color produced is linear with aldehyde concentrations up to 300 mg/liter using n-octanal and trans-2-hexenal as a mixed standard. The test is applicable to aldehyde determinations in orange essence provided proper sample dilution is made before analysis.

INTRODUCTION

THE NEED for criteria to evaluate the quality and strength of orange essence and other citrus flavoring materials prompted the search for analytical methods to assay major groups of organic compounds. Orange essence is an aqueous mixture containing carbonyls (Attaway et al., 1962), alcohols and volatile organic acids (Attaway et al., 1964). It also contains terpene hydrocarbons, oxygenated terpenes of the general formula ($C_{10}H_{18}O$) and esters (Stanley, 1958; Wolford et al., 1962). The proportion of these components governs both the strength and quality of essences and, in turn, of the products to which they are added (Wolford et al., 1969).

2 methods for the assay of saturated and unsaturated aliphatic aldehydes in aqueous orange essence have been reported by Attaway et al. (1967). The development of a simple method for determination of total aldehydes including saturated, unsaturated and aromatic aldehydes in citrus essences, applicable for use in both research and industry, was explored. The present paper describes a new colorimetric procedure for determination of total aldehydes in aqueous solutions based on a spot test described by Feigl (1966).

EXPERIMENTAL

Reagents

1. 1000 mg/liter standard mixture of n-octanal and trans-2-hexenal (1:1 w/w) in 80% ethanol.
2. 0.5% n-hydroxybenzenesulfonamide (HBS) in 95% ethanol.
3. N KOH.
4. 1% $FeCl_3$ in 2 N HCl.

Samples of orange essence were obtained from commercial processing plants in central Florida.

Procedure

After several trials with various HBS concentrations and various timings of the reaction, the following procedure was adopted for optimum linearity, stability and reproducibility of the color developed.

10 ml test solution was pipetted into a 50-ml Erlenmeyer flask and 1 ml 0.5% HBS added, followed by 1 ml N KOH. After a 10-min reaction time 1 ml 1% $FeCl_3$ was added and 5 min allowed for color stabilization, before reading light absorbance of the final colored complex with a Fisher II electrophotometer using a 525-mμ green filter.

In the alkaline medium, the HBS ($C_6H_5SO_2NHOH$) splits into benzenesulfinic acid ($C_6H_5SO_2H$) and a nitrosyl radical (NOH). The latter forms a hydroxamic acid (R-CONHOH) with the aldehyde which, in the acidic medium produces a brown-purplish inner colored complex with the ferric ions.

Table 1—Effect of HBS volume and concentration on final color development and stability.

HBS volume and concentration	Optical density (525 mμ filter) Time after adding $FeCl_3$ (min) 5	10	15
1 ml 0.5%	0.285	0.285	0.282
1 ml 1.0%	0.433	0.395	0.371
1 ml 2.0%	0.466	0.387	0.329
2 ml 2.0%	0.369	0.269	0.186

RESULTS & DISCUSSION

EXAMINATION of the absorption spectrum of the final colored solution with a Beckman DK-2 spectrophotometer revealed an absorption peak with maximum absorption at 510–525 mμ (Fig. 1). Orange essence exhibited a similar absorption spectrum and absorption maximum (Fig. 1). The selection of n-octanal and trans-2-hexenal as a mixed standard in this study was based on their reported presence in Florida orange essences (Attaway et al., 1962).

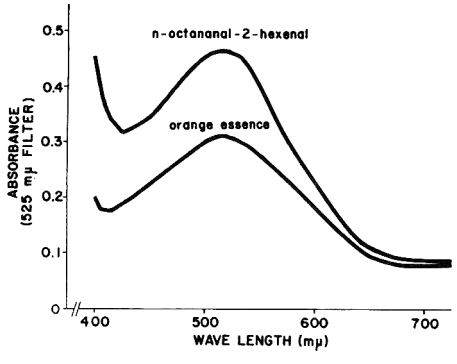

Fig. 1—Absorption spectra of a mixture of n-octanal and trans-2-hexenal and a sample of orange essence tested with HBS.

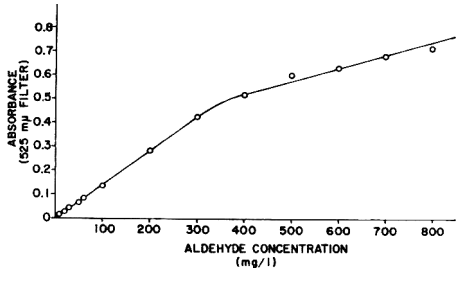

Fig. 2—Standard curve for estimation of total aldehyde concentration in aqueous solutions.

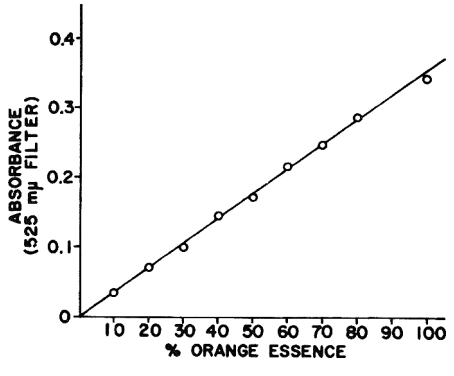

Fig. 3—Effect of dilution of orange essence on detection of aldehydes and linearity of color development with HBS.

Table 2–Detection of a known mixture of n-octanal and trans-2-hexenal in orange essence.

Test solution[1]	Optical density (525 mμ filter) Dilution factor 5	10
A. 500 mg/liter Aldehyde	0.115	0.056
B. Orange essence	0.198	0.099
C. 500 mg/liter Aldehyde in orange essence	0.302	0.156

[1] Solution A was obtained by mixing equal volumes of 1000 mg/liter aldehyde mixture and orange essence.

Table 3–Effect of reaction time on color intensity and stability.

Reaction time (min)	Optical density (525 mμ filter) Time after adding $FeCl_3$ (min) 5	10	15	20
0	0.052	0.049	0.047	0.047
5	0.169	0.163	0.164	0.162
10	0.247	0.239	0.238	0.239
15	0.258	0.251	0.251	0.252

The amount of HBS used in the test was found to have a profound effect on both color development and stability (Table 1). Samples containing 200 mg of n-octanal and trans-2-hexenal were analyzed in triplicate for total aldehydes. The optical density was read at various time intervals after addition of $FeCl_3$.

Color intensity increased with increasing concentrations up to 1 ml 2% HBS, but declined with 2 ml 2% HBS. Amounts greater than 1 ml 0.5% HBS resulted in relatively rapid fading of color. Maximum color stability was attained when 1 ml 0.5% HBS was used.

Linearity of color development

The test was conducted on a series of standard solutions ranging in concentration between 5 and 800 mg/liter. Linear relationship between concentration and optical density was obtained up to 300 mg/liter aldehyde (Fig. 2). Above that level the slope changed, although the relationship remained linear at the new slope up to 800 mg/liter aldehydes.

The relationship between aldehyde concentration and color intensity was also examined in orange essence. A sample of blended mid-season orange essence was diluted with distilled water and the test conducted on the various dilutions. Proportional increases in absorbance were associated with increased concentration of the essence (Fig. 3).

In another experiment a known amount of authentic aldehyde mixture (n-octanal and trans-2-hexenal) was added to an equal volume of orange essence sample and the test carried out 3 times on the aldehyde mixture and essence, both separately and combined at 2 dilution levels. Results indicate quantitative increase in optical density as a result of the aldehyde addition. The sum of optical densities obtained with the aldehyde mixture and essence samples separately was nearly equal to that obtained with the 2 samples combined at both the 5- and 10-fold dilutions.

Table 4–Minimum detectable level for representative aldehydes with the HBS test.

Aldehyde	Minimum detectable level (mg/liter)	Aldehyde	Minimum detectable level (mg/liter)
I. Saturated		II. Unsaturated	
Acetaldehyde	2	trans-2-Hexenal	10
n-Butyraldehyde	10	Citronellal	24
n-Valeraldehyde	5	Citral	50
α-Ethylbutyraldehyde	5	Furfural	10
n-Octanal	5	III. Aromatic	
1-Decanal	2	Phenylacetaldehyde	10

The length of time allowed between addition of KOH and $FeCl_3$ was found to have a profound effect on color intensity but very slight effect on color stability (Table 3). The test was conducted with a mixture contaning 200 mg/liter n-octanal and trans-2-hexenal. Reaction time varied from 0 to 15 min and time after addition of $FeCl_3$ between 5 and 20 min.

Optical density values increased as the reaction time increased from 0–10 min. At 15-min reaction time, only slight increase in color intensity was observed (Table 3).

Limits of detection

The minimum detectable level of various aldehydes representing the saturated, unsaturated and aromatic groups was determined (Table 4). A series of 500-1000 mg/liter standard stock solution of several aldehydes was prepared. Increasing dilutions were made and tested until no color could be detected. Linearity of color and optical density was exibited with all the aldehydes tested.

Specificity of the HBS test

Specificity of the test for aldehydes and the possibility of interference by other components was examined. Standard solutions, ranging in concentration between 100 and 1000 mg/liter methyl-ethyl ketone, amyl alcohol, linalool, ethyl-butyrate, d-limonene and valencene were prepared and tested. No color was detected even at the relatively high concentrations tested.

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PHYSICAL AND CHEMICAL STABILITY OF SOYBEAN OIL-FILLED MILK

SUMMARY—Filled milks were formulated from fresh skimmilk, vegetable oils and emulsifiers. The filled milks were pasteurized at 170°F for 8 sec, homogenized at 500/2,500 psi and cooled to 36°F. Studies on lipid oxidation and flavor score were made during storage at 40°F for 7 days. Soybean oil-filled milk, prepared from lightly hydrogenated soybean oil, was quite acceptable when evaluated organoleptically at 24-hr intervals of approximately 1 wk. Thiobarbituric acid and peroxide values revealed that very slight oxidation had occurred during storage for approximately 1 wk at 40°F. Monoglyceride emulsifiers with varying degrees of saturation were used to stabilize the emulsion of soybean oil in skimmilk. The more unsaturated monoglycerides tended to impart bitter flavors to the milk when used at a concentration of 0.1% (based on weight of product) and also were less efficient emulsifiers when compared to saturated monoglycerides. Development of a very undesirable sulfide-like odor and flavor occurred under extremely high pasteurization temperatures. The degree of off-flavor produced was directly proportional to time and temperature of heating.

INTRODUCTION

ACCORDING to the National Dairy Council (1968) a large proportion of the filled milks on the market contain coconut oil, which is normally inexpensive and stable to oxidation when compared to milk fat. In addition, this oil has desirable melting-point characteristics similar to milk fat.

There is no sound nutritional basis for substitution of milk fat with coconut oil in the preparation of a filled milk. Brink et al. (1969) and Rice (1960) indicate

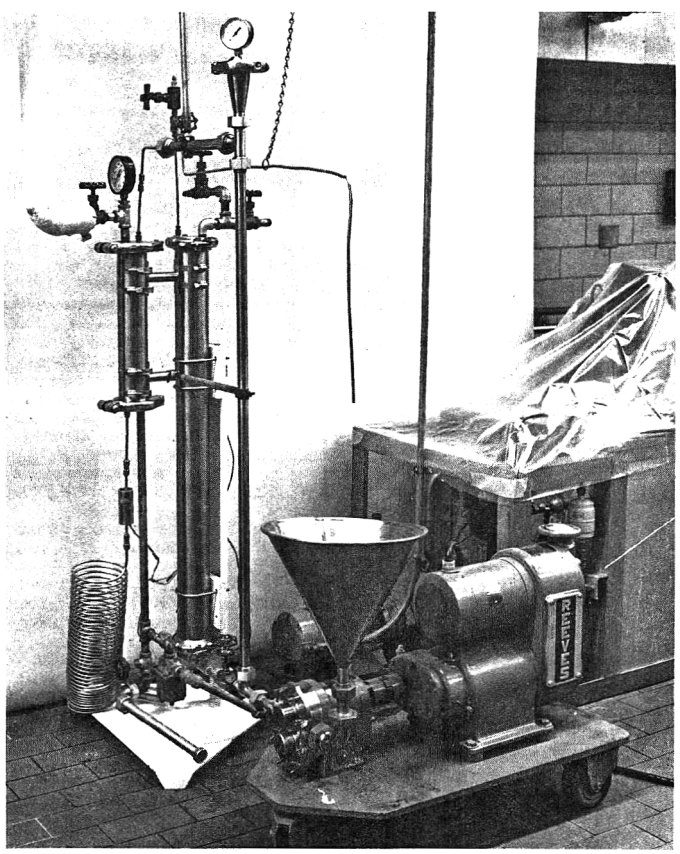

Fig. 1—High-temperature, short-time (HTST) heat exchanger used to pasteurize filled milks. Unit is equipped with variable-speed positive displacement pump, an 8-sec holding coil, with thermocouples at either end and cooling section.

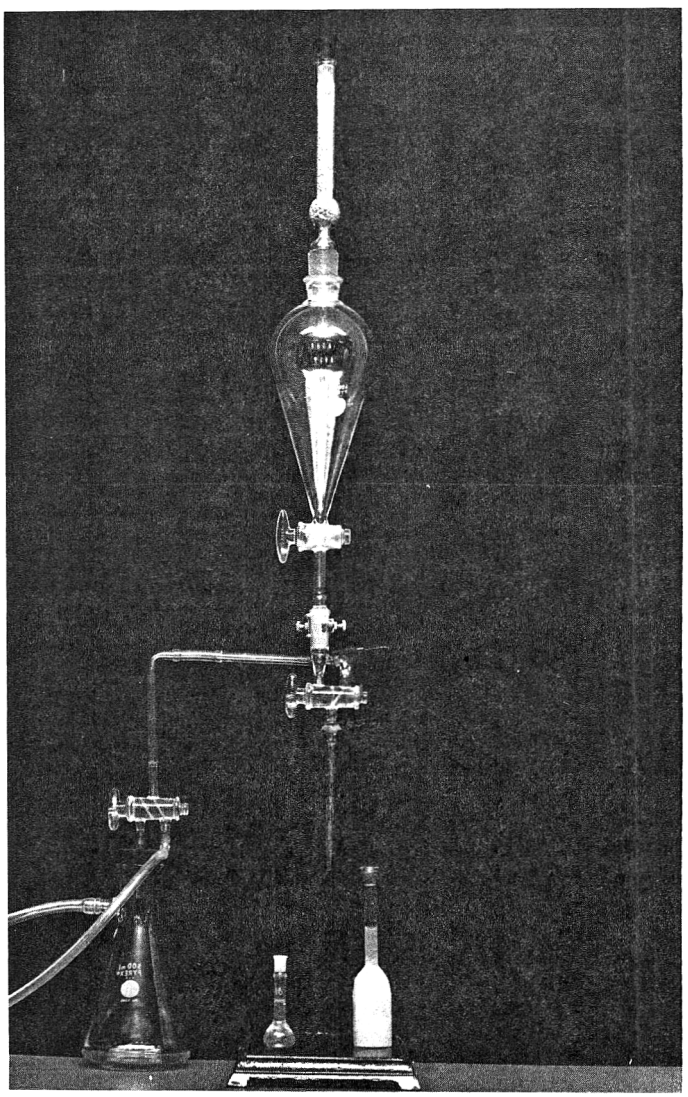

Fig. 2—Semiautomatic peroxide apparatus for delivering aliquots of lipid from extraction bottle to peroxide flask.

that coconut oil is an inferior replacement for milk fat with respect to the essential fatty acids, particularly linoleic, required for growth and maintenance of dermal integrity.

The purpose of our study was to prepare a filled milk from a fat or oil that would be reasonably stable to oxidation, yet contain significant quantities of unsaturated fatty acids, including the essential fatty acids. Preliminary studies indicated that lightly hydrogenated soybean oil was a satisfactory substitute for milk fat. Early studies by Thomasson et al. (1966) showed no abnormal changes in rats fed 3 types of hydrogenated soybean oil. Turpeinen et al. (1960) reported that replacement of milk fat by soybean oil in the human diet resulted in a decrease in serum cholesterol which was statistically highly significant, though not large. Cholesterol, among other things, has been implicated as being conducive to atherosclerosis; lowering of serum cholesterol may or may not reduce the incidence of this condition.

EXPERIMENTAL

Preparation of filled milk samples

Filled milks were formulated from fresh skimmilk, emulsifiers and vegetable oils. The oils studied were safflower, corn, cottonseed, peanut, olive and lightly hydrogenated soybean oil. All samples were forewarmed to 130°F, pasteurized at 170°F (unless otherwise indicated) with a high-temperature short-time (HTST) heat exchanger equipped with an 8-sec hold (Fig. 1), homogenized at 500/2,500 psi and cooled to 36°F.

Peroxide values

Peroxide values were determined by a procedure similar to that described by Stine et al. (1954), with 2 modifications: Fat was extracted by the butanol-salicylate de-emulsification technique of Pont (1955); a semiautomatic device was designed for transferring aliquots of extracted fat from the reaction vessel to the 10-ml volumetric flask. This apparatus (Fig. 2) consists of 2, 3-way valves, vacuum flask, nitrogen source and a reservoir for the benzene-methanol solvent mixture. By turning the 2, 3-way valves in the proper sequence the lipid sample can be drawn into the 0.5-ml Ostwald-Folin pipet, rinsed into the 10-ml volumetric flask and diluted to volume with the benzene-methanol solvent. Nitrogen can then be used to evaporate the residual solvent from the pipet in preparation for the next sample.

Thiobarbituric acid values (TBA)

The method of King (1962) was modified to accommodate a substantially larger sample of filled milk. After the protein was precipitated with trichloroacetic acid, 50 ml of clear supernatant were reacted with TBA. The interfering yellow pigments were separated by the procedure of Yu et al. (1962). Absorbancy of the pigments formed by the TBA reaction was measured at 532 mμ on a Beckman Model DU-2 spectrophotometer.

Gas-liquid chromatography

The fatty acid composition of the soybean oil was determined by GLC of the methyl esters formed by the method of McGinnis et al. (1965). A Beckman Model GC-5 dual-column instrument was used to resolve the esters. 2, 6-ft by 1/8-in. stainless steel columns were packed with 20% DEGS on Chromosorb W (80/100-mesh). The columns were operated isothermally at 185°C while the detector was maintained at 250°C.

Emulsifiers

4 commercial monogyceride emulsifiers (A, B, C and D) with iodine values of 3, 22, 40 and 85, respectively, were used at concentrations of 0.05, 0.1 and 0.2% (based on formula weight) to stabilize the soybean oil/skimmilk emulsion. The homogenization efficiency of the accept-

Table 1—Organoleptic evalution of fresh filled milk prepared from some common oils.

Oil used	Organoleptic evaluation of filled milk	
	Acceptability	Oxidized flavor[1]
Corn	NO	+++
Cottonseed	NO	++
Peanut	NO	+++
Olive	NO	++
Safflower	NO	+++
Soybean	YES	±

[1] +++Pronounced; ++ moderate; + slight; ± very slight.

Table 2—Fatty acid composition of coconut oil, soybean oil and lightly hydrogenated soybean oil.

Fatty acid	Weight percent		
	Coconut[1]	Soybean[1]	Lightly hydrogenated soybean[2]
Saturated			
Caproic (6:0)	Trace	–	–
Caprylic (8:0)	7.9	–	–
Capric (10:0)	7.2	–	–
Lauric (12:0)	48.0	–	–
Myristic (14:0)	17.5	0.4	–
Palmitic (16:0)	9.0	10.6	7.5
Stearic (18:0)	2.1	2.4	1.9
C_{20} and higher	–	2.4	–
Total	91.7	15.8	9.4
Unsaturated			
Palmitoleic and lower	–	1.0	–
Oleic (18:1)	5.7	23.5	52.7
Linoleic (18:2)	2.6	51.2	37.0
Linolenic (18:3)	–	8.5	1.8
Total	8.3	84.2	91.5

[1] Bailey's Industrial Oil and Fat Products. Ed. Swern, D. 3rd ed., 1103 pp., plus XIII. Interscience Publishers, Inc., N.Y.

[2] Analysis by L.R. Dugan, Jr., Department of Food Science, Michigan State University, East Lansing.

Table 3—Comparative accuracies of an analytical transfer pipet and a semiautomatic device for delivering aliquots of lipid into a peroxide flask.

Measurements	Transfer device studied	
	Semiautomatic	Analytical transfer
Number of samples tested	20	20
Mean delivery weight[1]	0.4031 g	0.3917 g
Standard deviation	0.0039 g	0.0061 g
95% Confidence limits	0.4031 g ± 0.0018 g	0.3917 g ± 0.0029 g

[1] 0.5-ml Ostwald-Folin pipet.

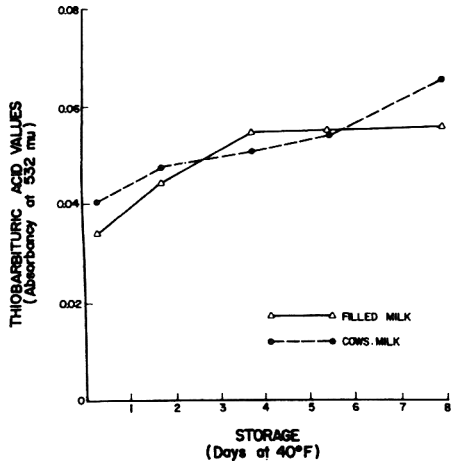

Fig. 3—Thiobarbituric acid values of fat extracted from cow's milk and soybean oil-filled milk during storage at 40°F for 8 days.

Table 4—Organoleptic evaluation of soybean oil-filled milk containing various levels of monoglyceride emulsifiers.

Chemical characteristics and quantity of emulsifiers used			
Monoglyceride	Iodine value	Percent[1]	Flavor noted in filled milk
A	3	0.05	No flavor criticism
		0.10	No flavor criticism
		0.20	Slightly bitter
B	22	0.05	No flavor criticism
		0.10	No flavor criticism
		0.20	Slightly bitter
C	40	0.05	No flavor criticism
		0.10	Slightly bitter
		0.20	Moderately bitter
D	85	0.05	Slightly bitter
		0.10	Moderately bitter
		0.20	Pronounced bitterness

[1]Concentration of emulsifier based on weight of product.

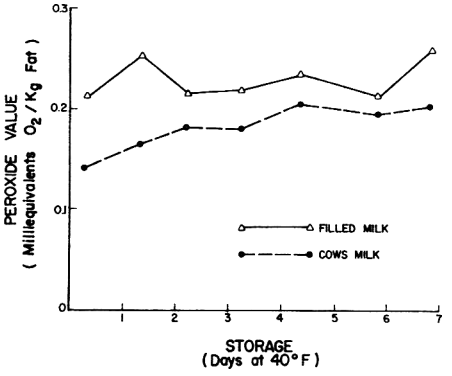

Fig. 4—Peroxide values of fat extracted from cow's milk and soybean oil-filled milk during storage at 40°F for 7 days.

Table 5—Homogenization efficiencies of soybean oil-filled milk prepared with types A and B monoglyceride emulsifiers.

Method of processing			Homogenization efficiency[1]	
Type of pasteurization	Order of processing	Times homogenized	Monoglyceride A	Monoglyceride B
Batch	Past.-Homog.	1	4.1	5.5
	Past.-Homog.	2	3.3	5.5
HTST[2]	Past.-Homog.	1	5.8	14.5
	Past.-Homog.	2	2.9	5.0
HTST[2]	Homog.-Past.	1	10.1	26.7
	Homog.-Past.	2	7.1	7.7

[1]Homogenization efficiency is the percent difference in the fat test of the top 100 ml and the bottom 900 ml of product stored in a 1000-ml volumetric cylinder for 48 hr at 40°F under quiescent conditions (maximum of 10% allowed in legal standard).

[2]8-sec holding time.

able emulsifiers (Type A and B) was determined by placing a representative sample of milk in a 1,000-ml volumetric cylinder and storing under quiescent conditions for 48 hr at 40°F. The percent difference between the fat test of the top 100 ml and the remaining 900 ml is termed homogenization efficiency and should not exceed 10% if the soybean oil in skimmilk emulsion is stable.

High-temperature, short-time (HTST) processing of filled milk

Undesirable sulfide-like odors and flavors were detected early in this research when filled milk was pasteurized under unusually high temperatures. To investigate the time/temperature relationship which might result in formation of these off-flavors, holding tubes were installed past the main heating chamber which permitted holding times of 8, 4 or 0.3 sec at the temperatures used for the HTST trials.

RESULTS & DISCUSSION

Preliminary studies

Filled milks prepared with corn, cottonseed, peanut, olive and safflower oils were rejected immediately after formulation due to objectionable oxidized off-flavors (Table 1). Oils used in this research were purchased in consumer-size bottles or cans at local supermarkets and exhibited the typical flavor qualities of a good salad oil. In addition to the off-flavors caused by oxidation, corn and peanut oils had a characteristic flavor that appeared to be further intensified when incorporated into the skimmilk. Of the oils evaluated, only the lightly hydrogenated soybean oil proved acceptable in a filled milk initially and during grading over 1 wk.

Studies with soybean oil-filled milk

Gas-liquid chromatographic analysis of methyl esters of the constituent fatty acids of soybean oil revealed the composi-

Table 6—Effect of high-temperature, short-time pasteurization on off-flavor development in filled milk.

Length of hold (sec)	Off-flavors produced at various temperatures (°F)			
	200	205	210	225
0.3	None	None	None	None
4.0	None	None	None	Very slight sulfide
8.0	None	Slight sulfide	Moderate sulfide	Very pronounced sulfide

tion reported in Table 2. For comparative purposes this table also contains typical data for nonhydrogenated coconut and soybean oils. This oil has been lightly hydrogenated and as a result the iodine value of 118 is somewhat lower than the average iodine value of 130 for a nonhydrogenated soybean oil. The flavor of this oil was essentially neutral.

Further organoleptic evaluation by a panel of 4 expert judges over 1 wk revealed that only a very slight increase in oxidized flavor had occurred. These results were further substantiated by chemical tests such as thiobarbituric acid (TBA) and peroxide values. A slight increase in the TBA value occurred during storage for 8 days in both the soybean oil-filled milk and cow's milk (Fig. 3). The peroxide values (Fig. 4) of these 2 products showed only slight fluctuations over 1 wk, with an over-all increase of 0.044 and 0.065 meq/kg of fat for the soybean oil-filled milk and cow's milk, respectively.

The comparative accuracies of the analytical transfer pipet and the semiautomatic device for delivering aliquots of lipid into a peroxide flask are shown in Table 3. The semiautomatic device has the advantage of being faster and requires only 1 pipet, thus reducing inherent variations of delivery when a series of individual pipets is used. Rinsing the residual lipid from the pipet with solvent also results in a higher and more uniform delivery weight.

Results of organoleptic evaluation of soybean oil-filled milk containing various levels of monoglyceride emulsifiers are shown in Table 4. Only type A and B emulsifiers proved to be acceptable when used at the recommended level of 0.1% based on the formula weight. Type C and D emulsifiers contribute some bitterness to the filled milk when used at 0.1%; this may be a function of unsaturation or the source of fat from which the monoglyceride emulsifiers were prepared, or both.

Homogenization efficiencies of soybean oil-filled milk prepared with types A and B monoglyceride emulsifiers appear in Table 5, and demonstrate that either emulsifier was acceptable when the samples were batch pasteurized and single homogenized. When HTST heat was used to pasteurize the filled milk, only the type A emulsifier proved acceptable when given a single homogenization. Use of the type B emulsifier required double homogenization to sufficiently stabilize the filled milk. If the filled milk were first homogenized, then HTST pasteurized, emulsion stability was reduced to an extent requiring double homogenization with either type A or B emulsifier. The soybean oil, emulsifier and skimmilk must be agitated constantly on the intake side of the homogenizer if single homogenization with the type A emulsifier is to suffice; otherwise, the soybean oil and emulsifier will separate, resulting in poor dispersion. The only recourse in this case is to double homogenize. To facilitate incorporation of the emulsifier in the filled milk, it is advisable to dissolve the emulsifier in the soybean oil prior to formulation of the filled milk. This requires heating of the oil when saturated monoglyceride emulsifiers such as A and B are utilized, since they are more difficult to dissolve. If the emulsifier is not completely dissolved in the oil, proper emulsion stability is not obtained upon homogenization.

The effect of varying time/temperature relationship during HTST pasteurization on off-flavor development in filled milk is illustrated by the data in Table 6. Off-flavor was evident at 205°F with an 8-sec hold and at 225°F with a 4-sec hold. Reduction of the holding time to 0.3 sec eliminated the off-flavors. This off-flavor should not be a problem in a commercial operation, since the temperatures used are generally not as great as those we used.

Resistance to oxidation of the soybean oil used can be attributed in part to the lower content of linolenic acid, which is prone to rapid autoxidation. Antioxidants (BHA and BHT) added to the oil after refining also help retard oxidation. Hydrogenation of this oil results in the conversion of some of the naturally occurring *cis* isomers of the fatty acids to geometric isomers which may be more resistant to oxidation.

Evidence has been presented that the geometric isomers of linoleic acid cannot serve as essential fatty acids, although they are utilized calorically. The amount of geometric isomers in the lightly hydrogenated soybean oil used was not determined.

Removal of milk fat from cow's milk results in loss of fat-soluble vitamins A, D, E and K in addition to some of the phospholipids adsorbed on the fat globules. Only the loss of vitamin A is of any great significance, since the other fat-soluble vitamins are present in minute quantities. Much fluid milk is now fortified with vitamin D and both vitamins A and D could also be added easily to a filled milk.

The major advantage of using soybean oil in filled milk lies in the high content of linoleic acid in this oil. On this basis soybean oil is nutritively superior to coconut oil or milk fat. At present, soybean oil is also one of the cheapest vegetable oils available and the economics of filled milk production would suggest that this is a logical choice of edible oil for an unsaturated oil-filled milk.

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LIPID AND OTHER COMPOSITIONAL CHANGES IN 9 VARIETIES OF SWEET POTATOES DURING STORAGE

SUMMARY—9 Varieties of sweet potatoes (Ipomoea batatas) *were harvested from controlled experimental plots, graded, cured 5 to 7 days and stored for 5 months at 15.5°C and 80–85% r.h. Centennial, Canbake, Georgia Red, Copperskin Goldrush, Gem, Coastal Sweet, Julian, Nuggett and Puerto Rico were analyzed after curing and after storage for °Brix, acidity, solids, total lipids, levels of nonphospholipids, cephalin, lecithin and fatty acid composition of each lipid. Variations in mean measurements were °Brix 13.5–15.7, solids 24.6–31.8, pH 5.92–6.06; acidity 0.134–0.178 and lipids 1.21–2.50. Each of these was significantly influenced by variety of potato and by storage. Most of the fatty acids in total lipids and lipid fractions were influenced significantly by variety and storage of potatoes. Although changes in many of these measurements were significantly correlated, it was concluded that most of the changes during this "ideal" storage were not particularly related to variety since very few variety-by-storage interactions were statistically significant.*

INTRODUCTION

CHANGES in the carbohydrate constituents of sweet potatoes during periods of storage after harvest have been fairly well documented [Arthur et al. (1955); Cooley et al. (1954); Hopkins et al. (1937) and Webb et al. (1949)]. Changes in lipid constituents during storage have not been studied as extensively, but have been documented somewhat by Tereshkovich et al. (1963), Fontenot (1966) and Boggess et al. (1967). Collectively these data show that some changes in fatty acids do occur during storage, with the last-named study demonstrating that changes occur primarily in total lipids, short-chain saturated fatty acids and possibly linoleic and linolenic acids. The data of this study were quite variable, especially when storage conditions were varied with 2 varieties of sweet potatoes. To study further the composition of several varieties under controlled storage conditions, additional research was initiated on 9 varieties of sweet potatoes stored under controlled temperature and humidity conditions. Arthur et al. (1955) showed that a storage temperature of 60°F was superior for maintenance of raw material. Lipid levels, fatty acids of different lipids and other chemical measurements were made on all varieties before and after a 5 months' period of storage.

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EXPERIMENTAL

9 VARIETIES of sweet potatoes were examined in this study. These varieties, covering most of those in the United States, were Centennial, Georgia Red, Copperskin Goldrush, Julian, Coastal Sweet, Gem, Nuggett, Canbake and Unit 1 Puerto Rico. They were grown under uniform conditions in experimental plots with commercially acceptable production practices. Samples of each variety in lots of 1 to 2 bushels were obtained from these plots at Experiment, Georgia, in November, 1966. Each lot was graded and the roots meeting USDA grade No. 1 were retained. These were cured for 7 days at 30°C and 85% r.h. and then sampled for laboratory analyses (cured). Following this curing period, part of the lot was stored for 5 months in controlled storage at 15.5°C and 80–85% r.h. and then sampled for analysis (cured-stored).

Samples taken at the end of the curing and after storage periods consisted of 20 roots selected at random; these were subdivided into 4 samples of 5 roots each. The 5 roots of each sample were washed, chopped, mixed and a 200-g portion homogenized and freeze dried by procedures previously described (Boggess et al., 1967). Total solids were calculated from the weight of the wet and freeze-dried materials.

A 20-g portion of the chopped sample was blended with 25 ml of distilled water and the pH determined using a Beckman Zeromatic pH meter. Another 20-g portion was blended with distilled water, transferred to a 600-ml beaker and brought to a volume of 500 ml. The total acidity was determined by titrating with 0.1 N NaOH to an endpoint of 8.2 on the pH meter. Another small portion of the chopped sample was pressed between filter paper, the pressed fluid recovered and used for measuring °Brix on the Abbé Refractometer.

A 25-g portion of each freeze-dried sample was extracted with chloroform-methanol as described earlier (Boggess et al., 1967) except a double extraction with 75 ml of chloroform-methanol (1:1 V/V) for mixing and 50 ml for filtering was used instead of the simple extraction. The filtrate was then transferred to graduated cylinders containing 100 ml of distilled water and allowed to form a biphasic system. Also, each mixture was allowed to stand overnight at 4°C to allow complete separation of the biphasic system. Lipids were recovered and separated into nonphospholipids, cephalin and lecithin by the column chromatography procedure of Carroll (1963). Each fraction and an aliquot of total lipids were analyzed for fatty acids as described earlier (Marion et al., 1967).

The data on total solids, pH, acidity, percent lipids, levels of lipid fractions and fatty

Table 1—Chemical measurements on 9 varieties of sweet potatoes.

	Variety									Storage	
				Copperskin		Coastal			Unit I	Date	
Measurement	Centennial	Canbake	Ga. Red	Goldrush	Gem	Sweet	Julian	Nuggett	P.R.	11-66	4-67
Lipid (%)	1.65	1.35	1.36	1.21	1.56	2.50	1.64	1.26	1.24	1.32	1.73
Nonphospholipid (%)	87.1	81.8	82.0	81.4	85.1	87.6	85.5	91.0	84.1	85.0	85.1
Cephalin (%)	8.5	11.5	11.1	12.2	9.7	7.6	9.7	6.4	9.7	9.7	9.5
Lecithin (%)	4.4	6.7	7.0	6.4	5.1	4.7	4.8	2.6	6.2	5.4	5.3
Brix[1]	15.0	15.7	14.2	14.8	13.5	14.4	14.0	15.4	15.0	14.3	15.0
pH	5.93	6.00	5.99	6.06	5.89	5.95	5.92	5.96	5.98	5.88	6.05
Total acidity[2]	0.164	0.142	0.134	0.140	0.134	0.142	0.178	0.152	0.149	0.144	0.153
Total solids (%)	27.9	31.8	27.9	27.1	24.6	31.0	27.6	31.0	29.0	29.3	28.0

[1]Brix = percent soluble solids determined on a refractometer.

[2]Total acidity as citric = $\frac{\text{ml base} \times N \times 0.64 \times 100}{\text{gram sample}}$.

acid composition were analyzed by computer to determine statistical significance of variety and storage influence. Correlations between all measurements were determined after varietal and storage effects were statistically removed.

RESULTS

THE LEVEL of total lipids, lipid fractions, total solids and measurements for °Brix, pH and total acidity are shown for 9 varieties of sweet potatoes in Table 1. Mean levels for all varieties are shown for these measurements after curing and after storage for 5 months. The statistical significance of variety, storage and variety-by-storage interaction for each of these measurements is shown in Table 2. Total lipids, expressed as a percentage of dry matter, varied from 1.21 in Copperskin Goldrush to 2.50 in Coastal Sweet. These levels were significantly affected by variety and were significantly higher after storage. When each lipid fraction (nonphospholipid, cephalin and lecithin) was expressed as a percentage of total lipid, there was significant varietal influence of the level of each, whereas storage had no effect at all. Of the other measurements, °Brix and total acidity increased with storage, whereas pH and total solids decreased. Each of these was significantly affected by variety of sweet potato. Of all the measurements shown in Table 1, only pH exhibited a significant variety-by-storage interaction.

In Table 3 the fatty acids of total

Table 2–Statistical significance of differences on chemical measurements of sweet potatoes.

Measurement	Variety	Storage	Var. × storage
% Lipid	**	**	NS
Nonphospholipid	**	NS	NS
Cephalin	**	NS	NS
Lecithin	**	NS	NS
Brix	**	**	NS
pH	**	**	*
Acidity	**	*	NS
Total solids	**	**	NS

NS = not significant; * and ** = statistical significance at the 5 and 1% levels of probability.

Table 3–Fatty acids of the total extract of 9 varieties of sweet potatoes.

Fatty acid		Variety									Storage	
	Extract	Centennial	Canbake	Ga. Red	Copperskin Goldrush	Gem	Coastal Sweet	Julian	Nuggett	Unit I P.R.	Cured	Cured-stored
Total lipid[1]												
	14:0	1.4	0.8	1.0	0.4	1.0	2.6	0.6	3.6	2.0	1.0	2.0
	14:1	0.2	0.2	0.2	0.5	0.2	0.4	0.2	0.7	0.4	0.4	0.3
	16:0	36.0	32.8	29.0	39.4	30.9	32.4	30.4	41.5	38.7	33.0	36.1
	16:1	0.5	0.9	0.6	0.5	0.5	1.2	0.4	2.2	0.5	1.0	0.6
	18:0	6.1	5.3	5.2	5.7	8.4	7.3	6.4	6.3	5.7	6.4	6.1
	18:1	1.4	1.2	1.5	1.4	1.6	1.4	1.8	1.9	1.6	1.8	1.3
	18:2	46.1	51.2	54.5	45.1	50.6	49.4	53.3	37.9	42.5	49.1	46.6
	18:3	8.2	7.6	8.0	7.1	6.6	5.3	6.9	5.9	8.6	7.2	7.0
Nonphospholipid												
	14:0	2.3	2.0	3.1	1.0	1.8	6.1	1.3	5.6	3.2	3.1	2.8
	14:1	0.4	0.3	0.4	0.4	0.2	0.7	0.4	0.8	0.5	0.5	0.4
	16:0	37.9	30.4	29.9	32.9	32.9	31.3	34.7	38.8	33.7	31.2	36.0
	16:1	0.6	0.4	0.7	1.0	0.5	1.9	0.4	1.7	0.8	0.6	1.2
	18:0	6.4	5.5	5.7	6.1	8.4	7.5	6.8	6.2	5.8	6.9	6.1
	18:1	1.7	1.7	3.1	2.1	2.3	1.6	2.9	2.4	2.2	2.9	1.5
	18:2	42.4	51.2	47.9	47.7	47.2	45.2	45.9	37.3	43.1	45.3	45.3
	18:3	8.4	8.5	9.3	8.8	6.6	5.7	7.7	7.2	10.6	8.8	7.4
Cephalin												
	14:0	0.2	0.4	0.5	0.3	0.2	1.9	0.2	2.2	0.5	0.4	1.1
	14:1	0.1	0.2	0.3	0.3	0.2	0.5	0.2	0.8	0.2	0.2	0.4
	16:0	33.5	30.3	37.2	37.6	31.8	26.7	34.9	33.2	40.4	33.9	34.0
	16:1	0.4	0.6	0.4	1.6	0.5	1.3	0.5	2.1	0.5	1.1	0.6
	18:0	7.0	6.3	5.3	5.5	7.9	7.3	7.4	6.7	4.9	7.4	5.6
	18:1	1.8	1.8	1.6	1.8	1.4	2.1	1.7	3.0	1.2	2.4	1.3
	18:2	49.4	54.1	48.9	46.3	53.2	55.5	47.9	46.0	45.6	47.7	51.6
	18:3	7.6	6.3	5.7	6.6	4.9	4.6	7.3	6.0	6.9	7.0	5.4
Lecithin												
	14:0	0.2	0.2	0.2	0.2	0.1	0.4	0.1	0.6	0.4	0.4	0.2
	14:1	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.6	0.3	0.4	0.2
	16:0	31.6	33.6	31.4	42.7	32.3	35.8	39.0	33.6	39.2	35.6	35.4
	16:1	0.9	0.4	0.5	0.6	0.4	0.7	0.4	0.8	0.5	0.6	0.5
	18:0	6.1	4.8	6.2	5.5	7.1	6.2	5.0	6.3	4.8	6.7	4.8
	18:1	2.8	2.2	3.3	3.4	2.6	3.9	2.3	7.1	2.6	4.8	1.9
	18:2	52.0	53.5	51.4	40.2	53.1	46.5	48.0	44.9	44.8	44.8	51.7
	18:3	6.2	5.2	6.7	7.2	4.2	6.0	5.0	6.2	7.5	6.7	5.4

[1] Myristic 14:0; myristoleic 14:1; palmitic 16:0; palmitoleic 16:1; stearic 18:0; oleic 18:1; linoleic 18:2 and linolenic 18:3.

lipids and the 3 lipid fractions are listed. Table 4 shows the statistical significance of the variations in fatty acid patterns. In total lipids, every fatty acid measured was significantly influenced by variety of sweet potato. However, only 14:0, 16:1 and 18:1 were significantly changed by storage. The 2 major fatty acids (16:0 and 18:2) were generally inversely related, with 16:0 varying from 29.0% in Georgia Red to 41.5% in Nuggett. Oleic acid (18:2) varied from 37.9% in Nuggett to 54.5% in Georgia Red. With storage, 14:0 and 16:0 increased in total lipids whereas 18:2 decreased, although not significantly.

The fatty acids of nonphospholipid resembled closely those of total lipids as would be expected since nonphospholipids (NP) constituted 85% of the total lipids. Variety significantly influenced all NP fatty acids except 18:1, and storage significantly influenced all NP fatty acids except 14:0 and 18:2. During storage, 16:0 increased whereas all the other fatty acids remained unchanged or decreased with storage. Cephalin, 14:0, 14:1, 18:0 and 18:2 were influenced significantly by variety and storage. Also, 18:1 and 18:3 were significantly decreased by storage. Variety-by-storage interactions were noted only in the 14:0 and 14:1 fatty acids. In lecithin only 14:1 and 18:1 were significantly different between varieties. These fatty acids plus 14:0, 18 0 and 18:2 changed significantly during storage. Each of these decreased with storage except 18:2 which showed a definite increase.

When all the data on chemical measurements were pooled and the influence of variety and storage was removed statistically, it was found that 104 correlations were statistically significant ($P < 0.05$). Of these 104 significant correlation coefficients, 26 were 0.50 or higher. These 26 are presented in Table 5. Some of these, such as negative correlations between 16:0 of total lipids and 18:2 of total lipids are very high and immediately apparent by inspection of the data in Table 3. Of the relationships between fatty acids and measurements such as lipid levels, °Brix, pH, solids and acidity, most are found in the cephalin fatty acids 16:0 and 18:0. Relationships of these measurements with lecithin fatty acids were mostly with levels of 18.2 and 18.3. °Brix was significantly and positively correlated with lecithin, pH and total solids. Percent lecithin was positively correlated with pH, as were acidity and total solids.

DISCUSSION

IN OUR previous research (Boggess et al., 1967) we reported that the level of total lipids and lipid fractions differed between the 2 varieties studied, suggesting that lipids are not uniform between varieties. Also that the possibility was mentioned that the lipids become more extractable as the respiring potatoes underwent compositional changes during storage. The work of Cooley et al. (1954) indicated that a moderate temperature of 55–60°F is necessary for successful storage of all of the sweet potato varieties tested, although there were marked varietal differences.

Table 4—Statistical analysis of storage and varietal effects on fatty acids of sweet potato lipids.

Lipid	F.A.	Variety	Storage	Var. × storage
Total extract	14:0	**	**	*
	14:1	**	NS	NS
	16:0	**	NS	NS
	16:1	**	*	**
	18:0	**	NS	NS
	18:1	**	**	NS
	18:2	**	NS	NS
	18:3	*	NS	NS
Nonphospholipid	14:0	**	NS	NS
	14:1	**	**	NS
	16.0	**	**	*
	16:1	**	**	NS
	18:0	**	*	NS
	18:1	NS	**	NS
	18:2	**	NS	*
	18:3	**	**	NS
Cephalin	14:0	**	**	**
	14:1	**	**	*
	16:0	NS	NS	NS
	16:1	NS	NS	NS
	18:0	*	**	NS
	18:1	NS	**	NS
	18:2	*	*	NS
	18:3	NS	**	NS
Lecithin	14:0	NS	*	NS
	14:1	*	**	NS
	16:0	NS	NS	NS
	16:1	NS	NS	NS
	18:0	NS	**	NS
	18:1	**	**	*
	18:2	NS	*	NS
	18:3	NS	NS	NS

NS = not significant; * and ** = statistical significance at the 5 and 1% levels of probability.

[1]The first number refers to the number of carbon atoms, the second to the number of double bonds. Each fatty acid expressed as a percent of total fatty acids.

[2]Fatty acid values for each variety are means of 4 cured and 4 cured-stored samples. Values for each storage period are means for all varieties.

Table 5—Correlations between measurements on sweet potatoes.

Measurement a	X	Measurement b	Coefficients
Total lipid 16:0		Total lipid 18:2	−0.94
Total lipid 16:0		Total lipid 18:3	−0.54
Total lipid 18:0		Total lipid 18:2	0.57
Nonphos. 16:0		Nonphos. 18:2	−0.72
Nonphos. 16:0		Nonphos. 18:3	−0.76
Nonphos. 18:0		Nonphos. 18:1	0.58
Nonphos. 18:2		Nonphos. 18:3	0.57
Cephalin 14:0		Cephalin 14:1	0.52
Cephalin 16:0		Lecithin 18:3	−0.51
Cephalin 16:0		Total acidity	0.59
Cephalin 16:0		Percent nonphos.	−0.79
Cephalin 16:0		Percent cephalin	−0.58
Cephalin 18:0		Percent lecithin	−0.63
Cephalin 18:0		°Brix	−0.66
Cephalin 18:0		pH	−0.89
Cephalin 18:0		Total solids	−0.61
Cephalin 18:3		Lecithin 14:0	−0.86
Cephalin 18:3		Lecithin 14:1	−0.74
Lecithin 18:2		Percent lipid	0.54
Lecithin 18:3		Percent lipid	0.71
Lecithin 18:3		Percent cephalin	0.70
Percent lecithin		°Brix	0.74
Percent lecithin		pH	0.60
°Brix		pH	0.52
°Brix		Total solids	0.59
Total acidity		Total solids	0.53

Note: 0.273 and 0.354 statistical significance at the 5 and 1% level of probability, respectively.

Hopkins et al. (1937) found there is some sugar accumulation at all temperatures studied (50, 55, 60, 65 and 70°F). The present work with whole potatoes indicates that the lipid and chemical changes were significantly affected by variety and were significantly higher after storage. But because the "ideal" storage conditions were used instead of lower temperatures, most of the changes were not particularly related to variety since very few variety-by-storage interactions were statistically significant.

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QUANTITATIVE METHODS FOR ANTHOCYANINS. 5. Separation of Cranberry Phenolics by Electrophoresis and Chromatography

SUMMARY—The mixed phenolics of cranberries were extracted by blending with a mixture of methanol (containing 10% water) and ethyl acetate. Anthocyanins were separated from the remainder of the phenolics by electrophoresis on paper. The part of the electropherogram bearing the anthocyanins was cut away. The remaining part of the paper was submitted to 2-dimensional chromatography. 4 well-defined spots of flavonoid glycoside were obtained.

INTRODUCTION

THE EXTRACTION of phenolic compounds from plant material has been discussed by Swain (1965). These compounds are most frequently extracted by the repetitive treatment of the plant tissues with aqueous alcohols. Probably the most commonly used solvents are methanol, ethanol and 2-propanol, either cold or boiling. In view of the relatively high water content of fresh plant material, initial extracts are diluted by physiological water and, therefore, contain a high proportion of the water-soluble components of the plant tissues. Subsequent extracts may carry components of a more lipophilic character. The combined extracts may, therefore, contain a rather heterogeneous mixture of impurities.

A further problem arises because the phenolic compounds are themselves extracted as mixtures which may contain simple phenolic compounds such as cinnamic acids derivatives as well as flavones, flavonols, anthocyanins and leucoanthocyanins. Examination of the phenolic compounds in such extracts normally involves 2 operations: separation of phenolics from other extraneous material and resolution of the mixture of phenolic compounds. The first operation may be accomplished by the selective precipitation of phenolics as lead complexes, by solvent extraction or by column chromatography using columns of ion exchange resin or polyamide (Fuleki et al., 1968a). Gallop (1965) has reported the use of columns of polyvinylpyrrolidone. Partial resolution of the mixed phenolics may occur during column chromatography but actual resolution is usually accomplished by repetitive chromatography on paper. 2 disadvantages are evident in these preliminary purification processes. First, a further series of operations is added to an already tedious process; secondly, compounds present in small proportions may be lost during the purification process.

Most of the aforementioned methods are efficient where only 1 type of phenolic compound is desired. They are very inefficient when it is desired to follow changes in several types of phenolics on the same sample. The following work was performed in attempts to develop methodology to follow quantitative changes in the yellow flavonoids, the red anthocyanins and the colorless cinnamic acids of cranberries. Because of the impurities introduced by the usual liquid extraction methods it was decided to extract the berries with a solvent designed to entrain a minimum quantity of nonphenolic compounds. After concentration, the extracts could be applied directly to paper for subsequent separations by a combination of paper electrophoresis and chromatography. Paper electrophoresis of phenolic compounds has been extensively reviewed by Pridham (1964). It is sufficient to say here that simpler phenolics and anthoxanthins have been separated on paper electropherograms with varying degrees of success. The most successful separations appear to have been with electrolyte systems containing complexing agents such as borate anion, the net effect being an increase in the anionic nature of the phenols. Alkaline electrolytes are most useful because they cause uncomplexed phenolic hydroxyl groups to become ionized. Markakis (1960) made fundamental paper electrophoretic studies of anthocyanins and confirmed the cationic nature of anthocyanins. He was able to show that at low pH values the pigments of Montmorency cherries could be separated into bands on paper electropherograms.

EXPERIMENTAL

Electrophoresis apparatus

A simple electrophoresis apparatus was

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Table 1—R_f values and fluorescence under ultraviolet light of cranberry phenolics.

Cranberry phenolics	Fluorescence	R_f values BAW	R_f values 10% Acetic Acid
A Quercetin-3-galactoside	Dark brown	0.36	0.31
B Myricetin-3-arabinoside	Dark brown	0.55	0.30
C Quercetin-3-arabinoside	Dark brown	0.77	0.36
D Quercetin-3-rhamnoside	Dark brown	0.56	0.41
P	Blue	0.89	0.44
Q	Blue	0.39	0.60
R	Blue	0.70	0.71
S	Blue	0.80	0.82
T	Blue	0.58	0.78
U	Blue	0.56	0.85
V	White	0.22	0.86

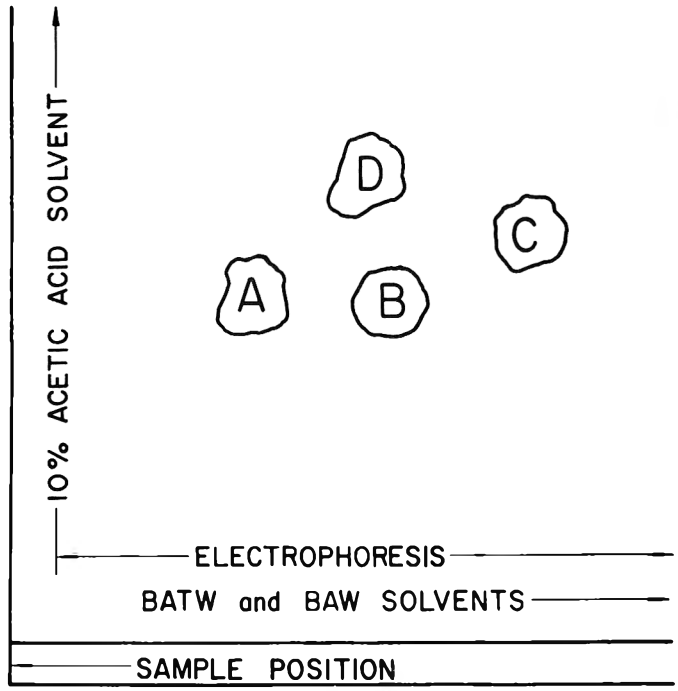

Fig. 1—Chromatogram of cranberry flavonoids after electrophoresis and 2-dimensional chromatography. A = quercetin-3-galactoside, B = myricetin-3-arabinoside, C = quercetin-3-arabinoside and D = quercetin-3-rhamnoside.

constructed from a ¼-in. Plexiglas (acrylic) sheet. The apparatus accommodated Whatman No. 3 chromatography paper 16 by 19 in. in size. Each paper was folded to form 2 panels 9.5 by 16 in. The paper was held in the apparatus in a tent-like form so that each 16-in. edge could dip into an electrode compartment which contained a platinum wire electrode. A 0.5-in.-long band of sample was streaked along the center fold 1.5 in. from the edge of the paper. Subsequent to sample streaking the paper was wetted with electrolyte by dipping in a large Shandon chromatography dipping trough. The paper for about 0.5 in. on either side of the center fold was allowed to remain dry. It was then placed on the apparatus with the edges dipping in electrolyte contained in the electrode compartments. When the band along the center fold was completely wetted by capillarity, a Plexiglas cover was placed over the apparatus and a potential applied across the electrodes. Potentials of between about 20 and 25 v per inch were found most suitable. A current of cold air was blown through the interior of the apparatus to cool it during electrophoresis. The air obviously did not enter the chamber containing the paper.

Preliminary experiments

Initial electrophoresis experiments with commercial samples of flavonoids, using borate buffers, showed that this method was not practicable for efficient separation of these compounds. The rate of migration was slow, separations were poor and, most important, there was appreciable oxidation of the phenolics. Electrophoresis of anthocyanins with 0.05 M formic acid electrolyte showed that these compounds would migrate but there was no separation into bands of individual anthocyanin pigment. However, experiments showed that by electrophoresis a separation could be achieved between anthocyanins which migrated towards the cathode and other phenolic compounds which remained stationary or moved slightly towards the cathode (probably due to electroosmosis). This observation was used as the basis for a method of separating flavonoid compounds of cranberries from several other chemical components of interest.

Extraction of phenolics from cranberries

A mixed solvent was used to reduce the impurities taken up from cranberries during extraction.

Petroleum ether boiling point 30–60° (150 ml) was placed in the cup of a 2-speed Waring Blendor and frozen cranberries (50 g) added. The mixture was blended for 3 min, 2 min at low speed and 1 min at high speed. After standing for 5 min the yellow-colored supernatant (containing carotenoid compounds) was decanted and discarded. The ground berries forming a gummy deposit at the bottom of the blender were blended again with petroleum ether (100 ml) in the same way as before. After standing for 5 min the ether layer was again decanted and discarded. A 100-ml volume of a mixture of methanol containing 10% water (75 vol) and ethyl acetate (25 vol), normal with respect to formic acid, was blended with the plant material. After 3 min of blending, the slurry was filtered using a Whatman No. 4 filter paper, Büchner funnel and a water aspirator and the filtrate collected. The residue and filter paper were replaced in the blender cup and blended as before with the methanol/ethyl acetate solvent (100 ml).

The processes of alternate blending and filtration were continued until all the red color had been extracted from the plant material. This took from 6 to 8 extractions with methanol/ethyl acetate solvent. Apart from the first extraction with the latter solvent, the filter pad was separated from the filter paper which was discarded, thus only the plant material was extracted. The extracts were combined, filtered and then concentrated at 30° under reduced pressure using a rotating flask evaporator. Concentration was continued practically to dryness, so that the residue was a very viscous liquid. The latter was extracted with 4 successive volumes (5 ml) of methanol containing 10% water. The extracts were collected and made up to 25 ml. The solution was centrifuged and it was then ready for streaking on chromatography paper.

Recent work at the Department of Food Science, University of Manitoba, has shown that it is beneficial to add a small quantity of sodium cyanide prior to the initial blending. This inhibits phenolase enzymes which might otherwise cause destruction of phenolics.

Electrophoresis and chromatography

A paper was prepared for electrophoresis as described in the section Preliminary Experiments. 200 μliters of the final 90% methanol extract of cranberry phenolics were streaked in the sample position. A cold air current was used to evaporate the solvent during streaking. The paper was dipped in 0.05 M formic acid electrolyte and placed in the electrophoresis apparatus. The cover was placed in position and a potential of about 480 v applied across the electrodes. Electrophoresis was allowed to proceed for about 10–12 hr, then the paper was removed from the apparatus and air dried in a fume hood. The paper was viewed under ultraviolet light and the position of the bands marked with pencil. At the starting line there occurred a yellow-brown band immobile to electrophoresis and chromatography, followed by a dark band of flavonoids and then a blue band of what were probably phenolic acids and finally the trailing edge of the anthocyanin band. A line was drawn across the paper, parallel to the center fold, at the junction of the blue band and the anthocyanin band and the paper cut along this line.

The paper bearing the flavonoids was

formed into a cylinder held by a stainless steel chromatography clip at its top with the phenolic compounds towards the bottom. The cylinder was developed in an upward direction with butanol/acetic acid/toluene/water solvent (BATW) 75:25:19:20, by standing it in a petri dish of the solvent contained in a chromatography tank. Development in the upward direction was continued until the solvent front was about 0.5–1.0 in. from the top of the paper cylinder. The cylinder was removed from the tank, dried in a current of air in a fume hood and chromatographed again in the same direction with butanol/acetic acid/water (BAW) 6:1:2 solvent. After air drying once more, the paper was formed into a cylinder so that upward development could be carried out in a direction perpendicular to the previous direction. A final development was then carried out with 10% acetic acid. After air drying, viewing under ultraviolet light showed the presence of 4 dark spots of flavonoid compounds (Fig. 1). The blue fluorescent band evident on the paper after electrophoresis was separated on the final chromatogram into 7 spots which gave a blue fluorescence under ultraviolet light. A spot which showed a white fluorescence was also noted. The R_f values of these spots and also those of the flavonoid are shown in Table 1. The R_f values, the fluorescence under ultraviolet light together with the change in fluorescence to blue-green on exposure to ammonia fumes suggested that the spots consisted of phenolic acid or phenolic acid derivatives. Identification of these spots awaits further research. All of the spots were clearly distinguishable from the spots of flavonoid compounds, since they occurred at positions on the paper well removed from the latter. The anthocyanins separated by electrophoresis could be cut from the remaining piece of the original paper. After elution and concentration they could be determined quantitatively by the methods of Fuleki et al. (1968b).

DISCUSSION

PUSKI et al. (1967) were able to isolate 5 flavonol glycosides plus the aglycone quercetin from aqueous methanolic extracts of cranberries. Using the method reported herein, only 4 glycosides were detected. The spots A, B, C, D, respectively, were quercetin-3-galactoside, myricetin-3-arabinoside, quercetin-3-arabinoside and quercetin-3-rhamnoside. The identity of the 4 spots was confirmed by chromatography of authentic compounds. The 2 other pigments (myricetin-3-digalactoside and quercetin) identified by Puski et al. (1967) were present in amounts too small to be determined by this method. The quercetin previously detected may have been present as an artifact. Swain (1962) has suggested that flavonoid aglycones do not occur in nature but probably result from enzymatic hydrolysis during the extraction process. Chromatographic examination of the preliminary petrol ether extract after concentration showed the complete absence of flavonoid glycosides and aglycones; phenolic acids also appeared to be absent from the extract. Careful chromatographic examination of the residue left after the final extraction with methanol containing 10% water failed to reveal undissolved flavonoid aglycone. However, if the ground berries were allowed to stand for 12 hr before extraction with methanol/ethyl acetate solvent, appreciable quantities of flavonoid aglycone could be detected on the final chromatogram. This suggests that Swain's observation is probably correct.

The techniques employed by Puski et al. (1967) were elaborate, time-consuming and not applicable to routine quantitative analyses. The more rapid electrophoretic/chromatographic method could be used for routine studies. Although quantitative studies were not carried out, the final chromatogram suggested that the method should be applicable to direct photodensitometry on paper or elution from the paper for spectrophotometric measurements.

The BATW solvent was used to produce an initial separation of flavonoids and also to produce compact spots. It was found to be a solvent of rather good powers of resolution for simpler phenolic compounds and it gave fairly good resolution of cranberry anthocyanins, though not as good as the benzene/butanol/formic acid/water (BBFW) solvent of Fuleki et al. (1968b). It had the advantage of being a single-phase system.

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A PENETROMETER TEST TO MEASURE MEAT TENDERNESS

SUMMARY—Attempts to relate mechanical measures of raw meat tenderness to cooked meat tenderness have generally been unsuccessful. Therefore, a study was designed to evaluate the use of a simple penetration test to predict cooked meat tenderness from the force required to penetrate the raw meat sample. An Allo-Kramer Shear Press was modified to function as a penetrometer by replacing the Standard Shear-Compression cell and shearing blades with a plate containing 5 needles. The needles were made from 1/8-in.-diameter drill rod and were semiblunt, having 0.007-in.-diameter land and 0.472/1.0-in.-taper. Using slices from the longissimus dorsi muscle of pork, various factors of the time-force curve were analyzed to determine which would give the best indication of tenderness. It was found that the maximum force value gave the best results. Regression analysis showed correlations significant at the 1% level between the force necessary to penetrate the raw sample versus both force necessary to penetrate the cooked sample and trained technological panel evaluations.

INTRODUCTION

TO THE consumer, the texture of meat is one of its most important properties. Taste panels are used extensively to measure texture. Since they are subjective and in many cases unwieldy, many attempts have been made with considerable success to use mechanical devices for providing objective measurements. However, attempts to relate mechanical measures of raw meat tenderness to cooked meat tenderness have generally been unsuccessful.

Many different mechanical devices for measuring meat tenderness have been described by Schultz (1957), Matz (1962), Pearson (1963) and Szczesniak (1963). The 2 most popular instruments which measure shear values of meat are the Warner-Bratzler Shear and the Kramer Shear Press. Burrill et al. (1962) and Pearson (1963) noted correlation coefficients between sensory methods for tenderness and the Warner-Bratzler Shear generally range from 0.60 to 0.85. However, Deatherage et al. (1952) reported an r value of 0.369 between Warner-Bratzler shear values and panel scores. The correlation was low probably because of the small tenderness differences exhibited by the samples.

Burrill et al. (1962) and Sharrah et al. (1965) found an r of .7 to .8 between the Warner-Bratzler Shear and the L.E.E. Kramer Shear press. According to Pearson (1963) the major advantage of the L.E.E. Kramer Shear appears to be the time-force curve and more shears per unit of meat.

Many workers have related cooked meat tenderness as determined by mechanical devices to sensory evaluation panels. Few investigators have tried to relate raw meat tenderness determined objectively with sensory panel tenderness evaluations of cooked meat. Warner (1928), Black (1931), McBee (1959) and Carpenter et al. (1965) failed to find any significant correlation between tenderness of uncooked and cooked samples of meat as determined by the Warner-Bratzler Shear. Carpenter et al. (1965), reporting tenderness values for the raw meat samples by the wedge tenderometer, denture tenderometer and grinder tenderometer showed little association with taste panel data.

The variability of tenderness in muscle from various pork carcasses is a problem noted by several investigators, even though it has generally been held pork is fairly uniform in tenderness. Batcher et al. (1960) noted wide variations in shear values of cooked muscles from different pork carcasses. They also found the anterior parts of the raw longissimus dorsi and biceps femoris muscles more tender than the posterior section. Tuomy et al. (1964) and Batcher et al. (1962) also showed that there is a large variability in tenderness in raw and cooked pork muscles. Tuomy et al. (1964) indicated considerable differences in the individual pieces of pork cooked under identical conditions.

Marbling score, grade and loin weight have been advanced as means by which the tenderness of the cooked meat could be predicted from the raw state. Batcher et al. (1962), Alsmeyer et al. (1966) and Palmer et al. (1958) found that marbling score alone is not a reliable indicator of cooked tenderness. Palmer et al. (1958), Fielder et al. (1963) and Alsmeyer et al. (1966) indicate that beef grade accounts for only a small percentage of tenderness variation found in taste panel evaluations of various cooked beef samples. Fielder et al. (1963) suggested that anatomical location of a muscle had a greater influence on tenderness than did the factors used in determining grade. Studies by Tuomy et al. (1966; 1967) showed there was a statistically significant correlation between loin weight and both the shear value in pounds and taste panel evaluation for tenderness of the cooked pork. However, the correlation coefficients were so low (approximately 0.3) it was evident a large part of the variation in tenderness was not being accounted for by the loin weight.

Bourne et al. (1966) described a Uni-

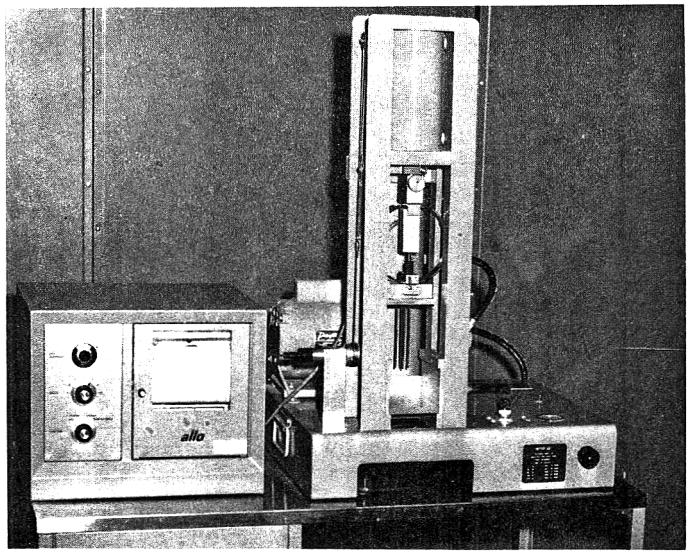

Fig. 1—An over-all view of the Allo-Kramer Shear Press with recording chart. The meat penetrometer is shown replacing the standard shear-compression cell.

versal Testing Machine. This machine can be fitted with the working parts of any food texture-measuring device that uses a linear movement. Chart recordings can be used for determination of maximum force, slope of force distance curve or measurement of work. Using the Universal Testing Machine and punches with varying areas and perimeters, Bourne et al. demonstrated that the force required to puncture a food depends on the area and perimeter of the punch and on the compressive strength and shear strength of the food being tested. The puncture force for different punches on the same food will be a function of both the area and perimeter of the punch used. The puncture force with the same punch on different foods will be a function of both the compressive strength and the shear strength of each food.

One of the problems of using a standard procedure of testing on raw meat is being unable to predict its tenderness after cooking. Further, most methods of mechanically determining tenderness on a raw sample destroy its integrity so the same sample cannot be meaningfully tested again after cooking. The "Precision" Universal Penetrometer, which did not destroy the sample, showed poor correlation to sensory panel (Pearson, 1963), even though Birmingham et al. (1966) and Hiner et al. (1941) found a good correlation to muscle and fatty tissue firmness.

In view of the work done with punches, a series of experiments was conducted using needles with an Allo-Kramer Shear Press.

EXPERIMENTAL

UNPUBLISHED data at this laboratory indicate that significant correlations between raw and cooked pork samples could not be obtained using the standard Allo-Kramer Shear Press. Preliminary work indicated that significant correlations could be obtained using a head for this Press containing 5 needles (1/8-in. in diameter) and a plastic base plate with holes for receiving the needles (Fig. 1 and 2). It took at least 5 needles to give enough resistance to make a meaningful time-force curve. Preliminary experiments indicated the semiblunt needle gave a higher correlation coefficient in comparing the penetration of raw and cooked pork chops than a fine-pointed or a blunt needle. The pointed needle and a blunt needle with a 3/16-in. diameter resulted in statistically nonsignificant r's in comparing raw to cooked pork chops. The 1/8-in. blunt needle gave an r = .3 significant at the 5% level. In the same preliminary work, the semiblunt needle had an r value of .7 between raw and cooked pork chops. As long as the land diameter of the semiblunt needle was maintained at .007 in., varying the taper from 0.236/1.000 to 0.951/1.000 in. seemed neither to hinder nor to help in determining the relationship between raw and cooked pork chops. As a result of these preliminary studies a semiblunt needle with a 0.007-in. diameter land and an intermediate taper of 0.472/1.000 in. of length was selected. Analysis of the time-force curve indicated that the maximum force value gave the best results.

The fresh bone-in pork loins were from Midwestern sources. They ranged from 14 to 25 lb. The weight range was varied in an attempt to obtain variations in muscle tenderness. All loins were trimmed to ½-in. backfat before weight was recorded. The weights of boned loin and the longissimus dorsi muscle also were recorded. The excised longissimus dorsi was shaped by stuffing into a 3-½-in.-diameter artificial casing. The stuffed loin was frozen and sawed into ½-in.-thick pork chops, then cut into 2-½-in.-diameter chops with a drill press and circular cutter.

Care was taken to keep the chops identified in order from anterior to posterior. Each group from 4 areas of the longissimus dorsi contained 3 chops. The 3rd chop in each group was used for another study. The 4 groups were as follows: a) posterior to the 2nd lumbar vertebra and anterior to the 6th lumbar vertebra, b) posterior 12th thoracic vertebra and anterior to the 2nd lumbar vertebra, c) posterior 8th thoracic vertebra and anterior to the 12th thoracic vertebra, and d) posterior 4th thoracic vertebra and anterior to the 8th thoracic vertebra.

The raw frozen chops were equilibrated to 40°F, penetrated raw and individually wrapped in aluminum foil. Identity of each chop was maintained throughout the study. After cooking, the chops were again penetrated. Penetration value in each case consisted of the average of 3 readings taken in different locations. Peak value from the time-force curve was used. We used 540 chops from 60 different loins. Of this

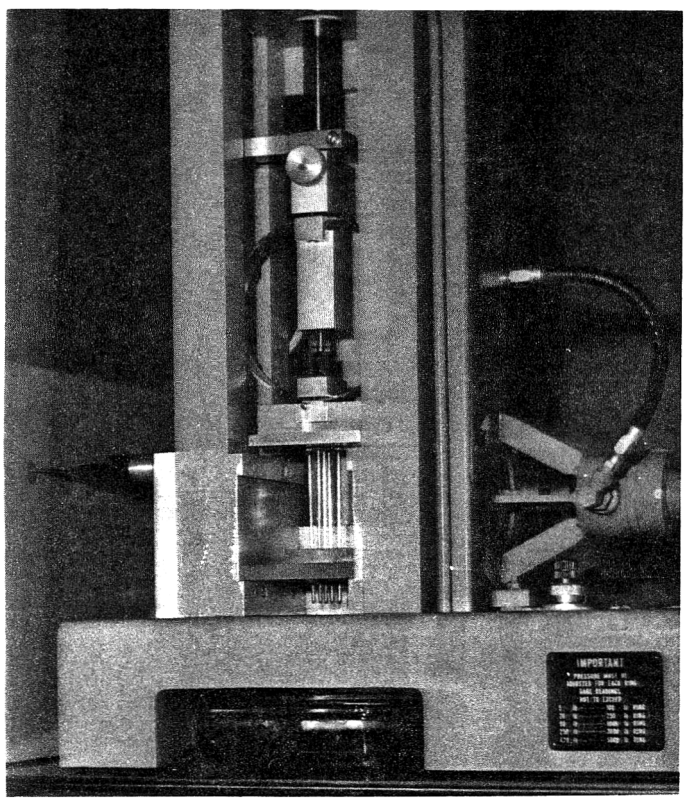

Fig. 2–A closeup of the meat penetrometer during penetration of a pork chop.

Table 1–Combined correlation values for chops steam cooked to 160, 180 and 200°F internally and evaluated by penetrometer and sensory panel.[1]

X	vs.	Y	N	R
Raw penetrometer		Taste panel	30	.6261**
Raw penetrometer		Cooked penetrometer	30	.7183**
Cooked penetrometer		Taste panel	30	.8618**

**Significant at 1% level.

[1] All pork samples furnished the taste panel had been cooked to an internal temperature of either 160, 180 or 200°F.

Table 2–Correlation coefficients of raw penetration values, cooked penetration values and taste panel tenderness scores for 30 loins. Chops were cooked at 460°F.

Comparison	N	R
Raw penetrometer vs. cooked penetrometer	240	0.6645**
Raw penetrometer vs. cooked penetrometer[1]	30	0.6982**
Raw penetrometer vs. taste panel[1]	30	0.5032**
Cooked penetrometer vs. taste panel[1]	30	0.6283**

**Significant at 1% level.

[1] The same 30 chops were penetrated raw, penetrated cooked and submitted to the taste panel. They are part of the 240 chops listed first.

number, 240 chops from 30 loins (2 from each of the 4 locations in each loin) were baked in an oven at 460°F for 20 min. 30 of these chops were evaluated by a trained technological taste panel as well as being penetrated. Another 240 chops from 30 loins were steam cooked at 6 lb pressure to internal temperatures of 160, 180 and 200°F in groups of 80. A further 60 were steam cooked to an internal temperature of 140°F. 60 cooked chops from the 240 steam cooked were evaluated by a trained technological panel—all of these were selected immediately posterior to the 8th thoracic vertebra (group c).

RESULTS & DISCUSSION

CORRELATION coefficients for various parts of the study are shown in Tables 1, 2 and 3. Range of correlations between raw penetration and cooked penetration is 0.5643–0.7511. Correlations between raw penetration and the taste panel were 0.6261 and 0.5032 and between cooked penetration and taste panel, 0.6283 and 0.8618.

Analysis of variance of raw penetration readings for loin weight and chop location is shown (Table 4). We found significant differences between loins and chop location. Further analysis by the method of Hicks (1956) shows that about 44% of the variation can be attributed to differences between loins, 31% to differences in chop location and 25% to the loin chop position interaction. Analysis of variance results when the same chops were penetrated after being cooked at 460°F for 20 min are also shown (Table 5). Again, the results indicated a significant difference between loins and chop location. However, in this case about 76% of the variation can be attributed to differences between loins, 9% to differences in chop location and 15% to the loin × chop position interaction.

Results of this study indicate that the penetrometer has promise for predicting cooked meat tenderness from the raw product and that further work is warranted (Table 6). Correlations obtained between raw and cooked pork tenderness are statistically significant and much better than correlations reported for other mechanical devices. Correlations obtained between cooked meat penetration and panel results for texture are equivalent to those reported for other mechanical devices. One of the principal advantages of the penetrometer is that it is essentially nondestructive, in that several readings can be taken in the same sample which then can be cooked, retested and finally submitted to a taste panel.

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Table 3—Correlation coefficients of raw penetration values vs. cooked penetration values with the chops cooked to 4 different internal temperatures in steam under 6 lb pressure.

Temperature (°F)	N	R
140	60	.7511**
160	80	.5643**
180	80	.5942**
200	80	.6773**

**Significant at 1% level.

Table 4—Analysis of variance of raw pork chops from 30 loins for differences in penetration.

Factor	Significance
Loins	**
Chops	**
Loin × chop interaction	**

**Significant at the 1% level.

Table 5—Analysis of variance of pork chops in Table 4 after cooking at 460°F for 20 min for differences in penetration values.

Factor	Significance
Loins	**
Chop location	**
Loin × chop interaction	**

**Significant at the 1% level.

Table 6—Typical data for comparison of meat penetrometer and technological taste panel evaluation of pork chops.[1]

Loin No.	Penetration force in lb raw pork chops	Penetration[2] force in lb steam-cooked chops	Technological[3] taste panel evaluation for tenderness
1	3.24	8.47	5.1
2	1.28	6.05	8.0
3	3.24	7.21	6.9
4	1.87	7.21	6.2
5	2.93	7.35	6.3
6	2.64	8.42	6.5
7	2.70	7.50	6.2
8	3.56	6.40	6.6
9	2.56	9.18	6.6
10	4.74	15.70	2.8
11	3.66	11.31	5.9
12	3.20	12.18	3.4
13	2.26	8.45	6.2
14	3.66	12.05	5.3
15	3.26	9.46	6.4
16	3.39	13.55	4.2
17	2.27	9.45	6.9
18	3.86	14.45	4.6
19	3.74	11.25	5.8
20	3.22	13.10	3.5
21	3.62	12.40	4.3
22	3.95	10.90	6.2
23	3.15	11.53	4.7
24	3.38	9.77	6.1
25	3.18	8.77	6.8
26	3.97	11.40	5.0
27	3.90	14.28	2.9
28	1.91	7.32	7.4
29	3.21	11.75	5.2
30	2.10	8.91	5.4

[1] All chops for meat penetrometer and sensory evaluation were taken immediately posterior to the 8th thoracic vertebra. The same pork chop was penetrated raw, penetrated cooked and sampled by the taste panel.

[2] Penetration values represent the average of 3 readings per pork chop.

[3] Taste panel ratings represent the average rating for tenderness by 10 panel members.

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COLORIMETRY OF FOODS. 2. Color Measurement of Squash Puree Using the Kubelka-Munk Concept

SUMMARY—A series of 10 squash purees was prepared with added canthaxanthin in increments of 0.3-ppm. The samples were evaluated visually for color differences under illumination of 7400°K and measured with a G.E. spectrophotometer with tristimulus integrator and a Hunterlab D25 colorimeter. Each sample was measured against a white and a black background in Plexiglass cells varying in thickness from 2 to 8 mm for both visual and instrumental experiments. The instrumental data were calculated in conventional and K/S ratios and correlated with theoretical and visual color rankings. The optimum visual rankings were found with a 5-mm thickness or greater, and a black background. The panels could rank samples differing in approximately 0.2 delta E units, calculated as $\Delta E = (\Delta L^2 + \Delta a^2 + \Delta b^2)^{1/2}$ *reasonably well with 5-mm thickness or greater, and no better with thinner cells, even though the* ΔE *values were much larger. Adequate rankings of the samples by either instrument required a multiple correlation with all three color parameters (X Y Z or L a b). Calculation of tristimulus data in terms of K/S ratios or K/S ratios at single wavelengths did not improve the correlations with visual or theoretical rankings.*

INTRODUCTION

CONVENTIONAL tristimulus and spectrophotometric methods for measurement of color have been developed for use with either opaque or transparent materials. However, many products are neither opaque nor transparent and the over-all visual impression is a combination of both the light reflected from the surface and that partially absorbed by the material and reflected from subsurface layers. Most foods are in this category. Interpretation of reflectance and transmittance data from translucent or semi-translucent materials has generally been handled by the "turbid media" concept. Equations for a number of applications of this theory developed by Kubelka and Munk are described in the book by Judd et al. (1963). Little and Mackinney (Little, 1964; Mackinney et al., 1966) discussed these applications to food materials.

The Kubelka-Munk concept involves reflectance measurements of thin layers of material with a white and a black background. From this, a reflectance value for infinite thickness can be calculated, as well as values for degree of light scattering and absorption. The interpretation of reflectance data in terms of both scattering and absorption was found by Little and Mackinney to be effective for separating samples of appleberry-applesauce mixtures. The concept was also applied to mixtures of applesauce and caramelized sauce and two series of milk samples (Mackinney et al., 1966).

The samples used by these authors were essentially of medium reflectance and low chroma. It was decided to test this concept with samples of high chroma (squash puree) and very small color differences.

MATERIALS & METHODS

THE SQUASH puree used in this work was commercial junior baby food puree. A series of samples was colored with a water-dispersible red carotenoid pigment (canthaxanthin, 10% dispersible beadlets, Hoffmann-La Roche Co.). In this manner a series of samples could be prepared in which the order of color change was known and the color differences were close to the visual threshold. Ten samples of colored squash puree were prepared for each series by adding stock solution (3 mg canthaxanthin/ml) such that the mixtures contained 0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4 and 3.0 ppm canthaxanthin.

The samples were placed in circular Plexiglass cells of 6.4 cm inside diameter with sample thicknesses of 2, 3, 4, 5, 6, 7 and 8 mm. The cells had a cemented bottom and a removable cover of Plexiglass.

The visual examinations were performed in a MacBeth Lablite booth (MacBeth Daylighting Corp., Newburgh, New York) under north-sky daylight (7400°K). 10 samples in cells of identical thickness were presented to the panelist for ranking in order of redness. The cells were

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

	Cell thickness—mm						
	2	3	4	5	6	7	8
Correlation	Correlation coefficient—r × 1000[1]						
Th[2] vs. V_B[3]	957	946	977	983	986	982	984
Th vs. V_W	935	922	940	976	971	957	946
	I.C.[4]						
V_B	12.0	12.0	8.5	7.0	6.5	8.0	7.5
V_W	12.0	15.0	8.0	10.0	10.0	10.5	9.5

[1] All correlation coefficients are the average of r or R values from three experiments.

[2] Theoretical ranking.

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

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

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

Cell thickness (mm)	Concentration of canthaxanthin (ppm) 0.3	0.9	1.5	2.1	3.0
	E[1]				
	Small illum. area and white background				
2	2.7	3.4	4.0	4.6	5.5
3	1.3	1.8	2.3	2.8	3.5
4	1.0	1.4	1.8	2.2	2.8
6	0.7	1.1	1.4	1.8	2.4
8	0.4	0.8	1.2	1.6	2.2
	Large illum. area and black background				
2	1.2	1.5	1.8	2.0	2.5
3	0.5	0.8	1.0	1.3	1.6
4	0.3	0.7	1.1	1.5	2.1
6	0.5	0.9	1.3	1.7	2.3
8	0.4	0.8	1.2	1.6	2.2

[1] Data obtained with a Hunterlab D25 colorimeter and calculated as $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$ = difference between readings for samples with added pigment and the controls.

ranked on both a black background and a white background (Munsell papers N2.25/ and N9/, respectively). 4 panelists with a good color theory background and superior color vision and color discrimination ability, as shown by the Farnsworth Munsell 100 Hue test and the ISCC Color Aptitude Test were used for the visual judgments. A small panel was chosen, because of the large number of samples. The experiment was repeated three times for a total of 1,680 judgments.

The instrument readings were obtained with a General Electric Recording Spectrophotometer (General Electric Co., Lynn, Mass.) operated with specular reflection included and pressed barium sulphate standards. A Davidson and Hemmendinger (Davidson & Hemmendinger, Inc., Easton, Pa.) Tristimulus Integrator provided the X Y Z data from the reflection curves. A Hunterlab D25 Colorimeter (Hunterlab Associates, Inc., Lee Highway, Fairfax, Va.) equipped with a 2-in.-diameter aperture and lenses to provide small or large area illumination was used to obtain L a b data through the vertical face of the cells. A pink standard tile (L = 74.5 a = 14.7 b = 7.2) was used to standardize the instrument. With both instruments, the samples were backed with Munsell white (N9/) and black (N2.25/) papers.

The equations used for calculating the K/S ratios were as follows:

$$a = \tfrac{1}{2}\left(R + \frac{Ro - R + Rg}{Ro\ Rg}\right)$$

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

$$R = a - b$$

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

where R = reflectance of sample with white background

R_o = reflectance of sample with black background

Rg = reflectance of white background

Table 3—Correlation of visual and theoretical rankings with data from G.E. Spectrophotometer.

Calculation	Cell thickness—mm 2	4	6	8
	Correlation coefficient—r or R × 1,000			
Th vs. X_B[1]	508	489	491	468
V_B vs. X_B	549	528	522	527
Th vs. X_B Y_B	676	883	966	925
V_B vs. X_B Y_B	727	853	964	933
Th vs. X_B Y_B Z_B	924	982	989	978
V_B vs. $X_B Y_B$ Z_B	940	967	981	980
Th vs. X_W Y_W Z_W	742	917	975	974
Th vs. (K/S)X	450	446	459	469
V_B vs. (K/S)X	510	479	487	497
Th vs. (K/S)X (K/S)Y	883	982	913	963
V_B vs. (K/S)X (K/S)Y	902	975	909	963
Th vs. (K/S)X (K/S)Y (K/S)Z	884	983	966	982
V_B vs. (K/S)X (K/S)Y (K/S)Z	904	981	960	980
Th vs. (K/S) 585	542	547	525	548

[1] X, Y and Z are tristimulus values obtained with a G.E. Recording Spectrophotometer. The subscripts B and W refer to black and white backgrounds. The prefix (K/S) refers to the K/S values calculated from G.E. X Y Z data by the Kubelka-Munk equations. Other symbols same as in Table 1.

Table 4—Correlation of visual and theoretical rankings with data from Hunterlab D-25 colorimeter.

	Cell thickness—mm 2	4	6	8
Correlation	Correlation coefficient—r or R × 1,000			
Th[1] vs. a_{Bl}	656	612	600	578
V_B vs. a_{Bl}	694	630	621	600
Th vs. a_{Bl} b_{Bl}	823	933	985	959
V_B vs. a_{Bl} b_{Bl}	850	909	983	958
Th vs. a_{Bl} b_{Bl} L_{Bl}	881	969	990	974
V_B vs. a_{Bl} b_{Bl} L_{Bl}	888	943	985	970
Th vs. a_{Wl} b_{Wl} L_{Wl}	826	981	937	969
Th vs. a_{Bs} b_{Bs} L_{Bs}	805	948	977	965
Th vs. a_{Ws} b_{Ws} L_{Ws}	838	961	935	964
Th vs. $(K/S)a_l$	697	598	574	589
V_B vs. $(K/S)a_l$	746	628	598	615
Th vs. $(K/S)a_l$ $(K/S)b_l$	913	986	983	975
V_B vs. $(K/S)a_l$ $(K/S)b_l$	926	985	976	976
Th vs. $(K/S)a_l$ $(K/S)b_l$ $(K/S)L_l$	923	987	984	984
V_B vs. $(K/S)a_l$ $(K/S)b_l$ $(K/S)L_l$	938	985	976	982
Th vs. $(K/S)a_s$ $(K/S)b_s$ $(K/S)L_s$	903	981	984	984
V_B vs. $(\tan^{-1} a/b)_{Bl}$	608	626	614	605
V_B vs. $[(K/S)_{al}$ $(K/S)_{bl}]$	770	669	587	643

[1] The subscripts B and W refer to samples with black and white backgrounds. The subscripts l and s refer to large and small areas of illumination. The letters L a b refer to tristimulus data from the colorimeter. Other symbols same as in Tables 1 and 3.

R_∞ = reflectance of infinitely thick sample
K = coefficient of absorption
S = coefficient of scattering

Color differences were calculated from Hunter L a b data by the equation Delta E = $[(L_1 - L_2)^2 + (a_1 - a_2)^2 + (b_1 - b_2)^2]^{1/2}$

Correlation coefficients (r and R) were calculated to show the relationship between theoretical ranking and visual ranking versus other color indices obtained from the instruments. 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 test the difference in z values for each pair of color indices. In cases where it was desired to test the effect of cell thickness, an F test was used to test the linear regression of each color index with seven thickness levels.

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

RESULTS & DISCUSSION

TABLE 1 presents data on the correlation of visual panel results with the theoretical order of the samples. All correlations were high, but the panel could obviously rank the samples more accurately when judged against a black background. Correlations were lower for cell thicknesses of 4 mm and less. For optimum visual judgments the sample thickness should be 5 mm or larger. This conclusion is also seen by the index of confusion (Table 1) for each cell thickness, since both were calculated from the same data.

In Table 2, data are presented on the size of the color differences evaluated by the panel in Table 1. The actual figures in Table 2 were calculated from a regression equation calculated from the delta E values for 10 pigment concentrations, to reduce the error of measurement. Only 5 of the 9 delta E values are presented for each sample thickness. The numerical value of the color difference varied with method of measurement and the two extremes are presented in Table 2. The data for large area and black background probably more closely approximate visual judgments. The panelists were evaluating samples with an average color difference of approximately 0.2 delta E units, as shown for thick cells with both types of measurement. With thin cells the color difference readings were higher, indicating that the first increment of color had a much larger effect than the additional increments. This effect was very obvious with the 2-mm cells and decreased as the sample thickness increased. The 2- and 3-mm cells actually resulted in an expansion of color space as reported by Little (1964). Logically, this should allow the visual panels to place the samples more easily in the correct order with the thinner cells, but this is contrary to the data in Table 1. One reason for this discrepancy may be that both instrumental and visual data were more erratic for the thinner cells, possibly due to small errors in cell dimensions. This effect is being checked with special precision cells of optical glass, which can be held to much closer tolerances.

Correlations of visual and theoretical rankings against spectrophotometric data are presented in Table 3. The correlations of theoretical rank vs. X are low. For theoretical vs. X Y the correlations are higher and for theoretical vs. X Y Z are higher still. This indicates that for this particular system, all three parameters of color are necessary, but the X and Y values are most important. Correlations of G.E. X Y Z data against the theoretical rank or the visual ranking were similar where the samples were measured with a black background. Measurement with a white background was less satisfactory for sample thicknesses of 5 mm or less. Samples of 6 mm or more in thickness approached infinite thickness and the background made little difference. Interpretation of the X Y Z data in terms of K/S ratios and correlation with theoretical or visual rank followed the same trends as discussed above. The transformation to K/S ratios did not improve the situation, possibly because when all three color parameters were used, the multiple correlation coefficients were already very high.

Correlations of visual and theoretical rankings against Hunter L a b are presented in Table 4. The correlations involving only Hunter a were low. Those involving a and b were much higher. When the L value was added, the correlation was improved, but not much. Apparently, the changes in this set of samples were mainly in the a and b values but all three were necessary for most accurate predictions. As before, the white background was less satisfactory than the black.

There was a difference between the correlation of theoretical rank and many of the instrument scores for the large and small modes of illumination. As judged by the z values calculated from the r or R values and used in a paired "t" test, the large area mode was superior to the small area mode. In these experiments the area of illumination in the large area mode was approximately 4.7 cm in diameter which was, in effect, the diameter of the instrument aperture. The small-area lenses were focused to illuminate an area of approximately 1.2 cm in diameter in the 4.7-cm opening. An opening larger than the illuminated area is preferable to minimize the light trapping effect, but even so the large area mode was superior.

Substitution of L a b data in K/S transformations was no advantage in sample thicknesses of 6 mm or greater. With thinner samples the K/S correlations were higher than the correlations with direct L a b data. However, this would be an advantage only where very small samples were available for measurement. Usually the sample size is not limiting for most food applications.

The K and S components of the Kubelka-Munk equations are functions of the wavelength of the incident light. The absorption component (K) usually has an optimum depending on the absorption curve of the particular colorants involved, and the scattering component (S) depends on particle size and varies with wavelength. In colorant formations the K/S ratios are usually calculated at individual wavelengths rather than by substitution of tristimulus data as shown here. Correlations of K/S ratios for 585 nm (the dominant wavelength for these samples) with theoretical rankings are shown in Table 3. It is obvious that use of the K/S ratio at an optimum wavelength for canthaxanthin in this system is more efficient than a simple correlation with one tristimulus parameter, but less efficient than multiple correlations with tristimulus data. Other wavelengths were even less efficient.

It would be anticipated that addition of a red pigment to an orange medium would result mainly in a hue shift. This is true to some extent, as shown by the high correlations of both theoretical and visual rankings with a combination of a and b (Table 4). However, a simple function of hue ($\tan^{-1}$ a/b) or the same function calculated in K/S ratios $[(K/S)_{a1} / (K/K)_{b1}]$ correlated to a lesser degree with visual rankings than the a + b function above. The L function does affect the correlation, particularly with the very small color differences in these experiments.

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COMPARATIVE STUDIES ON THE NITROGEN SOLUBILITY OF MUNG BEANS, PEA BEANS AND RED KIDNEY BEANS

SUMMARY—The effect of pH and some salts on the extraction of nitrogenous matter from mung beans, pea beans and red kidney beans has been studied. The nitrogen solubilities of these beans were found to be strongly pH-dependent. The amount of nitrogen extracted at alkaline pH is greater than that produced at either neutral or acidic pH. Minimum points of nitrogen dispersion occurred at pH 4.0. Salts dispersed more nitrogenous constituents from the beans than did water. Dilute solutions of sodium chloride, sodium sulfate, calcium chloride and magnesium chloride were found to have an inhibitory effect on the dispersion of the nitrogenous matter of the beans. Alkaline salts such as sodium carbonate, disodium phosphate and sodium citrate appeared to be fairly effective dispersing agents, and the exact amount of nitrogen extracted was dependent upon the concentration of the salt solution.

INTRODUCTION

A REVIEW of literature reveals very little information on the extraction of nitrogenous constituents from mung beans, pea beans and red kidney beans. Results of extraction studies made by Pusztai (1965) have shown the importance of conditions such as pH and ionic strength of the dispersing media on the amount of total nitrogen and proteins extracted from kidney beans. The extraction of proteins is incomplete below pH 7, and the exact amount of proteins extracted depends upon the pH and the ionic strength of the solvent. Above pH 7, the ionic strength has little effect on the dispersibility of the nitrogenous components of the seed, as practically 100% extraction of nitrogen can be achieved between pH 7 and 9 at low or moderate ionic strength.

Sodium chloride, according to Osborne (1924), is the most widely used salt for extracting proteins from various seeds. Smith et al. (1938) studied the effect of neutral salts on the extraction of soybean proteins; their results indicate that neutral salt solutions never peptize as much nitrogenous matter as water. Smith et al. (1959) have also shown that concentrations of NaCl in the ranges 0–0.1 M have opposite effects on the solubilities of radish seed and soybean proteins. In this range, the solubility of the latter drops sharply from nearly 90 to less than 50%, whereas values for radish seed rise sharply from 20 to nearly 50%. It is thus desirable to study the dispersing effects of pH and a variety of salt solutions on the nitrogenous constituents of mung beans, pea beans and red kidney beans.

MATERIALS & METHODS

CERTIFIED red kidney beans (*Phaseolus vulgaris*) and pea beans (*Phaseolus vulgaris*) were purchased from Agway, Inc. (Geneva, New York). Certified mung beans (*Phaseolus aureus*) of the common, olive-green variety were supplied by the Specialty Food Corporation (Johnson City, New York). For these investigations, all the seeds were ground through the 1B screen (in which the diameter of the screen openings is 0.045 in.)of a Fitz mill Model D (W. J. Fitzpatrick Company, Chicago, Illinois).

Extraction procedure

5 g of ground beans and 200 ml of dispersing solution were placed in a 500-ml Erlenmeyer flask and shaken in a Gyrotary shaker, Model G25 (New Brunswick Scientific Company, New Brunswick, New Jersey) for 1 hr. All extractions were carried out at 25°C. The dispersions were filtered through Whatman No. 2 V folded filter paper and the filtrates then analyzed for the amount of total nitrogen extracted by a slight modification of the standard micro-Kjeldahl method (AOAC, 1960), in which mercuric oxide and potassium sulfate were replaced with a Kjeldahl tablet containing sodium sulfate and selenium as the catalyst (The British Drug Houses, Ltd., Poole, England).

The total nitrogen content of mung beans, pea beans and red kidney beans was found to be 3.98, 3.33 and 3.85%, respectively. Results of extractions are expressed as percent total nitrogen extracted from the beans. Values presented in Table 1 are indicative of the analytical precision for those graphically shown in Figures 1, 2, 3, 4, 5, 6 and 7. The moisture contents, as determined by the vacuum oven method (A.O.A.C., 1960), of mung beans, pea beans and red kidney beans were found to be 6.17, 10.81 and 7.49%, respectively.

RESULTS & DISCUSSION

THE CURVES in Figure 1 show that the solubilities of the nitrogenous constituents of mung beans, pea beans and red kidney beans, as determined over a wide range of pH values using dilute HCl and NaOH solutions in appropriate concentrations, are strongly pH-dependent. It can be seen that the amount of nitrogen extracted from the beans at alkaline pH is greater than that produced at either neu-

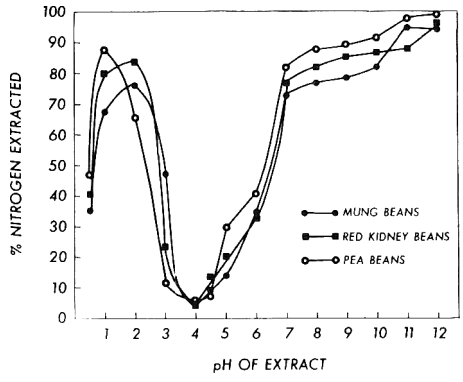

Fig. 1—Effect of pH on extraction of total nitrogen from mung beans, pea beans and red kidney beans.

Table 1—Extraction of nitrogenous constituents from mung beans, pea beans and red kidney beans by sodium chloride.

NaCl conc M	% Total nitrogen extracted[1]		
	Mung beans	Pea beans	Red kidney beans
0.010	62.60 ± 0.50	57.00 ± 0.30	38.30 ± 0.20
0.025	52.10 ± 0.20	53.75 ± 0.35	35.00 ± 0.10
0.050	44.35 ± 0.65	63.10 ± 0.30	38.05 ± 0.15
0.075	49.85 ± 0.15	68.90 ± 0.00	49.30 ± 0.70
0.100	53.85 ± 0.95	76.10 ± 0.10	57.45 ± 0.05
0.250	69.65 ± 0.05	79.55 ± 0.65	69.30 ± 0.20
0.500	71.05 ± 0.25	81.00 ± 0.50	70.25 ± 0.35
0.750	72.25 ± 0.15	78.75 ± 0.15	71.60 ± 0.40
1.000	66.70 ± 0.50	73.75 ± 0.35	66.10 ± 0.80

[1] Values represent mean of duplicate extractions ± over-all variations between samples.

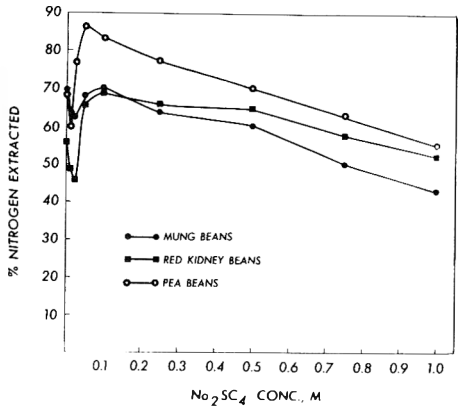

Fig. 2—Total nitrogen extracted from mung beans, pea beans and red kidney beans by sodium sulfate.

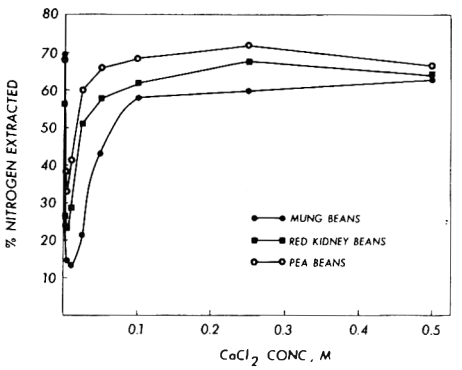

Fig. 3—Total nitrogen extracted from mung beans, pea beans and red kidney beans by calcium chloride.

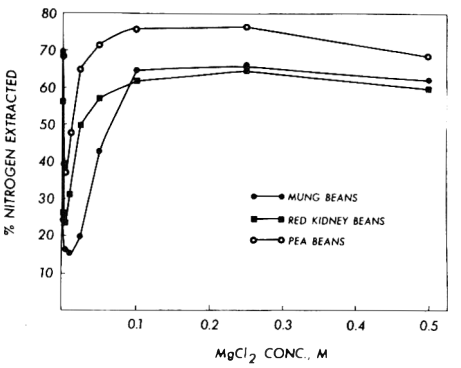

Fig. 4—Total nitrogen extracted from mung beans, pea beans and red kidney beans by magnesium chloride.

tral or acidic pH, and that the proteins of the beans have a common point of minimum dispersion at pH 4.0. The pH of the resulting water extracts of mung beans, pea beans and red kidney beans was found to be 6.59, 6.69 and 6.60, respectively. The pH-dispersion curves for these beans thus are similar to the patterns described for soybeans (Smith and Circle, 1938), navy beans (Evans et al., 1963) and kidney beans (Pusztai, 1965). The nitrogen solubility of some wild leguminous seeds as a function of pH has also been reported recently by Pant et al. (1969). Results in Figure 1 thus indicate that a large amount of the nitrogenous constituents of mung beans, pea beans and red kidney beans can be extracted either by dilute NaOH or dilute HCl at the pH of maximum dispersion, and that a major portion of this soluble nitrogenous matter can be precipitated by careful addition of acid or alkali to lower the pH to 4.0.

The amount of total nitrogen extracted from mung beans, pea beans and red kidney beans by water was found to be 69.8, 68.5 and 56.3%, respectively. Extraction of the nitrogenous constituents of the beans by different NaCl concentrations is shown in Table 1. It is evident that more nitrogen is extracted from pea beans by NaCl than from either mung beans or red kidney beans. Mung beans show a minimum dispersion at 0.05 M, whereas both pea beans and red kidney beans have a common point of minimum dispersion at 0.025 M. The solubilities of the nitrogenous constituents of mung beans, pea beans and red kidney beans reach maxima at 0.75, 0.5 and 0.75 M, respectively. Sodium chloride thus disperses more nitrogenous constituents from the beans than does water. In their extraction work, Smith et al. (1938) found that NaCl and other neutral salts disperse less of the nitrogenous matter from soybean meal than is dispersed by water and that soybeans have a minimum point of dispersion at 0.04 N for NaCl. Smith et al. (1959) reported that increasing the concentration of NaCl from 0–0.1 M does not have the same effect on the solubilities of radish seed and soybean proteins. In this range, the solubility of the latter drops sharply from nearly 90 to less than 50%, whereas values for radish seed rise sharply from 20 to nearly 50%. The radish seed solubility curve continues to rise to a maximum of 80% at 0.68 M, from whence it drops slightly as the concentration is raised further.

The solubilities of the nitrogenous constituents of mung beans, pea beans and red kidney beans in different Na_2SO_4 concentrations are shown in Figure 2. The solubility curves rise to maxima at 0.1, 0.05 and 0.1 M for mung beans, pea beans and red kidney beans, respectively. Increasing the concentration of this salt beyond the previously mentioned values results in a sharp decrease in its dispersing power. It is noted that the amount of nitrogen extracted from pea beans by Na_2SO_4 is much greater than that from either mung beans or red kidney beans.

Dilute solutions of either $CaCl_2$ or $MgCl_2$ are capable of inhibiting the dispersion of soybean proteins (Smith et al., 1938). In this work we have also observed

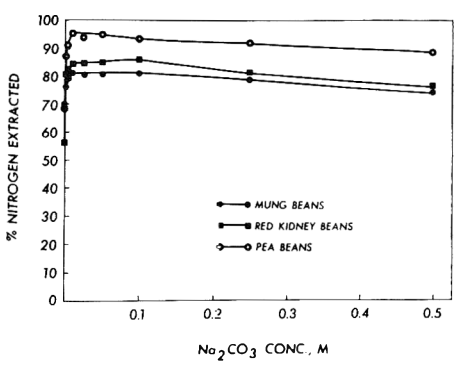

Fig. 5—Total nitrogen extracted from mung beans, pea beans and red kidney beans by sodium carbonate.

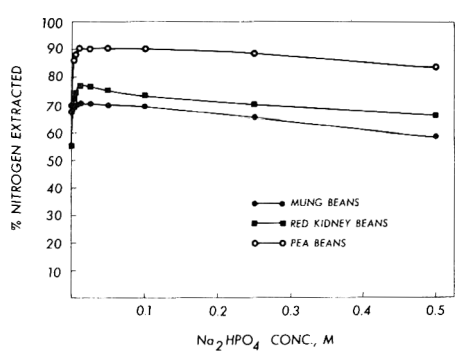

Fig. 6—Total nitrogen extracted from mung beans, pea beans and red kidney beans by disodium phosphate.

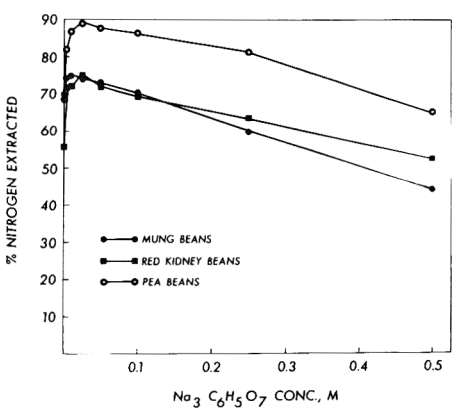

Fig. 7—Total nitrogen extracted from mung beans, pea beans and red kidney beans by sodium citrate.

that dilute solutions of both $CaCl_2$ and $MgCl_2$ lower the extraction of nitrogenous constituents from mung beans, pea beans and red kidney beans. Results in Figure 3 show the extraction of nitrogenous constituents from these beans by different $CaCl_2$ concentrations. Both pea beans and red kidney beans have a common point of minimum dispersion at 0.005 M, whereas the minimum dispersion of mung beans occurs at 0.01 M. The least amount of nitrogen extracted from mung beans, pea beans and red kidney beans was found to be 12.8, 33.7 and 23.3%, respectively. The effect of $MgCl_2$ on the extraction of nitrogenous constituents from these beans is illustrated in Figure 4. It is apparent that mung beans have a minimum point of dispersion at 0.01 M, whereas both pea beans and red kidney beans have a common point of minimum dispersion at 0.005 M. The least amount of nitrogen extracted from mung beans, pea beans and red kidney beans was found to be 14.9, 36.7 and 23.5%, respectively. In their extraction work, Smith et al. (1938) found that soybeans have a minimum point of dispersion at about 0.02 N for both $CaCl_2$ and $MgCl_2$.

The solubilities of the nitrogenous constituents of mung beans, pea beans and red kidney beans in 3 basic salts, Na_2HPO_4, $Na_3C_6H_5O_7$ and Na_2CO_3, have also been studied; results are shown in Figures 5, 6 and 7. Increasing the concentration of these salts causes a gradual increase in the amount of nitrogen extracted from the beans until the solubility curves reach maxima and then drop slightly. These data indicate that these salts are fairly effective dispersing agents for extracting nitrogenous constituents from the beans used in this study. Pusztai (1965) has also found that the ionic strength of the solvents has little effect on the dispersibility of the nitrogenous components of kidney beans under alkaline conditions, as practically 100% extraction of nitrogen can be achieved with alkaline buffers at low or moderate ionic strength.

It is concluded from the results obtained in this work that the nitrogenous constituents of mung beans, pea beans and red kidney beans have solubilities similar to those of the leguminous seeds previously studied (Smith and Circle, 1938; Smith et al., 1938; Evans et al., 1963; Pusztai, 1965; Pant et al., 1969).

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STUDIES ON AROMA OF CURED HAM

SUMMARY—Cured and uncured hams, raw, cooked or cooked-smoked, were analyzed for free amino acids. A number of amino acids decreased on curing. Total free amino acid concentration increased on cooking—slightly in uncured hams and to a much larger extent in cured hams. Smoking resulted in negligible change in total amino acid concentration of hams, although a number of individual amino acids were affected. Aqueous extracts and diffusates of cured and uncured hams were differentiated by a trained panel on the basis of aromas produced on heating. Smoke aroma could be detected in cured smoked samples but only with difficulty, if at all, in uncured smoked samples. Precursors of basic meaty aroma are water extractable from all hams examined, whereas components or precursors of cured and smoky aroma may be extracted from hams with chloroform-methanol. Gas chromatography of volatiles developed on heating of ham diffusates and lipid extracts was carried out and the odors of the components separated were observed. Some variations among the patterns of volatiles from the 6 types of hams studied were observed, but no single component had a meaty or cured aroma.

INTRODUCTION

THE CURING of ham has been investigated extensively in the past, with most of the work reported dealing with the formation and changes of color. Many of these reactions have been elucidated (Wilson, 1960; Lawrie, 1966). Cured ham may be easily distinguished from uncured ham on the basis of flavor. However, relatively little is known about the interaction of the cure components with meat components reflected in the modification of meat flavor due to curing.

Only a few reports dealing with flavor constituents of cured hams are available (Lillard et al., 1969; Ockerman et al., 1964; Cross et al., 1965). These indicate that cured ham flavor is found in the volatiles obtained by vacuum distillation of ham. Many of the volatile components of cured ham thus far identified have also been found in uncured ham as well as in other types of meat (Landmann et al., 1966; Solms, 1968). There has been no indication that any specific component or components of cured ham flavor possess the characteristic "cured" aroma.

According to Lillard et al. (1969) the flavor compounds and flavor precursors of country-cured hams were not water soluble. On the other hand, Baker (1961) reported that the freeze-dried water-soluble fraction of fresh pork yielded a strong ham flavor after curing and cooking. A variety of techniques for the curing of hams is being used in the food industry today. Consequently, "cured flavor" has not been clearly defined, and some variations in cured flavor may be attributed to the method of preparation.

In the present work we have studied various fractions of ham to isolate and identify the cured-ham aroma and to follow the changes occurring in pork during curing, cooking and smoking. In particular, analysis of the free amino acids in hams was undertaken as a possible indication of the processes leading toward the formation of ham flavor.

EXPERIMENTAL

Preparation of hams

The study involved 18 hams. The samples were prepared in 3 sets of 6 hams consisting of uncured raw (UR); uncured cooked (UC); uncured cooked and smoked (US); cured raw (CR); cured cooked (CC) and cured cooked and smoked (CS).

Raw hams and raw, cure-pumped hams, average weight 6 kg each, were obtained from a commercial source and aged 48 hr before processing. All samples referred to as cooked were heated in a Model ESQ-1 SH Smokehouse (Drying Systems Co., Div. of Michigan Oven Co.) without smoke at 120°F for 2 hr, 140°F for 2 hr, 160°F for 4 hr, 180°F for 2 hr and 190°F for 1.5 hr. The internal temperature of all hams at the end of this heating program was 150°F. The same heating program was used in preparing the smoked samples with the addition of smoke generated from commercial sawdust using a Meat Packers of America Junior Model Smoke Generator. The samples were smoked for 9.5 hr with a 2-hr drying-off period without smoke at the beginning of the heating program.

Preparation of aqueous extracts of ham

Steaks ½-in. thick were taken from each ham for organoleptic evaluation. After removal of as much visible fat as possible the remainder of the lean meat in each ham was ground twice to ensure a homogeneous mixture for analyses. 200 g of ground ham were homogenized with 200 ml of cold deionized water for 2 min in a blender. The mixture was centrifuged at 6,870 × g and the insoluble residue extracted twice with 100 ml of water each time. The combined supernatants, cooled to 2°C, were filtered through glass wool to remove suspended fat.

Preparation of ham diffusates

An aliquot of the aqueous extract (350 ml) was dialyzed against 350 ml of deionized water for 64.5 hr with 4 changes of water, using 7.5-cm cellulose casing (Union Carbide Co.) previously washed with water to remove glycerin. The dialysis procedure was carried out at 4°C. The combined diffusates (1,750 ml) were lyophilized, dissolved in deionized water to a final volume of 100 ml and stored at −18°C.

Preparation of chloroform-methanol extracts of ham

Lipid fractions of the ground hams were prepared by the extraction procedure of Folch et al. (1957).

100 g of meat were homogenized with 500 ml of chloroform-methanol (2:1, v/v) for 3 min in a blender. The slurry was filtered through Whatman No. 1 filter paper previously washed with the solvent and the residue extracted with an additional 500 ml of chloroform-methanol. The combined extracts were evaporated in vacuo at 30–35°C after washing with 200 ml of water to remove traces of water-soluble material. Extracts of uncured samples were pale amber in color and yielded an amber oil on removal of solvent. Extracts of cured samples were wine red in color and on evaporation yielded an amber oil containing particles of dark-brown pigment in suspension.

Analytical methods

Analyses for amino acids and other ninhydrin-reactive compounds were performed according to Spackman et al. (1958) using a Phoenix automatic amino acid analyzer.

Qualitative analyses of ham diffusates were carried out by thin-layer chromatography on Eastman Chromagram sheets 6064 (cellulose) and 6061 (silica gel) using the solvent systems: (A) n-propanol-ammonia (70:30 v/v) and (B) formic acid-butanone-t-butanol-water (15:30:40:15 v/v). Compounds on chromatograms were detected as described previously (Zaika et al., 1968).

Gas chromatography

All samples were heated in a Loenco Model 260 pyrolyzer and the volatile compounds introduced directly into the chromatographic column of an F and M Model 810 gas chromatograph by means of a valve. A 6-ft by ¼-in. stainless steel column packed with 15% Carbowax 20M TPA on 60-80-mesh Gaschrom P was used with helium flow of 63 ml/min. Column temperature was maintained at 70°C for 5 min, then programmed at 8°/min to 180°C and held at that temperature for a total analysis time of 60 min. The column effluent stream was split before passage into the flame ionization detector to permit evaluation of odors of components eluted from the column. Ham diffusate (0.5 ml) was used for analysis. The sample solution was introduced into a pyrex U-tube 8 cm long and 0.4 cm id and lyophilized. The dry material was heated in the pyrolyzer unit at an oven temperature of 250°C for 2 min and injected into the column for 2 min. Lipid fractions were treated in a similar manner.

Odor evaluation

Aroma was developed by heating 1.0 ml of

Table 1—Panel evaluations of odors obtained on heating ham extracts and diffusates.

	Extracts						Diffusates					
	UR	UC	US	CR	CC	CS	UR	UC	US	CR	CC	CS
Smoky			(√)			√						√
Chalky							√	√	√	√	√	√
Brothy	√	√	√	√	√	√	√	√	√	√	√	√
Cured				√	√	√				√	√	√
Salty				√	√	√						
Sweet				√	√	√				√	√	√
Porky	√	√	√									
Ham				√	√	√				√	√	√
Roast meat	√	√	√	√	√	√	√	√	√			
Burnt							√	√	√	√	√	√

aqueous sample or 0.1 ml of lipid fraction in a 10-ml beaker at 250°C on a calibrated hotplate. The aromas formed at different stages were noted, i.e., on warming, during boiling, at or just before dryness, at browning and after browning. A 5-member panel was trained over a period of 3 wk to recognize the aromas produced on heating and pyrolyzing whole pieces of ham and the diffusates of water extracts of ham. A standardized vocabulary was developed for the aromas of each type of preparation. After describing the aromas of unknown samples, differences in panelists' descriptions were resolved by referring to the standard preparations. Difficulties arose in obtaining uniform descriptions, because the odors change continuously during heating and the times of sniffing by the panelists were not synchronized. Furthermore, differences in heating rates occurred as a result of variations in temperature among the hotplates and variations in thickness of the beaker glass.

RESULTS & DISCUSSION

CURED and uncured hams could be identified readily by taste and odor on pan frying. All of the uncured ham steaks (UR, UC, US) were judged to have the odor and taste of pork chops or roast pork. The flavor of cured samples (CR, CC, CS) was identified with ham. Smoke was readily detected in CS samples. Only faint smoke flavor was detected in the outside portion of the US ham samples and none on the inside portion of the ham steaks.

In the present work it was found that aqueous extracts of cured and uncured hams (with or without smoking) could be identified by heating and observing the aromas produced (Table 1). For smoke-treated samples the smoke odor was quite noticeable in the cured ham extracts but could not be identified with certainty in uncured samples. The smoke aroma was evident at the beginning of the heating period and probably consisted of easily volatilized compounds dissolved in the extract.

When the aqueous extracts of hams were dialyzed, the diffusates from the cured hams (CR, CC, CS) gave, on heating, a cured-meat aroma, whereas the diffusates from uncured hams (UR, UC, US) gave an aroma of cooked meat (Table 1). The dialysate fractions containing the higher molecular weight components yielded only faint noncharacteristic odors on heating. Differences in odor of cured and uncured extracts (or diffusates) were most noticeable during the boiling stage. On heating to dryness (browning) the odors obtained were essentially those of roast meat for all of the samples, with the cured samples having sweeter aromas (possibly due to compounds originating from added sugar in the cure solution).

Qualitative analysis of ham diffusates was carried out by thin-layer chromatography. Not many differences were noted between cured and uncured samples. Cured samples contained a large amount of an iodine-reactive compound as shown on the thin-layer chromatogram. This material also reacted with bromphenol blue-methyl red indicator, which is characteristic of a basic compound. Creatinine content increased to some extent in the cooked samples as compared to the raw. This was in agreement with results obtained by Macy (1966). No significant differences in the purine derivatives (inosinic acid, inosine, hypoxanthine) were noted.

Amino acid analysis of ham diffusates is shown in Table 2. Ratios for each amino acid were calculated (Table 3) to determine whether there are any effects on these compounds due to curing, cooking or smoking. A number of differences in the ratios of free amino acids of the 6 types of hams were noted. The difference was considered meaningful if it was greater that 20% (an arbitrary value) of the denominator values in Table 3.

Curing resulted in a 26% decrease of total free amino acids of raw hams. A decrease of free amino acids in meat due to curing is not unexpected. These could be converted to α-hydroxy acids by way of the VanSlyke reaction, in which the amino group may interact with nitrous acid formed from sodium nitrite in the cure. There was a significant reduction of

Table 2—Free amino acids[1] and nitrogenous compounds in hams.

	UR	CR	UC	CC	US	CS
Taurine	2.92	2.40	3.66	4.32	3.73	3.78
Urea	2.39	1.48	2.05	2.16	4.09	5.13
Aspartic Acid	0.068	0.29	0.11	0.087	0.12	0.084
Threonine	0.50	0.34	0.46	0.47	0.52	0.56
Serine	0.68	0.56	0.84	0.69	0.82	0.90
Glutamine[2]	0.99	0.54	1.17	1.36	0.59	0.58
Proline	0.45	0.36	0.55	0.46	0.58	0.61
Glutamic Acid	0.75	0.45	1.21	0.94	1.38	1.12
Glycine	1.76	1.04	2.20	1.51	1.95	1.87
Alanine	3.22	1.96	3.72	3.01	3.35	3.66
Valine	0.35	0.45	0.65	0.54	0.66	0.48
Methionine	0.16	0.10	0.18	0.18	0.18	0.16
Isoleucine	0.24	0.24	0.36	0.38	0.40	0.34
Leucine	0.47	0.44	0.73	0.65	0.74	0.64
Tyrosine	0.23	0.22	0.30	0.29	0.32	0.27
Phenylalanine	0.22	0.18	0.29	0.28	0.33	0.26
Ornithine	0.068	0.031	0.050	0.035	0.11	0.43
Ethanolamine	0.12	0.046	0.14	0.066	0.10	0.81
Ammonia	2.43	1.91	3.09	4.46	3.38	3.11
Lysine	0.38	0.38	0.59	0.58	0.58	0.59
Histidine	0.16	0.12	0.26	0.26	0.32	0.34
Anserine	0.33	0.21	0.45	0.40	0.52	0.27
Carnosine	10.86	8.21	12.31	12.01	12.25	10.11
Total	29.75	21.96	35.37	35.14	37.02	36.10

[1] μmoles/g meat.

[2] Includes asparagine.

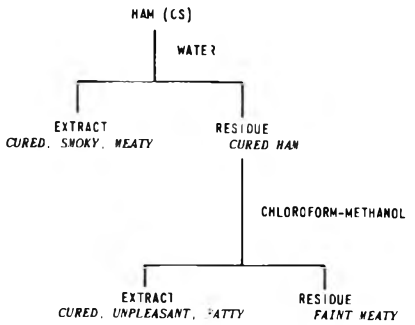

Fig. 1—Extraction of ham flavor.

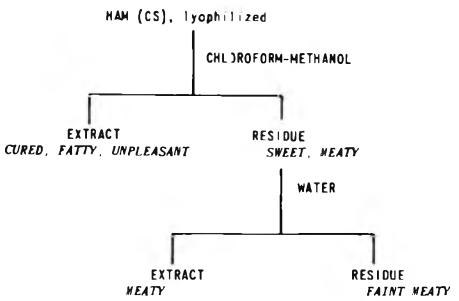

Fig. 2—Extraction of ham flavor.

Table 3—Ratios of free amino acids and nitrogenous compounds in hams.

	UR/CR	UR/UC	CR/CC	UC/US	CC/CS	UC/CC	US/CC
Taurine	1.22	0.80	0.56	0.98	1.14	0.85	0.99
Urea	1.62	1.17	0.68	0.50	0.42	0.95	0.80
Aspartic Acid	0.24	0.64	3.29	0.90	1.04	1.22	1.40
Threonine	1.46	1.06	0.73	0.89	0.84	1.00	0.93
Serine	1.21	0.81	0.81	1.01	0.76	1.21	0.91
Glutamine	1.82	0.84	0.40	1.20	2.36	0.86	1.02
Proline	1.23	0.82	0.80	0.95	0.75	1.20	0.94
Glutamic Acid	1.65	0.62	0.48	0.87	0.83	1.29	1.23
Glycine	1.69	0.80	0.69	1.12	0.81	1.46	1.04
Alanine	1.64	0.87	0.65	1.11	0.82	1.23	0.92
Valine	0.78	0.54	0.84	0.99	1.13	1.21	1.38
Methionine	1.51	0.89	0.57	1.01	1.12	0.97	1.08
Isoleucine	0.99	0.67	0.65	0.91	1.09	0.96	1.15
Leucine	1.05	0.64	0.69	0.98	1.02	1.12	1.16
Tyrosine	1.05	0.79	0.76	0.93	1.08	1.01	1.16
Phenylalanine	1.24	0.75	0.63	0.89	1.06	1.04	1.24
Ornithine	2.19	1.36	0.88	0.46	0.08	1.42	0.25
Ethanolamine	2.59	0.85	0.70	1.33	0.08	2.13	0.13
Ammonia	1.27	0.79	0.43	0.91	1.43	0.69	1.09
Lysine	0.97	0.63	0.66	1.02	0.99	1.02	0.99
Histidine	1.31	0.62	0.48	0.83	0.76	1.01	0.92
Anserine	1.57	0.73	0.53	0.87	1.47	1.14	1.93
Carnosine	1.32	0.88	0.67	1.00	1.19	1.02	1.21
Total	1.35	0.84	0.63	0.96	0.97	1.01	1.03

most of the amino acids in CR samples as compared to UR samples, particularly urea, threonine, glutamine, glutamic acid, glycine, alanine, methionine, ornithine, ethanolamine and anserine. However, aspartic acid and valine increased.

Cooking generally resulted in an increase of free amino acids in hams. The total free amino acid content increased 19% for uncured and 60% for cured hams compared to the corresponding raw hams. Carnosine, the major amino constituent derived from cured as well as uncured ham, accounted for 26 and 29% of total increases on cooking in uncured and cured hams, respectively. Taurine, glutamic acid, glycine, iso-leucine, leucine, tyrosine, phenylalanine, ammonia, lysine, histidine and anserine increased both in cured and uncured hams. Valine and aspartic acid also increased in uncured hams but ornithine decreased. More extensive changes due to cooking were noted for amino acids in cured hams. Thus, the contents of urea, threonine, glutamine, proline, alanine, methionine and ethanolamine were higher in CC samples than in CR samples. Aspartic acid was, however, decreased in the cured samples on cooking. The total levels of amino acids in cured and uncured cooked hams were comparable (UC total/CC total = 1.01), although there were variations in individual amino acids. Amino acids, important constituents of the water-soluble fraction of meat, probably contribute toward formation of meat flavor on heating by interaction with carbohydrates and degradation into volatile compounds and browning products. Macy et al. (1964) reported that amino acids in pork diffusate decreased 20% on heating at 100°C for 1 hr. Larger decreases were found for beef and lamb diffusates. However, according to Usborne et al. (1968), cooking of pork l. dorsi muscles to an internal temperature of 77°C resulted in a general increase of free amino acids, presumably due to degradation of proteins. In a similar study with beef, Macy (1966) reported that amino acids increased on cooking except threonine, serine, glutamic acid, histidine and arginine, which decreased. On the other hand, Zoltowska (1967) reported that in pork l. dorsi samples, canned and cooked at 95, 103 and 121°C, the free amino acid content decreased as compared to raw samples, particularly under high temperature.

In our study the over-all increase in amino acids observed on cooking may be due to protein hydrolysis caused by heating. Since the hams were subjected to rather low internal cooking temperature (66°C), destruction of amino acids originally present in raw ham through reactions with carbonyl compounds may not have been significant.

Table 4—Some volatile components from ham fractions.

R_t (min)	Uncured ham	Cured ham
Diffusates		
12.0		Small, buttery, sweet
15.3	Small, acidic	Large, acidic
19.0	Small, green	Large, green, haylike
21.0	Large, greasy, nutty	Small, greasy
Lipid extracts		
12.9	Medium, earthy, plantlike	Medium, waxy, aromatic
13.4	Large[1], moldy	
14.5	Large[2], greasy, rotten	Large[2], greasy, rotten
18.6	Small, haylike, cheesy	Large, oily, sweet
20.5	Small, stale fat	Large, pleasant, pastry

[1]Raw samples.
[2]Cooked and cooked-smoked samples.

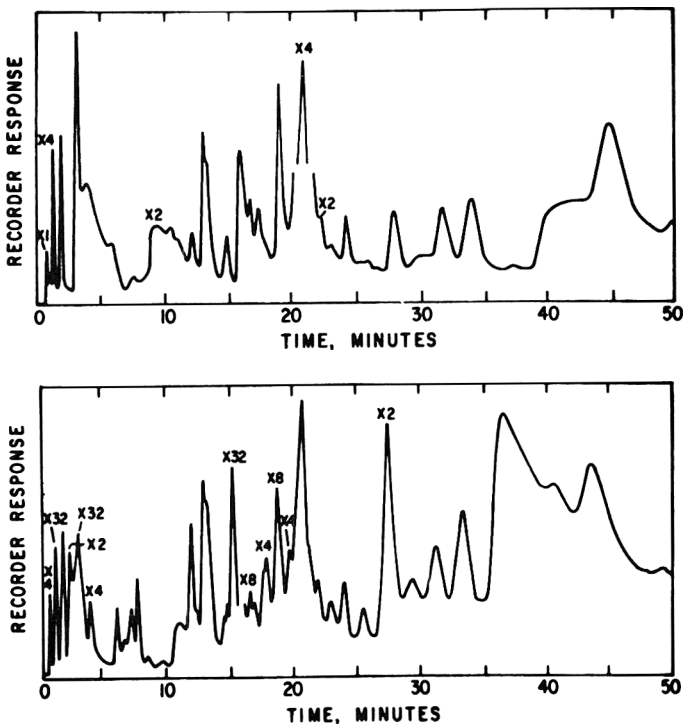

Fig. 3—Gas chromatogram of volatiles from (top) uncured cooked ham diffusate, and (bottom) cured cooked ham diffusate.

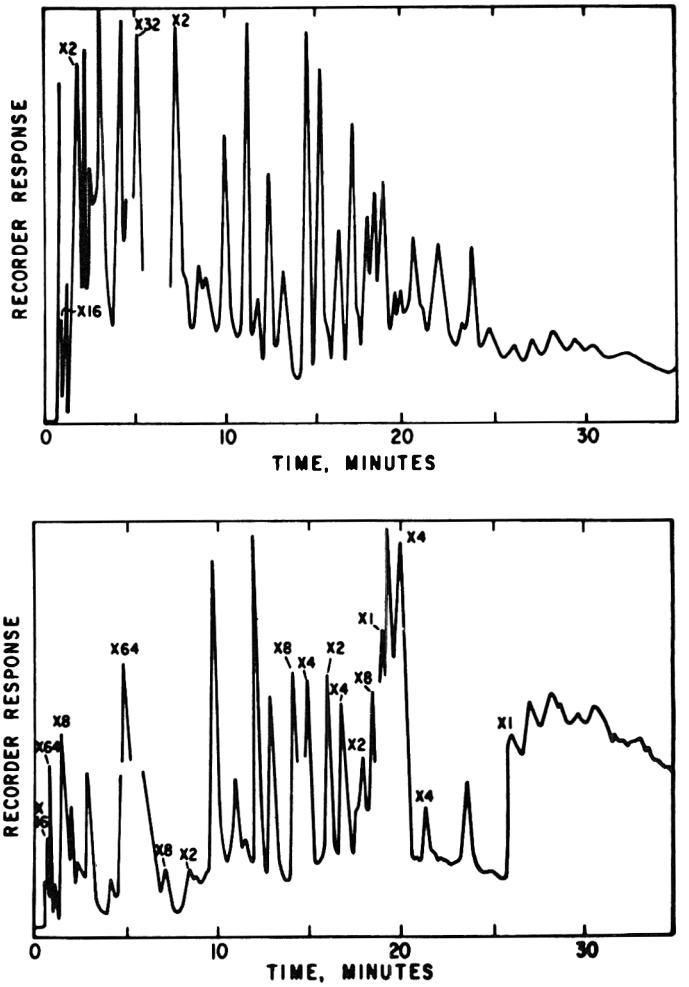

Fig. 4—Gas chromatogram of volatiles from (top) uncured cooked ham lipid extract, and (bottom) cured cooked ham lipid extract.

The effect of smoking on the free amino acids of ham was not so extensive as that of curing or cooking. The content of total free amino acids in smoked hams was 5% higher in uncured and 3% higher in cured hams compared to the corresponding cooked hams. Total levels of amino acids in US and CS hams were quite similar. However, meaningful differences were found for a number of individual amino acids. In uncured samples there was an increase on smoking in urea and orthinine and a decrease in glutamine and ethanolamine. In the cured samples urea, serine, proline, ornithine, ethanolamine and histidine were increased; glutamine, ammonia and anserine were decreased.

Free amino acids in hams have not been studied extensively. Grau et al. (1958) found, qualitatively, higher concentrations of free amino acids in cured, smoked and smoked-cooked meat as compared to fresh meat. Lillard et al. (1969), using a gas chromatographic method, found a much higher level of a number of amino acids in country-cured hams from various commercial sources than in fresh hams. Most of the studies reported probably include aging effects which would lead to protein degradation and a resulting increase in free amino acids in cured hams. Consequently, at present it is difficult to draw any comparison between present and past results concerning the effect of curing on the free amino acids in hams.

The water-insoluble residues from the extraction of cured samples still yielded cured ham aromas on heating. The possibility exists, therefore, that cured odor components or precursors are not water soluble and may be associated with the lipid phase.

Ham samples were extracted with a mixture of chloroform-methanol to yield the total lipid fractions. Cured samples could be readily distinguished from uncured samples when the lipid fractions were heated and their odors observed. Smoke odor could also be detected in the lipid fraction of cured smoked ham (CS). Although the aqueous extracts of cured hams yielded cured, hammy odors, these odors were more intense in the chloroform-methanol-soluble fractions, indicating that cured odor components or precursors were essentially in the lipid phase.

To investigate this further, a sample of cured smoked ham (CS) was divided into two parts: One was extracted first with water and then with chloroform-methanol (Fig. 1); the other was extracted first with chloroform-methanol and then with water (Fig. 2). The various fractions thus obtained were submitted to the panel for odor evaluation. Figure 1 indicates that some of the cured flavor can be extracted with water, but the main portion of cured flavor is soluble in chloroform-methanol. When the ham was first extracted with chloroform-methanol, a cured-ham, fatty aroma was obtained on heating. A meaty aroma was obtained from the water extract of the insoluble residue (Fig. 2). The characteristic cure, as well as smoke, odor was not detected. These results indicate that precursors of the basic meaty aroma are water soluble, whereas precursors of the cured-ham flavor are associated with the lipid phase.

The individual components of ham aroma were examined to determine whether significant differences in the patterns of volatiles existed between cured and uncured ham samples and, if possible, to isolate a component having a cured odor. Therefore, volatile compounds derived from ham diffusates and

ham lipid extracts were separated by gas chromatography. As the components emerged from the chromatographic column their odors were observed.

Representative gas chromatograms of volatiles from ham diffusates and lipid extracts are shown in Figures 3 and 4, respectively. In general, the differences between the 6 types of samples were quantitative rather than qualitative. Complex chromatograms were obtained, with 35–40 peaks for the diffusates and 40–50 peaks for the lipid fractions. It was noted that the material in a diffusate was a good representation of meat aroma, since chromatograms of lyophilized diffusate and lyophilized whole ham were quite similar. Very volatile compounds from ham diffusates possessed unpleasant sulfury or amine-like odors and occurred in both cured and uncured samples. Compounds of intermediate volatility, eluting between 10 and 25 min, had the most interesting odors, in many instances quite intense even though they were present in rather small amounts. A number of components were described as having a green, plant-like odor. The patterns of less-volatile compounds (eluted after 25 min) were similar for cured and uncured samples and the odors in this region were described as burnt or charred.

Many of the volatile components derived from the lipid fractions were described as waxy, fatty, fried, green and aromatic. As in the case of the diffusates, no single component had a cured, meaty or smoky odor. In general, more agreeable aromas were found in the volatiles of cured ham lipid fractions than in uncured samples, although the patterns of peaks on the chromatograms did not differ to a large extent. Cooked samples exhibited a higher level of volatiles than did raw samples.

Some of the outstanding features in the pattern of volatiles derived from ham diffusates and ham lipids are summarized in Table 4. We feel that compounds of intermediate volatility are important contributors toward meat flavor as well as better indicators of the differences between cured and uncured hams. Work is now in progress in our laboratory to isolate and identify the constituents of ham aroma and to further elucidate the effects of curing, cooking and smoking on the flavor of ham.

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GROWTH OF SALMONELLA AT LOW pH

SUMMARY—The growth of salmonellae was observed to occur at pH values as low as 4.05 ± 0.05. The growth-limiting pH was dependent on several factors, most important the acid molecule itself. Additionally, the effect of temperature, relative oxygen supply and level of inoculum was studied. The salmonellae could not be "trained" to grow at a lower pH by sequential transfer at near-optimum pH values.

INTRODUCTION

THE OPTIMUM pH for the growth of salmonellae is generally accepted to fall between pH 6.5 and 7.5. Although the organisms rapidly proliferate in this range, they are also able to grow readily in more acidic environments. The minimum pH at which salmonellae are able to initiate and sustain growth is not well defined and will vary depending on the serotype, the temperature of incubation and the nature and composition of the growth medium.

Prost et al. (1967) reported that pH values below 4.5 had a killing effect. Since the acid used to adjust the pH was not specified, the implicit assumption is that this was inconsequential. Dernby (1921) stated that minimum pH values permitting growth of *Salmonella typhosa*, *S. paratyphi* A and *S. paratyphi* B were pH 6.2, 4.5 and 4.5, respectively. More recently, Stokes et al. (1957) cited the inability of salmonellae to grow below pH 5.0. In contrast to these values, Levine et al. (1940) reported that *S. aertrycke* grew in broth acidified to pH 4.5 with lactic acid and pH 4.0 when hydrochloric acid was used.

Table 1—Minimum pH at which Salmonellae would initiate growth under optimum laboratory conditions.[1]

Acid	pH
Hydrochloric	4.05
Citric	4.05
Tartaric	4.10
Gluconic	4.20
Fumaric	4.30
Malic	4.30
Lactic	4.40
Succinic	4.60
Glutaric	4.70
Adipic	5.10
Pimelic	5.10
Acetic	5.40
Propionic	5.50

[1]Tryptone–yeast extract–glucose broth was inoculated with 10^4 cells per milliliter of *Salmonella anatum, S. tennessee* or *S. senftenberg.*

Ayres (1966) demonstrated the lethal properties of low pH toward Salmonella when 10^6-10^7 cells of 6 strains were quickly destroyed in lemon juice (pH 2.3) and lime juice (pH 2.5). The salmonellae were more tolerant of tomato juice (pH 4.3–4.4) and survived up to 30 days, depending on the temperature of storage. More recently, Goepfert et al. (1969) studied the sensitivity of salmonellae to the volatile fatty acid series.

Several different acids are more or less commonly used as acidulants in the food industry. The behavior of salmonellae in the presence of each of these has not been studied previously. The purpose of this study was to determine the minimum pH value as determined by these acidulants that would permit the initiation of growth by *Salmonella* in laboratory media. Further, the influence of temperature of incubation, relative oxygen supply and level of inoculum was studied, as this interrelationship affected the growth response.

EXPERIMENTAL

Test organisms

Single strains of *Salmonella anatum, S. senftenberg* and *S. tennessee* were obtained from the Food Research Institute collection. Stock cultures were maintained on nutrient agar slants at room temperature. Transfer of the stock cultures was made at approximately 6-month intervals. Working cultures were transferred daily in trypticase soy broth and incubated at 37°C.

Enumeration

Viable cell counts were made by surface plating in duplicate 0.1-ml aliquots of the appropriate dilutions on nutrient agar plates. The plates were incubated at 37°C for 24 hr before counting colonies. All dilutions were made in 0.1% peptone water.

pH determinations

The medium used to determine the minimum pH value that would permit growth consisted of 1% tryptone, 0.5% yeast extract and 0.1% glucose. This basal medium was autoclaved at 121°C for 15 min before addition of predetermined quantities of sterile solutions of the acid in question. The pH of a small portion of the broth was measured with a Radiometer phm 26 pH meter (Medtron Inc., Chicago, Ill.) before inoculation of the remainder of the medium.

Inoculum

A 24-hr trypticase soy broth culture served as the source of the inoculum. This culture was diluted in 0.1% peptone water to achieve the desired initial level of inoculum. A 0.1-ml volume of the appropriate dilution was added to 10 ml of the acidified broth. For the temperature study the inoculated tubes were tempered to the test temperature in a water bath before incubation in a desktop incubator. When agitation was required, this was by a reciprocal shaker (New Brunswick Corp., New Brunswick, N.J.) equipped to handle culture tubes.

RESULTS

VARIOUS FOOD acidulants were tested to learn the minimum pH at which the 3 serotypes of *Salmonella* would initiate growth. For this experiment, the level of inoculum was $1-3 \times 10^4$ cells/milliliter in the acidified broth. Growth was taken to mean an increase in cell number of at least 1 log over the initial load of organisms. Table 1 summarizes the minimum pH values at which 1 or more of the serotypes could initiate growth. Where

Table 2—Interaction of oxygen, pH and inoculum level on growth of Salmonella senftenberg at 30°C.

pH	Inoculum (cells/ml)	Aerated	Static
4.1	10^2	–	–
	10^4	+	–
	10^6	+	–
4.2	10^2	+	–
	10^4	+	+
	10^6	+	+
4.3	10^2	+	+
	10^4	+	+
	10^6	+	+

there was a difference between serotypes, the magnitude of this difference never exceeded 0.1 pH unit. It can be seen that there is a considerable difference in the "permissivity" of the various acids. The acids can be separated into 3 groups on the basis of the tolerance of the salmonellae to these molecules. The most permissive group includes tartaric, hydrochloric and citric acid in which growth was initiated at values as low as pH 4.05 ± 0.05. The intermediate group included fumaric, gluconic, glutaric, lactic, malic and succinic acids. Growth was initiated within the range pH 4.20–4.70, depending on the individual acid. The most restrictive class was comprised of adipic and pimelic acids and the short-chain volatile fatty acids, acetic and propionic. The minimum pH at which salmonellae would grow in these acids was pH 5.10 for the dicarboxylic acids and pH 5.40 and 5.50 for the latter 2 acids, respectively.

After establishing the minimum pH at which salmonellae would grow, a single acid was selected and the effects of other parameters measured as they influenced the minimum pH value. A citric acid–sodium citrate buffer (0.1 M) system was used to poise the pH at the desired levels between pH 3.9 and 5.0. The level of inoculum was then varied to obtain low, medium and high initial numbers (10^2, 10^4 and 10^6 cells/milliliter) in the test solutions. Additionally, the effect of aeration was imposed on the interaction of level of inoculum and pH. Results of a typical trial are summarized in Table 2. *S. senftenberg* grew at pH 4.1, provided the culture was aerated and a high initial inoculum was used. The effect of aeration was further evident at pH 4.2, where growth could be initiated at low levels only when the culture was shaken. At or above pH 4.3 there was no difference in the growth response whether low or high initial inocula were used or whether incubation was static or with agitation. The same growth responses were obtained for *S. anatum* and *S. tennessee* above pH 4.2. Below this value, very slight and possibly insignificant differences between the 3 strains were noted.

The initial cell number was varied between 10^2 and 10^6 per milliliter for the study of the influence of incubation temperature on the minimum pH at which the salmonellae would grow. Static incubation was used throughout this phase of the study. The temperatures were 16, 25, 32, 37 and 43°C. The interrelationship of level of inoculum and incubation temperature is shown (Table 3). It can be seen that these salmonellae were most tolerant of low pH at 25–32°C, and that the minimum pH at which growth was initiated increased at temperatures below and above this range. At pH values that would not allow multiplication of the salmonellae, death of the cells occurred. The rate of death was dependent on the incubation temperature. The rate was most rapid at the higher temperature, as would be expected. The shape of the growth curves obtained at the pH value, where an increase in cell number occurred only when 10^4 or 10^6 organisms were the initial inoculum, showed a dramatic decrease (2–3 logs) in viable cells, followed by a recovery period and subsequent proliferation. These data indicated that perhaps a small fraction of the cell population was more acid tolerant than the remainder. Inoculations to fresh media at borderline pH value from the progeny of this tolerant population resulted in identical growth curves, thereby negating the development of a tolerant population. Additionally, all attempts to "train" a serotype to grow at a lower pH by numerous sequential subcultures at low pH failed.

Table 3–Interaction of temperature, pH and initial cell numbers on growth of Salmonella anatum in laboratory media adjusted with citric acid.

	16°C	25°C	32°C	37°C	43°C
Level of inoculum	L M H[1]	L M H	L M H	L M H	L M H
pH 3.9	– – –	– – –	– – –	– – –	– – –
4.0	– – –	– + +	+ + +	– – –	– – –
4.1	– – –	+ + +	+ + +	– – –	– – –
4.2	– – –	+ + +	+ + +	– – –	– – –
4.3	– +[2] +	+ + +	NT	+ + +	– – –
4.4	+ + +	NT[3]	NT	+ + +	– – –
4.5	+ + +	NT	NT	+ + +	– – +
4.6	NT	NT	NT	NT	+ + +

[1] L = 10^2 cells/ml; M = 10^4 cells/ml; H = 10^6 cells/ml.
[2] + = An increase of at least 1 log over the initial cell number.
[3] NT = Not tested.

DISCUSSION

SEVERAL OF the data just presented merit further discussion. First, it should be emphasized that although growth was observed at pH 4.05 ± 0.05, this occurred under ideal conditions for salmonellae. A nutritionally favorable medium was used, free from natural inhibitors and competing microflora that would be present in a food material. Moreover, the temperature of incubation was suitable and the water activity (a_w) quite high. It is impossible to extrapolate the behavior that occurred in laboratory media to a food product or environmental situation that might exist in a food processing establishment. The limiting pH values obtained can be helpful if it is remembered that they were derived under ideal (for the salmonellae) laboratory conditions and, as such, would represent the absolute minima below which no growth of salmonellae will occur. Therefore, a processor could feel safe in the knowledge that salmonellae would not grow in a given product if it were more acid than the limiting pH values presented above. It is quite likely that in any given food material the combination of parameters (e.g., O/R potential, water activity, temperature, etc.) would act synergistically to prevent the growth of salmonellae at pH values considerably higher than those just discussed.

It should be mentioned that the study of the growth behavior of salmonellae in the various acids tested was restricted to measuring the initiation of growth rather than the continuation of growth. The broth media were adjusted to the test pH but not buffered at that value. Consequently, active growth by the inoculum would result in alteration of the pH (usually an increase) to a considerably different and ever-varying value. For this reason, it is impossible to draw any conclusions about the growth rate or final cell number at any given pH. It has been assumed that by using stationary phase cells as the inoculum a greater challenge, i.e., initiation of growth, has been exerted on the cells than if actively metabolizing organisms were used. It is possible that had the latter been used, slightly lower limiting pH values might have been obtained.

The varying degree of "permissivity" exhibited by the acids tested is of interest. These data should in part help to explain the variance in the values of the growth-limiting pH described in the literature. The data should emphasize the difficulty that can be encountered by presuming that information obtained under one set of conditions would apply to all other circumstances. Obviously, if the pH value obtained for acetic acid was taken as the limiting value considerable error would be introduced.

It was expected from previous studies (Goepfert et al., 1969) that the volatile

fatty acids would be among the most restrictive. However, the relatively high limiting pH for acetic and propionic acids is in variance with the value of pH 4.9 for acetic acid reported by Levine et al. (1940). The remaining 2 acids in the "restrictive" group were not expected to show an anti-*Salmonella* effect; perhaps more investigation of the saturated dicarboxylic acids is warranted.

The behavior of salmonellae in the presence of high acidity is seen to be a function not only of the type of acid but also of the incubation temperature, level of inoculum and relative oxygen tension. The effect of aeration and initial cell number was relatively minor, the magnitude of the differences in limiting value introduced by varying these parameters being less than 0.1 pH unit. The effect of temperature was more pronounced. These serotypes evidenced an optimum range of temperature with regard to tolerance of acidity. This finding was not unexpected, in view of reports by others (Mossel, 1963) that organisms are best able to cope with adverse environmental parameters when other parameters are optimum or very nearly so.

Finally, we were unable to "train" any of the serotypes to grow at a lower pH by repeated subculture in an acid environment.

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SUGAR-AMINO ACID INTERACTION IN THE DIFFUSATE OF WATER EXTRACT OF BEEF AND MODEL SYSTEMS

SUMMARY—Diffusates of water extract of beef and model systems containing amino acids or sugars, or both, in approximately the same concentrations as in beef diffusate were heated at 125°C for up to 60 min. Quantitative analyses of amino acids and sugars were performed. A brothy odor developed on boiling the diffusate (30 min of heating), but there were minor changes in amino acid concentrations except for arginine. After pyrolysis (45 min of heating), roast aroma and brown color developed and there was 40–60% loss of most amino acids. Glucose and fructose concentrations were only slightly affected during boiling, but ribose decreased about 25%. On pyrolysis, sugars completely disappeared. Amino acids heated alone decreased in concentration about 25% and sugars heated alone decreased approximately 20 to 55% on pyrolysis. A sugar-amino acid mixture simulating beef diffusate suffered greater losses in concentrations of amino acids on pyrolysis than did the diffusate. No pattern of changes in amino acid or sugar concentrations was detected to account for the aroma of roast meat on pyrolysis. In beef diffusate, sugars appear to be limiting and a portion of the amino acids remains after pyrolysis. When excess sugar is added, free amino acids disappear completely and the ratio of the amount of glucose disappearing to amino N disappearing levels off at about 4:1.

INTRODUCTION

THE INTERACTION of amino acids and carbohydrates, or α-dicarbonyl compounds, results in the formation of brown pigments. The chemistry of this browning reaction has been covered in a number of reviews (Hodge, 1967; Reynolds, 1963, 1965). Concomitantly with pigments, other products that may be odorous are formed. Since amino acids and requisite reducing compounds are present or can be formed readily in foods, and since browning occurs as a result of heating, the aromas of various food products have been linked to products of the browning reaction (Pinto et al., 1966; Horvat et al., 1962; Moores et al., 1964; Linko et al., 1963). In meat products, involvement of the Maillard, or browning, reaction with flavor has been mentioned by Hornstein et al. (1960), Wood (1961) and Macy et al. (1964b). However, for the most part this has been based on indirect evidence, as no specific reaction, or reaction product, has been reported to yield a meat aroma. A number of investigators have reported on aromas derived from heating sugars and amino acids under several conditions of temperature and pH. The descriptions of these aromas by Herz et al. (1960), Barnes et al. (1947) Kiely et al. (1960) and Rothe et al. (1963) mention cheese, potato and bakery odors, but the only reference to a meaty aroma is from the reaction of cysteine and sugar. El'Ode et al. (1966), on the other hand, describe aromas of several amino acids and sugars heated at 180°C in terms of broth or meat. Wood (1961) did obtain a meaty aroma on heating glucose and a mixture of amino acids in the concentrations in which they were present in a meat extract, but quantitative data were not reported. In recent years the only study on the quantitative changes resulting from heating amino acid and sugar constituents in meat extracts has been that of Macy et al. (1964b). These investigators also reported that after heating for 1 hr in a boiling-water bath a typical brothy aroma and a brown color developed in extracts of beef, pork and lamb.

Our studies on meat flavor have been directed toward the characterization of the strong roast beef or broiling steak aroma that develops on the pyrolysis of water extracts of meat (Wasserman et al., 1965). We have reported that these solutions, which give rise to dark-brown pigments on pyrolysis, contain amino acids and sugars (Zaika et al., 1968). However, there is no information available on quantitative changes occurring among these constituents under pyrolysis conditions. This study reports the effects

of heating on the components of diffusates of beef extracts and model systems containing the constituents of the diffusate, and the involvement of amino acids and sugars in the development of a characteristic roast aroma.

MATERIALS & METHODS

Materials

The preparations used included beef diffusates prepared from water extracts of beef muscle as described previously (Wasserman et al., 1965), model systems of amino acids and sugars in approximately the same concentrations in which they occur in beef diffusate and a mixture of solutions of amino acids and sugars to give a simulated beef diffusate.

Methods

4-ml samples of the various solutions, in 20-ml beakers, were heated for 30, 45 and 60 min in an air oven at 125°C. The volume in the beakers was great enough to ensure that liquid was still present in the sample at 30 min. Data for material analyzed at this time interval represent the composition of the solutions as they exist in the boiling state. At some point between 30 and 45 min the water completely evaporated and the 45-min samples were dry. The solid matter in the beakers was various shades of brown, depending on the contents. Very little change occurred during the next 15 min. The 45- and 60-min samples were considered to be pyrolyzed. Beakers were removed at appropriate times, the contents transferred to volumetric flasks and diluted to 10 ml with deionized distilled water. Since samples taken to dryness contained brown pigments that interfered with the analyses, all samples were treated with 0.5 g of a mixture of celite:charcoal (2:1) to remove color. Different amounts of amino acids and sugars are removed by adsorption of this mixture; therefore, all calculations are based on the concentrations present in similarly treated unheated samples. Investigation also showed that the concentration of component adsorbed was not dependent on its concentration in the solution; thus, changes found in components as a result of heating were not a reflection of the clarification process. The adsorbent material was removed by centrifugation and filtration through a Millipore filter. The colorless, clarified solutions were stored at −18°C until analyzed.

Decolorized solutions were analyzed quantitatively for amino acids by the Piez-Morris 1-column system (1960) using a Phoenix Analyzer. Total amino N was determined by the ninhydrin method of Cocking et al. (1954). The specific glucose oxidase technique was used according to the instructions of the Worthington Biochemical Corporation for analysis of glucose, and fructose analysis was carried out by the procedure of Bacon et al. (1948). The ribose determination using orcinol according to the method of Uehara et al. (1965) is not specific and must be corrected for concentrations of glucose and fructose if they are present in the solution.

The aromas of the solutions were characterized by several members of the laboratory familiar with the aroma of pyrolyzed beef diffusate. Differences in aromas were so obvious formal panel evaluation was not carried out.

RESULTS

THE AROMA of beef diffusate after 30 min of heating was typically brothy; the 45- and 60-min samples had a characteristic burnt-meat aroma–sharp and acrid.

Table 1—Effect of heating at 125°C on the concentrations of amino acids and sugars in a diffusate of a water extract of beef.

	Heating time–min			
	0	30	45	60
	(μM/ml)			
Cysteic Acid	0.023	0.036	0.034	0.034
Taurine	3.868	3.602	1.534	1.399
Urea	6.818	5.217	3.022	3.335
Serine	0.554	0.551	0.345	0.322
Glutamic Acid	0.464	0.472	0.212	0.176
Proline	0.209	0.213	0.168	0.199
Glycine	1.078	1.074	0.417	0.363
Alanine	3.993	3.806	2.703	2.571
Valine	0.392	0.386	0.271	0.243
Methionine	0.098	0.098	0.042	0.041
Iso-leucine	0.257	0.204	0.145	0.137
Leucine	0.378	0.365	0.228	0.207
Ornithine	0.213	0.166	---	---
NH_3	3.318	3.717	1.542	1.088
Lysine	0.171	0.159	0.083	0.054
Anserine	0.972	0.783	0.823	0.651
Carnosine	4.958	4.614	3.117	2.823
Asparagin + threonine	0.979	0.743	0.231	0.220
Arginine	0.243	0.097	0.097	0.092
Amino N	6.098	5.145	2.630	2.416
Sugars				
Glucose	5.503	5.584	0.044	0.009
Fructose	3.136	2.764	0.032	0.023
Ribose	1.066	0.833	0.013	0.010

The effects of heat treatment on the amino acid and sugar contents of beef diffusate are shown in Table 1. In the boiling state (the first 30 min of heating) only minor changes were observed except for the 60% decrease in arginine and the 57% increase in cysteic acid, probably as a result of cysteine oxidation. Large changes occurred on pyrolysis, as shown by the 45-min sample. There was a loss of 40–60% of most of the amino acids with a 77% loss of the asparagin-threonine complex being the greatest decline. Total amino N showed an average loss of 15% on boiling and a 60% decline after pyrolysis.

Glucose, fructose and ribose are present in beef diffusates. The phosphorylated forms of these sugars have also been identified (Zaika et al., 1968) but are present in such low concentrations that it was decided to investigate the changes in the parent sugars only. After 30 min of boiling the greatest loss was in ribose; glucose was apparently unaffected. This agrees with previous reports that of these 3 sugars the most reactive in the browning reaction is ribose, and glucose the least (Pomeranz et al., 1962; Casey et al., 1965). On pyrolysis, all of the sugars disappeared very rapidly as shown by the 45-min samples. There was very little further change in either amino acids or sugars on heating the beef diffusate the extra 15 min from 45 to 60 min.

There is a tendency to consider only the possible interaction between amino acids and sugars as the source of the brown pigments and odorous compounds that form on pyrolysis. However, Merritt et al. (1967) have shown that pyrolysis of amino acids alone resulted in the formation of odorous components, and many investigators have reported on the complex nature of the compounds formed on heating sugars (Hodge, 1967). It is possible that these reactions may be occurring simultaneously with, but independently of, amino acid-sugar interaction in diffusates.

An amino acid mixture containing these compounds in approximately the same concentrations found in beef diffusate was heated as just described. The aroma at 30 min was reminiscent of potato, but after pyrolysis there was no odor and no brown pigment formation. Amino acid changes are shown in Table 2. In general, there are only a few changes in amino acid concentrations in the 30-min sample; cysteic acid, since no cysteine was added to the system, declined 30%, glutamic acid declined about 25% and the methionine concentration was halved. On pyrolysis (45- and 60-min samples) some

Table 2—Effect of heating at 125°C on the concentrations of amino acids in a model system.

	Heating time—min			
	0	30	45	60
	(μM/ml)			
Cysteic Acid	0.087	0.062	0.061	0.063
Taurine	3.380	3.380	2.798	2.778
Urea	5.180	4.893	3.578	3.210
Serine	0.380	0.390	0.353	0.325
Glutamic Acid	0.318	0.243	0.200	0.205
Proline	0.803	0.748	0.720	0.728
Glycine	1.960	1.985	1.790	1.830
Alanine	3.140	3.193	2.920	2.983
Valine	0.318	0.310	0.285	0.288
Methionine	0.045	0.023	0.024	0.018
Iso-leucine	0.055	0.053	0.048	0.050
Leucine	0.230	0.220	0.150	0.148
Ornithine	0.188	0.243	0.230	0.118
NH_3	2.500	2.353	1.835	1.943
Lysine	0.195	0.260	0.183	0.125
Anserine	0.083	0.275	0.245	0.253
Amino N	5.183	4.002	3.963	3.963

Table 3—Effect of heating at 125°C on the concentrations of sugars in a model system.

	Heating time—min			
	0	30	45	60
	(μM/ml)			
Glucose	3.482	3.328	2.981	2.793
Fructose	2.287	2.220	2.044	1.240
Ribose	0.821	0.652	0.429	0.344

Table 4—Effect of heating at 125°C on concentrations of amino acids and sugars in a model system simulating beef diffusate.

	Heating time—min			
	0	30	45	60
	(μM/ml)			
Cysteic Acid	0.104	0.083	0.033	0.021
Taurine	4.276	1.392	1.235	1.198
Urea	10.249	8.057	1.955	1.182
Serine	0.470	0.456	0.171	0.136
Glutamic Acid	0.310	0.286	0.096	0.041
Proline	0.943	0.909	0.551	0.325
Glycine	2.438	2.237	0.669	0.487
Alanine	3.661	3.674	1.654	1.442
Valine	0.362	0.351	0.429	0.269
Methionine	0.048	0.036	---	---
Iso-leucine	0.065	0.068	0.038	0.031
Leucine	0.261	0.255	0.075	0.086
Ornithine	0.211	0.259	0.099	0.107
NH_3	2.638	2.019	0.546	0.570
Lysine	0.231	0.128	0.182	0.154
Anserine	0.432	0.312	0.176	0.159
Carnosine	4.411	4.402	1.919	1.949
Asparagin + threonine	0.749	0.760	0.262	0.214
Arginine	0.136	0.076	0.061	0.071
Amino N	7.90	6.550	2.250	1.777
Sugars				
Glucose	3.552	3.372	---	---
Fructose	1.846	1.735	0.069	0.069
Ribose	4.379	2.614	0.185	0.019

further losses were noted, but many amino acids were present in 80–90% concentrations of their initial levels. There was, however, a definite decrease in amino acid concentration as a result of pyrolyzing these compounds alone.

Results of heating individual sugar solutions, in approximately the same concentration in which they appear in the diffusate, at 125°C for various periods of time are shown in Table 3. After 30 min of heating the greatest loss, 26%, was in ribose; there was essentially no effect on fructose and glucose. On pyrolysis, larger amounts of sugar disappear, with ribose disappearing in greater quantity than fructose and glucose being least affected. Even after 60 min of heating there was no browning in any of the sugars and the only aroma was a slight caramel odor in glucose solutions. The lack of caramelization in dried samples may be because the temperature was not great enough.

The 2 model systems, amino acids alone and sugars alone, were combined to give a solution approximating the composition of beef diffusate. After 30 min of heating there was no particular aroma, but the pyrolyzed material in the 45- and 60-min samples developed a rich, sweet, grainy aroma. It was not like that of beef diffusate. The changes occurring on heating this model solution are shown in Table 4. Major losses were observed for taurine, lysine and arginine during the boiling phase, but after pyrolysis drastic changes occurred in concentrations of most of the amino acids. Between 80 and 90% of the cysteic acid, taurine, urea, glutamic acid and glycine disappeared; 50–60% of the remaining amino acids also disappeared after 60 min of heating. Lysine and valine, with only 35 and 25% loss, respectively, were present in greatest concentration. The pattern of the disappearance of the sugars and amino N was similar to that observed with the diffusate: Glucose and fructose were relatively unaffected by boiling, whereas approximately 40% of the ribose disappeared. All of the sugars disappeared on pyrolysis. The amino N level at 60 min of heating reflects the greater amino acid loss in the model system, with lower values than those present in beef diffusate after the same heating period.

Although none of the sugars remain in both beef diffusate and its model system following pyrolysis, the loss of amino N was less complete, with 40% left in the diffusate and 22% in the model. It was of interest to determine the effect of increasing the sugar concentration on the aroma produced and on the equilibrium of the system. To simplify the procedure, glucose alone was added to beef diffusate and model solutions in concentrations of approximately 5, 10, 25, and 70 x the amount found in the diffusate. The data in Table 5 report the changes in glucose and amino N after heating at 125°C for 60 min. On the assumption that the breakdown of glucose alone occurred independently of the sugar-amino acid interaction, even in the presence of amino acids, the loss due to glucose (column 2) was subtracted from losses in the diffusate and model systems. The values thus obtained (columns 5 and 6) presumably

Table 5–Effect of glucose concentration on the disappearance of glucose and Amino N on pyrolysis.

(μM/ml)

	Glucose loss					Amino N loss[2]		μM Glucose / μM Amino N	
1	2	3	4	5[3]	6	7	8		
Conc[1]	Gluc. alone	Gluc. + AA	Beef diff.	3-2	4-2	Gluc. + AA	Beef diff.	5/7[4]	6/8
1	---	---	4.07	---	4.07	---	11.73	---	0.35
5 ×	0.80	17.67	24.83	16.80	24.00	13.87	15.67	1.2	1.5
10 ×	11.37	42.33	54.87	30.87	43.50	15.43	20.20	2.0	2.1
25 ×	10.49	85.77	105.00	75.33	94.57	16.17	24.47	4.6	4.0
70 ×	146.87	---	238.63	---	91.77	---	22.83	---	4.0

[1]Concentration based on glucose content of beef diffusate: 4.07 = μM/ml.

[2]Beef diffusate contained 22.23 μM/ml Amino N and the model system contained 16.47 μM/ml Amino N. The Amino N was calculated as leucine by the ninhydrin method.

[3]Columns 5 and 6 were obtained by subtracting column 2 (glucose alone) from columns 3 and 4.

[4]These values were obtained by dividing column 5 by column 7 and column 6 by column 8.

represent glucose disappearance due to reaction with amino acids. With increasing concentration of glucose the amount of sugar disappearing increased, reaching a plateau at some concentration between 10 and 25 × the initial concentration. The loss of amino N also increased with additional glucose, reaching 100% loss at some level between 10 and 25 × the initial sugar concentration. The amount of glucose apparently reacting with a mole of amino N was calculated (columns 9 and 10, Table 5). In the basic beef diffusate 0.35 mole of glucose reacted with 1 mole of amino N. Increasing glucose concentration appeared to result in an increase in the ratio, leveling off at a final value of about 4 moles of sugar reacting with each mole of amino N.

DISCUSSION & CONCLUSIONS

BOILING beef diffusate for 30 min produces changes in components that yield a brothy aroma. However, the only significant modifications in the amino acid and sugar contents are losses in arginine and ribose. (Complete arginine loss was reported by Thaler et al. (1963) in roasting coffee.) It is possible that, if indeed amino acids and sugars are involved in aroma formation, the smaller losses in iso-leucine, ornithine, anserine and asparagin + threonine might be important, because at this time the composition of the aroma is unknown and trace quantities of a high flavor-impact component could be present.

Large changes occur on pyrolysis of beef diffusate with the production of a variety of aromas progressing through a characteristic roast beef aroma to an undesirable acrid, burnt odor. All of the amino acids enter into the reactions, with varying degrees of losses, although the over-all participation results in a 60% loss of amino N. From the data it is difficult to determine whether there is any pattern to the loss in amino acids. None of the amino acids appears to be specifically related to the aroma produced.

The loss of amino acids in beef diffusate reported by Macy et al. (1964b) compares favorably, percentage-wise, with the losses observed in these studies for the 10 amino acids directly comparable. However, it should be noted that while their preparation, when heated in a boiling-water bath, developed a brothy odor and browned extensively, it is not clear whether their solution had evaporated to dryness or still contained water after the 60-min heating period. On the basis of amino acid loss it appears from both this study and that of Macy et al. that the changes are related to a condition in which there is little or no water and brown pigments eventually form. The main difference between the data reported here and those of Macy et al. is in the sugars; all of the sugars in this study were completely utilized, whereas a substantial concentration of glucose, in particular, remained in their preparation.

Amino acids alone break down to some extent on pyrolysis. Merritt et al. (1967) have shown that some of the breakdown products were similar to those obtained from the Strecker degradation. More important, they demonstrated with dipeptides that the components produced were dependent on the order in which the amino acids were linked. Thus, the use of model systems of individual amino acids may not give the desired information with respect to heating meat or meat extracts. Although no particular aroma was noted in pyrolyzed preparations, the contribution of this reaction must be kept in mind in evaluating the over-all browning reaction products.

The presence of carbohydrate accelerated the breakdown of amino acids in both the beef diffusate and the model system. It is of interest to note that the breakdown was more complete in the model system; the diffusate may contain other components that have a protective action toward amino acids, or the form in which amino acids exist in the diffusate may hinder the reaction.

Although sugars did not brown under the conditions of our experiment, even when heated in the dry state, losses occurred. The rate of loss indicates that not only under browning conditions, as reported by others, but in pure solutions as well, the rate of decomposition, in decreasing order, is ribose > fructose > glucose. The significance of this rate of reaction is not immediately apparent except possibly in establishing the time sequence of the development of aromas. The interaction between sugars and amino acids is very evident in the formation of brown pigments and the increased reduction of sugar concentration in the presence of amino acids.

Glucose caramelization occurs even in the presence of amino acids. This can be deduced from the sweet, caramel aromas developed in the solutions containing large excesses of sugar. If it is assumed that caramelization, or sugar-sugar, reaction occurs simultaneously with sugar-amino acid reaction, correction for the former gives the approximate amount of sugar destroyed by reaction with amino acids. In beef diffusate about 0.35 mole of sugar reacts with a mole of amino N. The data of Macy et al., recalculated, indicate the glucose/amino N ratio is approximately 0.15, but not all of their

glucose was utilized in the reaction. At the level where molar concentrations of amino acid and glucose were approximately equal, the ratio was a little greater than 1.0. In solutions containing an excess of glucose the ratio of glucose/amino N disappearance was greater than 1.0, finally leveling off at a ratio of 4 moles of glucose used per mole of amino N. Reynolds (1965) reviewed the presence of diketose amines and di-D-glucosylamine in heated mixtures of sugars and amines, where 2 sugar molecules effectively fill the available active sites on the amino N. The fact that 4 moles of glucose will ultimately react with a mole of amino N suggests that the sugar moiety of a possible diglucosylamine is broken down and replaced. Reynolds (1965) also reported that in model systems of glucose and glycine, where the initial molar ratio was 8:1, the ratio of glucose lost to free amino groups lost reached 6:1. However, it is of interest to note that Wood (1961) reported the loss of 6 moles of amino acid per mole of sugar lost in a model system.

There is no doubt that the interaction of amino acids and sugars, or other reactive components, produces browning in diffusates of meat extracts, but there is still no identification of the components of the meat aroma formed or the mechanism of the reactions leading to their formation.

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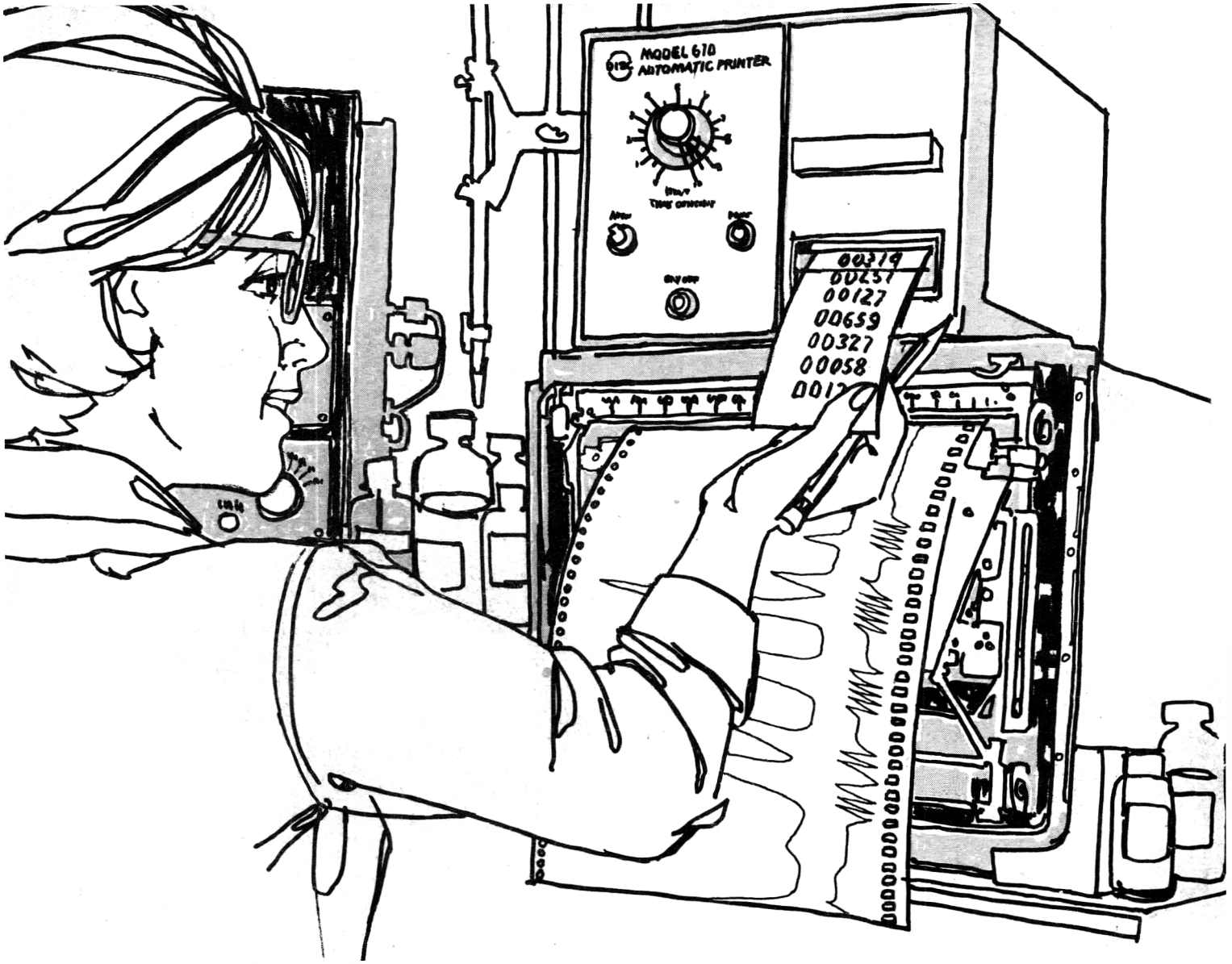

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