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IFT
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JOURNAL of FOOD SCIENCE

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ABSTRACTS:
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MEMBRANE STRUCTURE AND CELLULAR DEATH IN BIOLOGICAL TISSUE. N. F. HAARD. *J. Food Sci.* 37, 504–512 (1972)–The "state of the art" of membrane structure is reviewed and discussed with emphasis on some new lines of experimentation on membrane proteins and lipids. Evidence is presented which suggests a relation between the fine structure of mitochondrial inner membranes and the process of oxidative phosphorylation. Loss in membrane functional capacity resulting from structural perturbation is discussed in the light of biochemical events in fresh foods such as fruits, vegetables and meats.

STRESS IN THE POSTHARVEST CELL: THE RESPONSE OF MITOCHONDRIA AND RIBOSOMES. R. J. ROMANI. *J. Food Sci.* 37, 513–517 (1972)–The 'life' of a fresh fruit or vegetable undergoing the stresses of storage and marketing is closely linked to the fate of its constituent cells. In turn, cells are dependent upon mitochondria for energy and on ribosomes as sites for protein synthesis. Described in this brief review are a series of experiments illustrating how the mitochondria and ribosomes in postharvest fruit tissues withstand, and in some instances, repair the damage of ionizing radiation. Significantly, the capacity of the cells to respond to stress and repair damage decreases rapidly with ripening thus establishing a connection between mitochondrial and ribosomal functions and the storage or shelf-life potential of fresh food commodities.

POSTHARVEST FRUIT PRESERVATION: PROTEIN SYNTHESIS, RIPENING AND SENESCENCE. D. R. DILLEY. *J. Food Sci.* 37, 518–520 (1972)–Maturation, ripening and senescence are clearly defined developmental stages for most fruits. Postharvest metabolism of fruits is dynamic, culminating in marked physical and chemical transformations which are essential in order to achieve optimum product quality. Existing and newly synthesized enzymes catalyze the biochemical reactions responsible for the changes. Protein synthesis is required for normal fruit ripening and these proteins are enzymes required for the ripening process. Synthesis of these enzymes precedes the marked biophysical changes of ripening and newly synthesized RNA is required. Ethylene, the ripening hormone produced by the fruit, is required to initiate the ripening syndrome and without ethylene synthesis and action, fruits which otherwise are ready to ripen continue to synthesize those proteins for which synthetic capacity already exists.

NATURAL ENZYME INHIBITORS IN PLANT TISSUES. R. PRESSEY. *J. Food Sci.* 37, 521–523 (1972)–Protein inhibitors of enzymes are common in plants. Although the first inhibitors were detected with mammalian enzymes, it has been demonstrated that some plant proteins function as inhibitors of endogenous enzymes in a variety of tissues. Reactivities of the inhibitors with enzymes are usually not species specific and often are not restricted to individual enzymes. This suggests that interaction of many plant proteins with mammalian enzymes is coincidental and these proteins may be involved in regulation of metabolic activity in plants. As additional natural inhibitors of enzymes are discovered in plants, they may be useful in food processing to control changes in color, texture and flavor of fresh products.

ENZYMIC ACTIVITY AND CONTROL AS RELATED TO SUBCELLULAR LOCALIZATION. H. O. HULTIN. *J. Food Sci.* 37, 524–529 (1972)–Enzymes may be controlled in many ways. Discussion in this paper concentrates on those methods related to location in the cell. Examples are given of compartmentation (separation of enzyme and substrate) and reversible association-dissociation of enzyme and subcellular structures. Binding of enzymes to particulate structures may modify their kinetic parameters. These modifications along with reversible association-dissociation of enzyme and particulate structures may control an enzymic sequence in the cell, provide maximal efficiency of function, or direct substrate to the desired metabolic pathway. To properly evaluate the contribution of an enzymic reaction in a food, consideration should be given to the cellular milieu in which the reaction takes place.

CELL DISRUPTION AND ITS CONSEQUENCES IN FOOD PROCESSING. S. SCHWIMMER. *J. Food Sci.* 37, 530–535 1972–The thesis is put forward that much of food processing and technology may be looked upon as the management of food cell disruption and its consequences. Cell disruption can create identity, or can be either beneficial or deleterious. It occurs, or countermeasures must be taken to prevent it, in the food processing chain leading from the freshly harvested plant or slaughtered animal to final consumer utilization. With the exception of physiologically normal cell disruption, ensuing metabolism is characterized by removal of restraints imposed by enzyme control mechanisms. Disintegration of cellular membranes, relocalization of enzymes, as well as decompartmentation lead to the accumulation of unique nonphysiological products, which may or may not be desirable. These generalizations are illustrated largely by examples of investigations of problems in food technology conducted by the author and his colleagues and with work associated with the U.S. Dept. of Agriculture.

THE EFFECT OF SODIUM NITRITE ON THE FLAVOR OF FRANKFURTERS. A.E. WASSERMAN & F. TALLEY. *J. Food Sci.* 37, 536–538 (1972)–The characteristic flavor of frankfurters was produced by incorporating sodium nitrite in the cure. Differences in flavor between frankfurters cured with or without sodium nitrite were readily detectable by the triangle test procedure. The presence of smoke did not interfere greatly with the ability of the judges to distinguish between the treatments. In a scoring test procedure, however, smoke significantly affected the flavor of the frankfurters and little difference was observed as a result of the presence of nitrite.

THE EFFECTS OF SOME POST-SLAUGHTER TREATMENTS ON THE MECHANICAL PROPERTIES OF BOVINE AND OVINE MUSCLE. P.E. BOUTON & P.V. HARRIS. *J. Food Sci.* 37, 539–543 (1972)–The combined effects of stretching (or preventing shortening) and aging on selected ovine and bovine muscles were investigated using compression, shear and tensile tests. No significant change in adhesion between fibers attributable to aging was found for muscles from either species. Fiber tensile strengths decreased significantly with aging. Longissimus dorsi showed greater decreases in shear values with aging than semimembranosus muscles. Stretching muscles by hanging carcass sides pre-rigor from the obturator foramen produced appreciable improvements in tenderness, accompanied by significant decreases in adhesion values, when compared with muscles from conventionally-hung sides. Adhesion values were found to be affected by the fiber contraction state.

POULTRY PRODUCT QUALITY. Carbonyl Composition and Organoleptic Evaluation of Mechanically Deboned Poultry Meat. P.S. DIMICK, J.H. MACNEIL & L.P. GRUNDEN. *J. Food Sci.* 37, 544–546 (1972)–Samples of mechanically deboned meat from broiler necks and backs, whole spent layers and turkey racks were analyzed following refrigerated storage at 3°C for 0, 3, 6 and 12 days. The raw materials from these three sources were either deboned immediately (conventional processing) or held in the processing plant for 5 days at 3–5°C prior to deboning (delayed processing). Raw and cooked meat samples were ana-

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lyzed for carbonyl composition as their 2,4-dinitrophenylhydrazones and quantified spectrophotometrically. Cooked samples also were submitted to an experienced taste panel for organoleptic evaluation. The major monocarbonyls present were alkan-2-ones, alkanals and alk-2-enals. No consistent patterns in the levels of total carbonyls and monocarbonyls were shown to occur during the refrigerated storage of the three deboned meat sources. Cooking, however, increased the concentration of these two classes of compounds about two-fold. The most dramatic change occurred in the level of alk-2-enals in the cooked samples after 12 days, increasing 3- to 10-fold. Elevated levels of alkanals were also noted following the extended storage period. Concurrently the panel members could discriminate between the treatment and control samples of deboned broiler meat following 12 days of storage. Lower flavor responses were observed with whole spent layer and deboned turkey meat following 6 days of refrigerated storage.

OXIDATIVE RANCIDITY IN RAW FISH FILLETS HARVESTED FROM THE GULF OF MEXICO. V.T. MENDENHALL. *J. Food Sci.* 37, 547–550 (1972)–Eight species of fish, harvested from the Gulf of Mexico, were filleted and stored at 1°C. TBA number, total plate count per gram (TPC/g) and sensory panel flavor scores were collected during 8 days storage. Oxidative rancidity as determined by TBA number was of most importance in mullet tissues. Increases in the TBA number concurrent with decreases in flavor scores were evident in mullet tissue before TPC/g exceeded 400,000. Further investigation showed that the rate of oxidation in mullet tissue may be affected by season of harvest and by evisceration and filleting of the carcass. Although icing the fish carcasses prior to filleting reduced TPC/g, the rate of oxidative rancidity was not influenced.

YIELD AND ACCEPTABILITY OF MACHINE SEPARATED MINCED FLESH FROM SOME MARINE FOOD FISH. D.L. CRAWFORD, D.K. LAW, J.K. BABBITT & L.S. MCGILL. *J. Food Sci.* 37, 551–553 (1972)–The yield and acceptability of machine separated minced flesh from orange rockfish *(Sebastodes pinniger)*, yellowtail rockfish *(Sebastodes flavidus)*, English sole *(Parophrys vetulus)*, Dover sole *(Microstomus pacificus)*, Pacific hake *(Merluccius productus)*, ling cod *(Ophiodon elongatus)* and true cod *(Gadus macrocephalus)* were determined. Machine separation yielded a 34.4–101.9% increase in edible flesh over hand filleting. Bone and skin separation from two rockfish and three "cod-like" species was found to be complete. Portions prepared from frozen blocks of minced flesh were shown to generally possess a lower moisture content and higher fat content. Trained and student flavor panels showed preference for intact flesh portions, but minced flesh portions from two rockfish and three "cod-like" species reflected a relatively high degree of acceptance. Portions composed of minced flesh from English and Dover sole gave unacceptably low panel scores reflecting odor and flavor components associated with skin that was not successfully separated. Addition of 0.3% sodium tripolyphosphate did not enhance the acceptance of minced fish portions. Minced flesh portions were shown to receive a higher level of acceptance by flavor panels when intact flesh portions were not included in evaluations. The investigation suggests that frozen blocks of minced flesh from most species of marine food fish may possess a level of consumer acceptance necessary to achieve success in market development.

DETERMINATION OF THERMAL CONDUCTIVITY VALUES OF FREEZE-DRIED EVAPORATED SKIM MILK. G.L. GENTZLER & F.W. SCHMIDT. *J. Food Sci.* 37, 554–557 (1972)–Values of the thermal conductivity of freeze-dried skim milk were determined using both a steady-state and a transient method and variation with pressure, temperature and specimen freezing rate was explored. Results showed the thermal conductivity value increased as the pressure and temperature increased but did not vary for the moderate variations in freezing rates employed. Values of thermal conductivity obtained in the transient method were in effect integrated values which took into consideration the pressure, temperature and moisture variation across the specimen, and were significantly greater than those found using the steady-state method although both showed an increase with increase in pressure.

HYDROGEN PEROXIDE OXIDATION AND COAGULATION OF EGG WHITE. D.W. SNIDER & O.J. COTTERILL. *J. Food Sci.* 37, 558–561 (1972)–High concentrations of H_2O_2 coagulate the proteins in liquid egg white (EW). A mixture of EW containing 10% solids and 6% H_2O_2 had a coagulation time of 5.5 hr (27°C, pH 8.6). Coagulation time was maximum at pH 6. Shortest coagulation times were observed at pH 4 and 11. Electrophoretic data indicated a gradual decrease in all proteins with increasing concentrations of H_2O_2. Lysozyme activity and iron-binding capacity were not affected by H_2O_2 oxidation for 2 hr. Cysteine, methionine, tyrosine, phenylalanine and histidine decreased when EW was oxidized with 6% H_2O_2 for 2 hr. Results suggest that the mechanism of coagulation by H_2O_2 involves the oxidation of several amino acids in the EW system.

CALLOSE FORMATION BY BRUISING AND HEATING OF TOMATOES AND ITS PRESENCE IN PROCESSED PRODUCTS. ELIAS D. DEKAZOS. *J. Food Sci.* 37, 562–567 (1972)–The β-1, 3-glucan (callose) formation in bruised mechanically harvested and heated green and red tomatoes as well as in canned whole tomatoes and tomato juice was demonstrated. Callose ranges from cytoplasmic deposits to cell wall components of various geometric forms. Identification of callose was made by fluorochrome reaction. Depositions of callose increase as a result of heat (45–50°C). Temperature has a decided effect on callose formation. Once formed the substance is stable to heat as witnessed in canned whole tomatoes. Callose formation is discussed in relation to the phenomena known as texture and consistency in tomato and tomato juice, respectively.

IMPROVEMENT OF THE TEXTURE OF DEHYDRATED CELERY BY GLYCEROL TREATMENT. J.W. SHIPMAN, A.R. RAHMAN, R.A. SEGARS, J.G. KAPSALIS & D.E. WESTCOTT. *J. Food Sci.* 37, 568–571 (1972)–This study was initiated to develop a method for the production of good quality dehydrated celery–which can be rehydrated to virtually its predehydration characteristics–with the aid of additives such as sugars, salts and glycerol. In initial tests glycerol gave best results and was chosen for these investigations. Transversely sliced celery sections were soaked in 0–100% glycerol solutions at 10% intervals and freeze dried or air dried following commercial practices. Sensory panel evaluations on flavor and texture of the rehydrated celery indicated that air-dried or freeze-dried celery equilibrated in 20–60% glycerol solutions prior to dehydration produced the best results. The quality of the air-dried product was better than the freeze-dried. Objective measurements of textural properties determined by mechanical compression tests indicated that rehydrated celery pretreated with 20% or more glycerol was of firmer textural quality than celery treated with lower concentrations. Histological studies showed extensive rupturing or deformation of the cell walls caused by freezing and dehydration of untreated celery, whereas glycerol prevented such damage since celery so treated resembled the fresh product. It is concluded that a glycerol pretreatment method for the dehydration of celery produces an improved quality rehydrated product.

FERROMAGNETIC PARTICLES IN FOODS. H.M. CUNNINGHAM & R. O'BRIEN. *J. Food Sci.* 37, 572–573 (1972)–A technique was developed to determine the number of ferromagnetic particles that may be removed with a magnet from foods that are subjected to abrasive processing procedures. 34 out of 61 foods, consisting mainly of cereals and cereal products, were found to contain ferromagnetic particles: 4 contained particles ranging from 0.5–1.5 mm in length; 16 from 0.15–0.5 mm and the remainder from 0.001–0.15 mm. Some of the metal particles in flours and cereals came from fortification with iron powder.

ISOLATION AND CHARACTERIZATION OF THE NATIVE, THERMALLY INACTIVATED AND REGENERATED HORSE RADISH PEROXIDASE ISOZYMES. S.S. WANG & G.R. DIMARCO. *J. Food Sci.* 37, 574–578 (1972)–Six isozymes were obtained from repeated chromatography of commercial horse radish peroxidase (HRP) on cellulose ion exchangers. Similarities of RZ (Reinheitzahl) values, optimum pH's and specific activities made it possible to categorize these isozymes into two groups. HRP isozyme C of one group is more heat resistant than isozyme A-1 of the other group. Spectral studies showed that thermal inactivation (280°F, 1 min) of HRP isozymes involved reversible changes. Sedimentation studies and Sephadex gel chromatography showed that during thermal inactivation HRP molecules aggregated to form dimers and trimers. During regeneration (70°F, 2 days) higher aggregates were formed with the majority of the dimers and trimers dissociating to form HRP molecules.

FLAVOR QUALITY AND STABILITY OF POTATO FLAKES. Volatile Components Associated with Storage Changes. G.M. SAPERS, O. PANASIUK, F.B. TALLEY, S.F. OSMAN & R.L. SHAW. *J. Food Sci.* 37, 579–583 (1972)–Samples of commercial and experimental potato flakes, stored under nitrogen for as long as 9 months at 73°F, showed little or no evidence of change in flavor or volatile composition associated with nonenzymatic browning reactions. Similar samples stored in air developed a hay-like off-flavor in 3–9 months, depending on the product, and showed increases in n-hexanal, other volatile products of lipid oxidation, and benzaldehyde.

EFFECTS OF ENVIRONMENTAL FACTORS ON THE OXIDATION OF POTATO CHIPS. D.G. QUAST & M. KAREL. *J. Food Sci.* 37, 584–588 (1972)–The effects of oxygen concentration, equilibrium relative humidity, extent of oxidation, light and temperature on the rate of oxygen uptake of potato chips are presented. It was found that the product entered a rapid oxidation period after an extent of oxygen absorption of 1.2–1.5 cm^3O_2 STP/g. This critical extent was independent of the environmental conditions and of the rate at which it was reached. Beyond this extent the product exhibited an unacceptable rancid odor. In early stages of oxidation, the peroxide value correlated well with the extent of oxygen uptake and could therefore be used as an index of oxidative deterioration. Deterioration can be retarded by packaging measures, such as proper choice of oxygen and moisture barriers and by shielding against light. Inert gas packaging results in extended storage life, provided the headspace oxygen concentration is less than 1% O_2.

THE CAROTENOIDS OF THE AVOCADO PEAR Persea americana, Nabal Variety. J. GROSS, M. GABAI & A. LIFSHITZ. *J. Food Sci.* 37, 589–591 (1972)–The distribution of carotenoids in the avocado fruit *Persea americana*, Nabal variety, was investigated. 24 carotenoids could be separated. β-Carotene, cryptoxanthin, lutein, isolutein, violaxanthin, chrysanthemaxanthin, trollichrome and neoxanthin were the major pigments. Lutein and its furanoxide together represented approximately half of the total carotenoid content. A new pigment for which the formula α-citraurin,3-hydroxy-α-apo-8'-carotenal is suggested, was found to occur naturally for the first time. A very polar pigment with its main absorption maximum at 437 nm was tentatively identified as trollein. Five minor pigments could not be identified. Free vitamin A could not be found. The content of vitamin A calculated from the present provitamins A was found to be 150 IU/100g fresh matter.

DEGRADATION OF ANTHOCYANINS AT LIMITED WATER CONCENTRATION. J.A. ERLANDSON & R.E. WROLSTAD. *J. Food Sci.* 37, 592–595 (1972)–Anthocyanin degradation in freeze-dried strawberry puree was studied as a function of relative humidity (RH) at 37°C in the dark. The rate of degradation increased with RH, the pigments being quite stable at a RH of 11% or below. Anthocyanins of blanched and unblanched fruit deteriorated at the same rate implying the degradative mechanism was chemical hydrolysis rather than enzymatic. Substitution of nitrogen headspace for air had little effect on the rate of anthocyanin degradation but reduced the degree of browning.

THE EFFECT OF GAMMA IRRADIATION ON THE ANTITHIAMINE ACTIVITY OF SKIPJACK TUNA. D.M. HILKER, N.Y-A. TANG & K-C. CHAN. *J. Food Sci.* 37, 596–598 (1972)–The effect of gamma irradiation on antithiamine activity (ATA) in Skipjack tuna (*Katsuwonus pelamis*) was studied under air and N_2 atmosphere. The ATA (μg thiamine destroyed/mg dry weight) of the water soluble fish extracts after irradiation under air atmosphere was increased at a dosage of 4 Mrad while under N_2 atmosphere the ATA was decreased. Irradiation had no effect on the ATA of heme protein fractions of the fish extract separated by $(NH_4)_2SO_4$ precipitation (70% and 80% saturation fractions). Water-extractable solids were decreased under air but not N_2 at 4 Mrad. The absorption spectra of the irradiated fish extracts showed an additional absorption peak at 615 mμ and decreased absorption at 635 and 500 mμ.

APPROACHES TO THE UTILIZATION OF FISH FOR THE PREPARATION OF PROTEIN ISOLATES. Isolation and Properties of Myofibrillar and Sarcoplasmic Fish Proteins. J. SPINELLI, B. KOURY & R. MILLER. *J. Food Sci.* 37, 599–603 (1972)–Factors relating to the preparation of functional protein isolates from fish were studied. Fish tissue was separated into myofibrillar, sarcoplasmic and nonprotein fractions. The sarcoplasmic proteins are severely denatured when freeze dried. They contain a major portion of the substances that adversely affect the organoleptic stability of the proteins. Myofibrillar proteins lose most of their native functional characteristics when dried, and even when treated with antioxidants, they gradually deteriorate organoleptically. Extraction of the myofibrillar fraction with polar organic solvents prevents organoleptic deterioration, but functional properties are destroyed. Co-drying the myofibrillar proteins with carbohydrates preserves the salt-soluble characteristics of these proteins for only short periods of time.

APPROACHES TO THE UTILIZATION OF FISH FOR THE PREPARATION OF PROTEIN ISOLATES. Enzymic Modifications of Myofibrillar Fish Proteins. J. Spinelli, B. Koury & R. Miller. *J. Food Sci.* 37, 604–608 (1972)–Fish protein isolates possessing functional properties were prepared by enzymic modification of the myofibrillar proteins of fish muscle. The proteins were partially hydrolyzed with a proteolytic enzyme and recovered as protein-phosphate complexes. The dried isolates were practically free of flavor and odor and emulsified over 200 times their weight of oil.

POST-MORTEM BIOCHEMICAL CHANGES IN THE MUSCLE OF GULF SHRIMP, Penaeus aztecus. G.J. FLICK & R.T. LOVELL. *J. Food Sci.* 37, 609–611 (1972)–The changes in ATP, ADP, AMP, IMP inosine (Ino), and hypoxanthine (Hx) were determined in the tissues of unexercised brown shrimp (*Penaeus aztecus*) by using polyethyleneimine cellulose on thin-layer chromatograms. Glycogen, lactic acid and orthophosphate were determined by colorimetric methods of analysis. The pH was measured with a pH meter. The various measurements were conducted immediately after slaughter and after subsequent ice-pack storage. The degradation of ATP followed the route: ATP to ADP to AMP to IMP to Ino to Hx. Glycogen decreased from 160 mg per 100g of tissue to 70 mg within 10 days of storage. Concomitant with the decrease in glycogen was an increase in lactic acid from 160 mg per 100g tissue initially to 370 mg after 10 days of storage. During 10 days of storage, pH rose continually from 7.39 initially to 8.17 and orthophosphate degraded from 3079 μg per 100g of tissue to 725 μg.

ABSTRACTS:
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STUDIES ON NUCLEOTIDE METABOLISM IN PORCINE LONGISSIMUS MUSCLE POSTMORTEM. R. TSAI, R.G. CASSENS, E.J. BRISKEY and M.L. GREASER. *J. Food Sci.* 37, 612–616 (1972)–Ion-exchange chromatography with a linear gradient system was used to quantitate nucleotide patterns in postmortem muscle of stress-susceptible and stress-resistant pigs. ATP, ADP, IMP with a trace of AMP and DPN^++DPNH were the four major peaks resolved in pre-rigor muscle. Muscle from stress-susceptible animals entered rigor, as judged by extensibility loss, between 0.5 and 1.0 hr while that from stress-resistant animals required 3–4 hr. Muscle from stress-susceptible animals had a lower ATP and higher IMP at 0 hr than did muscle from stress-resistant animals. Between 0 hr and 3 hr the concentrations of ATP, ADP and DPN^++DPNH decreased rapidly while the concentration of IMP and inosine + hypoxanthine increased. Changes from 3 hr to 144 hr postmortem were of lesser magnitude and much slower. The activities of AMP deaminase, adenosine deaminase, adenine deaminase and 5′-nucleotidase were determined during postmortem time in the muscle. AMP-deaminase activity was higher in the muscle of stress-resistant animals at 3 hr and later postmortem.

RETENTION OF 2-PROPANOL AT LOW CONCENTRATION BY FREEZE DRYING CARBOHYDRATE SOLUTIONS. J.M. FLINK & T.P. LABUZA. *J. Food Sci.* 37, 617–618 (1972)–The retention of 2-propanol by freeze drying carbohydrate solutions has been determined for initial 2-propanol concentrations of 100 and 200 ppm. It is shown that the retention of 2-propanol increased when the freezing rate or sample thickness is decreased. There was essentially no influence of the 2-propanol concentration for the two levels utilized. The influence of the freeze-drying platen temperature was mixed which reflects differing responses of the different model systems. These results are in qualitative agreement with results which have been presented earlier for the same model system at a much higher initial 2-propanol concentration (7,500).

PROPERTIES OF A FUNGAL LACTASE. G.B. BORGLUM & M.Z. STERNBERG. *J. Food Sci.* 37, 619–623 (1972)–Lactase, β-galactosidase E.C.3.2.1.23, produced by *Aspergillus foetidus* hydrolyzed β-galactosides exclusively at optima of 60°C and pH 3.5–4.0. The pure enzyme was stable at pH 4–8 and the crude preparation at pH 2.2–8. The enzyme required no metal ion for stability or activity. Mercuric ion inhibited lactase, but phenylmercuric acetate had no effect. Chelating agents did not stimulate the enzyme indicating little sensitivity to trace heavy metals. Nitrophenyl thiogalactoside, galactonolactone and galactose were strong inhibitors. The molecular weight by gel filtration was 126,000. Enzyme concentrations suitable for industrial use catalyzed up to 74% lactose hydrolysis in 72 hr.

PERCEPTUAL ATTRIBUTES OF THE TASTE OF SUGARS. H.R. MOSKOWITZ. *J. Food Sci.* 37, 624–626 (1972)–Equally sweet concentrations of sorbitol, glycerol, arabinose xylose, galactose, sorbose, maltose, lactose and sucrose were compared to the flavor of glucose and fructose. Ss were asked to make "magnitude estimates" on a ratio scale, of the overall "flavor difference" between equally sweet concentrations of different sugar pairs. The median estimates were analyzed by a nonmetric, multi-dimensional analysis that placed the sugars into a geometrical space, so that the flavor difference between sugar pairs corresponded to their distance. Two dimensions were apparent in the perceptual space, the dimensions of viscosity-fluidity and primarily sweet taste vs. sweet and side tastes. The distribution and variability of sugars in the space was maximal at high sweetness levels and minimal at low ones.

A RAPID SALT-CURING TECHNIQUE. M.L. ANDERSON & J.M. MENDELSOHN. *J. Food Sci.* 37, 627–628 (1972)–A rapid method is reported for salt-curing fish by comminuting fillets in saturated salt solution to which enough excess salt has been added to maintain saturation, draining off fluid lost through syneresis and drying in a vacuum drum dryer or moving air. Use of a meat-bone separating device to obtain the comminuted fish is suggested for an efficient industrial process based on the method.

CONTROL OF CHLOROPHYLL AND SOLANINE SYNTHESES AND SPROUTING OF POTATO TUBERS BY HOT PARAFFIN WAX. M.T. WU & D.K. SALUNKHE. *J. Food Sci.* 37, 629–630 (1972)–This paper describes a simple and effective method of inhibiting chlorophyll and solanine formation and sprouting and weight loss of potato tubers–a serious storage and marketing problem–by a combined waxing/heating treatment. Low temperatures (60° and 80°C) did not significantly inhibit chlorophyll and solanine syntheses; intermediate temperatures (100° and 120°C) were more successful and higher temperatures (140° and 160°C) most successful. While waxing at 60° and 80°C stimulated sprouting, waxing at 100°C and above inhibited it and significantly reduced weight loss of tubers during storage. Data presented support the conclusion that the proper combination of hot waxing (>120°C for ½ sec) and subsequent storage at lower temperature (5°C) should effectively control chlorophyll, solanine and sprout formation as well as weight loss of potato tubers.

A METHOD FOR CLEAN-UP OF RAW POTATO EXTRACTS FOR REDUCING SUGAR DETERMINATION. J.A. KINTNER & R.G. ARNOLD. *J. Food Sci.* 37, 631–632 (1972)–Raw potato extracts were prepared for reducing sugar analyses by treatment of ground potato tissue with decolorizing charcoal followed by centrifugation. Peeling and enzyme inactivation pretreatments were unnecessary. The cleaned-up extract can be analyzed using any routine method, such as anthrone or copper reduction colorimetry. The method provides a rapid means of clean-up, and the resulting sample is sufficiently free of interfering substances to allow accurate determination of low levels of reducing sugar without extensive pretreatment.

EFFECT OF HANGING POSITION ON SOME PROPERTIES OF DRY SAUSAGE. W. E. TOWNSEND & C. E. DAVIS. *J. Food Sci.* 37, 633 (1972) – The effect of hanging position during drying on some properties of dry sausage was studied. More shrinkage occurred in sausages hung in the horizontal position than in those hung in the vertical position. A lower incidence of hollow centers was noted in sausage hung in the horizontal position.

METMYOGLOBIN REDUCTION AND FORMATION IN BEEF DURING AEROBIC STORAGE AT 1°C. D.A. LEDWARD. *J. Food Sci.* 37, 634–635 (1972)–A high concentration of metmyoglobin (metMb) was formed in beef slices 2.0 ± 0.5 mm thick during storage in 1% O_2. During subsequent aerobic storage at 1°C the reduction of this metMb was highly correlated ($r = -0.94$, $P < 0.001$) with the accumulation of metMb in slices 15 ± 1 mm thick. No significant correlation was found between the metMb reducing activity of anaerobically stored minced beef following ferricyanide oxidation (MRA) and the accumulation of metMb during aerobic storage at 1°C.

EVALUATION OF A METHOD TO DIFFERENTIATE BETWEEN NONFROZEN AND FROZEN-AND-THAWED MEAT. P. VANDEKERCKHOVE, D. DEMEYER & H. HENDERICKX. *J. Food Sci.* 37, 636–637 (1972)–Experiments were carried out to evaluate the effectiveness of an electrophoretic method to differentiate between nonfrozen and frozen-and-thawed porcine muscle based on the presence of only a GOT_S band with nonfrozen meat and GOT_S + GOT_M bands with frozen-and-thawed meat. Contrary to previously published data, results show the method may be inappropriate for porcine meat.

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A SYMPOSIUM . . . complete in this issue

BIOCHEMICAL CONTROL SYSTEMS IN FOOD TISSUES
INTRODUCTION

IT HAS LONG BEEN recognized that cellular metabolism is mediated by enzymes, and much is known of the specific reactions intrinsic to cell types. One of the most important frontiers in molecular biology is the realization of those factors which control enzyme catalyzed reactions in the cellular environment. The bulk of man's food is comprised of variable cells; hence, the control of enzymic reactions in food tissues is extremely important in determining the quality of the foods we eat. Very often, conditions are introduced in postharvest or postmortem tissues which create a stress condition on the cellular milieu; e.g., the loss of oxygen supply to the excised muscle or the termination of solute transfer into the detached fruit. The response of any cellular system to such stress is largely dictated by enzymic reactions and those factors which regulate their nature and direction.

It has been recognized for centuries that the quality of food depends on the individuality of the tissue type as well as the treatment that it receives, and it is now equally clear that these effects are a function of gene dictation as well as the response of existing enzymes. One who has observed the transition of a green banana (astringent, low sugar, little aroma) to a fully ripe state (sweet, mellow, aromatic) in a few days or has observed the contractility of freshly excised muscle, must be aware of the dynamic nature of those edible tissues.

In this symposium, we focus on the nature of food tissues as dynamic entities, emphasizing modes of enzymic control which are of importance in food quality. No attempt is made to cover all the types of regulation which are important, but rather to give some examples which will illustrate the complex nature of the mechanisms involved and the types of considerations necessary in understanding the dynamic nature of food tissues.

The first paper (Haard, 1972) deals with the structure and composition of cellular membranes. An understanding of the function and structure of these membranes is necessary in understanding their breakdown and the catastrophic consequence thereof, such as loss of cellular energy reserves and destruction of subcellular compartments.

The second paper (Romani, 1972) deals with the ability of fruit tissue to respond to stress, especially as related to their propensity to repair or regenerate the cellular machinery required for energy transduction and protein synthesis. The requirement of such tissue to maintain an appropriate energy balance and to carry on with protein synthesis is further illustrated in the third paper (Dilley, 1972). The fact that fruits not only are capable of synthesizing proteins but do it in a very specific way related to the ripening process, indicates the high degree of control operative in this type of food tissue.

The presence of enzyme inhibitors in plant tissues has been known for a long time. The suggestion made in the fourth paper (Pressey, 1972) of this Symposium, that this phenomenon is of importance in controlling native plant enzymes, has important implications in food handling and the documentation of the relationship of invertase and invertase inhibitor to accumulation of reducing sugars in potato tubers lends credence to the suggestion. The fifth paper (Hultin, 1972) suggests that interactions between enzymes and subcellular particulate structures may be important in controlling enzymic activity. Such interactions may be different under postmortem or postharvest conditions than in their previous state and certainly would be different in macerated tissues. Emphasis is given to the control of glycolytic enzymes; their importance in connection with pH decline in postmortem muscle is well documented but little understood.

The final paper (Schwimmer, 1972) concerns the general problem of cell disruption and its effects not only on quality but also on the nature of the food produced. It is suggested that the field of food science and technology can be considered as the management of food cell disruption and its consequences. Many of the consequences are undoubtedly related to phenomena described both here and in the earlier papers.

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Pressey, R. 1972. Natural enzyme inhibitors in plant tissues. J. Food Sci. 37: 521.

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SYMPOSIUM: Biochemical Control Systems
MEMBRANE STRUCTURE AND CELLULAR DEATH IN BIOLOGICAL TISSUE

INTRODUCTION

THE STATE OF the science of membranes represents somewhat of a paradox. At present, we have no clear picture of the molecular ordering in biological membranes. The subject is wrought with contradictory data and wide differences in interpretation. However, an understanding has emerged that the structure of biological membranes delimit their respective functions. Regulation of gene expression, enzyme action and the direction of metabolic flow are dependent on cellular compartmentation; the balance of modifiers, cofactors, hormones and other metabolites; the association of enzymes with superstructure; and the energy state of the cell. The latter are a direct reflection of the structure and function of cellular membranes. The nature of biochemical control mechanism in plant and animal tissues is, therefore, closely allied to the science of membranes.

In this treatise selected aspects of the "state of the art" of membrane chemistry are reviewed. Emphasis is given to the delicate relationship between mitochondrial membrane structure and the energy transduction process of oxidative phosphorylation. The mitochondrial membrane is a most appropriate subject with our interest in foods. Storage disorders in fresh fruits, vegetables, cereals, meats and fish are surely a result of damage to membranes involved with energy transduction. Other cellular membranes, such as the sarcotubular system, nuclear, lysosomal membrane, etc., are likely to be of importance to biochemical control in specific tissues.

STATE OF THE ART

Structure of biological membranes

The structure of biological membranes has been reviewed extensively (Korn, 1969; Branton, 1969; Staehelin and Probine, 1970; Green et al., 1970; Vanderkooi and Green, 1971; Vandenheuval, 1971). The conclusions of authors who advocate one or another model have been at extreme ends of the imagination spectrum. Differences in interpretation of data have been so great that "in many respects our knowlege of membranes today stands where our knowledge of DNA stood in 1945" (Branton, 1969).

Composition of biological membranes

The composition of biological membranes of subcellular components from different life forms has been well established (Table 1). The principle constituents are lipids and proteins which may vary in both proportion and qualitative nature depending on the membrane source. While membranes of the myelin sheath contain excessive lipid (4 parts to 1 part protein) the membranes of gram positive bacteria of mammalian organelles may contain as much as 4 parts protein to 1 part lipid. It is generally agreed that covalent linkages between lipids and protein are not important for maintaining membrane integrity. While covalent bonding may exist, the dissolution of most membranes only requires conditions which perturb weak polar and apolar interaction and not the cleavage of covalent bonds. There is, however, little agreement on how proteins and lipids are oriented and interact in membranes.

Observed similarities in membrane constituents led Robertson to propose the "unit membrane" model which attempts to describe the molecular ordering of proteins and lipids (Robertson, 1960). The unit membrane concept, illustrated in Figure 1, is supported by various lines of evidence and has dominated the thinking of most membrane researchers. The unit membrane is an extension of the paucimolecular model suggested earlier by Danielli and Davson (1935). These models picture membranes as a continuous bilayer of phospholipid stabilized by hydrophobic interactions and covered with proteins in the extended or β-confor-

Table 1—Protein and lipid content of membranes[a]

Membrane	Protein / Lipid (wt/wt)	Cholesterol / Polar lipid (mole/mole)
Myelin	0.25	0.7–1.2
Chloroplast lamellae	0.80	0
Endoplasmic reticulum	0.70–1.2	0.03–0.08
Plasma (liver cell)	1.0–1.4	0.3–0.5
Bacteria (gram pos.)	2.0–4.0	0
Mitochondrial inner	3.6	0.02–0.04

[a]Adapted from Korn (1969)

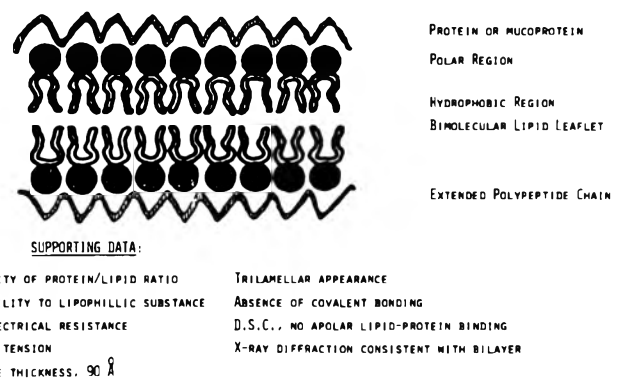

Fig. 1—Unit membrane model.

mation via electrostatic association. Data obtained from polarization optics, x-ray diffraction, differential scanning calorimetry, electron microscopy, surface tension measurements, chemical analyses and phospholipid model systems have been interpreted as consistent with this arrangement (see Branton, 1969 and Vandenheuval, 1971 for review) although others have argued that such results are either consistent with alternative models; are the reflection of various types of preparative artifact; or are misinterpretation of data (see Staehelin and Probine, 1970 and Korn, 1969 for review).

Membrane structure

Over the past 10 years and particularly the past 5 years, many investigators have become disenchanted with the unit membrane model. Physical and chemical properties of membranes are not always interpreted as consistent with a lipid bilayer interacting with extended protein by polar forces. Circular dichroism, optical rotary dispersion and infrared spectroscopy measurements suggest that little, if any, membrane proteins exist in the β-conformation, and that membranes in general contain substantial amounts of protein in helical and random coil conformation. There is also evidence that membrane proteins are associated with the acyl chains of fatty acid residues of phospholipids and not with the polar residues as suggested by the unit membrane model. Furthermore, certain membranes can be dissociated into discrete lipoprotein repeating units and all membranes appear to show globular structure when fractured by freeze etching or when viewed by high resolution electron microscopy. Other information, too numerous to mention here, has been found inconsistent with the continuous bilayer concept of membrane structure.

Examples of the many models which have been proposed are shown in Figure 2. Current models may be roughly divided into four categories: (1) protein-lipid-protein trilayers; (2) lipid-protein-lipid trilayers; (3) lipoprotein repeating units; and (4) dynamic models where the membrane constituents are considered to be under constant flux. A fundamental difference between a protein-lipid-proteins model, such as the Danielli-Davson model, and a lipid-protein-lipid type such as that proposed by Benson (1966), is the nature of interaction between phospholipid and protein. In the former, proteins are associated with the polar moieties of the lipids and in the latter interaction is hydrophobic. Other models envision lipoprotein arrays stabilized by both polar and apolar interactions (i.e., Vanderkooi and Green, 1971) and appear to be most consistent with experimental findings.

Unfortunately, none of the proposed models have built-in the fine points required to explain the functional capabilities of membranes. While the phospholipid bilayer can account for the passive permeation of lipophilic molecules through membranes, there is little evidence relating membrane structure to dynamic processes. Vandenheuvel (1971) has recently presented a plausable explanation for active transport through the bilayer. This point will be discussed later.

Why the inability to agree on something as appearingly simple as the gross molecular ordering of proteins and lipids in membranes? Of most importance is the fact that investigators have worked with membranes of widely different source and physiological capability. There is a tendency to suppose an underlying ubiquity to biological membranes structure, but specialization of structure for appropriate functional capability may result in lack of such similarity and hence, a universal model may be inappropriate. The purity of membrane fractions studied are often in question and probably is the explanation for much of the contradicting evidence. Many of the tools used to study membrane structure, such as x-ray diffraction, optical rotary dispersion, circular dichroism, nuclear magnetic resonance and infrared spectroscopy are averaging techniques and as such may be unable to detect fine points of membrane structure. Electron microscopy has been used to characterize membrane structure; the trilayer appearance of membranes was a principal reason for Robertson's proposed "unit membrane" structure. The interpretation of which molecular regions become electron dense on staining is open to question and moreover, many have argued that fixation techniques cause gross changes in the molecular orientation of membrane constituents. While eloquent spokesmen have argued that all data can be interpreted as consistent with one model, it remains to be demonstrated that any interpretation can provide a fundamental understanding of how membranes carry out their respective functions. The following lines of experimentation also indicate the inadequacy of current information of membrane structure.

Membrane proteins

The proteins in membranes have received less study than lipids. Most models of membrance structure emphasize the importance of lipids and do not dictate a primary role for proteins in structure and function. From the point of view of food tissue, there is little one can specifically relate to membrane proteins. It is known that increased disulfide bonding of membrane proteins is associated with adaptation of certain plant tissues to low temperature stress (McGown et al., 1969; Morton, 1969; Kuraishi et al., 1968) and lack of such adaptation of membrane structure may relate to the chilling dis-

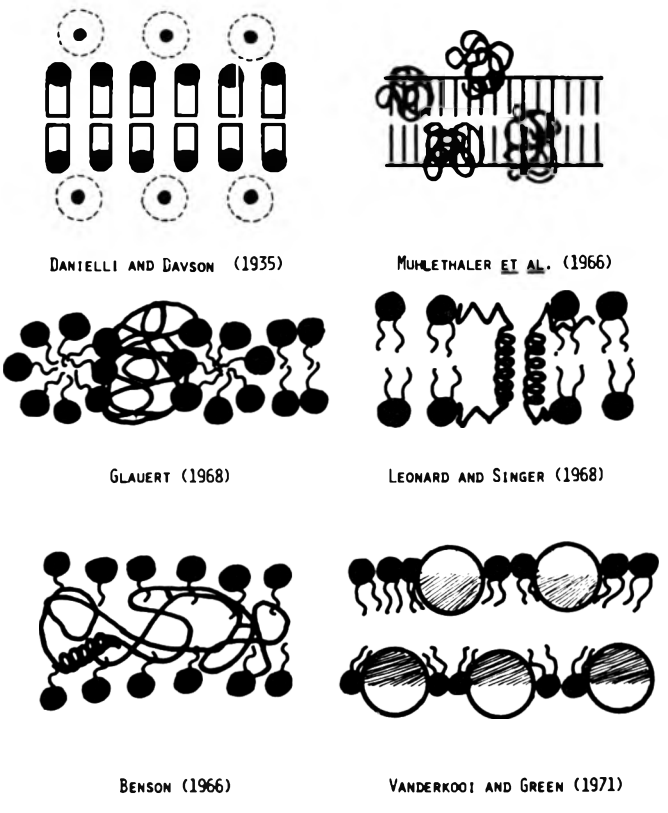

Fig. 2—Examples of membrane models.

orders which occur in most fruits and vegetables.

Once proteins are separated from the lipoprotein matrix, they become extremely difficult to fractionate by known techniques of protein chemistry. It has been suggested that membrane proteins go into a "molecular rigor mortis" to describe their intractable properties when separated from lipids (Branton, 1969). In 1961 an apparently pure fraction, which represented about 30% of the protein from membranes, was isolated from the membranes of beef heart mitochondria. The membrane protein had a snow white color, no apparent enzymatic activity and the capacity to bind phospholipids by hydrophobic interaction and to associate with cytochromes and mitochondrial enzymes by polar attraction. The view emerged that this protein served to organize the lipid and enzyme components of membranes and that phospholipids were bound to protein by hydrophobic rather than polar interaction. This was an ammonium sulfate fraction from bile salt-solubilized membranes and was called "structural protein" (Green et al., 1961). A number of findings over the following 7 years pointed to the reality of the concept that structural protein was an organizer element in membranes generally. These findings were as follows:

Homogeneity. Various lines of evidence were consistent with the conclusion that structural protein, as isolated, was a single protein species. Sedimentation analysis, free boundry and gel electrophoresis, N-terminal analyses, immunological properties, stoichiometry of binding and solubility properties were all consistent with the homogeneity of this fraction (Lenaz et al., 1968a).

Ubiquity. Different laboratories reported the presence of structural proteins, at 25–50% of membrane protein, in membranes obtained from various sources including mitochondria, chloroplasts, microsomes, plasma membranes, sarcotubular membranes and erythrocyte ghosts; and it appeared that this fraction was a universal component of biological membranes (Green et al., 1968).

Cytoplasmic code. Various groups studying protein synthesis by isolated mitochondria via mitochondrial DNA expression reported that structural proteins, or an analoquous fraction, was consistently the one in which labeled amino acids were incorporated (Newport et al., 1967). A thesis developed that cytoplasmic coded protein served as template for nuclear coded enzymes and lipids.

Structural protein from mutant yeast strains. Through extensive investigation, Woodward and Munkres (1966) showed that defects in oxidation of malate by respiratory mutant yeast could be related to a replacement of one amino acid in mitochondrial structural protein. Further studies provided that structural protein served to modulate malic dehydrogenase activity.

In 1966, it was observed that structural protein, as isolated from the membranes of beef heart mitochondria, separated into four major electrophoretic zones in polyacrylamide gels of high resolving power (Lenaz et al., 1968a). The observed heterogeneity was demonstrated to be real and not electrophoretic artifact, and it became obvious that the entire concept of structural protein with all the implications to its role as an organizing element and a modulator of membrane enzymic activity had to be reevaluated.

Later studies (Green et al., 1968; Lenaz et al., 1968b; Blair et al., 1968) showed that structural protein from beef heart mitochondria was a mixture of four species with near identity in amino acid composition, primary amino acid sequence, molecular weights and solubility properties. Structural protein, as isolated, was not derived from the membrane continuum but from readily detachable protein sectors of the inner and outer membranes of mitochondria (Haard and Lenaz, 1967; Green et al., 1968). Several species of structural protein have been identified with the ATPase complex of mitochondria (Senior and MacLennon, 1970).

Further investigations showed that additional proteins, derived from the membrane continuum itself, could be isolated from mitochondrial membranes (Silman et al., 1967; Haard and Lenaz, 1967). All of these membrane proteins exhibit similarities in amino acid composition (Table 2), primary amino acid sequence as judged by tryptic peptide maps

Table 2—Similarity of amino acid distribution

Protein[b]	Membrane source[c]	Number of amino acid residues[a] Δ mole % (< 1)	Δ mole % (> 1)	Δ mole % (> 2)
SP	HBHM	16	1	0
SP	HBLM	16	1	0
SP	HBHM, Outer	15	2	0
CP	HBHM, Inner	14	3	1
Cyt. A	MITO., Inner	6	11	5
Cyt. C	MITO., Inner	5	12	10
MDH	MITO., Outer	9	8	3

[a]Differing from SP species 2 by indicated mole percent
[b]SP = structural protein fraction; CP = core protein fraction; MDH = malic dehydrogenase.
[c]HBHM = heavy beef heart mitochondria; HBLM = heavy beef liver mitochondria.

Table 3—Common peptides—membrane proteins

Protein[a]	Source	Fraction	Components on gel	Tryptic peptides	Peptides corresponding to standard[b]
SP	Beef heart muscle	Mitochondrial ATPase	1	25–29	(Standard)
SP	Rabbit muscle	Sarcoplasmic reticulum	1	25–29	22–25
SP	Beef heart muscle	Whole mitochondria	4	32–36	20–24
SP	Beef heart muscle	Outer mitochondrial membrane	2	24–28	16–20
SP	Beef liver	Whole mitochondria	4 (major)	30–35	15–18
SP	Beef liver	Outer mitochondrial membrane	3 (major)	32–38	17–20
CP	Beef heart muscle	Complex IV	1 (major)	15	11
CP	Beef heart muscle	Complex III	1 (major)	24	18

[a]SP = structural protein; CP = core protein.
[b]The number of tryptic peptides, resulting from digestion of either cytochrome C (horse heart, Sigma Chemical Co.), bovine serum albumin (fraction V, Armour Pharmaceutical Co.) and glutamic dehydrogenase (bovine liver, calbiochem) common with those of the standard, ranged from 3 to 6.

(Table 3), molecular weight (Table 4), and tertiary structure as judged by circular dichroism (Urry et al., 1967). Evidence is consistent with the conclusion that there exists a class of proteins in membranes which are structurally homologous and which account for approximately 50% of membrane protein. The unique feature of membrane proteins may be a bimodiality in binding capacity such as that exhibited by phospholipids. Unfortunately, there is no definitive evidence that this class of membrane proteins are or are not active enzymes in their native milieu.

Table 4—Average molecular weights of membrane proteins[a]

Protein[b]	Source[c]	Components on gel	Avg Molecular wt.
SP	HBHM	4	60,000–65,000
SP	HBHM outer membrane	2	62,000–63,000
SP	HBHM ATPase	1	60,000–63,000
SP	HBLM	4	60,000–65,000
SP	HBLM outer membrane	3	59,000–62,000
CP	Complex III	1	50,000–51,000

[a]Determined by sedimentation velocity and equilibrium methods.
[b]SP = structural protein; CP = core protein.
[c]HBHM = heavy beef heart mitochondria, HBLM = heavy beef liver mitochondria, ATPase = adenosine triphosphatase.

Availability for hydrolysis. Another line of research on membrane proteins has been to determine their availability for hydrolysis by proteolytic enzymes. If proteins are at the outer surface of membranes, such as suggested by a protein-lipid-protein trilayer model, one would expect them to be readily available for attack by proteases. Studies have shown that the outer faces of mitochondrial membranes are completely resistant to hydrolysis by enzymes such as trypsin and bacterial pronase (Haard, unpublished). When the inner faces of these membranes are exposed to proteolytic enzymes by the sonicating of mitochondria, approximately 50% of the membrane proteins is readily hydrolyzed (Fig. 3). If phospholipids are extracted from mitochondria, the membranes are readily digested by proteases. These results suggest an assymetry in the orientation of protein on the two sides of membranes and are inconsistent with both type of symmetrical trilayer models discussed earlier.

Much additional study of membrane proteins is necessary before we can provide an acceptable picture of membrane structure and understand its relation to function. It is not yet clear whether noncatalytic proteins play a structural role in membranes, but evidence has accumulated that the bulk of membrane proteins have a unique primary structure. Further elucidation of this protein conformation may provide valuable insight into the structure of membranes and their biological properties.

Membrane lipids

The lipids of membranes have been characterized fairly well with respect to chemical structure (Table 1) and have been extensively studied as model systems for membranes. The polar lipids, principally phospholipids in higher life forms, are characteristic for different sources of membrane. The nature of fatty acid residues on the phospholipid glycerol may also be different depending on the membrane source. For example, the mitochondrial membranes of chilling-sensitive plant tissues have a greater distribution of saturated fatty acids in their phospholipids. It has been suggested that chilling injury of plant tissues results at temperatures where such membranes lose flexibility and hence, functional capacity (Lyons et al., 1964; Raison et al., 1971). Other lipids, such as sphingolipids, sphingosine and cholesterol, are also major constituents of specific membranes.

Approximately 95% of beef heart mitochondrial lipids are phospholipid and phosphatidylcholine represents about 40% of lipid phosphorous (Fleischer et al., 1963). The enzyme phospholipase C, which cleaves phosphorylcholine from lecithin, has been used to determine the availability of the polar heads of phospholipid for hydrolysis (Fig. 4). One would not expect a membrane of the protein-lipid-protein sandwich type to lose phosphorylcholine when exposed to the enzyme phospholipase C. The polar ends of phospholipids are pictured as readily available for hydrolysis in the lipid-protein-lipid sandwich models. The logic of this approach is further illustrated in Figure 5. When intact mitochon-

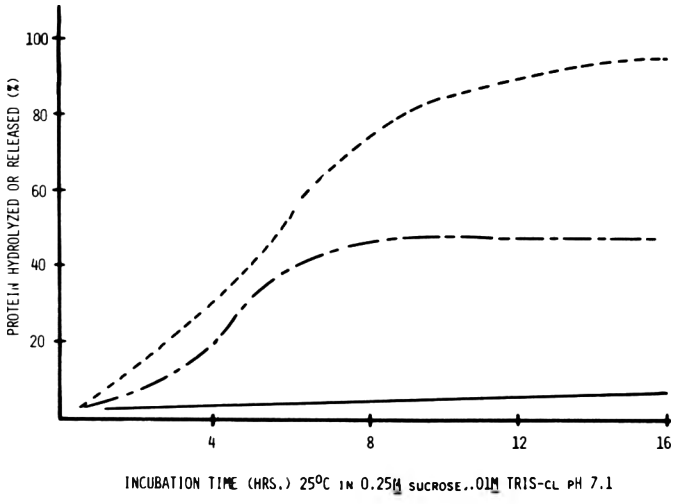

Fig. 3—Protein released or hydrolyzed as a result of trypsin digestion of intact beef heart mitochondria (——); sonicated beef heart mitochondria (— — — — —); and from lipid extracted beef heart mitochondria (— — —).

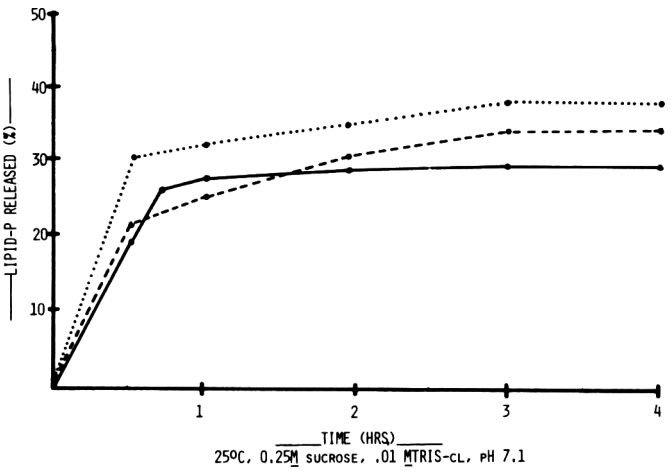

Fig. 4—Release of lipid phosphorous from membranes of beef heart mitochondria (——); electron transfer particules from beef heart mitochondria (ETP_h) (. . . . . .); and elementary particles (EP_1) from beef heart mitochondria (— — — —) as a result of phospholipase C digestion.

dria were digested with phospholipase C, all of the lecithin phosphatidylcholine was released into the supernatant while all protein and diglyceride remained with the membrane fraction (Vail et al., 1971). Similar results were obtained when mitochondria were sonicated in the presence of the enzyme or when they were prefixed with glutaraldehyde prior to digestion. While these findings are most consistent with lipoprotein association of the type assumed with lipid-protein-lipid trilayers, it appears that the enzyme was available at both sides of the membrane. Thus, while conclusions regarding symmetry vs. assymetry cannot be made, the results are powerful evidence that the continuity of mitochondrial membranes is not highly dependent on polar association of phospholipid and protein.

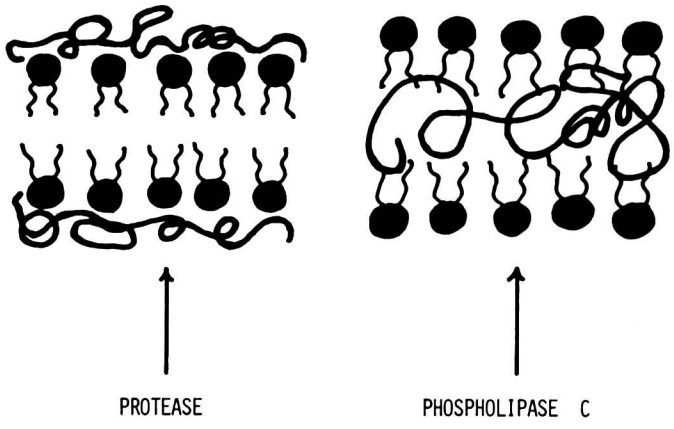

Fig. 5—Availability of phospholipid and protein for hydrolysis in alternative trilayer membrane models.

The appearance of phospholipase C-digested mitochondria in the electron microscope differed somewhat from those incubated without the enzyme (Fig. 6). Mitochondria digested with the enzyme until 40% of the lipid phosphorous was released (complete hydrolysis of lecithin) appeared somewhat swollen, and the outer membranes were usually discontinuous. Large, electron-dense boils were evident on the outer surfaces of most mitochondria, although these were seldom present when the membranes were first prefixed with glutaraldehyde. When these dense bodies were observed at high

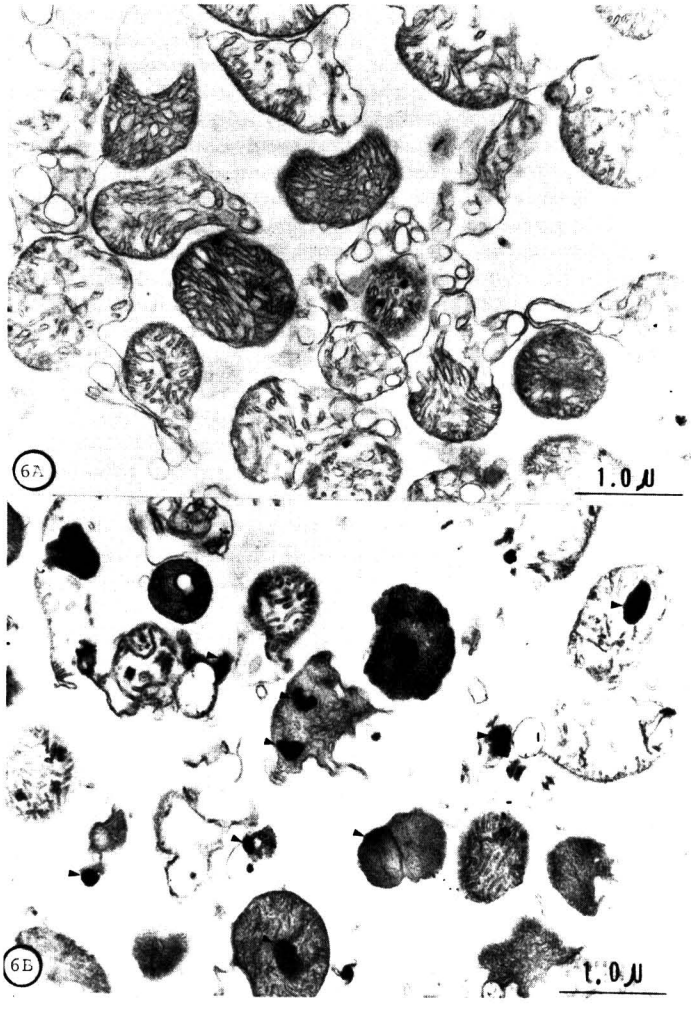

Fig. 6—Electron micrograph showing (A) control and (B) phospholipase C digested mitochondria after 1 hr incubation at 25°C, in 0.25M sucrose and .01M Tris-cl, pH 7.1.

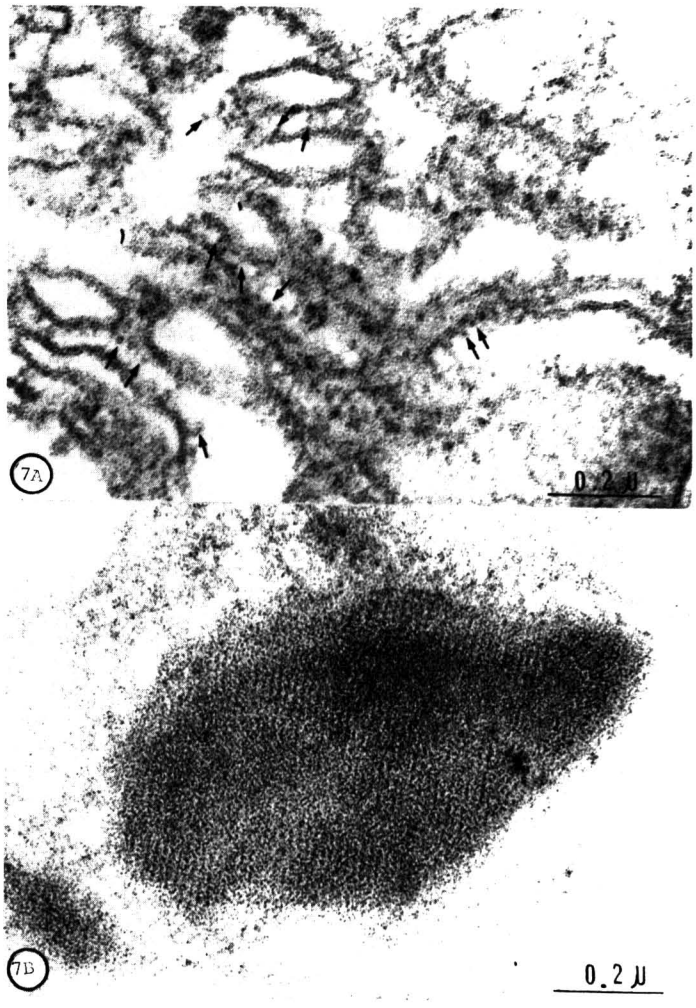

Fig. 7—Electron micrograph of phospholipase C digested beef heart mitochondria showing A, 100Å particles associated with the inner membranes and B, paracrystalline structure of electron dense boils.

magnification (Fig. 7B), a paracrystalline structure was revealed. Other evidence suggests these regions are masses of diglyceride (Vail et al., 1971). More important, the fine structure of mitochondrial membranes showed much greater detail after digestion. A trilamellar appearance was evident only after digestion and 100Å particles could be observed on the matrix side of the inner membrane (Fig. 7A). Such particles are normally seen only in disrupted mitochondria after negative staining but are rarely observed in positively-stained mitochondria.

Membrane structure and function

During the past few years, a thesis has developed relating the configurational changes of mitochondrial membranes with the functional state of mitochondria during oxidative phosphorylation (Harris et al., 1968; Penniston et al., 1968; Green and Young, 1971). The cycle of configurational changes which mitochondria undergo are illustrated in Figures 8A–11A. Little, if any, fine structure is observed in the membranes of such micrographs. Mitochondria were prefixed with glutaraldehyde in these functional variations and digested with phospholipase C. Phosphorylcholine was completely released throughout, but the membrane fine structure observed after digestion showed marked differences in the different configurational variations (Fig. 8B–11B). The 100Å particles were not evident on nonenergized membranes, appeared coalesed in the energized state and were quite distinct on membranes in the energized-twisted state (Fig. 12). These findings give further impetus to the theory relating conformation changes in membrane proteins to the process of ATP synthesis. The 100Å particles associated with the inner membranes would appear to be the ATPase complex described earlier (Kagawa and Racker, 1966). It is tempting to speculate that this complex is associated with the phospholipid milieu during the energized state and with protein in the membrane matrix during electron transfer when phosphoryl acceptor is not available.

Cellular death in the postharvest and postmortem cell

The progression of biochemical events in plant and animal cells is basically a manifestation of gene expression (ontogenic development) or a response to injury or stress. Senescence or cellular death in detached fruits and vegetables appears to be a consequence of developmental expression. Gene expression is necessary for the normal course of fruit ripening and senescence in plant tissues generally (Varner, 1961). The normal course of physiological breakdown in such tissues is a highly ordered process requiring a functional and integral metabolic machinery. What of the cells comprising animal tissues after slaughter? Gene dictation of cellular elimination is a vital element of organismal health and survival prior to slaughter. Cells are continually being replaced in many tissues; indeed, the nature of the tissue, age of

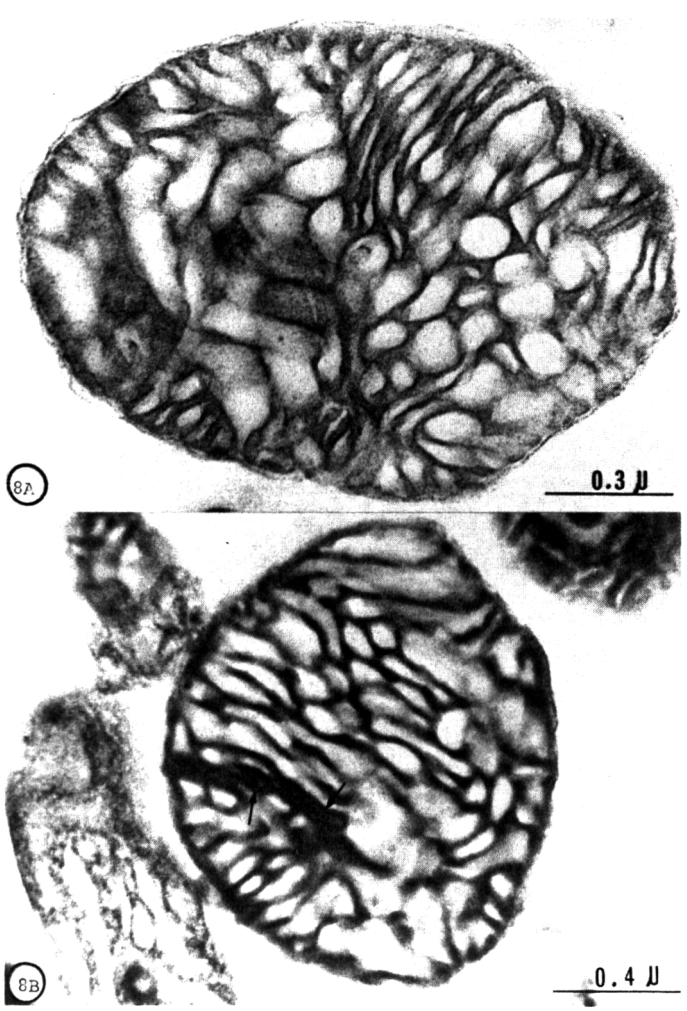

Fig. 8—Electron micrograph showing mitochondria fixed in nonenergized state and incubated for 1 hr at A, sucrose-buffer and B, sucrose-buffer and phospholipase C.

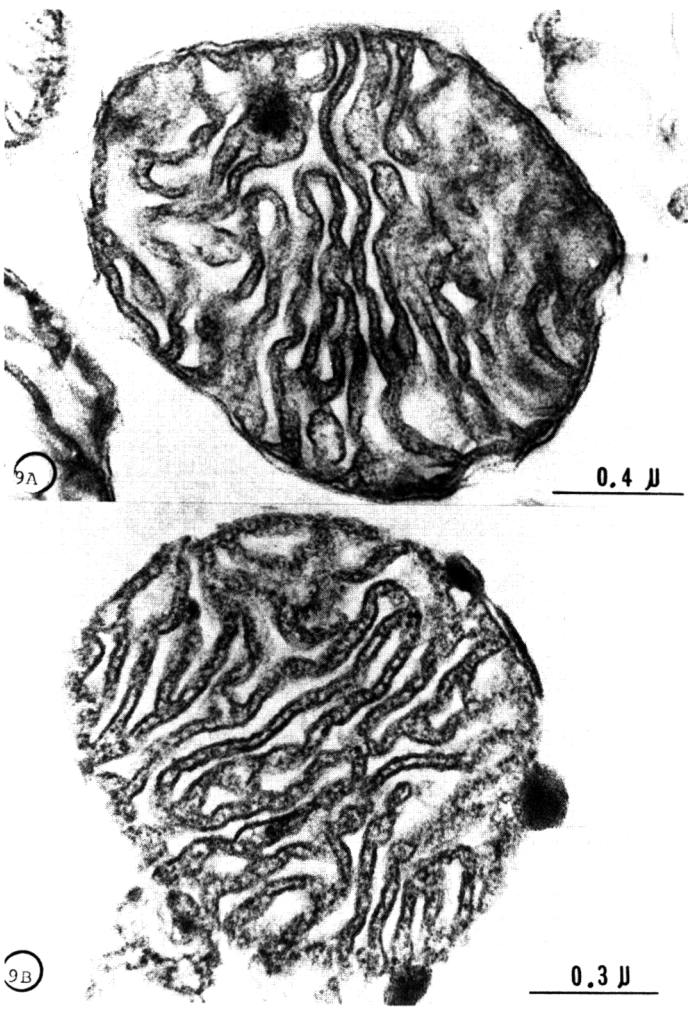

Fig. 9—Electron micrograph showing mitochondria fixed in energized state and incubated for 1 hr in A, sucrose-buffer and B, sucrose-buffer with phospholipase C.

the organism and the dietary and environmental considerations may determine the extent of gene-directed cell turnover. Accordingly, it might be expected that the levels of hydrolytic enzymes in a given animal tissue postmortem relates to such antemortem factors.

The direction of cellular metabolism may also be the result of a stress condition. Plant tissues maintained at improper storage conditions may undergo accelerated senescence as a result of injury or may fail to develop desirable attributes such as occur with the ripening of fruit. The cells of animal tissues such as excised muscle are under continual stress when the supply of O_2, nutrients, etc., cease, and the nervous and hormonal systems of control become inactive. It may well be that gene expression dictates events during the initial minutes of stress which ultimately influences cellular autolysis.

It would appear that biological membranes influence the course of cellular metabolism in tissues regardless of the initial trigger. Biological membranes are a primary locus of cellular disruption and death. The compartmentation of cells is altered and energy transduction capacity degenerates when membranes lose integrity. Developmental expression as well as wounding response are directly dependent on the balance of hormones, cofactors, salts, etc., and stress conditions, such as O_2 debt. These result in a rapid collapse in capacity to carry out active processes, notably oxidative phosphorylation. Biochemical evidence has pointed to this delicate relation for some time. Dislocation of mitochondrial oxidative phosphorylation has been linked to storage disorders in fruits and vegetables when exposed to ionizing irradiation (Romani and Yu, 1968), water vapor deficit (Haard and Hultin, 1968; Miller et al., 1971), and low temperature (Lenaz et al., 1968c; Raison et al., 1971).

Other modes of energy transduction are also dependent on the delicate balance between structure and function. The undesirable phenomenon of "thaw rigor" which occurs when certain muscles are rapidly frozen has been related to the malfunction of the sarcotubular membranes (Lawrie, 1968). In addition, differences in sarcotubular membranes, both qualitative and quantative, have been likened to the differences in post-mortem character of red and white meats. The transport functions of membranes have also been related to hormonal action (Haard, 1971b) and may provide an understanding of the controls on cellular death occurring in food tissues generally. The well-known effects of ethylene gas on fruit ripening (McGlassen, 1970) and other physiological processes, such as the accumulation of reducing sugars in potato tubers (Haard, 1971a), is likely to be associated with the permeability properties of membranes (Saikai, 1970).

Elucidation of biological membrane structures will lead to more basic knowledge of membrane functional properties and a better understanding of membrane-dependent biochemical controls which occur in tissues as a result of injury or developmental expression.

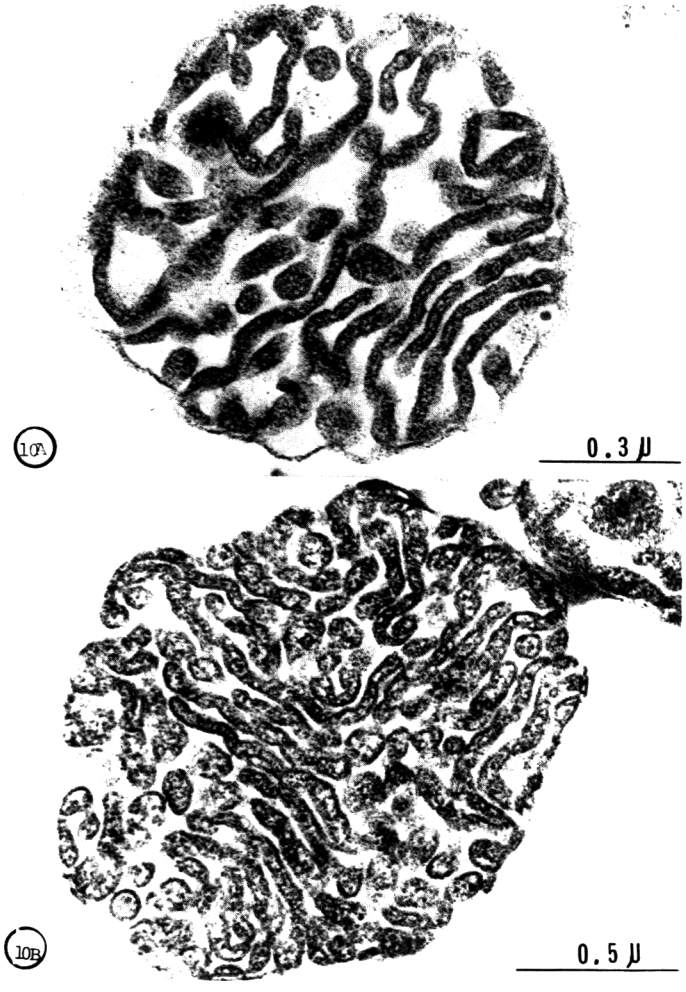

Fig. 10—Electron micrograph showing mitochondria fixed in energized twisted state and incubated for 1 hr in A, sucrose-buffer and B, sucrose-buffer with phospholipase C.

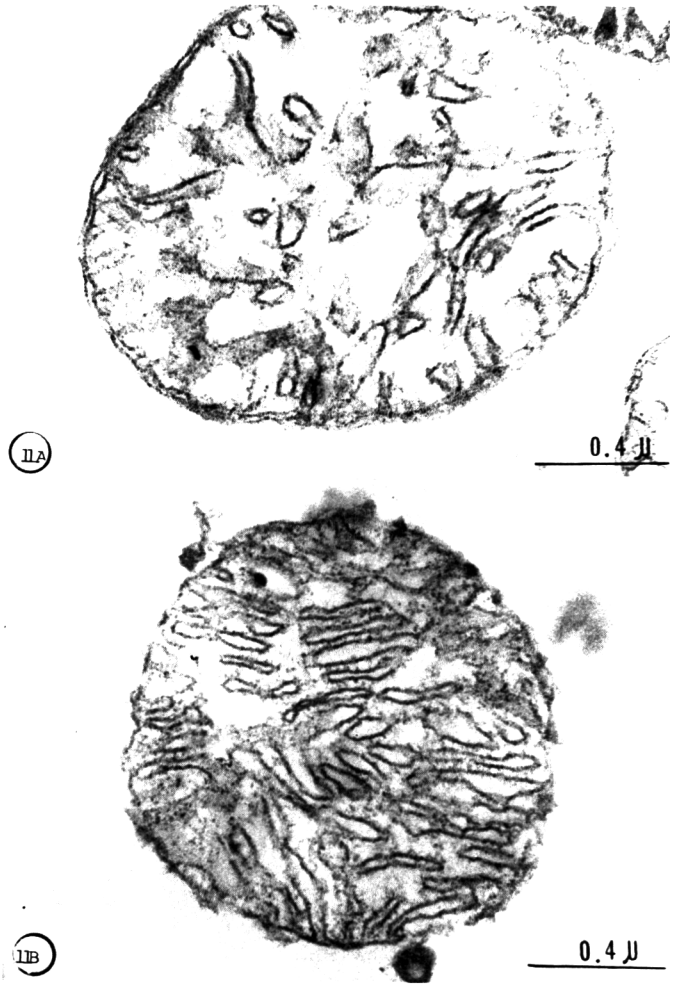

Fig. 11—Electron micrograph showing mitochondria fixed in the orthodox configuration and incubated for 1 hr in A, sucrose-buffer and B, sucrose-buffer with phospholipase C.

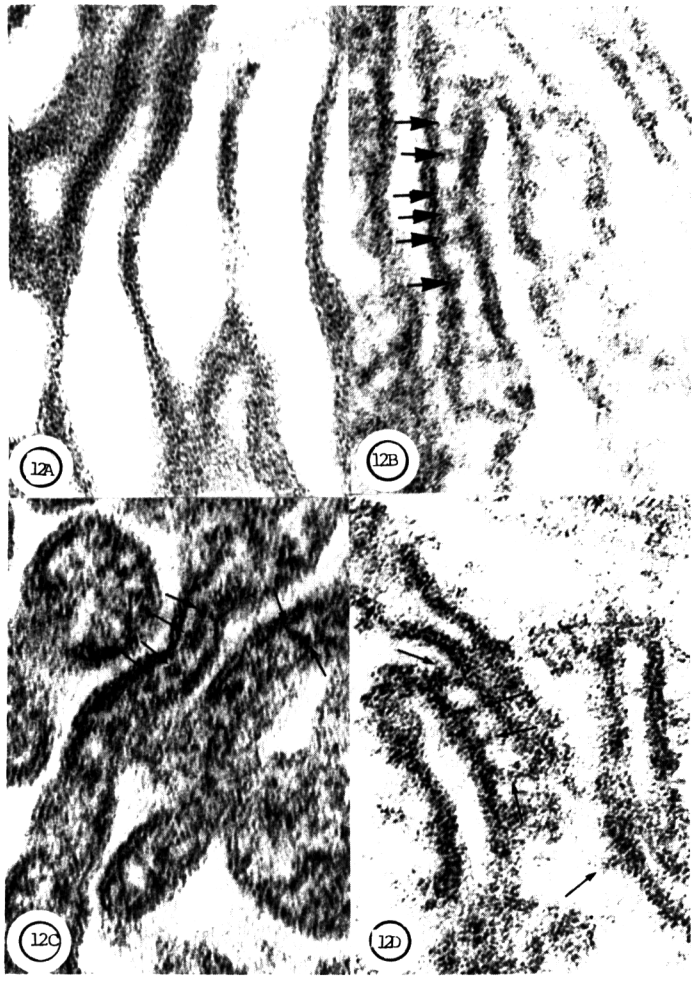

Fig. 12—Electron micrograph showing inner membrane of mitochondria after digestion with phospholipase C; A, nonenergized state; B, energized state; C, energized-twisted state; and D, orthodox state. Arrows indicate presence of 100Å particles.

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Electron microscopy was performed by Dr. Jerry Vail, Dept. of Microbiology, University of Guelph, Canada.

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SYMPOSIUM: Biochemical Control Systems

STRESS IN THE POSTHARVEST CELL: THE RESPONSE OF MITOCHONDRIA AND RIBOSOMES

INTRODUCTION

A DISTINGUISHING characteristic of the postharvest cell is its ability to complete a complex series of predetermined physiological transitions. This is of practical significance as most fruits and some vegetables are harvested when mature, but still unripe. A large number of changes, e.g., loss of some pigments and gain of others, softening, accumulation of sugars, synthesis of characteristic flavor components, must all take place in a reasonably normal manner if the final product is to be suitable for processing or marketing in fresh form.

It is a further characteristic of postharvest cells that these rather delicate physiological transitions must often take place under stress. Stress in multiple and recurrent forms is the dominant condition imposed on postharvest cells. Harvesting, severing the unripened fruit or vegetable from the parent plant or removing it from life supporting soil, results in an initial stress. Handling, storage, packaging and other stressful manipulations are often endured before the fruit or vegetable is either processed or consumed as fresh produce. How successfully the cells respond to these postharvest conditions may determine the quality and market value of the final product.

There have been periodic reviews dealing with many aspects of postharvest physiology and biochemistry (Biale, 1951, 1960; Hansen, 1966; Pentzer and Heinze, 1954; Ulrich, 1952, 1958). In this paper, emphasis will be placed on mitochondria and ribosomes, and the relationships of these intracellular organelles to the response of fruit or vegetable cells to postharvest stress. It will be a limited discussion drawing principally upon experiments conducted in our own laboratory with reference to only some of the many significant findings in other laboratories throughout the world.

LIFE PROCESSES IN THE POSTHARVEST FRUIT OR VEGETABLE, AND THEIR CONTROL

DURING THE 1920's and 30's, Kidd and West (1925) and Kidd (1935), among others, demonstrated the direct relationship between metabolic activity and the performance of postharvest tissues in storage or in transit. Their observations have been the basis for much of the technological progress in the field of postharvest physiology during the past 40 yr. Successful technological developments and a considerable extension of postharvest life has depended largely on the *external control* of metabolic rates, such as that achieved by lowered temperatures, modified atmospheres (Claypool and Allen, 1948), and removal or exposure to stimulatory hormones.

Advances in biochemistry and molecular biology over the past 10 yr now further the prospects of *internal control* and modulation of metabolism. Maturation and ripening are part of a continuous process composed of integrated sequential events. Control at points along this process could influence not only its overall rate but its very character with direct consequences on the nature and quality of the final product.

One form of internal control is derived from the restraint or enhancement of enzymic action as the result of allosteric effects, feedback or feedforward mechanisms, and the existance of multiple forms of enzymes. Other papers in this series discuss these aspects. Another form of internal control is implicit in the expression of genetic traits, such as those that may predetermine how quickly a tissue will ripen, how early it can be harvested, or how well it performs under given storage conditions. Molecular reactions whereby genetic traits coded by DNA are *transcribed* into RNA and thereafter *translated* into active proteins have been reasonably well defined, particularly in microbial organisms. The control of protein synthesis at these points of transcription and translation is the subject of much current biochemical research.

Protein synthesis per se has been of long standing interest to postharvest physiologists, as noted in the early work of Hulme (1954) and in subsequent studies as those by Richmond and Biale (1966), Romani and Fisher (1966), Frenkel et al. (1968) and Brady et al. (1970). Evidence indicates that the rates and direction of protein synthesis will affect the physiological transitions important to postharvest tissues. It should be noted, however, that protein synthesis requires energy and functioning assembly sites. Moreover, since all harvested fruit or vegetables are now isolated and self-contained organs, all energy must be derived internally. Herein lies the basis for a third form of internal control, that dependent on the status of energy sources and protein assembly sites within the cell, or more specifically, the status of the mitochondrial and ribosomal systems.

EXPERIMENTATION WITH MITOCHONDRIA AND RIBOSOMES

A FEW GENERAL comments may be helpful in visualizing the nature of research with intracellular organelles. Mitochondria are spherical or slightly oblong, seldom exceeding 2–3 microns (ca. 0.0001 in.) in diameter. Ribosomes are considerably smaller, about 1/100th the size of mitochondria. In a typical plant cell these organelles are found in the thin layer of cytoplasm compressed between a rigid cell wall and a vacuole replete with acids and phenolic substances deadly to the very sensitive organelles.

To "isolate" mitochondria, the cell walls are crushed by a physical means in the presence of a buffer, sucrose, and protective agents. Unbroken cells and fine debris remaining in the homogenate after passage through cheesecloth are removed by centrifugation at ca. 2,000 × G. Another centrifugation of the supernatant fraction at 10,000 × G conveniently packs the mitochondria in a pellet at the bottom of the tube. To "wash" the organelles, the pellet is resuspended in buffered medium and the sequence of low and high speed centrifugation repeated once again. Thus, after having been expelled from the protective cytoplasm, exposed to acids and phenolics, and compressed with forces several thousand times gravity, the mitochondria are expected once again to respire, consume oxygen, extract energy from metabolites, and conserve it in the form of energy rich adenosine triphosphate (ATP).

Analogous methods of differential centrifugation are utilized in the isolation of ribosomes. Since ribosomes are not subject to osmotic shock, maceration is done in the frozen state to achieve further protection from enzymic attack. Debris, including mitochondria, are removed from the homogenate by centrifugation

at about 30,000 x G and the ribosomes and polysomes then pelleted at forces of 105,000 x G or higher.

Reference to specific aspects of these methodologies are given later. The general observation, however, is that isolated organelles have undergone harsh treatment and are ultimately examined in an unnatural milieu. Organelle behavior in vitro may be thus quite diverse from their performance in the cell. There is thus an overriding need, as emphasized by Hall and Palmer (1969), to understand the interrelationship between organelles, cytoplasm, and overall cell function. In the application of intracellular research to postharvest physiology there is the further need to understand the response of intracellular organelles to the stress imposed on postharvest cells.

Mitochondria in postharvest tissues

In the pioneering work of Millerd et al. (1953), the climacteric rise in avocados was attributed to an uncoupling of oxidative-phosphorylation resulting in a "runaway" or nonpurposeful increase in metabolism. On the other hand, Pearson and Robertson (1954) suggested that increased protein synthesis during ripening called for additional energy resulting in a purposeful increase in metabolism and the climacteric rise.

Utilizing the isolation techniques then available it appeared that mitochondria degenerated with progressive ripening (Romani and Biale, 1957). However, a subsequent refinement of techniques (Lance et al., 1966) permitted the observation that mitochondria equally active and equally capable of conserving energy could be isolated from both pre- and postclimacteric fruit. With further improvements e.g., use of polyvinylpyrrolidone (PVP) (Hulme et al., 1964) and careful pH control during maceration (Ku et al., 1968; Padwal-Desai et al., 1969; Romani et al., 1969) active mitochondria were also isolated from such high acid tissues as tomatoes, pears and apples. In most instances, the mitochondria were equally active whether the parent tissues were ripe or unripe.

Thus, the past 15 yr of mitochondrial research has largely substantiated the early postulates by Hulme (1954) that ripening is a positive, energy-requiring process. Perhaps such a finding was to be expected. If a tissue is to progress successfully through a multitide of physiological changes in order to mature and ripen, abundant energy and functional mitochondria are prime requisites.

Is one to conclude that there are no differences in the mitochondria of unripened and ripened cells? Results of experiments discussed below indicate that a difference does indeed exists, not that normally sought, but one perhaps more critical to the life processes in postharvest tissues.

Stress, compensation and aging. Further investigation of the role of mitochondria in postharvest cells was guided by the analogy often drawn between ripening and aging, and by the concept of 'compensatory action' proposed by Samis (1966). The latter is based on the premise that a cell cannot continue its normal life processes and begins to age and degenerate when it can no longer compensate for stress. As discussed above, stress is a condition often met by the postharvest cell.

An essential to further research was a convenient source of uniformly distributed intracellular stress. One was readily apparent in radiation experiments then underway. Ionizing irradiation stresses the postharvest cell, as is obvious from the increase in respiratory activity (Fig. 1) that occurs immediately upon exposure to radiation (Romani, 1964). The rise in respiration also indicates that mitochondria are involved.

Because of its physical nature, radiation has special attributes as a stress tool. Mitochondria are extremely small but radiation effects are molecular and hence smaller yet by many orders of magnitude. It has been calculated (Romani, 1966) that 100 Krad, a radiation dose reasonably well tolerated by the fruit tissue, will result in approximately one million ionizing events in each mitochondrion. One can be assured that each and every mitochondrion within an irradiated fruit or vegetable will have been equally stressed, i.e., equally damaged at the molecular level. Moreover, gamma radiation of the energy obtained from Co^{60} has the potential to penetrate whole fruits thus permitting uniform stress in all cells (Romani, 1967).

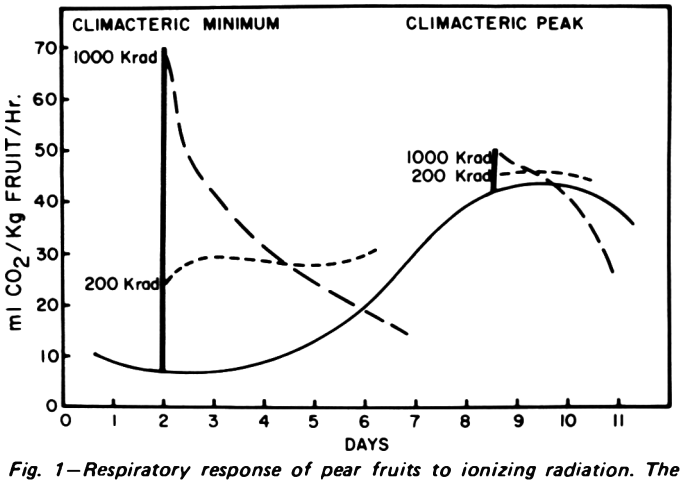

Fig. 1—Respiratory response of pear fruits to ionizing radiation. The intensity of the response during exposure (heavy bar) is dependent on both radiation dose and physiological (climacteric) state of the fruit. With time after exposure to 200 Krad the respiratory trend recovers to a quasi normal pattern; after 1000 Krad there is no recovery leading to a rapid decline in respiratory activity and cell death. Respiration: Normal (——), during exposure (▬▬), after 200 Krad (---), after 1000 Krad (— — —). Taken from Romani (1964), and Romani et al. (1968).

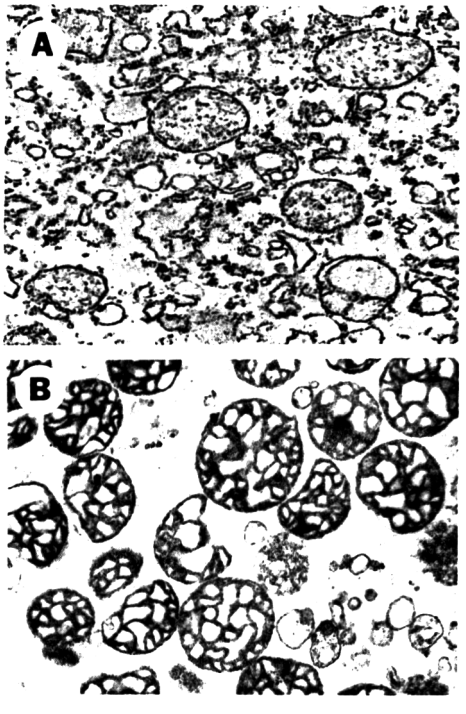

Fig. 2—Mitochrondria isolated from pear (A) and avocado (B) fruit. The avocado mitochondria are from a 'washed' preparation and are in the energized state (state 3). The pear mitochondria were not recentrifuged, i.e., 'washed', and are accompanied by numerous other vesicles and debris. (Magnification: ≃ 16,400X.)

In the experiments discussed below irradiations were performed in a cobalt-60 gamma irradiator calibrated with ferrous sulfate dosimetry (Romani et al., 1963).

Mitochondrial response in stressed fruit cells. The following data were gathered in an effort to answer the question: "Do fruit (or vegetable) mitochondria have the capacity to compensate for stress (damage), and if so, is there a change in this capacity during the postharvest phase?"

Mitochondria isolated from pear and avocado fruit are shown in Figure 2. Methods for the isolation of mitochondria have been given in detail elsewhere (Lance et al., 1966; Romani et al., 1969).

The efficiency of isolated mitochondria is assessed by monitoring their consumption of oxygen and noting the changes in rate that occur following stoichiometric additions of ADP. A schematic representation of such an assay is shown in Figure 3. The base rate (state 4), increases to a higher rate (state 3) upon the addition of ADP. As defined by Chance and Williams (1955), the respiratory rate in the presence of ADP divided by that in the absence of ADP, i.e., state 3/state 4, gives the respiratory control ratio (RCR). Intact, well integrated, highly functional mitochondria have an RCR of 3, 4 or higher, signifying that they oxidize maximally only if useful work can be produced, that is, if energy can be conserved by phosphorylating the added ADP. The respiration rate in coupled mitochondria is constrained by the absence of a phosphate acceptor. However, if injured or otherwise rendered less efficient, the oxidative and phosphorylative activities become uncoupled, respiration is no longer restrained, and the mitochondria will oxidize maximally even in the absence of ADP thus utilizing cellular food (substrate) without producing useful energy.

It has been shown (Romani and Yu, 1968) large doses of ionizing radiation, in the order of 1,000 Krad, are required to injure and uncouple mitochondria in intact fruit. This is evidenced by the performance of mitochondria (second trace in Fig. 3) isolated immediately after exposure of the fruit to radiation. However, if isolation of the organelles is delayed for 24–48 hr after the irradiation of the fruit, the mitochondria are once again coupled signifying that compensation has taken place and radiation damage has been repaired (third trace, Fig. 3).

Split doses, a classical technique in radiobiology, can be used to substantiate the occurence of recovery and radiation repair. Data from an experiment utilizing this technique are summarized in Figure 4. As shown by the open bars, a 500 Krad dose administered to the whole fruit caused a partial loss in respiratory control whereas 1000 Krad resulted in almost completely uncoupling the mitochondria. However, if the dose is split into three 500 Krad dose increments administered at 24-hr intervals, the mitochondria extracted *after* the third dose increment are still capable of conserving energy, i.e., still "coupled," even though they have been exposed to a total dose of 1500 Krad. One can conclude therefore, that recovery and repair must occur in the 24-hr periods between dose increments.

We now return to the question posed at the onset: Are there essential differences in mitochondria of progressively older, more ripened postharvest cells? An answer in terms of mitochondrial response to stress is evident in a comparison of the open and closed bars in Figure 4. When mitochondria are isolated immediately after exposure of pear fruit to 500 or 1000 Krad doses (first 3 sets of bars) the radiation dependent decrease in respiratory control is nearly similar whether the mitochondria are part of an unripened (pre-climacteric) or ripened (peak-climacteric) cell. These results merely substantiate prior evidence that the immediate functional qualities of the mitochondria do not change with ripening. However, when split doses are employed (4th and 5th set of bars) the stage of ripeness has a profound effect. Whereas mitochondria in preclimacteric fruit recover from damage in the interval between dose increments and tolerate re-

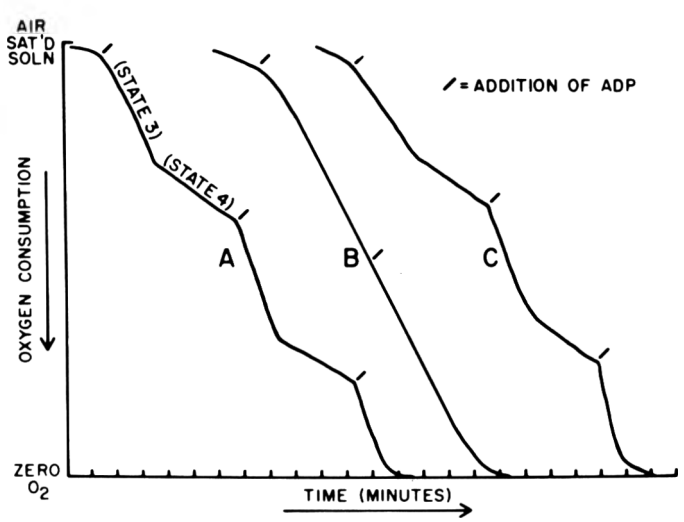

Fig. 3—Polarographic measurement of oxygen consumption by mitochondria. Respiratory control is evidenced by the increased respiratory rates (state 3) following stoichiometric additions of ADP, and return to base rate (state 4) when the ADP supply is exhausted. Uncoupling of mitochondria and loss of respiratory control (B) occur as a result of exposing the intact fruit to ionizing radiation. Respiratory control is recovered (C) if the isolation of mitochondria from irradiated fruit is delayed 24 hr. For experimental details see Romani et al. (1968).

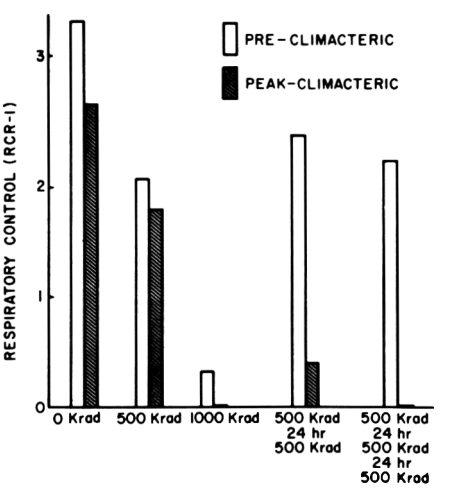

Fig. 4—Mitochondrial recovery of respiratory control in vivo as revealed by split doses and as affected by climacteric state of the pear fruit when exposed to ionizing radiation. Open bars: Respiratory control of mitochondria isolated from fruits that were irradiated in the unripe, preclimacteric stage. Cross-hatched bars: Respiratory control of mitochondria from ripening pear fruits that were irradiated when near the climacteric peak. Abscissa: Radiation dose administered either in one exposure or in 500 Krad increments with 24 hr intervening. The organelles were isolated and assayed for respiratory control immediately after the final dose. Ordinate: Respiratory control. In the absence of respiratory control, the respiration rate in state 3 equals that in state 4 and the RCR (state 3/state 4) = 1. Taken from Romani et al. (1968).

peated 500 Krad doses of ionizing radiation, the mitochondria in ripened fruit do not. A connection between this finding and the postharvest life of fruit or vegetable products is implicit. The capacity of ripening cells to withstand stress, to repair injury and return to a normal pattern of development, may be severely limited by the stress response of their energy-producing machinery.

Ribosomes in postharvest tissues

A dynamic ribosomal system is required to assemble the proteins and enzymes called for in the ripening processes and in the stress corrections essential to the life of postharvest tissues. Thus, ribosomes, along with mitochondria, constitute an organelle system with a "control" function in the postharvest cell.

In translating the genetic message into protein the ribosomes attach to, and move sequentially along the messenger RNA (m-RNA). It is generally held that the rates of protein synthesis are roughly proportional to the number of ribosomes; one (monomer), two (dimer), or many (polymer), that are attached to a given m-RNA. A decrease in polymers has been observed in aging microbial systems (Li and Umbrit, 1966), ripening seeds (Sturani and Cocucci, 1965), and senescent leaves (Strivastava and Arglebe, 1967). The increase in polymers reported in so-called "aging" tissue slices (Leaver and Key, 1967) is likely an injury or stress response to the slicing and not a true aging phenomenon.

Ribosomes were isolated from fruit tissue by Ku and Romani in 1966. To circumvent the harmful effects of phenolics, vacuolar acids, and degradative enzymes, the tissue along with buffer and protective agents, are macerated under liquid nitrogen and the pH carefully monitored and adjusted as the homogenate begins to thaw. After differential centrifugation and sedimentation at 105,000 × G, the ribosomes are separated into the monomers, dimers and higher polymers on sucrose density gradients.

Electron micrographs of pear and fig ribosomes are shown in Figure 5.

Sucrose density gradient separation of the organelles from pear fruit revealed a large proportion of single ribosomes (monomers) with smaller amounts of dimers, trimer, and higher polymers (Fig. 6). The density gradient profiles of ribosomes isolated from postharvest cells changed very little during ripening and the climacteric sequence with the exception of a small decrease in the polymer fraction (Ku and Romani, 1970). This may be seen by comparing the optical density profiles in Figure 6A and B. A small decrease in number of polymers was also noted in ripening figs (Marei and Romani, 1971).

It should be noted that the methods of isolation, especially from plant tissues characteristically high in nucleases, severely limit the reliance on quantitative estimates. Rates of ribosomal synthesis and renewal provide a more reliable index to the status of the ribosomal system in postharvest cells. As shown by the rates of ^{14}C-adenine incorporation into ribosomal RNA (dashed lines, Fig. 6AB), the capacity of the postharvest cell to synthesize ribosomes is markedly affected by fruit age. Freshly harvested fruit tissues support a continuing ribosomal synthesis. In contrast, cells from similar tissue but now ripened and at the climacteric peak, demonstrate little or no ribosomal synthesis. One can surmise that these cells have reached their final point of development and no longer required the formation of new machinery for protein synthesis.

The transition from a dynamic, constantly renewed intracellular condition to one that is relatively static is again dramatized by the use of radiation stress. Although much smaller than mitochondria, ribosomes are still sufficiently large relative to ionizing events that one can be assured of uniform damage and stress at the radiation doses used in our studies. In the unripened preclimacteric cell, radiation doses of 250 Krad stimulate ribosomal synthesis. In contrast, a similar dose applied to ripened fruit elicits no response (Ku and Romani, 1970). These effects of radiation on rates of ribosomal synthesis are summarized in Figure 7. The

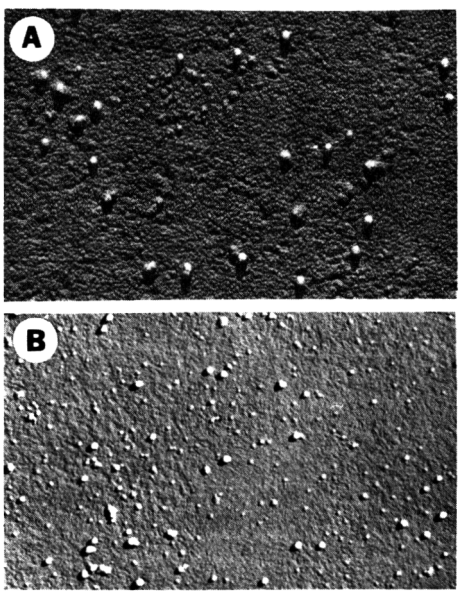

Fig. 5—Ribosomes from pear (A) and Fig (B) fruits. Whole ribosomes are approximately 200 angstroms in diameter. The smaller particles, more numerous in B, are 40 and 60 S subunits. Magnification: A ≅ 32,600X B ≅ 22,200X.

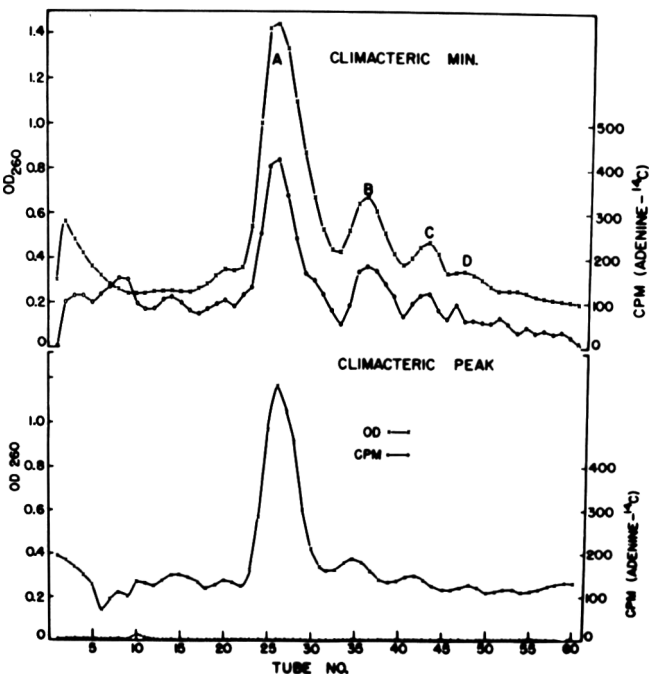

Fig. 6—Ribosomal population isolated from pear fruits at the climacteric minimum (upper graph) and climacteric peak (lower graph). Solid lines represent the quantitative distribution of monomers (A), dimers (B), trimers (C), and higher polymers (D and beyond). Dashed lines represent the incorporation of ^{14}C-adenine (during a 6-hr period) e.g., rate of ribosomal synthesis in vivo. (For details see Ku and Romani, 1970.)

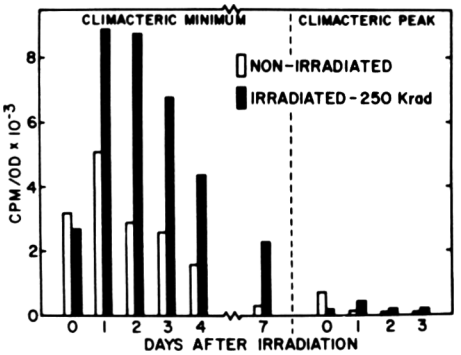

Fig. 7—Rates of ribosomal synthesis in normal and irradiated pear fruits. The fruits were irradiated at either the climacteric minimum (left graph) or the climacteric peak (right graph). (For details see Ku and Romani, 1970.)

findings point to a critical transition in the ribosomal system of the postharvest cell, from a state capable of recovery to one that is no longer capable of repairing injury.

Important to our present study is the fact that radiation doses in the order of 1000 Krad inactivate a large percentage of the ribosomes (Kucan, 1966). Hence, if the irradiated fruit or plant cell is to remain viable it must replace or repair the injured ribosomes, that is, compensate for stress. In this context it is of interest that the increased ribosomal synthesis resulting from 250 Krad of ionizing radiation persisted for several days (Fig. 7). It has also been well demonstrated that irradiated pears remain atypically firm and green for an extended period of time. Since many of the ripening changes have been badly distorted by radiation, the final product is by no means a desirable food commodity but ripening as such has been deterred and the life of fruit, though highly atypical, has been extended.

Thus, forms of stress, e.g., radiation, exposure to high temperatures, cold injury, may actually delay ripening in some tissues. The major deterrant to a successful adaption of such potentially useful phenomena is the fact that a normal pattern of ripening is not resumed, or put differently, the stress is not compensated.

SUMMARY & CONCLUSIONS

A LOSS OF compensatory potential in the mitochondrial system means that cellular responses requiring extra energy can no longer take place beyond some point in the postharvest life roughly coincident with the climacteric peak. While it is true that in most fruit (e.g., pears, bananas, avocados) ripening and associated developmental processes are essentially terminated at the climacteric peak a need to respond to the stress of handling, processing and marketing often still remains. The greater the tissue capacity for response and repair of damage and the longer this capacity can be maintained, the more numerous are the options for the maximum and most efficient utilization of fruit and vegetable products.

Similar considerations apply to the ribosomal system. Only if the machinery for the manufacture of new enzymes and other proteins remains vital can the transitions associated with ripening, or with a successful response to postharvest stress, take place.

In summary, the essential "life" properties of fresh produce suggest that the functions of mitochondria and ribosomes are significantly related to the practical problems faced by the fruit and vegetable industries. The technological application of this relationship remains speculative. Whether tests for mitochondrial vigor, as used in the selection of hybrids (McDaniel, 1971; Sarkissian and Srivastava, 1969) can also be used in assessing the storage and ripening characteristics of fruits and vegetables remains to be seen. However, a greater understanding of the requirements for energy and for protein synthesis in postharvest fruit and vegetable tissues should lead to an improved technology and help to make optimally processed or distributed fresh fruits and vegetables more widely available.

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SYMPOSIUM: Biochemical Control Systems
POSTHARVEST FRUIT PRESERVATION: PROTEIN SYNTHESIS, RIPENING AND SENESCENCE

INTRODUCTION

A REVIEW of the role of enzymes in fruit ripening was recently published (Dilley, 1970). Summarized here are some essential features of protein synthesis in relation to fruit ripening.

PHYSIOLOGICAL AND BIOCHEMICAL CHANGES OF FRUIT RIPENING

MANY OF the physiological and biochemical changes of fruit ripening have been extensively studied over the past 40 yr. These studies have provided qualitative and quantitative information on several important constituents undergoing change during ripening and senescence in relation to a wide range of postharvest environmental conditions. It has been only recently that attention has been focused on the relation of protein metabolism to fruit ripening. The recent emphasis in this area stems largely from the observation of Hulme (1948) that a net increase in protein N accompanied ripening of apple fruits. His observation has been verified in several other fruits and is particularly significant in that up until this time it was commonly assumed that the biochemical processes of ripening were exclusively catabolic in nature. During the past several years many anabolic processes have been elucidated during ripening such as the synthesis of pigments, lipids, nucleic acids and, in fact, a multitude of other syntheses requiring the expenditure of energy during a period in the life of fruit which formerly was characterized as one of general cellular deterioration. Reconciling these observations with the accelerated respiration rate of fruit during ripening, Biale (1964) proposed that the onset of the respiratory climacteric signaled the approach of senescence in fruits. Accordingly, most investigators in the field of postharvest physiology currently view ripening as a dynamic and orderly process initiated by ethylene, the ripening hormone. While ripening normally occurs in fruit after growth ceases, it is a natural consequence of developmental changes during growth. A fruit is physiologically "ready" to ripen when it has gained the required potential. This in turn is governed by the interaction of the genetic makeup with environmental and chemical stimuli during growth and development. It is implied from this that the specific biochemical reactions involved in fruit metabolism undergo change with fruit development. Specific enzyme activities may be expected to increase or decrease in response to a number of factors which may regulate enzyme content or activity at specific stages of fruit development.

Protein Synthesis

It is logical to assume that the increase in protein N over the course of fruit ripening may represent enzymes which may be directly involved in ripening processes. The activity of many enzymes has been found to change in fruits as they ripen. An increase in apple fruit malic enzyme activity during ripening was demonstrated by Dilley (1962) and Hulme and Wooltorton (1962). The enzyme is considered to contribute a portion of the CO_2 evolved during the respiratory climacteric from the decarboxylation of malic acid. At that time there was insufficient evidence to conclude that the increase in activity was the result of induced enzyme synthesis. In fact, it has only been quite recently that evidence has been provided for induced synthesis of the enzyme in fruits (Frenkel et al., 1968).

Specific synthesis coupled with selective degradation of enzymes allows differentiated cells, such as those of a fully developed fruit, to alter metabolism in response to changes in their physical and chemical environment. Without capacity for selective degradation the cell would be able only to add new enzymes as they are needed and then only as long as the supply or synthesis of amino acids is maintained.

Several investigators have speculated that the increase in protein synthesis rate, the net increase in protein content and increased activity of several enzymes during the respiratory climacteric was the result of synthesis of enzymes involved in the various ripening reactions. An increase in enzyme activity is not evidence of protein synthesis since this can result from activation of an already existing protein, disappearance of an inhibitor of the enzyme or an artifact arising from differential extractability from tissue of markedly different physical or chemical nature. An increase in protein content is not proof of an increase in enzyme synthesis rate since this may result from a decrease in degradation rate. Furthermore, a net increase in protein content is not necessary. In fact, the total protein content may decrease while the synthesis rate of certain enzymes increases. An increase in rate of amino acid incorporation into protein is a measure of the dynamics of the system but does not imply that specific protein synthesis is taking place or even that the proteins being synthesized are directly involved in the ripening reactions. New ribonucleic acid synthesis, particularly m-RNA, immediately preceding or accompanying protein synthesis is not required if the RNA involved is stable. Thus, inhibition of RNA synthesis does not necessarily inhibit synthesis of particular enzymes.

Relationships to fruit ripenings. To establish the relationship of protein synthesis to fruit ripening certain criteria must be met. It must be demonstrated that protein synthesis is essential and that the proteins synthesized include those involved in the ripening reactions. This is important since an increased rate of protein synthesis may be only indicative of the increased metabolic rate of the tissue during ripening and that the cells are merely making more of the same enzymes which were present prior to the onset of ripening.

Evidence of amino acid incorporation into protein of avocado fruit at the early climacteric stage, but sharply falling thereafter, led Richmond and Biale (1966a) to propose induction of enzymes which catalyze the climacteric and final breakdown of the cell. Other investigations (Richmond and Biale, 1966b; Frenkel et al., 1968; and Brady et al., 1970) have quite conclusively shown that protein synthesis activity increases at the onset of ripening and that if synthesis is inhibited, ripening does not occur.

Davies and Cocking (1967) investigated protein synthesis in tomato fruit locule tissue and found two main phases of protein synthesis activity during development. The first phase occurred during the first 5 wk of development while the chlorophyll content per cell was increasing. The second phase was during the 5th and 6th week coinciding with the onset

of the respiratory climacteric after which both protein content per cell and respiration rate per unit protein declined. An earlier study by Davies and Cocking (1965) revealed an increase in alpha-amylase activity associated with the period of rapid starch hydrolysis in tomato fruits. During the climacteric in tomato fruits (which precedes marked visual ripening changes) they found that mitochondrial and "soluble" protein synthesis rates increased 7-fold while the chloroplast protein synthesis rate remained about steady.

MSU's research

The following discussion summarizes our research on ripening in relation to protein synthesis. Our investigations revealed that protein synthesis was required for normal ripening of Bartlett pears and that the proteins synthesized early in the ripening process were, in fact, enzymes required for ripening (Frenkel et al., 1968). These studies were conducted with intact pear fruits employing techniques (Frenkel et al., 1969) essential to demonstrate synthesis of specific proteins.

Reduced atmospheric pressure. If ripening was delayed by subjecting pear fruits to reduced atmospheric pressure, a technique employed by Burg and Burg (1965) to demonstrate the dependency of ethylene for ripening, protein synthesis was likewise delayed. This demonstrates that ethylene is required to induce ripening (softening) and that one of the processes of ripening is protein synthesis but does not prove the dependence of ripening on protein synthesis.

Treatment with cycloheximide. This question was answered by showing that ripening was inhibited when protein synthesis was blocked by treating pear fruits with cycloheximide and that ethylene did not induce ripening under this condition. Intact pear fruits at various ripening stages were infiltrated with various inhibitors and protein and nucleic acid precursors employing 0.3–0.5M mannitol as a carrier solution. This procedure provided a means to incorporate sufficient ^{14}C-phenylalanine into fruit tissue to establish incorporation into distinct proteins subsequently separated by acrylamide gel electrophoresis. Flesh softening, chlorophyll degradation, respiration and ethylene synthesis were not adversely affected by mannitol infiltration. Infiltration with water apparently destroyed the osmotic balance of the tissue and prevented normal ripening.

Cycloheximide at 10 μg/ml prevented ripening when administered to fruits at the early climacteric stage (unripe), but was progressively less effective at later ripening stages. Flesh softening, chlorophyll degradation and ethylene synthesis were severely inhibited by cycloheximide treatment of early climacteric fruit and supplemental ethylene treatment (1000 ppm) did not overcome this. The progressive diminution of cycloheximide inhibition of ripening when applied at progressive ripening stages suggests a causative relationship of protein synthesis to ripening. Thus, at the pre- or early-climacteric stage the proteins involved in the ripening reactions were either not present or were in limited supply and cycloheximide prevented their synthesis. When cycloheximide was applied to fruits which had begun to ripen (approximately 1/3 ripe) ripening proceeded at a reduced rate compared to the controls indicating that limited synthesis of the enzymes involved in ripening had already taken place. Cycloheximide treatment of fruits in a nearly ripe condition allowed fruits to ripen to the same extent as controls indicating that enzyme content was not limiting at this stage. A similar situation was found following treatment of preclimacteric fruits with Actinomycin-D, a potent inhibitor of RNA synthesis. Ethylene treatment did not reverse the complete inhibition of ripening imposed by Actinomycin-D.

The dependency of ripening on protein synthesis as judged by the effect of cycloheximide on the physical parameters of ripening was born out by data on ^{14}C-phenylalanine incorporation into the fruit proteins. Although cycloheximide inhibited ^{14}C-phenylalanine incorporation into the fruit proteins at all stages of ripening, ripening became less dependent on protein synthesis as ripening progressed. Thus the proteins synthesized early, but not late in ripening development, include the enzymes whose concentration is rate limiting. These data support the well-known fact that once ripening has been initiated the process cannot be reversed, but can only be retarded by low temperature and controlled atmosphere storage. This also may partially explain the decreasing effectiveness of gamma radiation in inhibiting pear ripening when applied to fruits at progressively later ripening stages (Maxie et al., 1966).

Double labeling experiment. A double labeling experiment conducted with partially ripened pears employing ^{3}H-uridine and ^{14}C-phenylalanine and Actinomycin-D (RNA synthesis inhibitor) or cycloheximide (protein synthesis inhibitor) revealed that (1) the fruits continued to ripen when RNA synthesis was inhibited but ripened more slowly than control fruit when protein synthesis was inhibited; (2) RNA synthesis was inhibited by both cycloheximide and Actinomycin-D; and (3) protein synthesis was greatly inhibited by cycloheximide but only slightly reduced by Actinomycin-D. Inhibiting RNA and protein synthesis in an unripe fruit completely inhibits ripening. Inhibiting RNA synthesis in a partially ripened fruit does not markedly alter the ripening rate indicating that the RNA required is synthesized quite early in the ripening process. Protein synthesis becomes less dependent upon newly synthesized RNA as ripening progresses. Thus a logical sequence of RNA and protein synthesis is evident during ripening but further studies would be helpful to elucidate the nature of the RNA requirement and the specific enzymes involved.

Synthesis of specific enzymes. Evidence of synthesis of specific proteins during ripening was obtained by administering ^{14}C-phenylalanine to pear fruits at various ripening stages, subsequently separating the proteins by acrylamide gel electrophoresis and determining the extent of amino acid incorporation into specific proteins. Amino acid incorporation into malic enzyme was studied in detail as its activity was found to increase markedly over the course of ripening. Capacity to synthesize specific proteins changed as ripening progressed. Certain proteins continued to be synthesized at the same rate while others increased or decreased.

Malic enzyme was found to be among the proteins showing an increase in rate of synthesis during the early stage of ripening. A rather clear indication of differential protein synthesis was observed for malic enzyme when enzyme synthesis was expressed as phenylalanine incorporation per enzyme unit at two stages of ripening. Phenylalanine incorporation into malic enzyme was approximately 3-fold greater in mid–as compared to early–climacteric pears.

Influence of ethylene. The point was made earlier that if pear fruits were maintained under conditions where ethylene was held below a physiologically active concentration ripening was delayed and so also was the normal increase in protein content. An experiment was conducted to examine the nature of proteins synthesized under the influence of ethylene (conditions conducive for ripening) compared to that in which ethylene was maintained below a physiologically active level in which case the fruits did not ripen. In the presence of sufficient ethylene to induce ripening ^{14}C-phenylalanine was incorporated into malic enzyme and other proteins at a greater rate as ripening progressed. However, when the ethylene level within the pears was reduced by ventilating them at reduced pressure, ripening did not take place and the fruit synthesized malic enzyme and other proteins at a low rate typical for unripe fruit.

CONCLUSIONS

IT IS CONCLUDED that normal ripening of pear fruit requires coordinated synthesis of enzymes involved in the various

ripening reactions. The synthesis of these enzymes take place, for the most part, during the early stage of ripening before marked physical changes become apparent in the tissue. Ethylene is required in physiologically active concentrations in order to initiate ripening. Without sufficient ethylene fruits which otherwise are ready to ripen continue to synthesize those proteins for which capacity already exists and the fruit remains unripe.

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SYMPOSIUM: Biochemical Control Systems
NATURAL ENZYME INHIBITORS IN PLANT TISSUES

IT HAS LONG been known that some plant proteins inhibit the action of certain mammalian enzymes. The first member of this group of biologically active proteins to be recognized was trypsin inhibitor from soybeans (Ham and Sandstedt, 1944). Since the discovery of soybean trypsin inhibitor, inhibitors of mammalian proteinases and glycosidases have been found in a variety of plant tissues. These proteins are often considered from a nutritional point of view and have been regarded as curiosities, but their roles in plants have not been identified. On the other hand, numerous reports in recent years have described systems in which plant enzymes are inhibited by endogenous proteins. This paper places special emphasis on the inhibitors of plant enzymes and shows that inhibition of enzymes by endogenous proteins represents a common regulatory mechanism in plants.

Plant inhibitors of mammalian enzymes

Of all the protein inhibitors, soybean trypsin inhibitor has received the most attention, and it has served as a model system for studies on protein inhibitor-enzyme interaction. Other trypsin inhibitors have been identified in numerous plant materials including lima beans (Tauber et al., 1949), kidney beans (Puztai, 1966), barley (Kirsi and Mikola, 1971), black-eyed peas (Ventura and Felko, 1966), corn (Hochstrasser et al., 1967), alfalfa (Ramirez and Mitchell, 1960), lettuce seeds (Shain and Mayer, 1965) and potatoes (Sohonie and Ambe, 1955). It is now known that multiple forms of trypsin inhibitors occur in some of these sources. Frattali and Steiner (1968) separated three trypsin inhibitors from commercial preparations of soybean inhibitor. Another inhibitor from soybeans, the Bowman-Birk inhibitor, has been found to be double-headed with dual, independent activity against both trypsin and chymotrypsin (Birk et al., 1967). Six different inhibitors have been isolated from lima beans, some of which inhibit both trypsin and chymotrypsin (Haynes and Feeney, 1967).

Other inhibitors of chymotrypsin are relatively common in plants. Balls and Ryan (1963) have crystallized a chymotrypsin inhibitor from potato juice. This particular protein inhibits fungal and bacterial proteinases, carboxypeptidase B, the proteolytic activity of trypsin, as well as, chymotrypsin (Ryan, 1966). It occurs in all parts of the potato plant except the xylem, flowers and seeds (Ryan et al., 1968). Ryan and Huisman (1967) found that this protein is synthesized in the leaves and subsequently translocated to the meristematic regions. Potatoes contain another chymotrypsin inhibitor which differs from inhibitor I in crystalline habit, composition, size, and reactivity (Ryan, 1966). A number of other plant tissues are known to contain inhibitory activity toward chymotrypsin including sweet potatoes, banana tubers, taro tubers and tomato roots (Ryan et al., 1968).

In contrast to the large number of known proteinase inhibitors in plants, only a few inhibitors of mammalian glycosidases have been identified. A protein in navy beans inhibits both trypsin and pancreatic amylase (Bowman, 1945). Kneen and Sandstedt (1946) identified inhibitors of pancreatic and salivary amylases in wheat, rye and sorghum. Thermolabile inhibitors of fungal pectinase have been detected in pears (Weurman, 1953) and unripe cucumbers (Bock et al., 1970).

The above examples of plant inhibitors of mammalian and microbial enzymes serve to illustrate the abundance and diversity of these proteins in plant tissues. What are the physiological functions of these proteins in their sources? Some workers feel that they represent protective mechanisms against microorganisms and insects. Evidence supporting this view has been recently presented by Albersheim and Anderson (1971). They extracted proteins from cell walls of red kidney bean hypocotyls, tomato stems, and suspension-cultured sycamore cells which completely inhibit the activity of polygalacturonases secreted by fungal plant pathogens. The proteins from the three sources are not identical because different levels are required to inhibit polygalacturonases from three species of pathogenic fungi. The differences in reactivities suggest that these inhibitors may participate in disease resistance.

On the other hand, interaction of many plant proteins with mammalian enzymes, for example, may be the result of a lack of complete specificity of the inhibitors for endogenous plant enzymes. A plant inhibitor may be reactive with not only its natural enzyme but also mammalian and microbial enzymes possessing structural similarities. At least some of the plant inhibitors detected with mammalian enzymes, therefore, may be involved in enzyme regulation in plants. Evidence has not been presented so far that soybean trypsin inhibitor inhibits soybean proteinases. Of course, there is no reason to restrict its possible role to inhibition of a proteinase because a different enzyme may actually be involved.

Inhibitors of plant proteinases

An example of an inhibitor effective for an endogenous plant proteinase is the system in barley. The seeds contain inhibitors for an *Aspergillus* proteinase, trypsin and a barley proteinase (Kirsi and Mikola, 1971). During germination, the inhibitor of barley proteinase is rapidly destroyed while the other two remain unchanged. In the case of lettuce seeds, it has been shown that the trypsin inhibitor is also reactive with an endogenous proteinase (Shain and Mayer, 1965). The mature seeds contain two proteinases and the trypsin inhibitor. Only one of the proteinases is inhibited by the inhibitor. The inhibitor decreases during germination of the seeds, resulting in increased activity of the inhibited enzyme. In both lettuce and barley, therefore, proteinase activity is increased during germination by a mechanism involving the destruction of an endogenous inhibitor.

Potato invertase inhibitor

There are now numerous examples of enzyme inhibitors in plants that have been detected in assays with endogenous enzymes rather than with mammalian enzymes. One of the first such inhibitors to be discovered, and probably the most intensively studied, is the invertase inhibitor in potatoes. The interest in the invertase system in potatoes stems from the economic importance of the relation between sugar accumulation in stored tubers and processing quality.

Early studies on the accumulation of reducing sugars in potatoes provided indirect evidence for the probable role of invertase but attempts to detect this enzyme were often unsuccessful. Schwimmer et al. (1961) found that cold-stored tubers contain a low level of invertase

activity. In a kinetic study of the weak activity in crude extracts, they observed deviation from linearity between invertase concentration and the rate of sucrose cleavage. Furthermore, the enzyme exhibited double pH optima, but this phenomenon disappeared when the crude extracts were diluted sufficiently. They correctly interpreted these results in terms of an endogenous inhibitor which accompanies the invertase in crude extracts.

A problem in studying protein inhibitor-enzyme systems is the separation of the two components. An assay for enzymatic activity in an extract known to contain an inhibitor as well as the enzyme yields only the amount of enzyme not combined with the inhibitor. Thus, a study of the changes in enzyme activity would not distinguish between variations in the level of the enzyme itself and fluctuations in the amount of the inhibitor. The ultimate goal in research on an inhibitor-enzyme system is the selective destruction of each component, permitting the measurement of the total levels of enzyme and inhibitor.

In my early studies on the invertase system in potatoes, (Pressey, 1966) I observed that vigorous blending of crude extracts increased the invertase activity. For a sample of cold-stored tubers, blending the crude extract at 37°C increased the activity 16-fold. Whereas the relationship between enzyme concentration and the rate of sucrose cleavage deviated from linearity for the unblended extract, a linear relationship was obtained after blending. Furthermore, the double pH optima typical for crude extracts was eliminated by the blending. It was clear that I had discovered a simple method for selectively inactivating the inhibitor in extracts of potatoes. This made it possible to measure basal invertase and total invertase in potatoes during development and storage. A method for selective inactivation of the enzyme was not devised and, therefore, only the amount of inhibitor exceeding the level of invertase was assayed in samples containing excess inhibitor.

During tuber development, the total invertase level is relatively low initially and decreases as the tubers mature (Pressey, 1969). Basal invertase activity does not appear during the entire growing season. The reason for the absence of free invertase is that the tubers contain an excess of invertase inhibitor. The excess inhibitor increases during tuber growth, and the level varies considerably with potato variety. Presumably while the potato is growing, sucrose is translocated to the tuber where it is converted to starch through the action of sucrose synthetase rather than invertase.

Mature tubers, therefore, contain a low level of invertase and a large excess of invertase inhibitor. During curing and storage at warm temperature, total invertase increases but the excess inhibitor persists (Pressey and Shaw, 1966). It is not until the tubers are placed in cold storage that dramatic changes in composition occur. In addition to the well-known accumulation of soluble sugars, total invertase increases sharply until the enzyme exceeds the inhibitor (Pressey and Shaw, 1966; Pressey, 1969). Reducing sugars are rapidly accumulated during this period of basal invertase activity. After several weeks the sugars reach a maximum and invertase begins to decrease until the basal activity is depleted and an excess of inhibitor develops. Furthermore, the changes in invertase, invertase inhibitor and sugars occur reversibly when the tubers are subjected to alternating cold and warm storage.

The results indicate a dynamic system consisting of an enzyme and a specific inhibitor. The enzyme concentration fluctuates, presumably by synthesis and destruction, above and below the level of the inhibitor in response to changes in temperature. It is not readily apparent why the enzyme is not simply synthesized and depleted to meet the cells requirements.

Potato invertase inhibitor has been highly purified and characterized (Pressey, 1967). It is a thermolabile protein with a molecular weight of about 17,000. Effectiveness of the inhibitor on potato invertase varies with pH. Inhibition is greatest at about pH 4.5, which is also the pH for optimal enzyme activity. Inhibition decreases on either side of pH 4.5, resulting in double optima of residual invertase activity at moderate levels of inhibitor. The inhibition is independent of substrate concentration and is identical for the substrates sucrose, raffinose and stachyose. The enzyme-inhibitor complex appears to be of low dissociability. The inhibitor does not inhibit yeast and Neurospora invertases. It is reactive with some but not all invertases from higher plants. The invertase activity in extracts of certain plants is partially inhibited, indicating that the inhibitor may be useful in identification of invertase isoenzymes.

Invertase inhibitors in other plants

Protein inhibitors of potato invertase have also been isolated from red beets, sugar beets and sweet potatoes (Pressey, 1968). The inhibitors from red beets and sugar beets are similar to the potato inhibitor in molecular weight, stability to heat and reactivity with invertases. The inhibitor from sweet potatoes has a slightly higher molecular weight and is considerably more stable to heat. It is more effective at higher pH than the other inhibitors, and it is the only one that inhibits invertase from sweet potato foliage. Kursanov et al., (1971) confirmed the presence of a natural inhibitor of invertase in sugar beet roots. They reported that this protein specifically inhibits invertase activity of leaf mesophyll, roots of seedlings and conducting bundle tissues of sugar beet plants.

Another inhibitor of invertase has been found in maize (Jaynes and Nelson, 1971). It occurs only in the endosperm and increases in concentration during seed development. The inhibitor appears to be a protein but is stable to boiling at pH 8. The molecular weight was estimated to be less than 75,000. Winkenbach and Matile (1970) reported that an inhibitor of invertase is present in senescing petals of sweet potato. This inhibitor is also a protein and its synthesis can be blocked by cycloheximide.

Enzyme inhibitors in ripening fruits

There have been several reports of the involvement of enzyme inhibitors in ripening of fruits. Reymond and Phaff (1965) observed that during purification of avocado polygalacturonase, the enzyme recovered exceeded the enzyme activity in the crude extract, indicating the presence of an inhibitor in the crude extract. They isolated the inhibitor from green avocados and demonstrated its reactivity with avocado polygalacturonase. The development of polygalacturonase activity in avocados during ripening may, therefore, involve the degradation of the inhibitor.

Mattoo et al. (1968) found that ripening of mangoes is accompanied by considerable increases in the oxidative enzymes catalase and peroxidase. Unripe mangoes contain a heat-labile and nondialyzable factor which inhibits both catalase and peroxidase isolated from ripe mangoes. The inhibitor disappears from mango slices treated with low levels of ethylene. This led Mattoo and Modi (1969) to conclude that ethylene promotes the inactivation of the enzyme inhibitor, and the removal of the inhibitor results in increased catalase and peroxidase activities.

The peroxidase and catalase inhibitor and an amylase inhibitor have been partially purfied (Mattoo and Modi, 1970). The purified inhibitors were separated into several components by chromatography on DEAE-cellulose. They found that some of the inhibitors were effective for both peroxidase and amylase, while others were specific for peroxidase or amylase. Furthermore, inhibitors isolated from bananas were effective for mango enzymes as well as banana enzymes.

Specificities of natural inhibitors

The natural enzyme inhibitors found in plants are, therefore, usually low molecular weight proteins. They are most frequently encountered in seeds and other storage tissues. Some of the inhibitors appear to be specific for individual

endogenous enzymes. However, many of the inhibitors are quite general in reactivity. Although there is no evidence that potato invertase inhibitor inhibits enzymes other than invertase, it is reactive with invertases from many other higher plants. Similarly, potato invertase is inhibited by invertase inhibitors from red beets, sugar beets and sweet potatoes. Some banana proteins inhibit both peroxidase and amylase from either bananas or mangoes. The common absence of specificity for a single enzyme and species-specificity for plant enzymes could explain the interaction of plant proteins with mammalian and microbial enzymes. As others have suggested, all of the proteolytic inhibitors detected in plants are probably involved in regulation of enzymes in their sources (Ambe and Sohonie, 1956). Definite roles have not been identified for most of these proteins, but Shain and Mayer (1965) have shown that the trypsin inhibitor in lettuce seeds is indeed an inhibitor of an endogenous proteinase.

Importance of natural inhibitors in foods

Some plant enzyme inhibitors have long been implicated in depressing the growth of nonruminants fed raw products such as soybean meal. However, Gertler et al. (1965) concluded that factors other than trypsin inhibitor may actually be responsible. Aside from the nutritional aspects of enzyme inhibitors, there are several ways that these proteins may be important in food processing. Fruit and vegetable quality may be maintained by storage conditions conducive to a favorably altered balance of inhibitors and degradative enzymes. Better storing varieties may be developed by selection for higher levels of enzyme inhibitors.

A possible application of enzyme inhibitors in food processing is the addition of isolated inhibitors to certain fresh products to control deterioration of quality. This approach would be most useful for highly perishable products such as refrigerated fresh peach slices (Heaton et al., 1969). At least three enzyme systems are responsible for the loss of quality. The enzymatic oxidation of polyphenols is an especially difficult reaction to control in not only peach slices but also other fruit and vegetable products. As more inhibitors are identified, enzymatic darkening and other reactions may be controlled by natural enzyme inhibitors rather than chemical preservatives that are currently used.

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SYMPOSIUM: Biochemical Control Systems
ENZYMIC ACTIVITY AND CONTROL AS RELATED TO SUBCELLULAR LOCALIZATION

INTRODUCTION

ENZYMES may be defined as protein catalysts. The unique 3-dimensional structure of proteins is responsible for the important properties of enzymes, viz., their high specificity and extremely efficient catalysis. In addition, the sensitivity of the conformation of enzymes to external influences such as temperature, pH, ions and metabolites, as well as that of protein concentration, opens the possibility of major modification of the catalytic activity of these agents, and many types of control have been observed. Some of these are listed in Table 1.

It is well-known that many enzymes may be inhibited either by their substrates or by the products of the reaction they catalyze. Enzymes may react with small molecules at sites other than the active site, such interactions leading to conformational changes in the enzyme thus effecting catalytic changes. These interactions are termed allosteric. Feedback inhibition is the inhibition of an initial enzyme in a metabolic sequence by an end-product of the sequence. Feed forward refers to the activation of a final enzyme in a sequence by an early metabolite of the sequence. Allosteric interactions can also occur through metabolites not related to the sequence with which the enzyme is involved, but rather to the physiological state of the cell, as reflected, for example, by the relative proportions of ATP, ADP and AMP.

Association-dissociation of an enzyme into subunits or polymers occurs as a function of enzyme concentration. The enzyme may have different kinetic properties in one form versus another, and thus its activity will be influenced by its degree of association (Agatova and Kurganov, 1966). Many enzymes are composed of subunits (Klotz, 1967) and thus can potentially exhibit this characteristic. If the subunits of an enzyme are dissimilar, the enzyme may exist in different forms, or isoenzymes. The properties of the isoenzymes will then depend on their subunit composition. If these isoenzymes can dissociate and reassociate in vivo, different isoenzymic forms may result. The process, which is termed "hybridization," could be controlled by environmental factors in the cell and could markedly affect enzymic activity.

The ability of the cell to synthesize and destroy its macromolecules according to its needs is well documented. An important example of how the synthesis and degradation of enzymes might be important in control functions is illustrated in an earlier paper of this series (Dilley, 1972).

One of the shortcomings of much of the work regarding enzymic control mechanisms is the fact that most of these mechanisms have been observed only in vitro (the synthesis and degradation of enzymes is an obvious exception), and

Table 1—Some enzymic control mechanisms

Product inhibition
Substrate inhibition
Allosteric interactions with small molecules
Feedback
Feed forward
Metabolic state
Association – dissociation
Isoenzymes (hybridization)
Synthesis – degradation

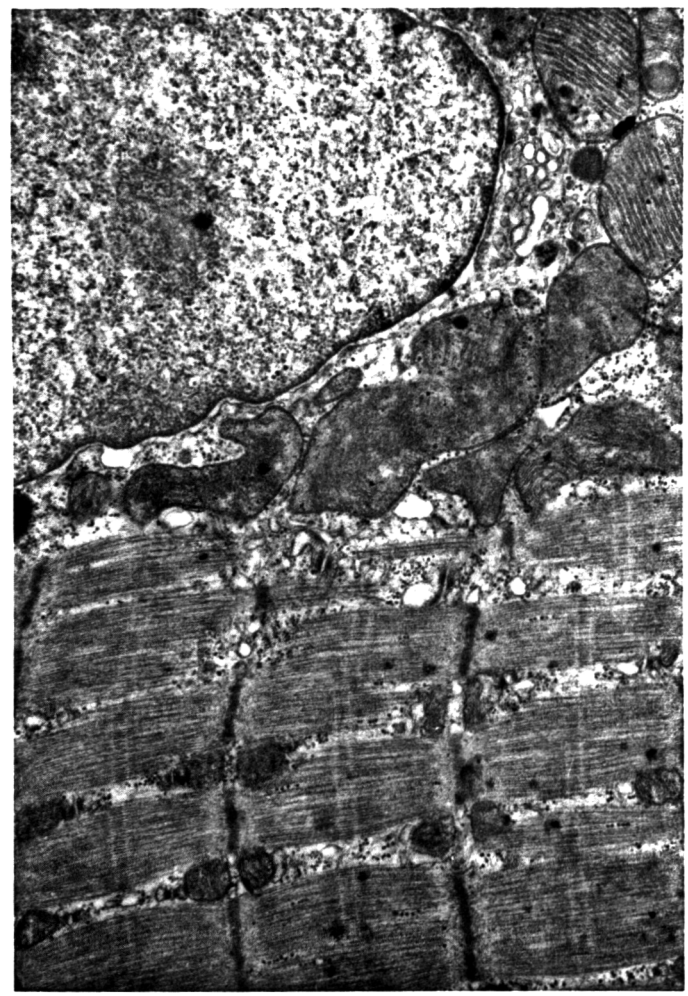

Fig. 1—Electron micrograph of skeletal muscle cell (40,000X). Courtesy of Dr. Dee Edington, Dept. of Exercise Science, Univ. of Massachusetts.

whether conditions in the cell would allow the same controls to operate in vivo is not known. In this paper I plan to discuss some possible ways in which the location of an enzyme in the cell might affect its functioning. The emphasis will be to illustrate general ways in which subcellular location can possibly affect enzymes, using examples where possible, and not to review all the work that has been performed on all enzymes.

In Figure 1 is illustrated the nature of the environment which will be discussed. This is a section through a muscle cell, but a similar degree of organization is observed in other cells. This is shown here to emphasize the point that any cell is comprised of large amounts of structural elements producing very large particulate surface areas which may interact with the enzymes.

Two major ways in which cellular location can affect enzymic activity are compartmentation and the reversible association of the enzyme with the particulate structures.

COMPARTMENTATION

COMPARTMENTATION can be defined as a restriction of movement of either enzyme or substrate due to the structural elements of the cell. It may be broken down into the following categories:

1. Association with subcellular membranes.
2. Isolation by membrane systems of the cell.
3. Organization into multiple systems with different subcellular locations.

Association with membranes

Some enzymes are associated with specific membraneous elements. For example, the enzymes of the electron transport chain of mitochondria are located in the inner membrane (Fig. 2) and function specifically there (Green and MacLennan, 1969). The physical juxtaposition of one enzyme to the others of the chain is of major significance in determining the efficiency of the sequence. Obviously, metabolites would have to be brought to the locale of the enzyme if it were to work on them.

Isolation by membranes

The concept of the cell as a bag of homogeneous ingredients is of historical interest only, but it may be appropriate to consider it as a bag full of little bags. Isolation of an enzyme within one of these little "bags" (subcellular compartment) could separate a soluble enzyme from its soluble substrate. As an example of how this type of compartmentation might work, let us consider a schematic diagram of the subcellular organelle, the mitochondrion (Fig. 2). It is comprised of outer and inner membranes, an intramembrane space and the matrix. Invaginations of the inner membrane are termed "cristae." If an enzyme is associated with this subcellular fraction, it might be able to utilize a substrate or be responsive to an inhibitor on only one side of one of the membranes because of permeability restrictions.

An example of this is mitochondrial glutaminase (Kovacevic et al., 1970) which catalyzes the deamination of glutamine. It reacts with glutamine only after penetration of the mitochondrial membrane by this substrate. The penetration of glutamine is rapid and does not control the rate of reaction. However, the enzyme is inhibited by the product of the reaction, glutamate, which does not readily pass through the membrane but builds up inside the mitochondrion and inhibits the reaction. The rate of this reaction is controlled in the intact mitochondrion, therefore, by the rate of removal of glutamate, either by a slow diffusion through the membrane or by its utilization by other enzymes.

Multiple enzymic systems

Another form of control which can be considered as compartmentation is when a cell has more than one system to carry out a particular reaction. For example, mitochondrial acyl-CoA synthetase, which catalyzes the reaction shown in Equation 1, consists of three separate systems. These vary as to location, i.e., whether they are "inside" or on the "outer surface" of the mitochondrion,

$$\text{ATP} + \text{fatty acid} + \text{CoA} = \text{AMP} + \text{P-P} + \text{acyl-CoA} \qquad \text{(Eq. 1)}$$

the source and type of energy (ATP or GTP), and response to activators and inhibitors (Rossi et al., 1968). Such a multiplicity of systems allows for a greater refinement in control of the reaction.

REVERSIBLE ASSOCIATION OF ENZYMES AND CELL PARTICULATES

Effect on kinetic properties of enzymes

The above considerations relate to the specific location of an enzyme in the cell, such location modifying the ability of the enzyme to interact with substrate or cofactor. The second major effect of the cellular milieu on an enzyme is the interaction of the enzyme with other components in the cell with subsequent modification of its properties. In our laboratory, we have shown that binding of chicken breast muscle lactate dehydrogenase (LDH) to subcellular fractions modifies both its velocity and the binding of its substrate pyruvate; on the basis of these modifications and the conditions under which they occur, we have suggested that reversible binding-solubilization of LDH in muscle may be an important control mechanism activating the enzyme during periods of stress (such as post-mortem conditions) and inhibiting it when it is advantageous to do so (Hultin et al., 1972).

We have also examined another glycolytic enzyme from chicken muscle, glyceraldehyde-3-phosphate dehydrogenase (Dagher, 1971). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can exist in equilibrium between the soluble and bound phases of homogenized muscle, as does LDH. In fact, GAPDH

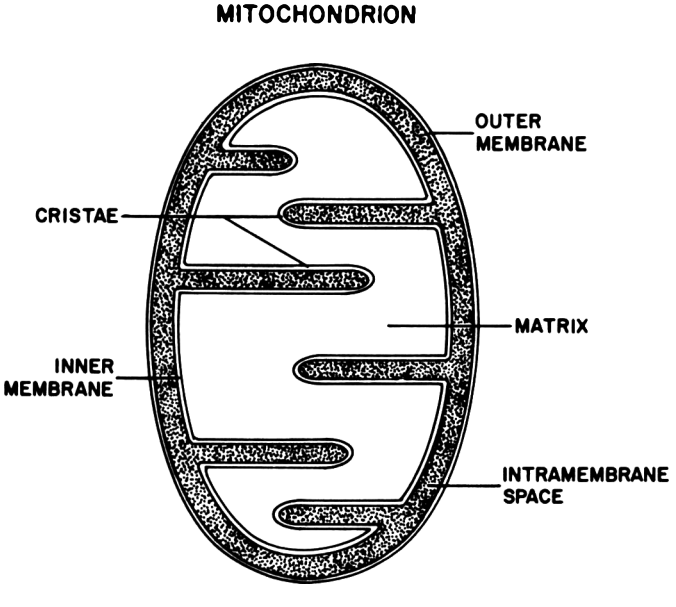

Fig. 2—Schematic diagram of the mitochondrion illustrating major morphological features.

responds in a manner similar to LDH towards many of the factors that solubilize LDH, e.g., the amount of enzyme in the soluble phase increases with increasing pH and with increasing ionic strength. GAPDH is also solubilized by its substrate, glyceraldehyde-3-phosphate (GAP) and other phosphorylated glycolytic intermediates, as well as NADH, but not NAD^+. Some kinetic parameters of GAPDH in the bound and soluble forms are shown in Table 2. These data were obtained with a stopped-flow technique at an enzyme concentration of 5 μM. Initial velocities were taken during the first 200 msec of the reaction. As with LDH, the maximal velocity obtained under conditions of substrate saturation was greater for the soluble enzyme compared with the bound. On the other hand, the K_M for GAP for the bound enzyme was about one-third that of the soluble, which means that the bound enzyme becomes relatively more efficient as the substrate concentration decreases. The inhibitor constant for NADH is low, and approximately equal, for both.

These data indicate that the soluble enzyme will function more efficiently at high concentrations of glyceraldehyde-3-phosphate. An increase in GAP does occur during glycolysis, as do other phosphorylated intermediates capable of solubilizing GAPDH (Lowry et al., 1964). Thus, the enzyme is solubilized under conditions where it is most efficient in the soluble form, viz., high GAP concentrations. The bound enzyme, on the other hand, appears to function better than the soluble at low concentrations of GAP, as indicated by its low K_M for this substrate, and would therefore work better at low glycolytic rates where substrate (GAP) concentration is low. In other words, reversible binding-solubilization of GAPDH in chicken muscle allows maximal efficiency of use of the enzyme under all conditions. This would not be possible with a strictly soluble enzyme.

It should not necessarily be expected that binding would consistently affect kinetic parameters of other enzymes in the same way. For example, work done by Arnold and Pette (1970) using aldolase with F-actin as the insoluble binding material showed an increase in V_{Max} and an increase in the K_M (fructose-1,6-diphosphate) on binding, which is just the opposite of what we have observed with GAPDH.

Effect on some control mechanisms

Another glycolytic enzyme, hexokinase, exists in part with the mitochondrial fraction of several types of cells (Crane and Sols, 1953; Rose and Warms, 1967; Spydevold and Borrebach, 1968). Solubilization of hexokinase (HK) is brought about by the same conditions and agents as that for the other glycolytic enzymes mentioned, i.e., pH, ionic strength, and certain cellular metabolites. It is solubilized by one of its substrates, ATP, and is very sensitive to solubilization by its product, glucose-6-phosphate (G-6-P). Properties of bound and soluble HK have been determined to a great extent, and illustrate the danger in extrapolating data from one system to another (Southard, 1970). Differences in V_{Max}, K_M and K_I values have shown no consistent patterns from one laboratory to another and from one tissue to another. Several factors could account for this. There are three HK isoenzymes which may vary with the state of metabolism of the animal; thus, different investigators may have studied different enzymes. Also, preparative techniques vary and give different degrees of integrity of the mitochondria. The integrity of the mitochondria could alter the ability of substrate or inhibitor to reach the site of action of a mitochondrial enzyme which in turn could affect the K_M or K_I values observed. Or these may simply be differences in hexokinases from different sources.

Although there is disagreement as to the particular type of inhibition obtained with G-6-P (England and Randle, 1967; Kosow and Rose, 1968; Moore, 1968), it is generally agreed that this potent inhibitor is more effective against soluble HK than the bound enzyme (Karpatkin, 1967; Kosow and Rose, 1968). Gumaa and McLean (1969) have used this fact, together with the observation of Kosow and Rose (1968) that the half time of solubilization of HK by G-6-P is 18 sec, plus their own observations on the rates of accumulation of glucose-6-phosphate, fructose-1,6-diphosphate, ATP and ADP to propose the following sequence of events in glycolysis and the role of binding-solubilization of HK in this sequence:

1. A rapid rate of glucose phosphorylation for 15 sec by bound HK, essentially independent of feedback inhibition by G-6-P.
2. Release of the bound HK by G-6-P.
3. Finally, a slow rate of glucose phosphorylation by soluble HK under control by G-6-P inhibition.

The rapid initial production of G-6-P is to speed up the system to get glycolysis moving fast. It would be self-defeating to have the G-6-P produced under these conditions inhibit its producer, HK. On the other hand, continued rapid production of G-6-P to a level greater than could be utilized by the rest of the glycolytic system (and using up available ATP) would be inefficient. In the above proposal, control of glucose phosphorylation is achieved by having the HK bound initially, and hence, relatively insensitive to inhibition by G-6-P. After G-6-P reaches the proper level, it solubilizes the HK, making it sensitive to control by G-6-P inhibition. In other words, binding-solubilization of HK and the time that it takes for this to occur, permits a smooth adjustment to changing cellular conditions. It is an example of the hysteretic effect discussed by Frieden (1970).

Table 2—Kinetic parameters for soluble and bound glyceraldehyde-3-phosphate dehydrogenase[a]

	Soluble	Bound
V_{Max}, nmoles/sec	29	7
K_M (GAP), mM	0.2	0.07
K_I (NADH), μM	3.8	3.5

[a]The concentration of enzyme was 5 μM.

Work in our laboratory may also shed some light on the regulatory role for the binding-solubilization cycle of mitochondrial HK. It is first necessary to review the concept of energy charge proposed by Atkinson (1968), which is defined by Equation 2.

$$\text{Energy charge} = \frac{(ATP) + 1/2\,(ADP)}{(ATP) + (ADP) + (AMP)} \qquad \text{(Eq. 2)}$$

Atkinson and his coworkers have found that many control enzymes are sensitive to this factor. In addition, the response of the enzyme is usually such that the activity of an enzyme involved with energy generation decreases with increased energy charge, and the activity of one involved with energy utilization increases with increased energy charge (Fig. 3). In the living muscle cell, the energy charge varies over the range 0.8–0.9 although this can drop rapidly postmortem.

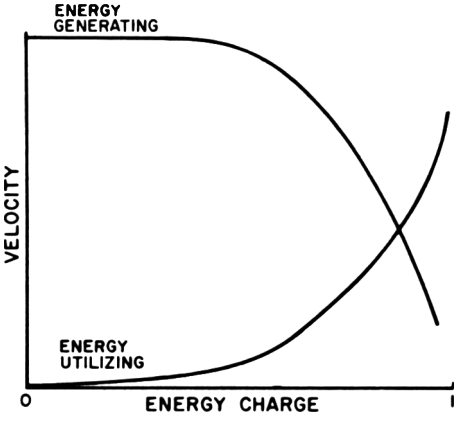

Fig. 3—Response of enzyme to energy charge in energy generating and energy utilizing systems as proposed by Atkinson (1968).

The response of bound and soluble mitochondrial HK from chicken skeletal muscle to energy charge obtained in our laboratory is shown in Figure 4. Over the physiological range, the bound HK is much more sensitive than is the soluble. This is a reflection of the greater inhibition of the bound enzyme by ADP than the soluble (Southard, 1970). The shape of the HK response curve to energy charge indicates that it takes part in an energy-utilizing pathway. This means that the reaction catalyzed by the enzyme contributes to a biosynthetic pathway, viz., one requiring a net expenditure of energy. The data illustrated in Figure 4 suggest that the G-6-P produced by the hexokinase-catalyzed reaction would be principally utilized for glycogen synthesis rather than glycolysis.

When HK takes part in glycogen synthesis, significant levels of G-6-P must be present since this compound activates the glycogen synthesis system. As mentioned earlier, the soluble enzyme is more sensitive to G-6-P than is the bound. Thus, the bound enzyme would be better suited for producing G-6-P for glycogen synthesis than the soluble. This suggestion might also relate to the association of the bound enzyme with the mitochondrion, which is the major source of ATP in the resting cell and, therefore, the energy source for glycogen synthesis. When glycogen stores are complete, there would be no need to manufacture more. If the energy charge were high, the enzyme would be active and waste ATP. It would also produce G-6-P, but this is a relatively poor inhibitor of bound compared to soluble HK. A possible explanation for the control of HK under these conditions would be the sensitivity of bound HK to energy charge. As ATP is utilized and the energy charge drops, the bound enzyme is inhibited. This could provide the control to maintain energy stores without at the same time wasting energy. I am suggesting that soluble HK takes part principally in glycolysis and is regulated by G-6-P, while the bound enzyme may be involved in glycogen synthesis and controlled by the energy charge. The particular location of this enzyme in the cell would affect not only its kinetic properties but also the metabolic sequence in which it is active.

A very real difficulty arises in the extrapolation of data obtained with purified enzymes in vitro which are usually assayed under conditions very different from those occurring in vivo. As an example of what might happen, we consider chicken muscle-type LDH whose kinetic properties, as I have previously mentioned, change on binding to subcellular fractions. LDH is usually assayed in the presence of NADH with no NAD^+ present. In the cell, however, there is usually much more NAD^+ than NADH. LDH forms a ternary complex with NAD^+ and pyruvate which is inactive. It has been suggested that the formation of this "abortive ternary complex" may serve a control function to shut off LDH activity at high pyruvate concentrations. Griffin and Criddle (1970) have presented evidence that the abortive ternary complex is formed by dissociation of tetrameric LDH into monomers which combine with NAD^+, and then with pyruvate in the rate-determining step. The ternary complex monomers then recombine to give inactive tetramers. If binding of LDH interferes with the process of dissociation-association, one could expect to see a differential inactivating effect of NAD^+ and pyruvate on bound LDH compared to the soluble enzyme.

Figure 5 shows results we have obtained on the inhibition of soluble and bound LDH by abortive ternary complex formation. The extent of inhibiton is different for the two forms of the enzyme, with soluble LDH being much more sensitive to inhibition. These data were obtained at 23°C. At a higher temperature (40°C), even greater differences are found. In fact, there is essentially no inhibition of bound LDH by pyruvate and NAD^+ at 40°C. Thus, binding of LDH to the particulate fraction of muscle may not only modify its kinetic properties, but may serve to protect the enzyme against inhibition under physiological conditions. The particular purpose of this phenomenon may be to leave the soluble enzyme subject to a sensitive control system during rapid glycolytic activity, which includes the possibility of its forming the inactive ternary complex, while allowing the bound LDH to operate even in the presence of high NAD^+ and pyruvate concentrations. The effect of temperature on the prevention of inhibition of LDH by ternary complex formation may have important implications regarding cooling rates and quality changes in poultry meat since this enzyme is important in the glycolytic sequence which is responsible for acid production postmortem.

The reversible dissociation discussed above for glyceraldehyde-3-phosphate dehydrogenase, hexokinase, and lactate dehydrogenase presumably would occur between the surface of cellular particulate fractions and the soluble cytoplasm. It is also possible that shifts between phases could occur within a given subcellular organelle. Rendon and Waksman (1969) provide an example of this in their study of mitochondrial aspartate amino transferase which can exist either associated with the inner mitochondrial membrane or in the soluble matrix. The distribution of the enzyme between the two areas is dependent on the metabolic state of the mitochondria, i.e., whether they are in the orthodox or condensed conformation (see Fig. 6). The orthodox represents the resting state and the condensed the respiring state of mitochondria. The conformation is dependent on the metabolites present as well as the ionic composition of the medium. Presumably aspartate amino transferase would behave differently depending on its location in the mitochondrion. The environment around the mitochondrion and its physiological state would be the factors determining the location of this enzyme.

SUMMARY & CONCLUSIONS

THE LITERATURE contains many examples of enzymes which are associated with subcellular structures. I have presented in this paper some suggestions as

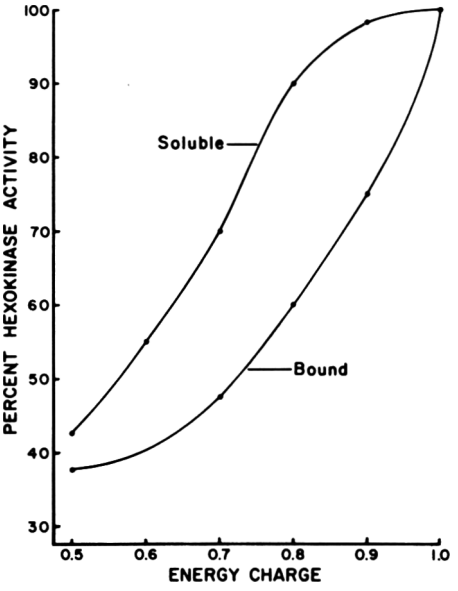

Fig. 4—Response of soluble and bound hexokinase from chicken breast muscle mitochondria to energy charge.

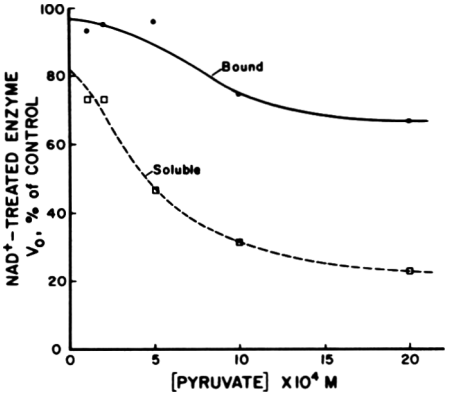

Fig. 5—Inhibition of soluble and bound chicken muscle LDH by NAD^+ at 23°C.

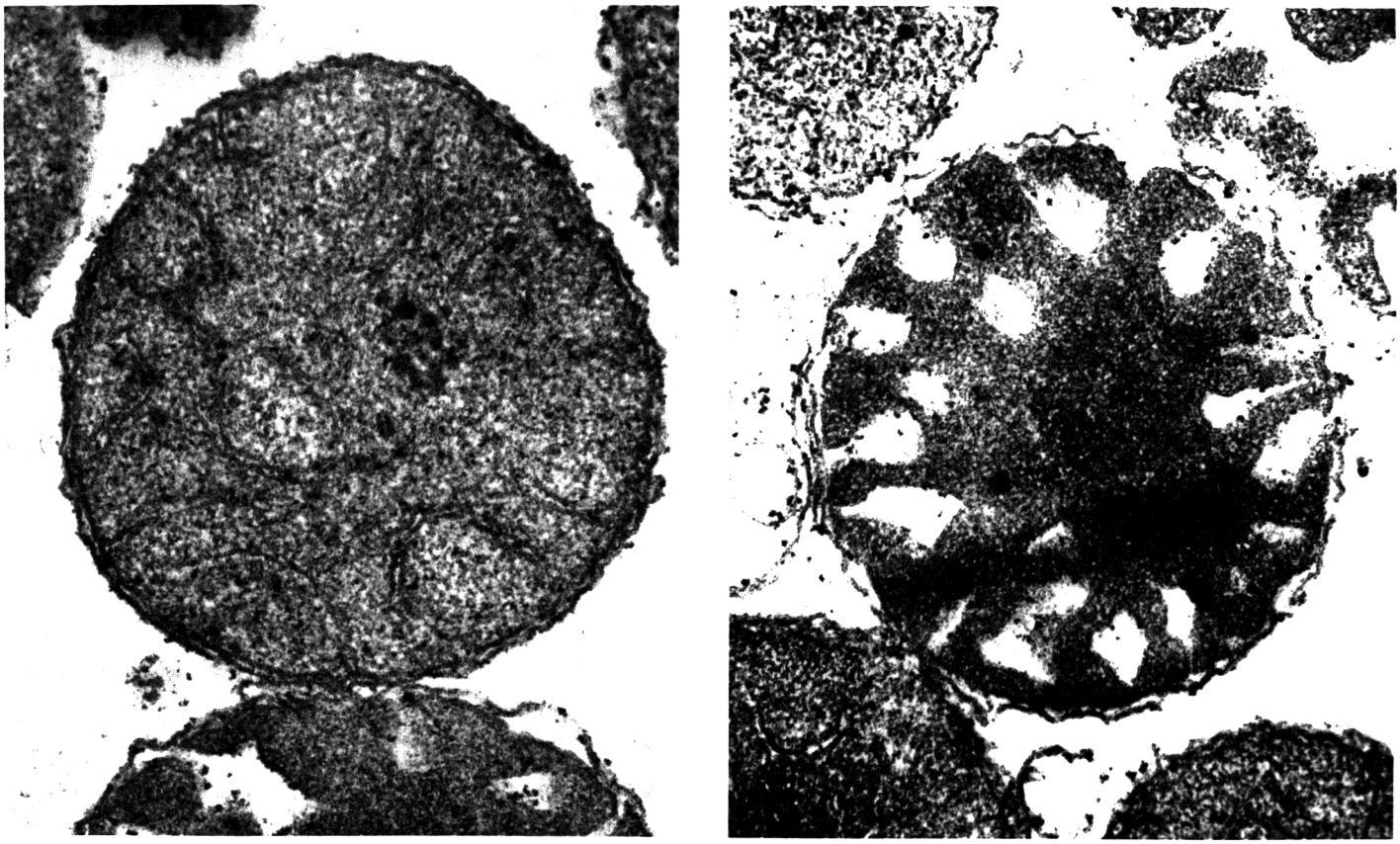

Fig. 6–Electron micrographs of mitochondria in orthodox (left) and condensed (right) conformations (47,000X).

to how these associations might affect the functioning of some selected enzymes. These suggestions have been both my own and those from the literature. Not one of the suggested mechanisms has been proved, and I have used them here only to show some types of modifications which are possible. Further research in this newly emerging field will be necessary before definitive statements can be made.

Evolutionary processes have designed enzymes to respond to factors which aid survival of the cell and the organism. Although conditions in tissues handled as foods are often different from those in the living organism, the enzymes are still subject to the same control mechanisms. Thus the rapidly changing conditions in a tissue like skeletal muscle postmortem are both a cause and effect of changing enzymic responses. Deletion of O_2, decrease in pH, redistribution of ions and change in concentration of metabolites can all markedly affect enzyme interactions with the particulate fraction, which in turn can modify the activity of the enzyme. It may be that studies relating postharvest or postmortem changes to extracted enzymic activities have little relation to what is occurring in vivo, and the enzymes must be studied under conditions more nearly physiological to properly assess their role in determining food quality.

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SYMPOSIUM: Biochemical Control Systems

CELL DISRUPTION AND ITS CONSEQUENCES IN FOOD PROCESSING

IT IS PERHAPS not too great an exaggeration to maintain the thesis that food processing and technology may be considered as the art and science of the promotion, control and/or the prevention of cellular disruption and its metabolic consequences at the right time and at the right place in the food processing chain. This is especially true if one considers internal as well as external cell damage. We believe that random sampling of recent food technology literature should uphold this point of view and have chosen our own work and that of our colleagues, for the most part, to support the ensuing generalizations concerning food cell disruption. In this context, cellular disruption with respect to foods can: (1) create identity; (2) improve quality; (3) be deleterious. The most obvious example of foods for which there is an absolute requirement for cell disruption for the creation of food identity are purees and juices. In order to transform the wheat berry into bread, it is subjected to a highly selective and carefully controlled process of cell breakage. In this process of milling the endosperm cells are disrupted without extensive disruption of the starch granules contained therein and in such a manner as to maintain the integrity of the aleurone layer, which becomes associated with the bran.

Cellular disruption

The classical illustration of improvement in quality brought about by cell disruption is that which occurs via the autolysis of muscle tissue, resulting in the liberation of lysosomal proteinases which contribute to the tenderness of meat.

Cell disruption is also essential for high quality dates (Coggins et al., 1968). As shown in Figure 1, the cells of both immature and of low quality dates are intact, as evidenced by the well-defined cell walls. On the other hand, in tree-ripened dates or in poor-quality dates upgraded by vacuum hydration (Huxoll and Maier, 1971, unpublished results) or by vacuum infiltration at elevated temperature as shown in Figure 1, the boundaries between cells are abolished or become indistinct. This cell disruption is accompanied by the activation of pectinases, polyphenol oxidase, and invertase as well as cellulase (Hasegawa and Smolensky, 1971). The activation of these autolytic enzymes converts a light colored, tasteless and turgid fruit into a dark, moist, soft product typical of a high quality date.

When beans are soaked and cooked prior to further processing, the cells, although internally disrupted, do not separate, so that the full nutritional value of the bean is not utilized. Kon and Wagner (1970) have shown that improved products can be obtained by controlled cell disruption at low pH's prior to cooking. Becker et al. (1972) showed that the consistency of tomato products can be elevated by extensive disruption leading to the formation of, and due to, brush heap cellulose fibers in the tomato puree.

Of course, cellular disruption can, in addition to creating identity and improving quality in foods and processes, indeed be quite deleterious when it occurs at the

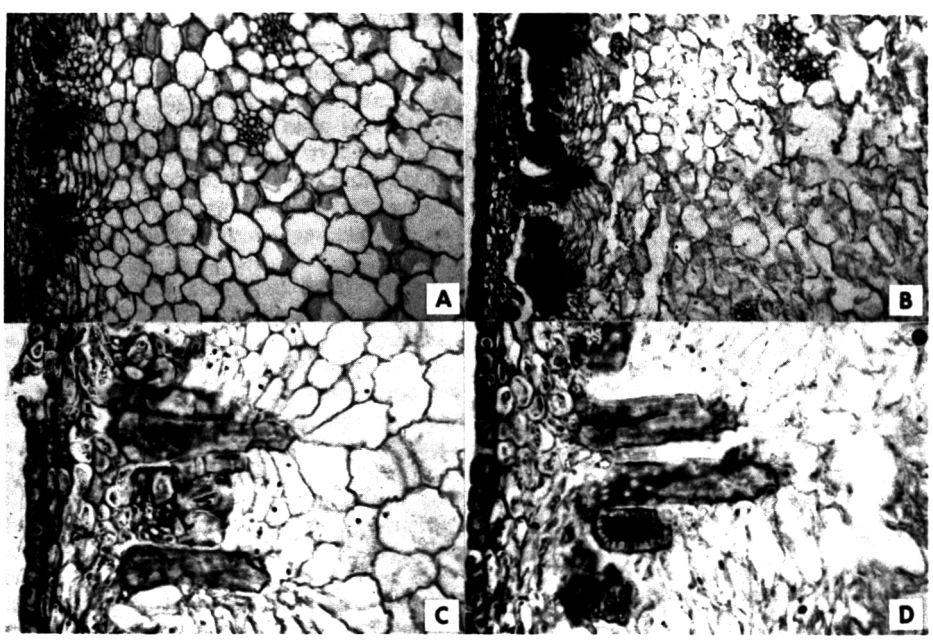

Fig. 1—Cell disruption improves date quality. Photomicrographs (21X) of cross section of date tissue located about midway from stem to stylar end of fruit: (A) Kimri stage of development, turgid and green; (B) Tree-ripe stage of development but not as ripe as a completely ripe date; (C) Number 2 dry grade of date; (D) Number 2 grade of date after filtration and incubation for 24 hr at 122°F. These photographs were supplied through the courtesy of Dr. C.W. Coggins, University of California, Riverside.

CELL DISRUPTION AND ITS PREVENTION IN THE FOOD PROCESSING CHAIN

Harvesting - Mechanical, Field Processing
↓
Transportation - Handling, Transfer, Biological
↓
PreProcessing - Conditioning, Controlled Atmosphere
↓
Processing - Freezing, Shear, Temperature
- Drying, Enzymes
↓
Packaging, Storage - Temperature, Container,
- N_2, Enzymes
↓
Distribution - Temperature, Handling
↓
Consumer - Mastication, Blends

Fig. 2—The role of cell disruption in the food processing chain.

wrong time, leading to undesirable changes in texture, flavor and nutritional quality. Stratagems consisting of countermeasures must be inserted into the food processing chain to circumvent these deleterious consequences of cell disruption.

Cell disruption and the food processing chain

Figure 2 outlines the occurrence of undesirable cell disruption in harvested food and also indicates countermeasures to prevent it. Deleterious consequences have already occurred prior to harvest via predators (including viruses, bacteria and fungi, as well as insects) and physiological changes such as overripening. Further cell disruption ensues upon harvesting. With the increasingly widespread adoption of mechanical harvesting, the incidence of cell disruptions has been considerably augmented. Thus, it has been estimated that as much as 30% of the commercial tomato crop in California, all harvested mechanically, is damaged due to bruising and splitting (Schultz et al., 1971).

Further cell disruption can occur between harvesting and the initiation of processing, much of it during transportation. In addition to further mechanical damage such as bruising and splitting, harvested produce is subject to microbial deterioration. Some 10–20% by weight of a tomato crop can be lost during this interval. An effective countermeasure is to process the tomatoes at the site of harvest, as was clearly demonstrated by Schultz et al. (1971).

Alternatively, one can interpose a preprocessing step such as controlled atmosphere storage of the produce to keep it in optimal condition for processing (Brody, 1970). In the future, application of appropriate, safe plant growth regulators may perform a similar function.

As indicated in Figure 2, many of the steps in food processing involve cell disruption. Again countermeasures (some of which are themselves alternative means of disrupting cells) have to be taken to prevent the deleterious consequences of cell disruption. Perhaps the most widely used countermeasure is blanching, which destroys deleterious enzymes. However, enzyme inactivation may be counteracted by the presence of substrate, optimal pH stability, low water activity, adsorption on the cellular debris (Schwimmer, 1969c) and regeneration of heat inactivated enzymes (Schwimmer, 1944). Regeneration, in addition to interfering with the test to measure the adequacy of blanching has posed a problem in HTST processes because, as shown in Figure 3, the extent of enzyme regeneration appears to be proportional to the heating rate.

In some foods, the consequences of cell disruption must be taken into account in packaging of the product either before or after processing. Thus, fresh produce can be bruised if the containers in which they are packed are not rigid enough to prevent the transmission of pressure to the bottom of the stack. Using low temperatures and excluding oxygen and reducing moisture can help prevent undesirable consequences of cell disruption. Thus, Fellers and Bean (1970) prevented lipase action in their high protein-fortified flour blend by reducing its moisture content and maintaining the latter at low levels by switching to water-impermeable packaging.

Agents of cell disruption (Table 1)

Heuristic considerations prompt us to list most of the known agents of cell disruption and to briefly cite applications of interest to food technologists. Shear forces, intentional or not, are, as has been previously indicated, met with frequently in the food industry. Whereas freezing is usually considered to disrupt cells via cell wall penetration by ice crystals, much of this sort of damage can be prevented by slow freezing (Mazur, 1969). In the latter case the cell damage which does occur resembles that occurring during dehydration; increased electrolyte concentration; deformation and reduction of cell volume; and, perhaps, most important of all, disruption of internal organelle membranes, upon which the viability, controlled metabolic activity, and turgor of the tissue depends. Among many factors contributing to the deterioration of quality upon cell disruption in foods are uncontrolled temperature fluctuation during the course of the food processing chain (Schwimmer et al., 1955).

As a countermeasure to the consequences of cell disruption due to heating and dehydration, we have shown that, to an appreciable degree, the shape and turgor of dehydrated vegetables can be maintained by adding to the food, prior to processing, a suitable polymerizable monomer. During processing, polymerization takes place and the resulting incorporated polymer imparts the above-mentioned desirable characteristics to the food (Schwimmer, 1969a).

Onion dehydration is an interesting process in relation to cell disruption management. The object is to minimize cell disruption during the early stages of drying so as to maintain compartmentation of the C-S lyase (Schwimmer, 1971a) and the principle flavor precursor, S-propenyl-L-cysteine sulfoxide (Schwimmer, 1969b) until the product is adequately dry. In the final product the enzyme reaction releases flavor promptly upon rehydration (Schwimmer et al., 1964). Further flavor can be developed by the addition of a suitable source of glutamyl transpeptidase, which releases the flavor precursor from gamma glutamyl bondage (Schwimmer, 1971b; Schwimmer and Austin, 1971a, b).

Physiological cell disruption is that which occurs during the life-cycle of the organism. In the case of plants, the onset of the climacteric and ensuing senescence signal a programmed schedule of progressive cell disruption. In the case of mammals, aging has been ascribed to cell damage via lipid peroxidation (Tappel, 1971). A brief mention should be made of osmotic shock, explosive decompression and ultrasonics. These are relatively mild laboratory procedures designed to

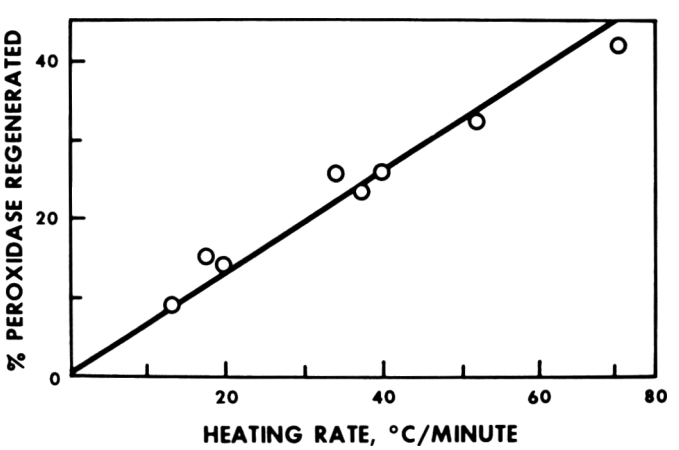

Fig. 3—Regeneration of turnip peroxidase as a function of heating rate (Schwimmer, 1944).

Table 1—Agents of cell disruption

Shear forces	Osmotic shock
Temperature change	Decompression
Dehydration	Ultrasonics
Physiological	Chemicals
Predators	Poisons
Radiation	Autolysis
	Exolysis

maximize organelle yield. These procedures may eventually find some applications in food processing in addition to manufacture of puffed cereals.

Predators include not only insects but also molds, bacteria and viruses. In each case a microecological system of challenge by the predator, such as secretion of cell-destroying enzymes and defense by the plant, resisting these incursions (Albersheim, 1965). Phenolics and their oxidation products formed in cells disrupted by the predator protect the cells of the surviving tissue by inhibiting the predator's macromolecular-degrading enzymes (Schwimmer, 1957; Bull, 1970).

The role of autolysis in date and meat processing has previously been mentioned.

A relatively novel agent of cell disruption which, I predict, will contribute significantly to food technology in the future is what I have termed "exolysis," the application to foods of external sources of cell-disruptive enzymes. A rather spectacular example is afforded by the sequential action of a cell-separating enzyme and a cellulase preparation results in loss of cell identity (Tomaya, 1969). Recently, Saunders et al. (1971) succeeded in removing the cell wall from the cells of the aleurone layer of wheat, thus rendering the nutritious component thereof available.

Organelles, compartmentation and enzyme functionality

It is now well established that enzymes are not scattered at random in the living cells but perform their catalytic functions as members of highly integrated control systems localized in highly specialized organelles and in well-ordered sequences. The author has recently discussed the implications of organelle integrity and function for food technology (Schwimmer, 1969c) including flavor improvement by cabbage mitochondria (Schwimmer, 1963). Since then some rather interesting addenda have been published. Thus, Melnick and Hultin (1971) showed that one of the products of trout muscle lactate dehydrogenase action solubilizes the enzyme and prevents binding of the soluble enzyme to particulate matter, presumably mitochondria. Transpeptidase in extracts of sprouted onion (Schwimmer and Austin, 1971b) and the flavor-producing enzyme in onion (Schwimmer, 1969b) are strongly associated with relatively small-sized particulate matter; 20,000 × G is required for complete centrifugation of activities. The activities can be released only by relatively strong salt concentrations. Recently, Heftmann (1971) and Caldwell and Grosjean (1971) have demonstrated the presence of lysosomes in tomatoes and in chicken skeletal muscle, respectively, and have shown that such cell-disruptive agents as freezing and thawing, high shear forces and detergents abolish latency, thus providing a rational for the mechanism of autolysis in both plant and animal foods.

An interesting instance of compartmentation is shown in Figure 4. Two sugar cane cells, each carrying out photosynthesis by its own distinctive mechanism, exist in the leaf side-by-side: one via the now well-known Calvin cycle (carboxylation of ribulose diphosphate) and the other via Hatch-Slack cycle (carboxylation of phosphoenol pyruvate; Hatch and Slack, 1966). Not only is there a compartmentation of enzymes, but as food scientists have long recognized, that there is a compartmentation of enzyme and substrate and that certain enzymes do not manifest their presence until the integrity of the cell is impaired; in the browning of food fruits and vegetables and the release of flavor of onions and horse radish, and probably tea. The nuances of flavor of many foods may arise from the interaction of the enzymes of the protoplast with the vacuolar constituents upon cell disruption.

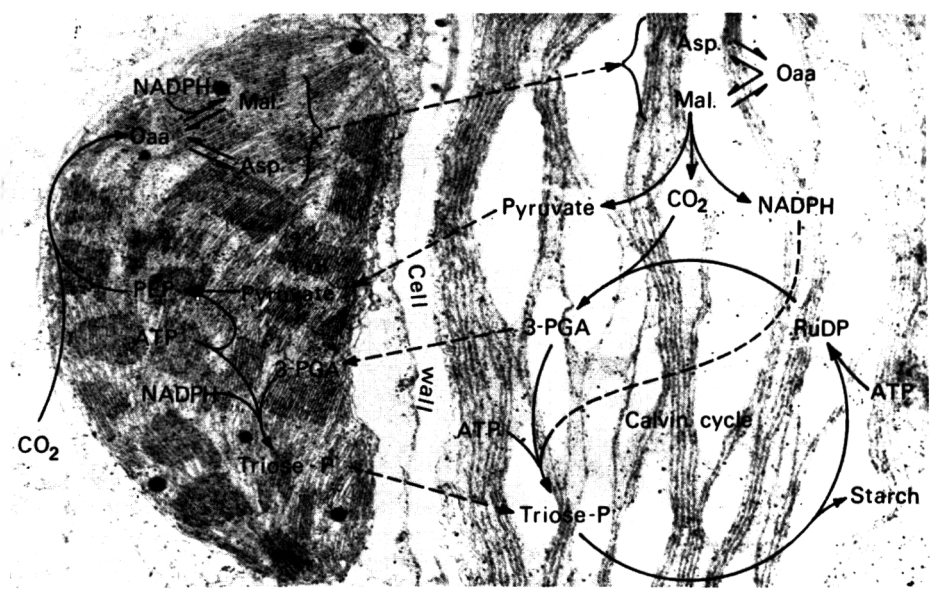

Fig. 4—Compartmentation of two photosynthetic pathways (Berry, 1971). Solid lines represent chemical transformation; dashed lines represent movement. The background electron micrograph shows adjacent bundle sheath and mesophyll cells of Sorghum sudanese.

In general, with the exception of physiological cell disruption signalling senescence, previously alluded to, cell metabolism ensuing cell disruption is characterized by removal of restraints imposed by phenotypic enzyme control systems.

On the other hand, cell disruption can influence metabolism in surviving cell at the level of genotypic control. Thus, ethylene, the harbinger of the de novo enzyme synthesis of enzymes involved in onset of the climacteric phase of fruit development also appears when certain food cells are bruised (Pratt and Goeschl, 1969). Glasziou (1969) has reviewed the extensive literature on the apparent derepression of enzyme synthesis following the slicing of fruits and vegetables. More recently, Guadagni et al. (1972) has shown an increased production of flavor substances in apples following peeling.

In disrupted cells, as previously mentioned, among the most sensitive structures which become nonfunctional are the membranes, especially those involved in protein synthesis, like the endoplasmic reticulum. Perhaps most important with regard to metabolic consequences of cell disruption are the mitochondrial cristae, site of oxidative phosphorylation, to make ATP, the driving force and source of the free energy required for biosyntheses. Thus, the quasi-chaotic, entropy-decreasing consequences of cell disruption lead to ascendancy of degradative reactions of hydrolytic, uncoupled oxidative, lyase and nonphosphate dependent transferase enzymes. Reductases and dehydogenases dependent on substrate levels of coenzymes, such as NAD, and ligases (all ATP dependent) are no longer functional due to destruction of ATP and the ATP regenerating machinery and coenzymes, by unrestrained phosphatase action.

Disintegration of cellular and organelle membranes, relocalization, redistribution and adsorption on cellular debris; as well as decompartmentation and interaction of enzymes and substrates which do not interact in the intact cell, as previously discussed, lead to the accumulation of intermediate metabolites and to unique nonphysiological products in foods. Among these products, the most easily discernible are colored. Some of the

off-colors which are identified with a food or with a problem in food processing are shown in Table 2.

The pototo-metabolism, cell disruption and processing—A case history

The interplay among metabolism, cell disruption and food technology in the food processing chain is aptly illustrated by the fate of the potato tuber. When potatoes are harvested they are subjected to enzymatic browning, occasioned upon bruising, due to the oxidation of polyphenols in the disrupted cells. Upon loading and shipping they are further exposed to jostling, resulting in occurrence of black spot (Weaver and Hautala, 1970). Simple but effective countermeasures to this deleterious consequence of cell disruption are to allow the potatoes to drop into a layer of water in a waterproof truck (Weaver et al., 1965) and by shipping the potatoes in bulk instead of in sacks (Weaver and Merril, 1965). Oxidative browning is also a problem in the pre-peeled potato industry and effective countermeasures have been proposed (Schwimmer and Burr, 1967). Among these is the application of ATP, which produces a reducing milieu via reductive dephosphorylation involving the mitochondria (Makower and Schwimmer, 1954). Paez and Hultin (1970) have studied potato mitochondria in some detail. Potato slices are experimental objects of choice for the study of the enzyme activation via gene depression in surviving plant tissue (Glasziou, 1969).

The other great problem in potato processing is nonenzymatic browning. Potatoes respond to stresses such as temperature extremes and ionizing radiations (Schwimmer et al., 1958), by converting part of their starch to sugar, with reducing sugars predominating in the case of low temperature storage. Upon subsequent processing, these reducing sugars, as is well-known, give rise to brown products. On the basis of the known enzyme reaction in potatoes, we have proposed the metabolic cycle shown in Figure 5 (Schwimmer, 1965). The question arises as to what triggers the conversion of starch to sugars. Most of the enzymes involved appear to maintain their normal activity. Paez and Hultin (1970) showed that potato mitochondria display a temperature response typical of that of enzyme reactions. [An exception, which accounts for the appearance of reducing sugar, is the sucrose-hydrolyzing enzyme invertase whose activity rises in cold-storage potatoes (Schwimmer, 1962; Rorem and Schwimmer, 1963). Pressey (1966) showed that expressed invertase activity is the resultant of the action among a specific invertase inhibitor, de novo enzyme synthesis and enzyme turnover.] The actual trigger may be, according to Ohad et al. (1971), the disruption and/or modification at low temperatures, of a membrance surrounding starch granules of freshly harvested or room-temperature-stored potatoes. This membrane disruption renders the granule susceptible to starch-degrading enzymes which, as shown in Figure 5, can give rise to reducing sugars.

Figure 6 displays electron photomicrographs of potato sections showing the protoplastid in immature tubers, which becomes a membrane-encased starch granule in the mature and room-temperature stored tubers. An intact membrane is no longer visible in low temperature-stored tubers. Thus, we have a "physiological" internal cell disruption functioning to protect the cell against the external stress of low temperature.

The traditional procedure for preventing nonenzymatic browning of potato products due to accumulation of reducing sugars is to condition them for about a week or two at room temperature. This leads to weight losses and can be quite erratic. Weaver and Hautala (1971) have developed an alternative means for removing sugars in the manufacture of fresh fries. A very thin crust of the potato strip is quick-frozen, thus disrupting internal membranes in the cells of the outer layer. This allows controlled leaching of the solutes including reducing sugars. This particular beneficial consequence of cell disruption eliminates the necessity for conditioning, widens choice of varieties, controls color and texture and reduces fat uptake in the finished processed potato.

Table 2—A rainbow of nonphysiological off-colors in foods

Color	Food	Substance of Correlative or Precursor
Violet-blue	Cauliflower	Flavonoid + S[a]
Green	Tuna	Metamyglobin-S-S-R[b]
Yellow-brown	Vegetables	Pheophytin (chlorophyll)
	Vegetables, fruits	Melanins (polyphenols)
	Meat	Metmyoglobin[a]
	Apple scald	Farnesene[c]
Pink	Onions	Propenyl cysteine sulfoxide[d]
	Pears	Cyanidin[a]

[a]Borgstrom (1968)
[b]Grosjean et al. (1969)
[c]Meigh (1969)
[d]Shannon et al. (1967)

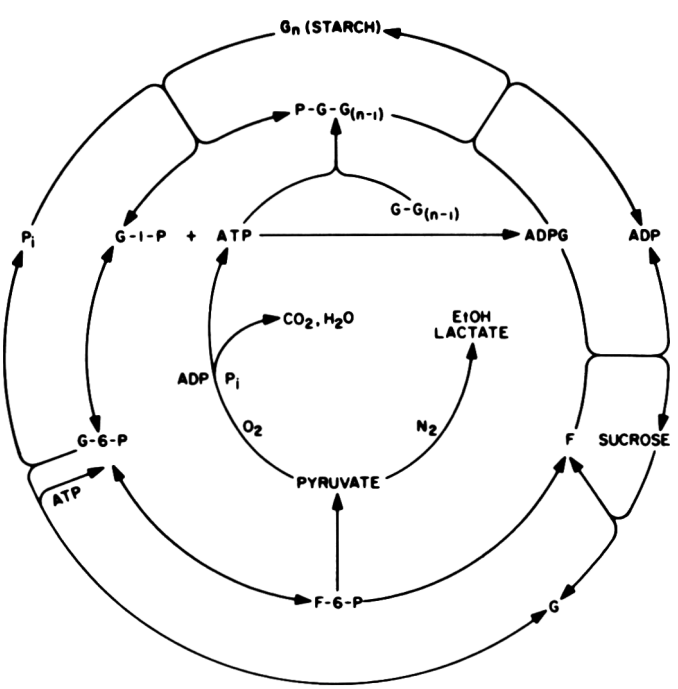

Fig. 5—The starch-sugar interconversion cycle in potatoes.

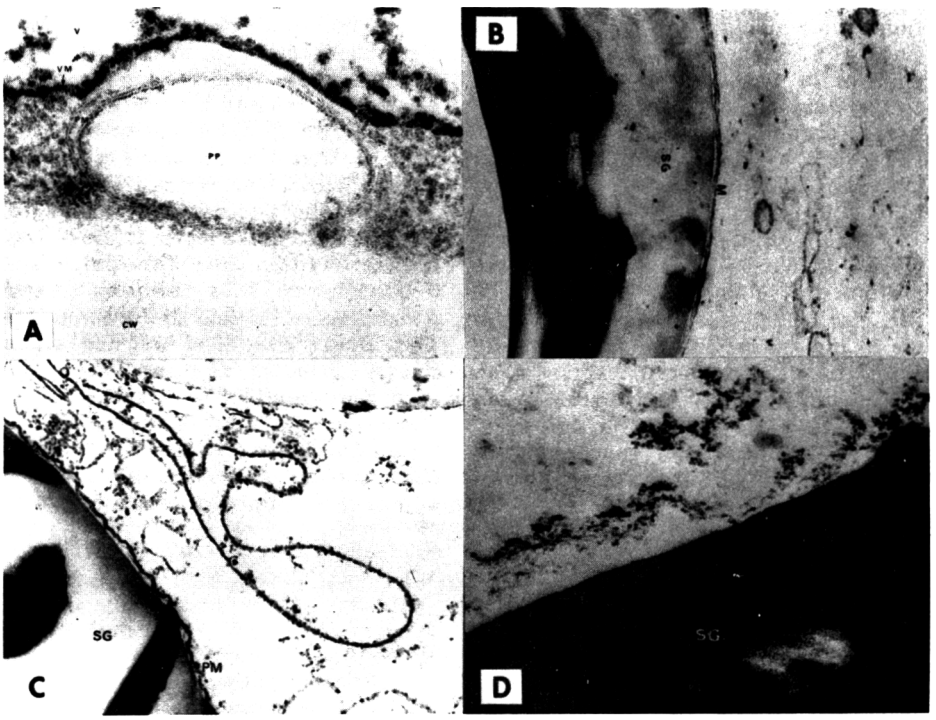

Fig. 6—Development and disintegration of starch-granule-associated membranes. (Ohad et al. 1971.) (A) Section through the cell of an immature potato tuber. Within the cytoplasm is a protoplastic (pp) surrounded by a double membrane (27,800X); (B) Section from a mature tuber immediately after harvest. A starch granule is seen surrounded by the intact plastic membrane, pm (6,400X); (C) Section from a tuber stored at 77°F for 31 days (13,900X); (D) Section through a tuber stored 40°F for 12 days. The membrane has disintegrated and moved away from the starch granule, whereas the vacuolar and cytoplasmic membranes remain intact (25,200X).

SUMMARY & CONCLUSIONS

WE TRUST that the foregoing discussion has established the validity of the thesis set forth at the outset that food processing may be considered as the management of cell disruption and its consequences. This is not a comprehensive treatise to prove the validity of the thesis set forth at the outset of this discussion but we believe this discussion, in which we confined most of our citations and examples to our work and that of our colleagues, constitutes strong confirmatory evidence for its validity, and that a scrutiny of the reader's own field of endeavor from the viewpoint herein presented would probably convince him of its universality.

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APPLIED SCIENCE and ENGINEERING

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THE EFFECT OF SODIUM NITRITE ON THE FLAVOR OF FRANKFURTERS

INTRODUCTION

THE CURING OF MEAT and comminuted meat products with salt and an alkali nitrate salt is an ancient process originally intended as a method of preservation. Changes in the color and appearance of the product occur in the process. In 1891 Polenski demonstrated that the nitrate in the cure was reduced to nitrite as a result of bacterial action. The red color of the product, which was considered a characteristic of cured meat, was found by Kisskalt (1899) to be formed in the presence of nitrite. Kerr and co-workers (1926) established the limits of sodium nitrite concentration in the cure that would yield a satisfactory product, and these limits are part of the legal requirements for cured meats in use today. The work of Kerr et al. (1926), however, was primarily directed toward the development of the cured color in the product. They noted that the flavor and quality of the products were equivalent to those prepared in the customary fashion, i.e., with nitrate only, but there was no investigation of the effect of the nitrite ion on the flavor of the product.

The relationship of nitrite to flavor was first described by Brooks et al. (1940) in a study of the use of nitrite in the cure of bacon and ham. Although they presented no taste panel data, these authors stated that the panel showed a preference for meat cured with nitrite. (It must be noted, however, that in a number of experiments very little differences in flavor were found among the variously treated bacons.) Barnett et al. (1965) reported on an extensive study of the factors affecting cured ham flavor. In a study on the concentration of nitrite in the pumping pickle, they found the panel had an equal preference for hams pumped with pickle containing the usual nitrite concentration (1.5g sodium nitrite per liter) and those in which pickle with 0.1g sodium nitrite per liter had been used. Recently Cho and Bratzler (1970) studied the effect of nitrite and smoke on the flavor of cured pork roasts, reaching the conclusion that more cured flavor was present in the roasts cured with nitrite.

The effect of nitrite on the flavor of frankfurters, a comminuted product of beef, pork and various spices, is reported in this paper.

MATERIALS & METHODS

FRANKFURTERS were prepared by a standard procedure according to the following formulation: lean beef–45%; pork (50% lean)–55%; and ice–25% of total beef and pork weight. The cure salts were added in the following quantities per pound of total meat: salt–11.4g; sugar–9g; commercial spice preparation–2.4g; sodium ascorbate–0.24g; sodium nitrate–0.574g; and sodium nitrite (when added)–2.070g. The frankfurters were cooked in a Dry-Sys smokehouse, using a 90-min program of increasing heat and controlled humidity. When smoking was desired, smoke, generated in a Mepaco apparatus from commercial hickory sawdust, was led into the smokehouse for the entire 90-min cooking period.

The frankfurters were stored at 5°C overnight and submitted to a taste panel for evaluation. The panel consisted of 23 judges who had been testing frankfurter flavor for several years. Frankfurters were heated for 5 min in water that had been brought to a boil, cut into ½-in. pieces and kept warm in a double boiler over hot water. Judging was carried out in individual booths under low intensity green light. While red light did cover up differences in color among the frankfurters, it was still possible to distinguish among them by the intensity of the dark color visible. This was alleviated to some extent by the use of the green light.

Types of tests used

Triangle test. The two samples to be compared were given as the odd sample an approximately equal number of times in a random fashion and were positioned randomly in the triangle to prevent sample or positional bias. Statistical significance of the results of these tests was determined from the table in the book by Amerine et al. (1965).

Scoring test. The panelists were presented with a standard of untreated frankfurter having a given value of "0" for poor flavor and were asked to score test samples for "frankfurter" flavor, on a scale of 0 to 10, compared to the standard. A duplicate of the standard was included as a hidden control. Analysis of variance was carried out as described in Steel and Torrie (1960).

Table 1—Triangle test evaluation of the flavor of frankfurters prepared with cure in which sodium nitrite was either present or absent

Experiment	Conditions	No. correct/ No. judges
1	Cooked, no smoke; + NO_2 vs. no NO_2	15/22***
2	Cooked, no smoke; + NO_2 vs. no NO_2	28/36***
3	Cooked, no smoke; + NO_2 vs. no NO_2	18/24***
4	Cooked, smoke; + NO_2 vs. no NO_2	11/17**
5	Cooked, smoke; + NO_2 vs. no NO_2	13/24*
6	Cooked, smoke; 50% NO_2 vs. no NO_2	12/17**
7	Cooked, smoke; 50% NO_2 100% NO_2	9/17NS

*p = .05; **p = .01; ***p = .001; NS = not significant

RESULTS & DISCUSSION

FRANKFURTERS prepared without sodium nitrite in the cure, and cooked but not smoked had an unpleasant grey color. Comments were made by the judges that

Table 2–Scaling test evaluation of the flavor of cooked or smoked frankfurters prepared with cure in which sodium nitrite was either absent or present

		Nitrite	
		No	Yes
Smoke	No	0.65[a]	3.85
	Yes	5.05	4.65

Analysis of Variance

Sources	df	SS	MS	F
Total	79	679.8		
Judges	19	155.3	8.17	1.63[NS]
Treatment	3	239.2	79.73	15.93**
Smoke vs. no smoke	1		135.20	27.01**
Nitrite vs. no nitrite	1		39.20	7.83**
Smoke × nitrite	1		64.80	12.95**
Error	57	285.3	5.00	

[a]Averages of scores of judges on a scale of 0 = no hot dog flavor, to 10 = excellent hot dog flavor
**Significant at p = .01; NS = not significant

Table 3–Scaling test evaluation of the flavor of cooked or smoked frankfurters prepared with cure containing various concentrations of sodium nitrite

		Nitrite		
		0	50%	100%
Smoke	No	1.18[a]	4.33	–
	Yes	5.25	5.59	5.02

Analysis of Variance

Sources	df	SS	MS	F
Total	84	472.92		
Judges	16	29.32	1.83	NS
Treatments	4	217.97	54.49	15.40**
Cooked vs. smoked	1		130.73	37.14**
0 vs. 50% NO_2 (cooked)	1		84.47	24.00**
Nitrite in smoked franks				
Linear	1		0.43	NS
Quadratic	1		2.36	NS
Error	64	225.61	3.52	

[a]Averages of scores of judges on a scale of 0 = no hot dog flavor, to 10 = excellent hot dog flavor
**Significant at p = .01; NS = not significant

this type of preparation had an unappetizing cooked pork flavor. When the frankfurters without sodium nitrite were smoked as well as cooked, the surface was brown (the intensity varying with degree of smoke applied) as a result of the deposition of smoke components. The interior of such franks, however, was still grey in color. The hardened, denatured protein skin of the franks could be removed easily. A red pigment was noted on the surface of the grey, underlying meat, which on analysis with the Cary Spectrophotometer was identified as nitrosomyoglobin. Smoke appears to contain sufficient oxides of nitrogen to penetrate the sausage casing into which the meat is stuffed and to react with myoglobin to form the nitrosated pigment.

A change in frankfurter flavor noticeable to the judges was brought about by eliminating sodium nitrite from the cure. Experiments 1 to 5 in Table 1 show that a statistically significant number of judges could distinguish between the flavors of franks prepared with or without sodium nitrite. While this was particularly true with the franks that were cooked only (Experiments 1–3) smoke did not prevent the selection of the correct odd sample, although the number of correct responses was somewhat lower (Experiments 4–6).

To quantitate the differences in frankfurter flavor indicated by the triangle test, a scoring procedure was applied. Frankfurters were prepared with and without sodium nitrite. One half of each batch was cooked only and the other half smoked as well. The results of triangle tests to detect differences in flavor among the samples are shown in Experiments 3 and 5 in Table 1. Table 2 shows the scores and analysis of variance for the flavors of the four preparations compared to a control sample of no nitrite–no smoke–treated frankfurter with a value of "0" for frankfurter flavor. In the absence of smoke there was a highly significant difference in flavor produced on the addition of nitrite. When the frankfurters were smoked, however, there was essentially no difference in the scores of the untreated and nitrite-treated franks. The analysis of variance demonstrated the statistically significant interaction between smoke and nitrite treatment.

The effect of nitrite concentration was explored by preparing frankfurters with no nitrite, with the full amount of sodium nitrite normally used (100% nitrite), and with half this concentration of sodium nitrite (50% nitrite). Half of each batch was cooked, the other half was cooked and smoked. Triangle tests showed that the judges could distinguish between the flavors of the franks with no nitrite and 50% nitrite, but although there was a trend to distinguish between the flavors of the franks treated with 50% and 100% nitrite concentration, the values were not significant at the 5% level (Table 1, Experiments 6 and 7). The frankfurters used in these tests were smoked; triangle tests with the franks that had been cooked only were not carried out since, on the basis of the previous tests, there was very little difficulty in distinguishing between untreated and nitrite-treated franks.

The scores of scaling tests of the flavors of these preparations, and the analysis of variance in the data, are shown in Table 3. In the absence of smoke there was a significant difference in the values assigned to the untreated and 50% nitrite-treated franks. (Although the 100% nitrite-treated, cooked-only franks were not tested it is anticipated that there would not be a significant difference between this preparation and the 50% nitrite-treated franks.) The presence of smoke resulted in similar scores (no significant difference) for the franks receiving no nitrite and those prepared with the two levels of sodium nitrite. The highly significant statistical difference among treatments was analyzed into single degree-of-freedom contrasts. The difference in scores between the cooked-only and the cooked and smoked samples is statistically significant. The scores of the flavors of the smoked franks treated with the various concentrations of nitrite were not significantly different but there appears to be a trend that suggests their relationship can be described by a quadratic expression, indicating the presence of a point of maximum flavor.

A consumer-type test was carried out on a group of visitors to the laboratory consisting of children and adults, male and female. They were requested to indicate their preference between a pair of smoked frankfurters prepared with and without nitrite in the cure; 44 out of 55 of those participating preferred the flavor of the frankfurter with nitrite.

It is interesting to note that in triangle Experiments 4–6 (Table 1) in which the frankfurters were smoked, and in the experiments with cured pork described by Cho and Bratzler (1970), the judges were able to detect the effect of nitrite on the flavor of the product; the application of smoke apparently did not affect the flavor. However, when the frankfurters were subjected to a scaling test (Experiment 3, Tables 1 and 2; Experiment 6, Tables 1 and 3), the flavors of the smoked products were judged approx-

imately the same whether or not sodium nitrite had been used in the cure. The triangle test is purely a difference test, indicating in this case that an effect—some effect—differentiates the two samples. The scaling procedure requires a value judgement of the flavor, taking into account all factors, psychic and physical, that enter into such a judgement. Thus, while it is of interest to know that a difference can be detected, it would appear to be of greater importance that the judges found no significant difference in the flavor of smoked frankfurters in the presence or absence of sodium nitrite.

It is also of interest to note that the presence of a commercial frankfurter spice formulation was not sufficient to impart a good frankfurter flavor in the absence of sodium nitrite in the cure.

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Reference to a brand or firm name does not constitute endorsement by the U.S. Dept. of Agric. over others of a similar nature not mentioned.

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THE EFFECTS OF SOME POST-SLAUGHTER TREATMENTS ON THE MECHANICAL PROPERTIES OF BOVINE AND OVINE MUSCLE

INTRODUCTION

THE IMPROVEMENTS in meat tenderness, produced by prolonged storage at temperatures above its freezing point, were once considered to be related to changes in the connective tissue, which was the structural component believed to be primarily responsible for the toughness or tenderness of the meat (Lehmann and Rumpf, 1907; Mitchell et al., 1927; Mackintosh et al., 1936). Since then the work of Locker (1960), Marsh and Leet (1966), Herring et al. (1965a, b; 1967a, b) and Davey et al. (1967) showed that the state of contraction of the muscle fibers greatly influenced tenderness, and the role of connective tissue was relegated to that of background toughness. From this work it is now established that both the fibers and their associated connective tissue contribute to toughness. The influence of aging upon both of these structural components must be taken into account when investigating the effects of aging.

There is considerable evidence that aging affects the meat fibers per se. Stromer and Goll (1967a, b) and Stromer et al. (1967) have reported aging-induced changes in bovine myofibrils. Fukazawa et al. (1969, 1970), Fukazawa and Yasui (1967) and Takahashi et al. (1967) found with chicken muscle that aging produced structural changes at the Z-line area. Davey and Gilbert (1967, 1968, 1969, 1970) and Davey et al. (1967) have reported similar changes in bovine muscle at the Z-line and in the I-bands, which could weaken the myofibrils.

Goll et al. (1970) reviewing the literature on postmortem changes in connective tissue, concluded that they were probably due to changes in the number or strength of the cross-bridges between connective tissue proteins. It was suggested that such changes with aging could cause some weakening or rupture of cross-linkages between collagen molecules, with resultant increase in the collagen solubility at temperatures of 50–80°C. Bate-Smith and Bendall (1956) expressed the view that during aging the collagen swelled under the influence of lactic acid, so that during subsequent cooking it softened more readily. de Fremery and Streeter (1969), however, found no significant changes due to aging in the thermal solubilization of connective tissue in chicken muscles. There have been few mechanical assessments of changes in connective tissue due to aging. Steiner (1939) used shear measurements along and across the muscle fibers, and found no significant aging effects which could be attributed to connective tissue. On the other hand Winegarden et al. (1952) found a small but significant decrease in the shear values of collagenous tissue strips with aging.

Davey et al. (1967) and Herring et al. (1967b) have shown that the level of tenderness achieved by aging is influenced by the state of contraction of the muscle fibers. In this present work the state of contraction has been varied by using the carcass hanging methods of Hostetler et al. (1970a, b) and Arango et al. (1970). The possible contribution of connective tissue to the toughness has been altered by using old as well as comparatively young animals. The effect of aging has been investigated using fiber tensile strength and adhesion measurements in addition to the more conventional shear and compression measurements.

EXPERIMENTAL

Aging experiments

In the first aging experiment a topside (semimembranosus muscle) and a strip loin (longissimus dorsi muscle) were removed 24 hr after slaughter from each of nine steers aged 2–3 yr. The muscles were wrapped in polyethylene sheet and stored at 0–1°C for a further 24 hr before each muscle was subdivided into three approximately equal pieces. Two parts were vacuum sealed in Cryovac bags according to commercial practice and aged at 0–1°C, for 2 and 4 wk for the loin, and 3 and 6 wk for the topside. The remaining samples were cooked 2½ days post-slaughter. The three aging treatments were assigned to the three muscle parts so that each treatment was represented by an equal number of intramuscular locations. Cooking temperatures used for samples from each aging treatment were 60° and 90°C.

The first aging experiment was intended to

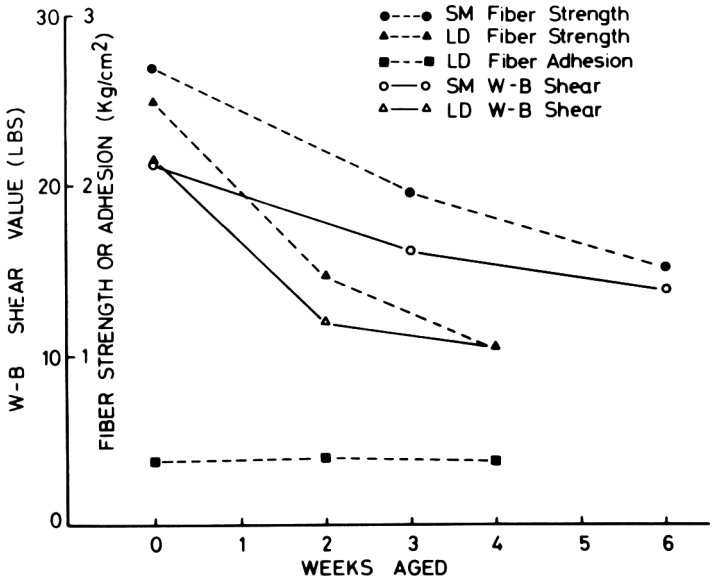

Fig. 1—Effect of aging at 0–1°C upon the fiber tensile strength and W-B shear force values obtained for beef longissimus dorsi and semimembranosus muscles, and upon fiber adhesion values for longissimus dorsi muscles, when cooked at 90°C for 1½ hr. Each point represents the mean of 6–8 observations per muscle averaged over the nine animals. Standard errors for semimembranosus and longissimus dorsi muscles are ± 1.10 and ± 0.66 for W-B shear force values and ± 0.2 and ± 0.1 for fiber tensile strength values. Standard error for adhesion values is ± 0.04.

show how the shear force, fiber tensile strength and adhesion measurements, of the muscles from normally hung carcasses, changed with aging time at 0–1°C. A second aging experiment was designed to (a) alter the contribution of the connective tissue by using animals of two different age groups and (b) to alter the contribution of the myofibrillar proteins by using muscles in different states of fiber contraction. Two groups of six animals each, aged 2–3 yr and 5–7 yr, were selected. A split plot statistical design was used. Each animal of each age group received two hanging treatments: one side had Achilles tendon suspension (normal or control method) while the other side was hung from the obturator foramen (aitch bone) using the method of Hostetler et al. (1970a, b). The same muscles were used as in the first aging experiment and each muscle from each side was subjected to three aging treatments (0, 2 and 4 wk). The muscles were sectioned, vacuum sealed in Cryovac and the parts distributed to the appropriate aging treatment, as in the first experiment, to compensate for within muscle variation. Two cooking temperatures, viz 60° and 80°C were used on samples from each aging treatment. These cooking temperatures were selected because it has been shown that at 60°C thermal contraction of the meat fibers is small while it is large at the higher temperature (Giles, 1969).

For the third aging experiment 18 Merino X sheep, aged 1–2 yr, were randomly selected from the slaughter line at a local abattoir. Within 1 hr of death, nine animals were hung from the Achilles tendon, and the remaining nine suspended from the pelvis with both back legs hanging freely. After storage at 0–1°C for 2 days the longissimus dorsi, semimembranosus and biceps femoris muscles were removed. One set of muscles was vacuum sealed in Cryovac and stored at 0–1°C for another 2 wk. The other corresponding set was cooked 2½ days postmortem.

Fiber contraction vs. adhesion measurements

The relationship between fiber contraction state and adhesion values was investigated by using the semitendinosus muscles from 10 steers aged 2–5 yr. One muscle was removed pre-rigor from each animal and split into two parts along the length of the muscle. One part was allowed to cold shorten by up to 30–40% of the rest length by storing at 0–1°C. For five animals the other part was held at room temperature for 8 hr before storing at 0–1°C and these shortened by about 10% of the rest length. The muscle sections for the remaining five animals were stretched by about 25% of the rest length by nailing to a plastic covered wooden board. The other (control) semitendinosus muscles were removed post-rigor from the remaining sides which were all conventionally (Achilles tendon) hung. All the samples were wrapped in polyethylene film and stored at 0–1°C for 6 days before cooking and using for adhesion measurements. States of contraction were assessed by measurement of sarcomere lengths.

Cooking methods

The cooking methods used have been described by Bouton et al. (1971). 180–200g samples shaped into blocks measuring approximately 12 × 6 × 4 cm were cooked for 1½ hr at 60°C and 90°C in the first beef aging experiment, and at 60°C and 80°C for the second experiment. Mutton samples weighing 130–150g were cooked at 75°C for 1½ hr. Beef semitendinosus muscles used for the contraction state vs. adhesion measurements weighed 150 ± 2g and were cooked at 80°C for 1 hr. Cooking conditions were standardized by keeping raw sample sizes approximately equal and by totally immersing, inside polyethylene bags, in water baths thermostatically controlled at the desired temperature. All samples reached the bath temperatures within an hour. The 90°C cooking temperature used for the first aging experiment was reduced to 80°C in the second as this was considered nearer the range encountered in conventional oven cooking.

Measurement of sarcomere lengths

Sarcomere lengths were determined, on raw samples removed just prior to cooking, after homogenizing several sub-samples in cold 0.08M KCl. The sarcomere length of a particular muscle was taken as the mean of 20–30 measurements on individual fibrils containing at least five sarcomeres. Samples from the aging experiments were measured three days after slaughter. No samples were frozen prior to measurements being made.

Mechanical measurements

The mechanical measurements were made with a Warner-Bratzler shear device, and with an Instron Universal Testing Machine (type TM-M), which was used for both compression and tensile tests. The Instron compression method has been described by Bouton et al. (1971). The tensile methods have been described by Bouton and Harris (1972). Double-bladed scalpel holders were used to cut rectangular sectioned samples of 1 sq cm (1.5 × 0.67 cm) with the meat fibers lying either lengthwise or perpendicular to the face of greatest area. The tensile measurements on the samples, which were held with the fibers oriented perpendicular to the strain, were considered to be a measure of the strength of the connective tissue holding the fibers together (Bouton and Harris, 1972). These measurements are referred

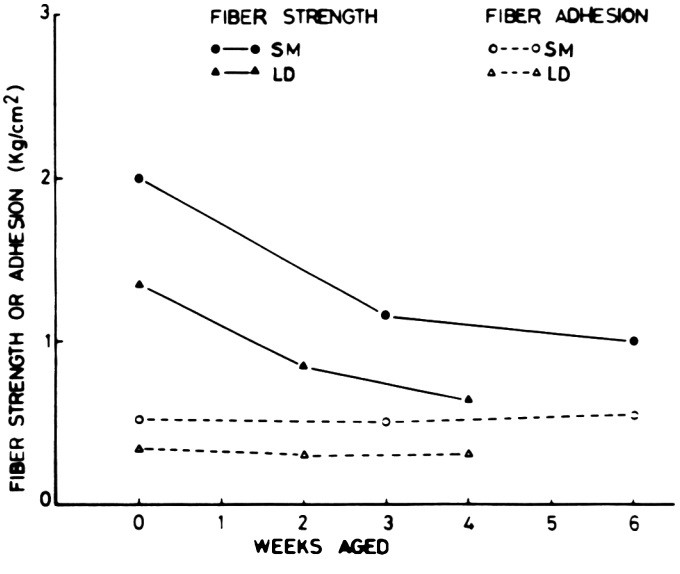

Fig. 2—Comparison of the changes produced by aging at 0–1°C on the fiber tensile strength and fiber adhesion values obtained for beef longissimus dorsi and semimembranosus muscles cooked at 60°C for 1½ hr. Each point on the graph represents the mean of 6–8 observations per muscle averaged over the nine animals. Standard errors for semimembranosus and longissimus dorsi muscles are ± 0.16 and ± 0.10 for fiber tensile strength measurements and ± 0.05 for the adhesion measurements.

Table 1—Treatment means and effective Standard Errors for Instron compression, W-B shear and fiber tensile strength measurements[a]

	Measurement					
	Instron compression		Fiber tensile strength		W-B shear force values	
Treatment	Young	Old	Young	Old	Young	Old
Hanging treatment						
Control	1.61	2.29	0.95	1.94	9.23	19.56
Stretched	1.41	2.00	1.71	2.58	8.40	13.76
S.E.	0.01	0.04	0.04	0.03	0.40	1.51
Aging						
0 wk	1.76	2.42	1.64	3.15	9.95	21.21
2 wk	1.49	2.02	1.21	2.03	8.42	15.26
4 wk	1.30	2.00	1.13	1.61	8.11	13.51
S.E.	0.03	0.05	0.05	0.05	0.50	1.02
Muscle						
SM	1.75	2.33	1.84	2.70	9.75	16.35
LD	1.28	1.97	0.81	1.82	7.91	16.98
S.E.	0.05	0.04	0.06	0.06	0.46	2.27
Temperature						
80°	1.61	2.16	1.31	2.51	9.71	18.84
60°	1.44	2.13	1.34	2.02	7.94	14.48
S.E.	0.03	0.03	0.02	0.04	0.49	0.67

[a]Measurements for semimembranosus (SM) and longissimus dorsi (LD) muscles, taken from beef sides hung from Achilles tendon and aitch bone, aged at 0–1°C for 0, 2 and 4 wk and cooked at 60 and 80°C for 1½ hr

to as adhesion measurements in the text. Tensile measurements on the samples with the fibers parallel to the applied strain were taken primarily as a measure of "fiber tensile strength" although there was some contribution from connective tissue. To avoid breakage in the pneumatically operated Instron 'jaws,' the effective cross-sectional area of these samples was reduced from 1.0 to 0.44 sq cm by preparing them in the dumb-bell shapes conventionally used for tensile testing.

RESULTS & DISCUSSION

Aging of beef–Experiment 1

The effect of aging on Warner-Bratzler shear values and measurements of adhesion and fiber tensile strength is shown in Figures 1 and 2. The effect of aging on longissimus dorsi muscles was greater than on the corresponding semimembranosus muscles, whether this effect was assessed by shear force or fiber tensile strength measurements. This difference in the rate of improvement in tenderness with aging was apparent when the samples were cooked at 60°C, where thermal contraction of meat fibers was small, and also at 90°C where such fiber shrinkage would be large, according to Giles (1969). Samples cooked at 90°C had greater tensile strength than those cooked at 60°C. This difference probably reflected the effects of the much higher cooking loss which occurred at the higher temperature. The fiber adhesion values were greater ($P < 0.001$) for the semimembranosus than for the corresponding longissimus dorsi muscles. Aging did not significantly change the adhesion between the fibers.

Aging of beef–Experiment 2

The results obtained for the mechanical measurements of Instron compression (chewiness), fiber tensile strength and Warner-Bratzler shear appear in Tables 1, 2 and 3.

The decrease in compression values due to aging were highly significant ($P < 0.001$) for both old and young animals. The stretching, due to hanging treatment of the sides, of the muscles significantly reduced the values obtained for both the young steers ($P < 0.05$) and for the old cows ($P < 0.01$). Muscles from the younger animals had significantly ($P < 0.001$) greater compression values when cooked at 80°C than when cooked at 60°C. The semimembranosus muscles had significantly ($P < 0.01$) greater values than the longissimus dorsi muscles.

The fiber tensile strength measurements showed that aging significantly ($P < 0.001$ for the old cows and $P < 0.01$ for the younger animals) reduced these values but the hanging treatment significantly ($P < 0.05$) increased the values for the semimembranosus muscle only. This is believed to be because the stretched semimembranosus muscle was stretched more by the hanging treatment than the longissimus dorsi muscle. These stretched semimembranosus muscles have more fibers per unit cross-sectional area than either the normal semimembranosus, the stretched or the normal longissimus dorsi muscles (about 27 per unit area for the stretched semimembranosus compared with 16–19 for the others, Bouton and Harris, unpublished work). The increase in fiber packing density might also be expected to increase the effective contribution of connective tissue to these measurements of fiber tensile strength.

The results from the Warner-Bratzler shear measurements showed very significant ($P < 0.001$) improvements in tenderness with aging. The hanging treatment significantly improved tenderness for both young animals ($P < 0.05$) and for the old cows ($P < 0.01$). The aging effect on shear force values was greater for the normal muscles than for the stretched muscles and was also greater for the longissimus dorsi muscles than for the semimembranosus muscles. The aging and stretching effects were more pronounced on shear force values after cooking at 80°C, when the muscles thermally contracted, than at 60°C, where contraction was small (Giles, 1969).

The mean sarcomere lengths obtained for the older animals were 1.83 ± 0.05 μm and 2.54 ± 0.05 μm for the control and stretched semimembranosus,

Table 2–Analysis of variance for Instron compression, fiber tensile strength and Warner-Bratzler shear force measurements[a]

Source of variation	D.F.	Mean squares: Instron compression	Fiber tensile strength	W-B shear force values
Animals (A)	5	0.1941**	0.0463	14.0218
Hanging treatment (T)	1	1.4641***	3.8351**	86.1803*
A × T	5	0.0159	0.0981	12.4691
Muscle (M)	1	7.8961**	7.0225**	501.7600**
M × T	1	0.0081	3.8481*	172.4844*
M × A	5	0.1310	0.1150	19.6512
M × T × A	5	0.1873	0.2381	15.4236
Aging (AG)	2	2.5305***	0.7108**	179.5180***
AG × M	2	0.2007**	0.0016	57.8839*
AG × T	2	0.1330	0.0034	39.9946*
AG × A	10	0.1227*	0.1117	19.6968
Error	32	0.0313	0.1004	11.7665
Cooking temperature (CT)	1	1.2358***	0.0023	450.1469***
CT × AG	2	0.0308	0.1590**	48.6155
CT × M	1	0.1078	0.0111	16.2678
CT × T	1	0.0007	0.0144	222.5070***
CT × A	5	0.0417	0.1972*	2.4865
Error	62	0.048	0.0178	17.3548

[a]Measurements on longissimus dorsi and semimembranosus muscles from young steers, hung from Achilles tendon and obturator foramen, then cooked at 60°C and 80°C after aging for 0, 2 and 4 wk at 0–1°C
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 3–Analysis of variance for Instron compression, fiber tensile strength and Warner-Bratzler shear force measurements[a]

Source of variation	D.F.	Mean squares: Instron compression	Fiber tensile strength	W-B shear force values
Animals (A)	5	1.0354**	0.5961*	385.4118
Hanging treatment (T)	1	2.9641**	2.8308**	4777.1136**
A × T	5	0.0929	0.0721	163.6426
Muscle (M)	1	4.9803**	5.4951**	58.0136
M × T	1	0.0008	3.1240*	109.9004
M × A	5	0.2766	0.3693	142.2759
M × T × A	5	0.1111	0.2355	370.1434
Aging (AG)	2	2.5617***	5.8535***	3115.3631***
AG × M	2	0.1689	0.1771	917.2401***
AG × T	2	0.0156	0.0294	370.1530*
AG × A	10	0.3012	0.1724	194.0054**
Error	32	0.1438	0.1156	49.6685
Cooking temperature (CT)	1	0.0103	1.6921***	2742.2677***
CT × AG	2	0.6417**	0.1531	686.7151***
CT ×M	1	0.0325	0.2836	94.7378
CT × T	1	0.0842	0.0001	1653.7778***
CT × A	5	0.0151	0.2792	17.0040
Error	62	0.1005	0.1050	32.6398

[a]Measurements on longissimus dorsi and semimembranosus muscles from old cows hung from Achilles tendon and obturator foramen, then cooked at 60°C and 80°C after aging for 0, 2 and 4 wk at 0–1°C
*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

1.89 ± 0.05 μm and 2.11 ± 0.05 μm for the control and stretched longissimus dorsi. Results for the younger animals were similar, viz. 1.80 ± 0.07 and 2.88 ± 0.07 for the control and stretched semimembranosus and 2.07 ± 0.03 and 2.28 ± 0.03 for the corresponding longissimus dorsi muscles. The aitch bone hanging method significantly stretched the semimembranosus ($P < 0.001$) and the longissimus dorsi ($P < 0.01$). The control longissimus dorsi muscles from the younger animals were significantly longer than in the older animals but there was no difference between the control semimembranosus muscles.

The results showed that, regardless of whether tenderness was assessed by a compression or by a shear method, the effect of stretching the longissimus dorsi and semimembranosus muscles by using the method of Hostetler et al. (1970a, b) was to give an initial tenderness (at 2½ days post-slaughter) equivalent to the tenderness of nonstretched muscles after about 2 wk aging.

Aging of mutton–Experiment 3

Hanging sheep carcasses from the pelvis, instead of from the Achilles tendon, significantly improves the tenderness, as assessed by compression ($P < 0.05$), shear force ($P < 0.001$) and adhesion ($P < 0.01$) measurements, of the longissimus dorsi, semimembranosus and biceps femoris muscles (Table 4).

Quarrier et al. (1971) have shown that hanging from the pelvis improved the tenderness of lamb longissimus dorsi muscles.

Sarcomere lengths for the three muscles, which are shown in Table 4, are mean values over the two hanging treatments and show that the longissimus dorsi (1.80–2.18 μm is stretched less by the hanging treatment than the semimembranosus (1.73–2.46 μm) and biceps femoris (1.79–2.40 μm) muscles. Aging significantly improves ($P < 0.001$) tenderness as assessed by compression and shear force values but has no significant effect on adhesion values.

As can be seen in Table 4 stretching and aging treatments both produce similar decreases in compression and shear force values but only stretching reduces adhesion values as well.

Adhesion measurements

Adhesion values obtained for the semimembranosus muscles from the second beef aging experiment were subjected to analysis of variance–see Table 5. Mean values, in kg/cm², for the older animals were 0.83 and 0.49 for samples from the control and stretched sides cooked at 80°C and these were significantly ($P < 0.01$) higher than the values of 0.68 and 0.34 obtained for the samples cooked at 60°C. Bouton and Harris (1972) reported an increase in adhesion values obtained for muscle samples cooked at 90°C over values obtained for samples cooked at lower temperatures or for raw samples. They attributed this effect to the influence of the marked thermal contraction of meat fibers which has been reported for cooking temperatures greater than 60°C (Giles, 1969). No significant difference attributable to cooking temperature was found for the younger animals although there was a highly significant difference in the mean values, 0.50 and 0.32, found for the muscles from the control and stretched sides. Adhesion values were, however, significantly ($P < 0.01$) greater for the older than for the younger animals and the stretching significantly reduced the values obtained for samples from both age groups. Aging again had no significant effect on adhesion values; a result in agreement with results from the first beef aging experiment reported here and from the mutton aging experiment.

Effect of fiber contraction state on adhesion values

Herring et al. (1967a) measured hydroxyproline content for muscles in different contraction states and found no significant relationship between contraction state and either collagen content or collagen solubility. Buck and Black (1968) suggested that increased tenderness in stretched muscle could be partly accounted for by a mechanical thinning of the connective tissue, and by greater thermal denaturation. The results from the second beef aging experiment and from the sheep experiment showed that stretching significantly reduced adhesion values. The results obtained in this present work for beef semitendinosus muscles in various contraction states are shown in Table 6. A direct comparison of all 10 cold shortened (1.4–1.5 μm sarcomere lengths) semitendinosus muscles with their controls (2.1–2.2 μm sarcomere lengths) showed that the more contracted

Table 4–Treatment means and effective standard errors for Instron compression, W-B shear, sarcomere length and adhesion[a]

Treatment	Measurement			
	Instron compression	W-B shear	Sarcomere length (μm)	Adhesion
Hanging treatment				
Achilles tendon	1.71	16.58	1.77	0.45
Pelvis	1.48	10.62	2.35	0.33
S.E.	0.06	0.64	0.03	0.03
Aging				
0 wk	1.75	15.70	–	0.37
2 wk	1.44	11.50	–	0.40
S.E.	0.03	0.35	–	0.02
Muscle				
LD	1.51	13.75	1.99	0.33
SM	1.53	14.70	2.10	0.34
BF	1.75	12.35	2.10	0.50
S.E.	0.03	0.44	0.03	0.02

[a]Data are for longissimus dorsi (LD) semimembranosus (SM) and biceps femoris (BF) muscles taken from sheep carcasses hung either from Achilles tendon or directly from the pelvic girdle, and aged for 0 and 2 wk at 0–1°C before cooking at 75°C for 1½ hr.

Table 5–Analysis of variance for adhesion values[a]

Source	D.F.	Mean Squares
Animal age	1	1.0729**
Animal (A)	10	0.0617
Hanging treatment (HT)	1	2.4675***
Animal age × HT	1	0.2377
A × HT	10	0.0555
Aging	2	0.0409
Aging × Animal age	2	0.0202
Aging × A	20	0.0540
Aging × HT	2	0.0713
Error	22	0.0267
Cooking temperature (CT)	1	0.1943**
CT × Animal age	1	0.1915*
CT × A	10	0.0363
CT × Aging	2	0.0060
CT × HT	1	0.0138
Error	57	0.0231

[a]Obtained for semimembranosus muscles from 2–3 yr old steers and 5–7 yr old cows hung from Achilles tendon and obturator foramen then cooked at 60°C or 80°C after aging for 0, 2 and 4 wk at 0–1°C

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table 6—Results obtained for sarcomere lengths and adhesion values of bovine semitendinosus muscles in different fiber contraction states

Experiment	Parameter measured	Control	Removed pre-rigor: Stored at 0–1°C	Removed pre-rigor: Stored at 22°C for 8 hr then stored at 0–1°C	Removed pre-rigor: Stretched and stored at 0–1°C	Std error
1	Adhesion (kg/cm²)	0.65[a]	1.08[b]	0.76[a]	–	0.09
	Sarcomere length (μm)	2.10[a]	1.43[b]	1.90[c]	–	0.04
2	Adhesion (kg/cm²)	0.76[a]	1.12[b]	–	0.65[a]	0.05
	Sarcomere length (μm)	2.24[a]	1.48[b]	–	2.56[c]	0.09

[a,b]Different superscripts in same line denotes that means are significantly different at least at $P < 0.01$ level. Each entry in table for adhesion values represents the mean of 10–20 observations averaged over five animals.

muscles had significantly ($P < 0.001$) higher adhesion values.

Since stretching decreases adhesion values and cold shortening increases these values relative to their controls, adhesion between the meat fibers was clearly affected by fiber contraction state.

CONCLUSIONS

ADHESION VALUES for both beef and mutton were not significantly affected by the process of aging. These results suggested that if changes occurred in the connective tissue, they were too subtle to be picked up by purely mechanical measurements. It was concluded that changes in connective tissue were unlikely to contribute significantly to the increase tenderness achieved during aging. Changes in connective tissue could, however, contribute to the increased tenderness achieved by stretching.

Once fiber toughness has decreased with aging the relative importance of connective tissue toughness increases. For muscles with high inherent connective tissue toughness it would be anticipated that they could remain tough even after aging. It has been shown that muscles when contracted have higher adhesion values than when stretched. Davey et al. (1967) and Herring et al. (1967b) have shown that fiber contraction state influenced the level of tenderness achieved with aging. Since connective tissue was also affected by contraction of the muscle fibers it could partly account for the failure of such contracted muscles to become as tender with aging as their stretched counterparts.

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POULTRY PRODUCT QUALITY. Carbonyl Composition and Organoleptic Evaluation of Mechanically Deboned Poultry Meat

INTRODUCTION

THE DEMAND for cut-up poultry has been steadily increasing during the past few years. Associated with this demand is the availability of large volumes of broiler necks and backs which in the past have been marketed as raw material for the pet food industry. With the introduction of commercial deboning machines, it became feasible to remove the meat from necks and backs for use in further processing operations thereby increasing the market values of these raw materials, and at the same time making the deboned poultry meat competitive with other types of raw meats. While the availability of this meat has opened new areas of utilization, several processing and quality control problems must be solved to fully utilize this meat. Because deboning machines can handle large volumes of raw material (up to a ton per hour) some plants might not need to operate on a daily basis. As a result raw material might spend up to several days in storage prior to the deboning operation. In addition to the obvious studies on quality and storage life of the deboned meat itself, it is important to be aware of any effects that might result from the storage of the raw materials.

The objectives of this study were to evaluate deboned meat from various types of poultry with regard to its keeping quality as measured by the carbonyl concentration, a measurement for autoxidation of lipids, and flavor evaluation by an experienced taste panel.

EXPERIMENTAL

Sample description

Samples of mechanically deboned poultry meat were collected for this study from two poultry processing plants. During the investigation several trials were conducted as outlined in Table 1. Attempts were made to duplicate the raw materials for each of the three groups of poultry types from (1) broiler necks and backs, (2) whole spent layers and (3) turkey racks; however, due to processing schedules in one plant this was not entirely feasible. This is evident in Trials 5 and 6 where there is a sex and age difference in the source of the turkey meat analyzed.

In each trial approximately 100 lb of comminuted meat were collected from a Bibun deboning machine during normal plant operations, subdivided into 10-lb aliquots, packaged in Cry-O-Vac bags, placed in chilled insulated coolers and immediately transported to the laboratory.

Treatment description

The raw material from each kind of poultry under test was divided into two groups: one was deboned immediately after chilling (referred to as conventional processing) and the other was held at the processing plant 5 days at 3–5°C prior to deboning (referred to as delayed processing). The effect of storage on the deboned meat from the two processes was observed by holding the deboned material in Cry-O-Vac bags in refrigerated storage at 3°C for 0, 3, 6 and 12 days.

Chemical analyses

The classes and concentrations of carbonyl compounds from raw deboned meat (Trials 1, 3 and 5) and cooked deboned poultry meat (Trials 2, 4 and 6) were determined following each of the storage periods. Approximately 50g of deboned meat was weighed, transferred into an Omni-Mixer jar and overlayered with 200 ml of carbonyl-free hexane. The resulting mixture was homogenized for 15 min and filtered. The residue was again homogenized for 5 min with 100 ml of carbonyl-free hexane and filtered. The combined filtrate was collected for reaction with 2,4-dinitrophenyl (DNP) hydrazine as outlined by Dimick and MacNeil (1970) and Thomas et al. (1971) and according to the method of Schwartz et al. (1962). The resulting derivatized carbonyl classes (total carbonyl, monocarbonyl, alkan-2-ones, alkanals and alk-2-enals) were identified and quantified spectrophotometrically at their respective absorption maxima and expressed as μmole per 10g fat. The amount of hexane extractable fat in each sample analyzed was determined by extracting a 50-g portion of deboned meat followed by drying under vacuum and weighing in a tared vessel.

Taste panel procedures

2 lb of deboned meat were heated to 163°C (≈ 15 min) while stirred with a rubber spatula in an uncovered teflon-lined electric skillet. Samples for carbonyl determinations were immediately placed in a glass beaker, covered and taken to the chemical laboratory for analyses. The remainder was presented to an experienced taste panel of 12 women according to the procedure outlined by MacNeil and Dimick (1970). A directional triangle test was used to identify the correct pairs and to establish taste preferences. The control samples were from the same source, held in a −32°C freezer and thawed the morning of the panel evaluation. An additional preference evaluation was obtained by using a 9-point hedonic scale. Chi-square and analysis of variance statistical techniques (Snedecor, 1956) were used to analyze the taste panel data.

RESULTS & DISCUSSION

Conventional processing

Concentrations of the carbonyl classes in the three raw deboned meat types following refrigerated storage at 3°C are presented in Table 2. Comparative data are presented in Table 3 for cooked deboned meat following raw meat storage at the same temperature. The data for the broiler necks and backs and whole spent layers may be considered replicates; however, data from turkey racks must be viewed in light of the sex and age differences which existed due to sample source. The concentrations of the total and monocarbonyl compounds in the raw deboned broiler meat (0-time sampling) were comparable to that found in raw skin (Thomas et al., 1971) from 8-wk old male broilers (ca. 46 and 10 μmole/10g fat, respectively). These investigators also found low amounts of alkan-2-ones associated with the skin of broilers as compared to that of turkeys, which in the present study, was also evident for raw deboned broiler meat when compared with meat from other kinds of poultry. Raw deboned meat from all three kinds of poultry contained saturated and unsaturated aldehydes in low concentrations (0.2–0.8 μmole/10g fat). These products of autoxidation, however, were not found

Table 1—Description of mechanically deboned poultry meat analyzed in this investigation

Trial	Type of poultry	Cut-up parts	Identification	Age (wk)	Sex	Avg live wt (lb)	Machine capacity (lb/hr)
1	Broiler	Necks & backs (1:2)	Commerical	7–8	♂	3–4	3500
2	Broiler	Necks & backs (1:2)	Commerical	7–8	♂	3.7	3500
3	Spent layer	Whole carcasses	White Leghorn	>52	♀	3–4	3500
4	Spent layer	Whole carcasses	White Leghorn	>52	♀	3–4	3500
5	Turkey	Racks	Broad breasted white	52	♀	17.8	2000
6	Turkey	Racks	Broad breasted white	24	♂	25.3	2000

in the fresh raw skin of chickens and turkeys (Thomas et al., 1971) and therefore may be a result of processing the deboned meat and/or associated with muscle tissue which makes up a portion of the meat emulsion.

The effect of cooking deboned meat dramatically increased the concentration of carbonyls (Table 3). At the 0-time sampling, the total carbonyls and monocarbonyls increased 2- to 3-fold as a result of cooking when compared to the concentration in raw deboned meat. Similarly, cooking promoted an increase in alkanal concentrations of fowl carcasses, increasing from 0.4 to 3.0 μmole/10g fat. The lack of alk-2-enals in the cooked fowl and turkey meat held in storage up to 6 days before cooking was surprising and the reason for their absence is unknown at present. No evidence of alk-2,4-dienals class of monocarbonyls was found in the raw or cooked samples in this study.

The effect of short-term storage at 3°C of raw deboned meat was negligible for all classes of carbonyls analyzed. However, when this material was cooked after being held in storage, dramatic increases in the concentration of the various carbonyls were noted. Deboned meat from turkey racks was the most unstable following 12 days of raw meat storage, containing 263 and 104 μmole/10g fat total carbonyl and monocarbonyl compounds, respectively. The concentrations were twice that found in the same material at 0-time storage. This increase can be partially accounted for by the increase in alkanals and alk-2-enals amounting to 22.5 and 10.2 μmole/10g fat, respectively, following 12 days of storage. The concentrations in the aldehyde classes of compounds remained relatively constant in the meat from spent layers and broiler necks and backs until the 12 days sampling period. The increase in these compounds from the 6 to 12 day storage samples in all three kinds of poultry may be indicative of autoxidative deterioration. The instability of the turkey deboned meat as compared to the chicken meat may be due to lower levels of the natural antioxidants, tocopherol, found in turkeys (Nutter et al., 1943). An examination of the carbonyl compounds associated with poultry skin fractions demonstrated the relative instability of turkey when compared to chickens (Dimick and MacNeil, 1970).

The taste panel evaluations of all samples tested are presented in Table 4. Panel members could not discriminate between control and conventionally processed treatment broiler meat samples at 6 days of storage but did indicate discriminatory ability after 12 days storage. With the delayed processed broiler samples the panel members could detect control-treatment differences after 6 days of storage in one test but when this test was repeated the 6-day differences were not indicated. The panel members could discriminate between the control and treated samples of spent layer meat and deboned turkey meat at 6 days of storage. A preference for the control fowl meat was evident at 6 days, whereas, the panel members could not determine a preference for deboned turkey meat until 12 days of storage. Mean hedonic scores showed lowered flavor responses for treated samples as compared to control for deboned meat from spent layers (Trial 4) and turkey racks (Trial 6) following 6 days of storage. Mean hedonic scores for broiler samples (Trial 1) remained relatively constant throughout the 12 days of raw storage following conventional processing.

Delayed processing

The effect of delayed processing, that is poultry parts held 5 days at 3–5°C prior to deboning, on the concentration of the individual carbonyl classes in raw meat throughout storage was similar when compared to conventionally processed raw deboned meat. Similarly, the holding treatment for the poultry parts did not increase the concentrations of the various carbonyl classes following storage and cooking of the deboned meat. These findings suggest that the autoxidative stability of the deboned meat as measured by the concentration of carbonyls, was not influenced measurably by holding the poultry parts for up to 5 days at 3–5°C, even though increased microbial activity has been shown to occur in

Table 2–Effect of raw storage on carbonyl composition of conventionally processed raw deboned poultry meat[a]

Storage at 3°C (days)	μMol/10g fat														
	Total carbonyls			Monocarbonyls			Alkan-2-ones			Alkanals			Alk-2-enals		
	B[c]	L[c]	T[c]	B	L	T	B	L	T	B	L	T	B	L	T
0	46.2	55.7	44.3	11.1	18.9	31.8	4.3	12.3	21.5	0.2	0.4	0.8	0.2	0.2	0.3
3	51.8	71.3	44.3	14.1	42.9	25.4	7.5	26.3	18.7	0.4	1.8	1.5	0.2	0.0	0.2
6	57.3	71.7	35.2	17.6	35.5	22.6	8.5	17.5	17.0	0.6	1.0	0.6	0.3	0.0	0.3
12	62.2	69.9	35.8	17.5	–[b]	25.3	7.9	–[b]	15.5	0.4	–[b]	1.6	0.2	0.0	0.5

[a]Mean of duplicate analyses
[b]Sample lost
[c]B = Broiler necks and backs, Trial 1; L = Whole spent layers, Trial 3; T = Turkey racks, Trial 5.

Table 3–Effect of raw storage on carbonyl composition of conventionally processed cooked deboned poultry meat[a]

Storage at 3°C (days)	μMol/10g fat														
	Total carbonyls			Monocarbonyls			Alkan-2-ones			Alkanals			Alk-2-enals		
	B[c]	L[c]	T[c]	B	L	T	B	L	T	B	L	T	B	L	T
0	91.7	154.1	138.8	38.3	47.3	54.0	22.3	23.7	25.2	0.2	3.0	1.9	0.3	0.0	0.0
3	123.3	144.8	90.4	70.9	48.8	35.6	55.5	24.3	20.4	0.5	2.6	0.0	0.7	0.0	0.0
6	55.9	173.7	108.1	25.5	74.0	37.3	19.5	26.1	25.5	0.2	3.7	4.9	0.3	0.0	0.0
12	81.4	141.8	262.9	22.5	32.5	104.2	12.3	–[b]	37.1	2.0	4.5	22.5	1.8	1.3	10.2

[a]Mean of duplicate analyses
[b]Sample lost
[c]B = Broiler necks and backs, Trial 2; L = Whole spent layers, Trial 4; T = Turkey racks, Trial 6.

Table 4—Taste panel evaluation of conventional (C) and delayed (D) processed cooked deboned poultry meat following raw storage at 3°C

Storage days	Trial	Triangle test		Preference for control		Mean hedonic score[a] Control		Treatment	
		C	D	C	D	C	D	C	D
Broiler necks & backs	1								
0		NS	NS	NS	NS	6.0	5.8	6.2	6.0
3		NS	NS	NS	NS	5.9	6.3	6.7	6.2
6		NS	NS	NS	NS	5.8	6.3	5.6	5.7
12		**	–[b]	++	–	6.3	–	5.0	–
Broiler necks & backs	2								
0		NS	NS	NS	NS	5.8	5.5	5.6	6.2
3		NS	NS	NS	NS	5.0	4.5	5.4	5.5
6		NS	NS	NS	NS	4.5	6.0	5.3	5.3
12		–	**	–	NS	–	5.3	–	3.8
Spent layers	3								
0		NS	NS	NS	NS	6.0	4.3	6.0	6.5
3		NS	NS	NS	NS	5.5	5.1	5.7	6.0
6		**	NS	+	NS	5.1	4.5	5.8	6.6
12		–	–	–	–	–	–	–	–
Spent layers	4								
0		NS	**	NS	NS	5.8	6.1	6.3	5.7
3		NS	NS	NS	NS	6.2	5.6	6.1	6.2
6		**	**	++	NS	5.6	5.4	2.4	4.9
12		–	–	–	–	–	–	–	–
♀ Turkey racks	5								
0		NS	**	NS	NS	6.1	5.4	6.3	5.8
3		NS	**	NS	NS	5.2	5.3	5.7	5.6
6		**	**	NS	+	5.4	5.2	5.9	5.8
12		**	–	++	–	5.2	–	3.4	–
♂ Turkey racks	6								
0		NS	**	NS	NS	5.6	5.7	5.8	6.0
3		NS	**	NS	++	5.3	5.8	5.4	3.5
6		*	**	NS	++	5.8	4.8	4.9	2.8
12		**	–	++	–	4.8	–	3.2	–

[a]Hedonic scores from 1, dislike extremely; to 9, like extremely
[b]–Not submitted to panel due to extremely disagreeable odor
*Significant discrimination between control and treatment at 5% level
**Significant discrimination between control and treatment at 1% level
+Preference for control samples at 5% level
++Preference for control samples at 1% level
NSNo significant discrimination between control and treatment

delayed processing studies (Ostovar et al., 1971).

The taste panel evaluations were also remarkably similar in both the delayed and conventional processing studies (Table 4). A notable difference was found however, in the delayed processed turkey meat where the panel members discriminated between the control and treated samples in all sampling periods. In addition, the panel members indicated a significant preference for the control after 6 days (♀ turkey racks) and 3 days (♂ turkey racks) of storage following delayed processing. However, the panel members did not indicate a preference for the control until after 12 days storage of conventionally processed turkey meat. The reason for the discrimination ability in the early storage samples of the delayed process is unknown at this time although the oxidative instability of turkey lipids could very well be involved. Great difficulty confronts the investigator in attempting to correlate subjective data (taste panel responses) with objective data (chemical analyses). Too many variables make up the total flavor response of a food system and therefore correlative data from limited parameters may lead to erroneous conclusions. For example, it has been shown that the volatile carbonyls contribute to the "chickeny" aroma of cooked chicken muscle, whereas, the sulfur compounds contribute to the "meaty" flavor (Minor et al., 1965). This study has demonstrated increased levels of saturated and unsaturated aldehydes following 6–12 days of storage of deboned poultry meat which may be responsible for the taste panel discrimination at those sampling periods. The taste thresholds for these aldehydes range from 1–0.01 ppm (Meijboom, 1964) which is below the concentrations found in this study. Preliminary evidence from our laboratory has demonstrated the increased concentrations of volatile sulfur compounds (H_2S, mercaptans and disulfides) in stored deboned broiler meat when compared to fresh deboned meat. Therefore, one must also consider the contribution of these protein degradation compounds to the overall flavor response.

CONCLUSIONS

THE KEEPING QUALITY of deboned meat, as measured by carbonyl concentration and organoleptic evaluation, is maintained up to 6 days at 3°C. After 6 days of raw meat storage, large increases in the concentration of carbonyls occurred in the cooked deboned meat. Concurrently, the panel members discriminated between the stored and control samples and indicated a lower hedonic flavor response for the stored samples. In general, deboned meat from turkey racks was least stable in storage, followed in order by whole spent layers and broiler necks and backs. No differences in storage stability were noted where poultry parts were deboned immediately or held 5 days at 3–5°C prior to deboning. However, as pointed out by Ostovar et al. (1971) from this laboratory, delayed processing should be avoided due to increased growth of psychrophilic and psychrotolerant microorganisms associated with this procedure.

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OXIDATIVE RANCIDITY IN RAW FISH FILLETS HARVESTED FROM THE GULF OF MEXICO

INTRODUCTION

TRADITIONALLY, oxidation of raw fresh fish muscle has not been considered a matter of great importance since most deterioration of the muscle tissue has generally been attributed to microbial spoilage (Olcott, 1962). Watts (1961) first reported on the incidence and rates of oxidation in the muscle tissue of several fish species from the Gulf. These species included mullet, mackerel, red snapper, red drum and sea trout. Significant increases in the TBA numbers of the muscle tissues were observed during refrigerated storage following a heat treatment of 60–80°C. Changes in TBA numbers were attributed to the cooking process which provided additional energy to the oxidation reaction. This conclusion agrees with that of Lea (1962) in his review of factors influencing the rate and course of the lipid oxidation reaction. No data are available, however, describing the extent of oxidation occurring in the muscle tissue of many of the Gulf species during harvest, processing and storage, prior to cooking and consumption. Factors which may affect the rate of oxidation include chemical composition of the carcass, season of harvest, handling procedures and conditions of storage. Data relating these factors to the oxidation reaction would be helpful in determining more effective methods of controlling rancidity. Previous studies have been designed to determine methods of preventing oxidative rancidity and controlling flavor changes utilizing various chemical compounds. The majority of these studies, related to fish species of the Gulf, were concerned with controlling oxidation in mullet *(Mugil cephalus)* tissue. Zipser and Watts (1961) have demonstrated the effectiveness of antioxidant mixtures, either alone or in combination with curing salts, to inhibit oxidation in ground cooked mullet. Saenz and Dubrow (1959) reported that butylated hydroxy toluene (BHT) was effective in controlling oxidative rancidity in whole frozen mullet fillets. BHT was also reported to be an effective antioxidant when used in smoked mullet (Thompson, 1962). Although these studies are of value as to methods of controlling oxidation, additional information is needed to accurately establish the effect of oxidative rancidity on the spoilage patterns of fresh uncooked fish. The purpose of this study was (1) to measure the extent of oxidative rancidity in various Gulf species and (2) to establish which factors associated with harvest, processing and storage affect the rate and course of the oxidative rancidity reaction and/or produce a significant change in flavor.

EXPERIMENTAL

Materials

Fish for this study were obtained from the West Coast of Florida either by direct harvest, purchase from local fishermen, or from local wholesale distributors. Size, post-mortem age and handling procedures were controlled only in those lots of fish which were captured by direct harvest. In some instances, direct harvest using commercial equipment was avoided because the quality of the fish that existed in the local market was of primary interest. All fish were held in ice and transported to laboratories at Gainesville, Fla. where they were eviscerated, filleted, skinned, washed and stored at 1°C. Each lot contained 25 fish. Samples were prepared by randomly selecting six paired fillets from each lot. The right fillets from each pair were pooled and used for chemical and microbial analysis. Left fillets were pooled for sensory panel samples.

Methods

Oxidative rancidity was determined using the 2-thiobarbituric acid method (Yu and Sinnhuber, 1957; Sinnhuber and Yu, 1958). Samples were prepared by adding 50g of tissue to 100 ml distilled-deionized water followed by blending at low speed in a Waring Blendor for 30 sec. Duplicate aliquots of the resulting homogenate were assayed for TBA number which was expressed as the mg of malonaldehyde per 1,000g of tissue.

A standard curve of the absorbance of several concentrations of malonaldehyde was prepared using 1,1,3,3-tetramethoxy propane (Eastman Chemicals). The extinction coefficient for the resulting malonaldehyde-TBA complex was 1.58×10^6 which was comparable to the value derived by Sinnhuber and Yu (1958) using 1,1,3,3-tetramethoxy propane as the source of malonaldehyde.

Differences in flavor response were determined using a sensory panel composed of twelve persons. Treated and control samples were cooked prior to sensory evaluation by broiling to an internal temperature of 160°F. Random coded samples of each treatment and a control were given a numerical score using a hedonic scale from Peryam and Gerardot (1952). Duplicate samples were scored for flavor and overall desirability.

Total plate counts were determined following standard methods (APHA, 1967). Plates were incubated 48 hr at 25°C. Growth was expressed as total plate count per g (TPC/g).

RESULTS & DISCUSSION

ALL OF THE FISH listed in Table 1 were collected at a single wholesale establishment. They had been previously purchased from local fishermen one day prior to collection and had been stored in the round, packed in ice at 2–3°C. After thorough washing they were filleted, placed on Teflon trays and stored at 1°C.

Muscle tissue from mullet and bluefish were the only samples which showed any consistent increase in TBA number (Table 1). Oxidative rancidity was evident in the mullet tissue after 3 days storage and in bluefish samples after 5 days. Average flavor scores decreased in both samples at 3 and 6 days, respectively. Total plate counts were between 1- and 3,000,000 per gram in the bluefish and between 400 and 370,000 per gram in the mullet tissue at 3 and 5 days, respectively.

Reduction of the flavor scores in most species which were studied was attributed to increases in TPC/g. Scores of the mullet fillets were unique, however, in that the initial reduction in flavor was probably due to oxidative rancidity rather than extensive bacterial growth. A reduction in flavor score of mullet from 5.5 to 4.5 was evident before TPC per g exceeded 500,000 in most cases. At a temperature of 1°C oxidation seems to be of greater importance in the flavor deterioration of fresh mullet fillets than microbial growth.

Because of the high susceptibility to oxidation evident in the mullet tissue and its economic importance as a food fish four lots of mullet were collected using commercial gear in an attempt to monitor the changes in TBA number immediately postmortem and to establish a more precise pattern of spoilage. Most of the fish were between 12 and 30 hr old at the time of the initial assay. Results of that experiment are given in Figure 1. As the storage time approached the 4th day TBA numbers of the mullet fillets began to increase rapidly, approaching a value of 10–12 mg. Concurrent with the sudden increase in TBA number was a simultaneous decrease in flavor score. Initially, the sensory panel's average flavor score for the cooked mullet tissue was 5.6. As the

TBA number approached a value of 4–5 the flavor score began to decrease until it reached a value of 3.5. At this point the TBA number had increased to at least 10 or above.

Sensory panel members were able to detect a change in mullet flavor as the TBA number approached a value between 4 and 6. The panel could not detect a flavor difference between fillets with TBA number of 8.0 and 15.0 indicating in their comments that both had a rancid flavor unlike the flavor of fresh mullet. These results suggest that the change in flavor response which occurs at the threshold level (4–6 mg malonaldehyde/1000g tissue) is probably of greatest importance since any increases in TBA number beyond the threshold had little influence on flavor.

Angalet (1971) found considerable seasonal variation in the lipid content of fresh mullet fillets. Lipid content average 4–5% during the period of August to January and 1–2% during the period of February to July. These data prompted a study to determine the effect of season of harvest on the susceptibility of mullet tissue to oxidation. Ten individual lots of mullet were captured during different months of 1970 and 1971. Availability of the fish limited the months of collection to those listed in Table 2. During 13 days storage at 1°C those fillets collected during the month of May did not show an increase in TBA number. Fish collected prior to May and during the regular commercial season from late summer to December showed a pattern of oxidative rancidity previously described in this paper.

Seasonal variation in the susceptibility of mullet tissue to oxidation cannot be attributed to total lipid content. Fillets with total lipids less than 2% have been shown to oxidize to the same extent as those containing 5% total lipid. Much of the seasonal variation in the rates of oxidative rancidity observed in mullet tissue may be due to physiological condition of the fish. During late November when the TBA numbers increased most rapidly most mullet from the collection area were in full roe. Variation may also be due to migration patterns which influence the diet of the fish to a great extent. Broadhead (1953) indicated that a major portion of the life of the mullet is spent in coastal bays and inlets. However, during summer many of the fish migrate into fresh water streams and inland waterways. During the spawning season (October to February) the mullet return to the salt water to spawn off-shore. Mullet collected during the month of May, when TBA numbers remained consistently below 2.0, were harvested from the mouth of a fresh water stream where it mixes with salt water of the Gulf. The effect of migration into fresh water, during the summer months, on the diet and susceptibility of the muscle tissue to oxidation as well as the effect of roe formation should be investigated further.

Hematin compounds have long been known to catalyze oxidative rancidity (Brown et al., 1957; Tappel, 1962; Zipser et al., 1962; Castell and MacLean, 1964;

Table 1–Average TBA numbers, TPC/g and flavor scores of fillets from selected fish harvested in the Gulf of Mexico

Gulf fish species	Days storage @ 1°C							
	1	2	3	4	5	6	7	8
Trout								
TBA No.	2.8	1.5	1.9	1.3	2.4	1.5	1.4	1.2
TPC/g × 10^{-3}	21	78	500	450	–	8,450	5,300	–
Flavor score	5.9	–	5.6	–	–	5.8	–	5.5
Grouper								
TBA No.	1.5	1.4	1.2	1.2	2.5	1.7	1.3	1.0
TPC/g × 10^{-3}	91	74	1,300	1,700	–	4,300	46,000	–
Flavor score	5.6	–	5.5	–	–	4.6	–	4.7
Blue Fish								
TBA No.	2.5	1.7	1.5	1.8	5.0	5.2	8.5	9.1
TPC/g × 10^{-3}	9	21	1,300	975	–	3,400	92,000	–
Flavor score	5.0	–	5.0	–	–	4.0	–	4.0
Grunt								
TBA No.	2.5	2.1	1.2	1.0	1.7	1.5	1.4	1.2
TPC/g × 10^{-3}	79	111	1,200	6,400	–	325,000	680,000	–
Flavor score	6.0	–	5.7	–	–	5.0	–	4.0
Whiting								
TBA No.	1.8	1.4	1.2	1.3	2.0	1.2	1.9	2.0
TPC/g × 10^{-3}	44	66	340	6,200	–	44,000	515,000	–
Flavor score	5.9	–	5.2	–	–	5.9	–	5.0
Mullet								
TBA No.	1.4	1.9	7.5	10.2	16.8	15.9	13.6	17.7
TPC/g × 10^{-3}	20	51	400	370	–	32,000	33,000	–
Flavor score	5.5	–	4.5	–	–	4.4	–	4.0
Black Sea Bass								
TBA No.	2.4	1.1	1.9	2.6	1.6	1.2	1.3	1.4
TPC/g × 10^{-3}	20	65	310	680	–	10,000	22,000	–
Flavor score	5.0	–	5.1	–	–	4.1	–	4.0
Mackerel								
TBA No.	1.2	2.5	3.5	3.2	2.8	2.0	3.1	2.8
TPC/g × 10^{-3}	62	62	515	6,900	–	104,000	340,000	–
Flavor score	5.0	–	4.9	–	–	5.3	–	5.2

Table 2–Average TBA numbers of mullet fillets collected during various months of the year and stored at 1°C

	Days storage												
Months	1	2	3	4	5	6	7	8	9	10	11	12	13
April	1.6	1.3	1.4	1.9	7.5	5.9	8.6	7.7			8.1		
May	1.0		1.4			1.7	1.4			1.8	1.6		1.5
July	1.6		2.1			2.4	8.1				9.1	8.4	
Aug.	2.3		2.4				7.8		10.9		13.3		11.6
Dec.	2.1		2.0	7.8			10.4				15.1		14.6

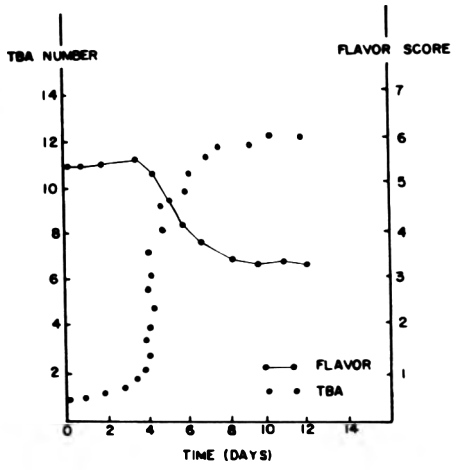

Fig. 1–Average TBA numbers and panel flavor scores of mullet fillets stored at 1°C.

MacLean and Castell, 1964; Castell et al., 1966). Even though the bleeding of mullet immediately postmortem reduces the hemoglobin content of the carcass, the effect of this practice on the rate of oxidation in the muscle tissue was not known. One-half of one lot of fish captured using commercial gear was bled immediately postmortem by severing the vessel connecting the heart to the gills and allowing the heart to continue pumping the blood from the carcass. These bled samples along with the remaining control samples were filleted, stored at 1°C and assayed for malonaldehyde at various intervals during a period of 13 days. There were no differences between the bled and nonbled samples as is evident from the data in Table 3.

Although bleeding postmortem did not reduce the rate of oxidation in the muscle tissue it did provide a more desirable carcass for processing. Bleeding reduced the quantity of blood remaining in the visceral cavity after death. This reduction of blood decreased the amount of contamination during the filleting process producing a more attractive fillet.

Commercially, the peak season of harvest for mullet in the Gulf occurs during November, December and January. It is common during this season for the particular catch of any one boat to exceed the amount of ice carried for the purpose of chilling the fish postmortem. The effect of the lack of the chilling process on the rate of lipid oxidation was studied by thoroughly icing one-half of one lot of fish captured following commercial practices. The remaining half of the lot remained untreated for 6 hours with an air temperature of 70°F. Data from Table 4 indicated that the lack of a chilling process immediately postmortem had little effect on the rate of oxidative rancidity. However, TPC/g was 50 times greater in the noniced sample after 6 days and 100 times greater after 9 days of storage. Even though the chilling process did not influence the rate of oxidative spoilage its importance in controlling microbial growth should not be overlooked or underestimated.

It has been a common commercial practice to hold mullet in the round during post-mortem storage with the viscera left intact during both frozen and refrigerated storage. To determine the effect of such a practice on the keeping quality of the muscle tissue 60 mullet captured using commercial equipment, were divided into three equal samples. One sample was filleted, a second was only eviscerated leaving the head and skin intact and the final sample received no treatment. All three samples were stored at 1°C for 15 days.

Holding the fish in the round does protect it from oxidative rancidity (Table 5). TBA numbers of the sample stored in the round remained static during storage while those of the filleted and eviscerated samples increased after 4 days. TPC/g was greatest in the muscle tissue of the filleted sample as would be expected due to increased handling and exposure prior to storage. Sensory panel flavor scores appeared normal for oxidizing tissue in the filleted and eviscerated samples, decreasing as the TBA number increased. However, flavor scores also decreased in those samples held in the round even though the TBA number remained below panel threshold (4–6 mg malonaldehyde/1000g tissue) and TPC/g did not exceed 300,000 in the fillets.

The major reason for the commerical practice of holding mullets in the round is to retain the quality of the roe which is prevalent during the peak harvest season. This practice was also found to be effective in controlling oxidation. Flavor scores of fish stored in the round, however, decreased similarly to those from tissues of filleted fish. Flavor changes in the fish stored in this manner may be caused by enzymatic activity and microbial growth in the viscera which adversely affects the adjacent muscle tissue. Further studies should be designed to determine the affect of the visceral enzymes and microorganisms on the flavor of the muscle tissue when stored in the round.

Although several million pounds of

Table 3–Average TBA numbers and flavor scores of bled and nonbled mullet fillets

	Time (days)			
Treatment	1	4	8	12
Bled fillets				
TBA No.	2.2	2.0	10.4	13.5
Flavor score	5.1	4.9	3.4	3.3
Nonbled fillets				
TBA No.	2.3	2.4	10.9	11.6
Flavor score	5.2	5.0	3.5	3.0

Table 4–Average TBA numbers and TPC/g of iced and noniced mullet

	Time (days)				
Treatment	1	3	6	9	13
Iced					
TBA No.	2.3	2.4	7.8	10.9	11.6
TPC/g			1,427	35,000	1,300,000
Noniced					
TBA No.	2.1	2.4	10.8	13.2	12.7
TPC/g	100	34	53,636	3,900,000	

Table 5—Average TBA numbers, TPC/g and flavor scores of filleted, eviscerated and untreated mullet

Treatment	Time (days)					
	1	4	6	8	11	15
Filleted						
TBA No.	1.6	2.5	2.4	11.2	8.1	8.4
TPC/g		70	436	4,000	9,545	27,180,000
Flavor score	5.1	5.3	4.8	4.2	3.2	3.1
Eviscerated						
TBA No.	1.6	2.3	2.4	3.6	4.8	9.6
TPC/g				854	1,127	182,727
Flavor score	5.1	5.8	5.4	5.6	4.7	3.0
Untreated						
TBA No.	1.7	1.4	1.7	2.2	1.9	1.7
TPC/g		50	9.8	2.272	213,636	280,000
Flavor score	5.2	5.2	4.6	4.9	3.8	3.3

mullet are harvested each year, the value per pound is one of the lowest among fish from the Gulf. When compared to other commercial species from the Gulf the mullet must be given special consideration since it has unique spoilage properties. Oxidative rancidity seems to be more important than microbial growth in the spoilage of mullet fillets under the conditions of this study.

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YIELD AND ACCEPTABILITY OF MACHINE SEPARATED MINCED FLESH FROM SOME MARINE FOOD FISH

INTRODUCTION

HAND AND MACHINE filleting of demersal and pelagic fin fish leaves a considerable quantity of edible flesh remaining on the carcass. Although this waste material is largely used for animal nutrition, it represents a significant loss of human food.

During the last 20 yr machines have been developed in Japan which remove edible flesh from bone and skin in a coarsely minced form (Tanikawa, 1963). These machines separate flesh through the squeezing and tearing action of a wide flexible belt moving against the outside of a rotating, perforated metal drum. The softer and less cohesive flesh is forced through the perforations of the drum by the pressure developed between the drum and belt rotating in the same direction, but at different speeds. Skin and bone remain behind on the belt.

Processing fish in this manner can greatly increase the yield of edible flesh and could, if applied, improve the economy of our domestic industry. Miyauchi and Steinberg (1970) have shown that the total yield of minced flesh from various species of fish ranges from 37–60% based upon round weight. This compares to a yield of 25–30% intact flesh using conventional filleting techniques on the same species. A flesh yield of from 16–22% can also be obtained from dressed and filleted fish (King and Carver, 1970).

In Japan, this coarsely minced flesh serves as a raw material for the preparation of "surimi," a basic ingredient in the manufacture of a major line of food products which include sausages, cakes and pastes (Amano, 1962). Utilization of minced flesh in the preparation of products designed for domestic markets offers considerable potential. Carver and King (1971) have demonstrated its use in the preparation of fish cakes and frankfurters and as a canned commodity.

Utilization of minced flesh in the form of frozen blocks to prepare sticks and portions offers an additional large potential market. This investigation was designed to develop additional information on the yield of minced flesh obtainable by machine deboning and skinning of important commercial species and to evaluate the comparative acceptance of portions prepared from frozen blocks of intact and minced flesh. Sodium tripolyphosphate (TPP) added to minced flesh to reduce drip loss was also evaluated as a means of improving acceptance.

EXPERIMENTAL

SAMPLE LOTS of iced round fish were obtained from local commercial sources. Frozen blocks were prepared with fillets, minced flesh and minced flesh with 0.3% added TPP. These treatments were prepared from the same lot of fish for each species. Intact flesh was separated using conventional hand filleting techniques. Minced flesh was obtained from thoroughly washed, eviscerated and beheaded fish using a Yanagiya Fish Separator ("miny" model) equipped with a perforated rotating drum possessing 4 mm openings. Larger fish from lots of some species were planked into smaller units to allow their introduction into the small laboratory separator. TPP added as a 12% (w/v) aqueous solution was mixed with the minced flesh for 3 min at slow speed using a mechanical mixer. Blocks were formed in 1 × 3.75 × 21-in. stainless steel trays and frozen at −30°F. These blocks were sawed into approximately 0.5 × 3.5 × 3.75-in. portions and vacuum sealed in moisture-vapor proof film. Portions were held at a −15°F for a period of no longer than 3 wk prior to evaluation.

The fish portions were battered and breaded in the frozen state using standard commercial products. The breaded portions were deep-fat fried from the frozen state at 375°F for 6–7 min and served hot in coded cups to judges seated in individual booths. Trained flavor panels judged portions for texture, juiciness and degree of off-flavor on an intensity scale ranging from 9, "highest affirmative value," to 1, "lowest value." Both flavor and student panels judged samples for desirability on a 9-point hedonic scale ranging from 9, "liked extremely," to 1, "disliked extremely."

The percentage of moisture and fat in portions was determined using AOAC methods (AOAC, 1965). All data were analysed by analysis of variance and the significance of means tested by the least significant difference (LSD) method.

RESULTS & DISCUSSION

YIELDS of flesh were found to range from 40.4–54.5% based upon the round

Table 1—Yield of flesh from some demersal and pelagic species of food fish passed through a laboratory model flesh separator

Species	Weight processed (kg)		Yield (%)			Fillet yield[c]
	Round	Dressed	Dressed	Flesh[a]	Flesh[b]	
Orange rockfish *Sebastodes pinniger*[d]	82.15	55.80	67.9	40.5	59.6	28
Yellowtail rockfish *Sebastodes flavidus*[d]	82.10	58.85	71.7	41.7	58.2	28
English sole *Parophrys vetulus*[d]	32.70	20.90	63.9	45.7	71.5	33
English sole *Parophrys vetulus*[e]	65.15	44.65	68.5	47.3	69.0	33
Dover sole *Microstomus pacificus*[d]	32.65	24.40	74.7	48.5	64.3	27
Dover sole *Microstomus pacificus*[e]	28.80	22.70	78.8	54.5	69.2	27
Pacific hake *Merluccius productus*[d]	69.75	39.25	56.3	44.7	79.4	30
Ling cod *Ophiodon elongatus*[d]	68.89	46.42	67.4	48.1	71.3	35
True cod *Gadus macrocephalus*[d]	71.34	37.55	52.6	40.4	76.8	28

[a]Based upon round weight
[b]Based upon dressed weight
[c]Olsen (1970)
[d]Processed heads off, eviscerated and planked
[e]Processed heads off and eviscerated

Table 2—Percent moisture and fat in fish portions

Species	Flesh condition	Moisture Mean[a]	Moisture S.D.	Fat Mean	Fat S.D.
Orange rockfish	Intact	79.22^{ab}	0.62	1.83^{ac}	0.79
	Minced	78.77^{a}	0.64	2.94^{b}	0.72
Yellowtail rockfish	Intact	80.42^{cd}	1.13	1.27^{de}	0.33
	Minced	79.76^{b}	0.28	2.15^{a}	0.28
English sole	Intact	85.89^{i}	0.29	0.42^{i}	0.11
	Minced	83.87^{j}	0.29	0.85^{fgh}	0.05
Dover sole	Intact	85.83^{h}	0.38	0.46^{i}	0.08
	Minced	84.93^{j}	0.25	0.92^{fgh}	0.11
Pacific hake	Intact	81.61^{fg}	0.61	1.43^{cd}	0.53
	Minced	81.27^{ef}	0.41	2.08^{a}	0.59
Ling cod	Intact	80.65^{de}	1.21	1.00^{ef}	0.22
	Minced	79.86^{bc}	0.38	1.55^{cd}	0.17
True cod	Intact	81.96^{g}	0.68	0.40^{i}	0.04
	Minced	82.03^{g}	0.42	0.29^{j}	0.08

[a]Mean values in a column with same exponent letter did not vary significantly ($P < .05$) from each other. (n = 6 randomly selected portions).

weight of fish processed (Table 1). This compared to a 27–35% yield by hand filleting, representing a 34.4–101.9% increase in edible flesh yield. Yields obtained in this study correspond closely to those reported by Miyauchi and Steinberg (1970) and King and Carver (1970). Large yield differences among species appear to be related to variations in the relative mass of anatomical features and are reflected in different yield relationships among species when round and dressed weight are used as a basis for calculation.

Removal of bone from the flesh of all species was complete. Skin was completely separated from the flesh of all species except the two species of sole. Exposing the flesh of these fish by planking prior to processing reduced the quantity of skin in the separated flesh, but unacceptable quantities still remained.

The fat, and to a lesser extent the moisture content, of portions composed of minced and intact flesh from the same lot of fish were shown to vary (Table 2). The moisture content of portions prepared from the minced flesh of yellowtail rockfish, English sole, Dover sole and ling cod were shown to be significantly ($P < .05$) lower than corresponding intact flesh portions. Conversely, the fat content of minced flesh portions was shown to be significantly ($P < .05$) higher for all species except true cod. Separation with machine settings designed for maximum yield removes a larger amount of fatty flesh close to the skin than filleting. The higher moisture content of intact flesh portions reflects moisture loss occurring during machine separation and formation of minced flesh blocks.

Trained flavor panels showed preference for intact over minced flesh portions in direct panel comparisons (Table 3). Panel preference for the texture and juiciness of intact flesh portions over those composed of minced flesh was shown to be minimal. Scores for the texture of minced flesh portions were significantly ($P < .05$) lower than portions of intact flesh only in the case of English sole processed whole and Dover sole processed planked. Scores for the texture of other species did not vary significantly ($P < .05$). Only scores for the juiciness of portions composed of minced orange rockfish flesh were shown to be significantly ($P < .05$) lower than their intact flesh counterpart.

Panel scores for the degree of off-flavor and desirability of portions composed of minced English and Dover sole processed either whole or planked were found to be significantly ($P < .05$) lower than intact flesh counterparts and generally disliked by panel judges. This result is a reflection of odor and flavor components somewhat peculiar to these species which are associated with skin that was not successfully separated by machine processing. Scores for the degree of off-flavor and desirability of portions composed of minced flesh from the 2 species of rockfish and 3 "cod-like" species were very acceptable. Scores for portions composed of minced yellowtail rockfish, Pacific hake and ling cod did not vary significantly ($P < .05$) from their intact flesh counterparts. Minced orange rockfish and true cod flesh portions received significantly ($P < .05$) lower scores.

Addition of TPP to minced fish did not generally enchance scores for the factors considered by the trained flavor panels. Only the texture of English sole

Table 3—Mean[a] flavor panel scores[b] for fish portions prepared from frozen blocks of intact and minced flesh

Factor	Flesh condition	Orange rockfish[d]	Yellowtail rockfish[d]	English sole[c]	English sole[c]	Dover sole[c]	Dover sole[d]	Pacific hake[d]	Ling cod[d]	True cod[d]
Texture	Intact	7.70^{a}	7.58^{a}	7.60^{a}	7.43^{a}	7.25^{a}	6.50^{a}	7.57^{a}	8.10^{a}	8.30^{a}
	Minced	7.08^{a}	6.95^{a}	6.58^{b}	7.30^{a}	6.08^{a}	4.60^{b}	7.00^{a}	7.30^{ab}	7.40^{ab}
	Minced + TPP	7.43^{a}	7.53^{a}	6.85^{a}	6.50^{a}	6.15^{a}	5.28^{b}	7.22^{a}	6.97^{b}	6.60^{b}
Juiciness	Intact	8.25^{a}	6.70^{a}	6.93^{a}	7.50^{a}	7.35^{a}	7.43^{a}	7.62^{a}	8.00^{a}	8.02^{a}
	Minced	7.38^{b}	6.98^{a}	6.98^{a}	7.23^{a}	7.33^{a}	7.38^{a}	7.37^{a}	7.62^{a}	7.77^{ab}
	Minced + TPP	7.23^{b}	6.93^{a}	7.08^{a}	6.98^{a}	6.88^{a}	7.48^{a}	7.37^{a}	7.32^{a}	6.95^{b}
Off-flavor	Intact	7.80^{a}	7.53^{a}	7.78^{a}	7.08^{a}	7.03^{a}	7.13^{a}	8.10^{a}	7.70^{a}	8.25^{a}
	Minced	6.80^{b}	7.00^{a}	5.73^{b}	4.63^{b}	3.88^{b}	3.85^{b}	7.62^{a}	7.77^{a}	7.32^{b}
	Minced + TPP	7.48^{a}	7.53^{a}	5.75^{b}	4.00^{b}	4.10^{b}	3.48^{b}	7.62^{a}	7.47^{a}	7.02^{b}
Desirability	Intact	7.40^{a}	6.80^{a}	7.00^{a}	6.55^{a}	6.38^{a}	5.93^{a}	7.45^{a}	6.92^{a}	7.42^{a}
	Minced	6.43^{b}	6.35^{a}	4.93^{b}	4.43^{b}	3.55^{b}	2.98^{b}	6.77^{a}	6.55^{a}	6.65^{b}
	Minced + TPP	6.93^{a}	6.90^{a}	4.90^{b}	3.88^{b}	3.43^{b}	2.80^{b}	6.78^{a}	6.35^{a}	5.75^{b}

[a]Mean scores with same exponent letter in a column for each factor did not vary significantly ($P < .05$) from each other. (n = 20)
[b]Scale: 9, "highest affirmative value," to 1, "lowest value"
[c]Processed dressed and whole
[d]Processed dressed and planked

Table 4—Mean student panel desirability scores[a] for fish portions prepared from frozen blocks of intact and minced flesh

Species	Judgments	Flesh form[d]		
		Intact	Minced	Minced + TPP
Orange rockfish[c]	109	6.95[a]	6.13[b]	6.24[b]
Yellowtail rockfish[c]	119	6.90[a]	5.84[b]	6.02[b]
English sole[b]	112	6.70[a]	4.58[b]	4.91[b]
English sole[c]	107	6.67[a]	4.46[b]	4.50[b]
Dover sole[b]	50	6.02[a]	2.96[b]	3.10[b]
Dover sole[c]	61	5.89[a]	2.98[b]	3.15[b]
Pacific hake[c]	156	6.21[a]	6.10[a]	6.28[a]
Ling cod[c]	156	6.45[a]	6.42[ab]	6.10[b]
True cod[c]	154	6.80[a]	5.66[b]	5.66[b]

[a]Scale: 9, "liked extremely," to 1, "disliked extremely"
[b]Processed dressed and whole
[c]Processed dressed and planked
[d]Mean scores with same exponent letter in a row did not vary significantly ($P < .05$) from each other.

Table 5—Mean panel scores[c] for fish portions prepared from frozen blocks of minced flesh evaluated with and without an intact flesh reference

Method of evaluation	Flavor panel[a]				Student panel[b]
	Texture	Juiciness	Off-flavor	Desirability	
No intact portion	7.33[a]	6.58[a]	7.23[a]	6.03[a]	6.03[a]
With intact portion	6.61[b]	6.08[a]	6.76[a]	5.19[b]	5.19[b]

[a]Mean scores with same exponent letter in a column did not vary significantly ($P < .05$) from each other. ($n = 60$)
[b]$n = 495$
[c]Mean scores for three replicate panel evaluations which included Pacific cod and true cod. Scale: 9, "highest affirmative value," to 1, "lowest value"

and the degree of off-flavor and desirability of orange rockfish was shown to be significantly ($P < .05$) improved. All other scores did not vary significantly ($P < .05$).

Student flavor panel scores for the desirability of minced flesh portions from the rockfish and "cod-like" species were shown to be good (Table 4). Scores for minced Pacific hake and ling cod did not vary significantly ($P < .05$) from their intact flesh counterparts. True cod and yellowtail rockfish yielded acceptable scores, but significantly ($P < .05$) lower than intact flesh portions. Scores for portions composed of minced flesh from English or Dover sole processed either whole or planked were significantly ($P < .05$) lower and unacceptable. Scores of less than 5.0 were considered unacceptable. These scores, as those obtained with trained flavor panels reflect unacceptable flavor and odor factors associated with skin which was not separated by machine processing. The addition of TPP to minced flesh did not significantly ($P < .05$) enhance student panel acceptance.

The absolute value of flavor and student panel scores for minced flesh portions was found to be higher when evaluations did not include a portion composed of intact flesh. The mean scores listed in Table 5 represent the results of three replicate trained and student flavor panels which include three different species of fish. Trained flavor panel scores for texture and desirability of minced flesh portions were shown to be significantly ($P < .05$) higher when an intact flesh portion was not included. Scores for juiciness and off-flavor were higher, but not significantly ($P < .05$). Student panel scores for desirability were significantly ($P < .05$) higher.

The economy derived from increased flesh yield by machine processing and the apparent level of acceptance shown for frozen blocks of minced flesh from most species seems to show a good potential for market development.

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DETERMINATION OF THERMAL CONDUCTIVITY VALUES OF FREEZE-DRIED EVAPORATED SKIM MILK

INTRODUCTION

IT IS NECESSARY to know the value of thermal conductivity when solving any drying rate equation for the freeze-drying process. Values of thermal conductivity for a limited number of different freeze-dried materials have been reported in the literature. However, values for freeze-dried evaporated skim milk have not appeared, and therefore were evaluated so that the drying rates for this material could be determined.

Various methods, both steady-state and transient, have been used to measure the thermal conductivity of freeze-dried materials. The steady-state method using a guarded hot plate is described by Harper (1962), Harper and El Sahrigi (1964) and Saravacos and Pilsworth (1965). The transient method which is based on a quasi-steady-state analysis of actual drying data is described by Lusk et al. (1964), Sandall (1966) and Massey and Sunderland (1967).

Both methods were used in this study to evaluate the thermal conductivity of freeze-dried evaporated skim milk. A guarded hot tube was used in the steady-state method instead of the guarded hot plate because of its compatibility with existing freezing and freeze-drying equipment.

The composition of the evaporated skim milk used in this work was 79.4% water, 10.8% milk sugar, 7.7% protein, 1.7% minerals and 0.4% milk fat.

EXPERIMENTAL

Apparatus for steady-state method

A photograph of the guarded hot tube and vacuum chamber and a sketch of the guarded hot tube are shown on Figures 1 and 2.

The guarded hot tube was constructed by cutting threads on a 0.312 in. ceramic-fiber glass tube. The resistance wire which formed the heater was wound around the ceramic-fiber glass tube in the thread grooves. The resistance wire was 0.005-in. diam., enamel constantan wire. Electrical insulation (0.003-in. glass tape) was then placed over the wound heater, and this assembly was fitted into a 3/8-in. aluminum tube. One main heater and two guard heaters were made in this manner. The three heaters were mounted in a tube configuration by means of nylon studs and washers. Plastic caps of "Castolite" plastic were cast on the ends of the tube so that the milk specimen would be 6 in. long with an outside diameter of 1 in. Two 0.005-in., copper-constantan thermocouples were attached to the main heater to measure its temperature and copper-constantan thermocouples were placed across the main heater and each guard heater to measure the temperature difference between them. In addition, two thermocouples were positioned to measure the surface temperature of the dried specimen.

The resistance of each heater was measured with and without leads with a Leeds and Northrup Model No. 5303 test set. A test was conducted to determine how the heater resistance changed with temperature. The results of this test indicated that the resistance changed less than 0.1% over the temperature range expected in the thermal conductivity tests.

The aluminum vacuum chamber was 12-3/4 in. long by 3-7/8 in. diam and consisted of three sections: a center section 6 in. long with a 1-1/4 in. hole through it, and two end sections. The chamber was designed to have the specimen positioned in the center section. The hole in the center section was painted with flat black lacquer paint.

The instrumentation consisted of three dc power supplies (two Harrison No. 6299A and Heath Kit No. IP-20) for the heaters of the guarded hot tube; an integrating digital voltmeter (Hewlett-Packard No. 2401C) for reading the voltages applied to the heaters; and a precision potentiometer (Leeds and Northrup, Model 7554, Type K-4), an electronic null

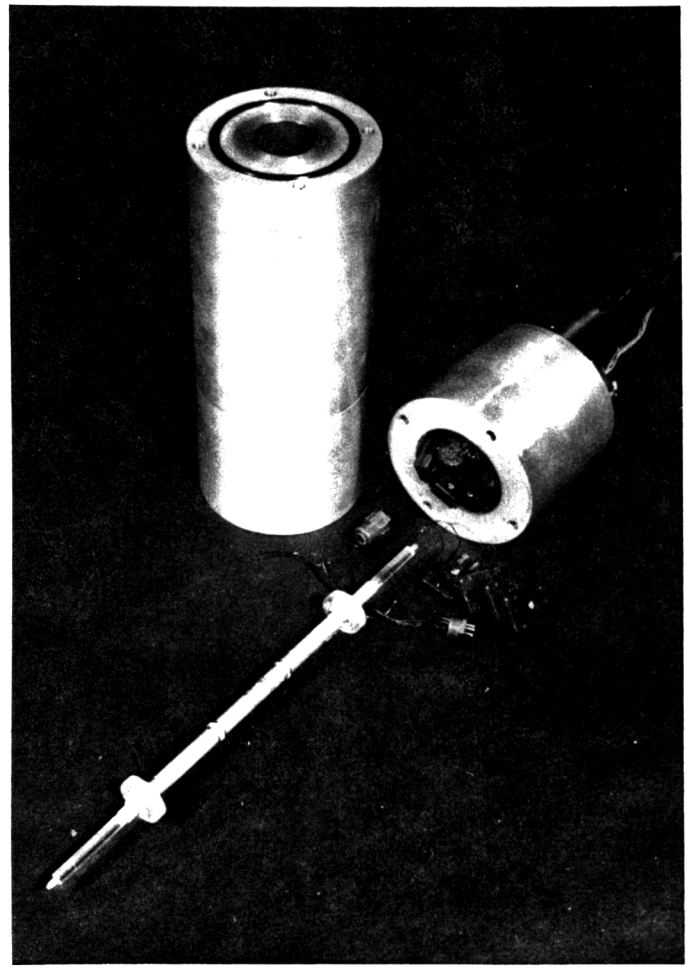

Fig. 1—Guarded hot tube and vacuum chamber.

[a]Present Address: A.M. Kinney, Inc., 2912 Vernon Place, Cincinnati, OH 45219

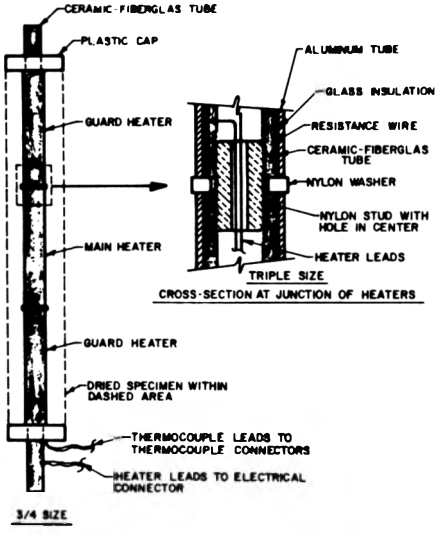

Fig. 2—Cross sectional view of the guarded hot tube.

detector (Honeywell Model 104WI) and a multipoint thermocouple switch for measuring the various thermocouple outputs.

Procedure for steady-state method

Before any tests were conducted, all thermocouples were calibrated against a glass thermometer which met ASTM specifications. The vacuum gage (Stokes Model TB-3) was calibrated for air against a McLeod gage (Consolidated Vacuum Corp., Type GM-100A).

For each test the guarded hot tube was placed in a freezing mold and a specimen frozen on it. The specimen was then freeze dried in a chamber which dried the specimen in a radial direction. After the specimen was removed from the drying chamber, positioning Plexiglas rods were screwed into the ends of the guarded hot tube and the tube was placed into the aluminum vacuum chamber. The thermocouple and heater leads were connected and the chamber was closed. The aluminum vacuum chamber with the specimen in it was then placed into a controlled temperature bath. The desired bath temperature, vacuum pressure and heater voltages were set. After the main heater and guard heaters were adjusted to essentially the same temperature, the specimen required approximately 1 hr to reach a steady state condition. However, the various temperatures and chamber pressures were not recorded until 6–7 hr later. Upon recording the data, the chamber pressure was changed and the specimen was again allowed to reach steady-state. This procedure was followed for the various pressure settings. The effect of temperature on the value of thermal conductivity was then determined by changing the bath temperature and repeating the series of tests at the new bath temperature. This basic procedure was followed for each test in this series which included specimens frozen at different rates.

Apparatus for transient method

The freeze-drying apparatus employed for the transient method dried the specimen in a radial direction. This apparatus essentially consisted of a vacuum chamber in which the specimen was surrounded by a cylindrical heater. The weight of the specimen was determined by using a load cell. This apparatus is described in detail in Gentzler (1970).

Procedure for transient method

The load cell in the drying chamber (Daytronic, Model 152A-1) was calibrated against laboratory standard balance weights before each test.

For each test the specimen holder was placed in the freezing mold and a specimen frozen on it. The specimen was then freeze dried in the chamber and weight readings were recorded throughout the drying cycle. During drying the chamber pressure and the temperature of the specimen's surface were held at a constant value. The tests in this series were terminated when the entire specimen reached the specimen's surface temperature. Drying chamber pressure, specimen freezing rate and drying surface temperature were varied from test to test.

The chamber pressure was obtained from water equilibrium pressure-temperature data (ASHRAE, 1967) and the recorded temperature of an ice bulb which was present in the chamber during the drying cycle. The ice bulb was 1 in. diam by 4 in. long and was made by freezing distilled water on a previously calibrated, 30 gage copper-constantan thermocouple.

Table 1—Variation of thermal conductivity of freeze-dried milk with pressure and temperature—Steady-state method

Test no.	Freezing time (min)	Chamber pressure (μ)	Temp. at r_i (°F)	Surface temp. measured (°F)	Surface temp. calc (°F)	Thermal conductivity (Btu/hr-ft.-°F) (a)	(b)	% Dif.
TC1	4.7	68	11.00	−13.06	−14.83	0.0124	0.0114	8.1
		225	5.68	−13.48	−15.89	0.0156	0.0136	12.8
		428	3.83	−13.58	−16.14	0.0171	0.0147	14.0
		645	2.64	−13.69	−16.24	0.0183	0.0156	14.8
		60	66.97	46.47	46.23	0.0146	0.0142	2.7
		195	63.89	45.68	45.26	0.0164	0.0158	3.8
		443	61.73	45.47	44.97	0.0184	0.0175	4.9
		638	60.71	45.36	44.89	0.0194	0.0185	4.6
TC2	4.7	60	10.98	−12.11	−14.81	0.0131	0.0116	11.4
		282	4.68	−13.37	−16.18	0.0168	0.0143	14.9
		482	3.00	−13.54	−16.36	0.0183	0.0154	15.8
		645	2.37	−13.54	−16.42	0.0190	0.0159	16.3
		65	66.58	46.81	46.01	0.0153	0.0145	5.2
		217	63.82	46.61	45.06	0.0176	0.0159	9.7
		468	61.58	46.00	44.81	0.0194	0.0177	8.8
		725	60.31	46.07	44.73	0.0213	0.0191	10.3
TC3	10.8	69	10.20	−13.05	−15.69	0.0133	0.0118	11.3
		315	4.02	−14.46	−16.93	0.0167	0.0145	13.2
		482	2.45	−14.73	−17.07	0.0179	0.0156	12.8
		637	1.74	−14.61	−17.13	0.0189	0.0161	14.8
		75	66.72	46.25	45.79	0.0151	0.0145	4.0
		300	63.02	45.89	44.86	0.0180	0.0167	7.2
		442	61.76	45.80	44.74	0.0193	0.0178	7.8
		660	60.45	45.44	44.66	0.0205	0.0192	6.3

[a]Calculated using measured surface temperature
[b]Calculated using calculated surface temperature

RESULTS & DISCUSSION

Steady-state

In the steady-state thermal conductivity test difficulties were encountered in keeping the thermocouple at the surface of the specimen. In some tests it became detached from the specimen altogether. Thus the temperatures indicated by the surface thermocouple were somewhat in question. Therefore, it was decided to also calculate the surface temperature by means of a steady-state heat transfer equation.

A heat balance from the outer surface of the skim milk to the chamber wall which accounted for two modes of heat transfer namely that of heat conduction and radiation yielded the following expression:

$$q = \frac{2\pi k_{air} L(T_o - T_c)}{\varrho n\, r_c/r_o} + \frac{A_o\, \sigma(T_o^4 - T_c^4)}{\frac{1}{\epsilon_o} + \frac{A_o}{A_c}\left(\frac{1}{\epsilon_c} - 1\right)} \quad (1)$$

where k is the thermal conductivity of the air as given in GE Design Data (1970), q is the heat output of the main heater, r is the radius, T is absolute temperature, A is surface area and ϵ is emissivity. Previously published work gave the value of 0.76 for the emissivity of freeze-dried beef (Sunderland, 1961) and the value of 0.82 for the emissivity of snow (Sparrow and Cess, 1970). In view of these values, it was decided to use the value of 0.80 for

Table 2–Effective values of thermal conductivity of freeze-dried milk calculated from drying data–Transient method

Test no.	Surface Temp T_o (°F)	Freezing time (min)	Chamber pressure (μ)	Thermal conductivity (Btu/hr-ft-°F)
V1	88.0	5.0	100	0.0328
V2	88.0	4.7	100	0.0341
V3	88.0	4.9	500	0.0414
V4	88.0	4.9	500	0.0389
V5	88.0	9.4	100	0.0291
V6	87.5	9.4	500	0.0387
V7	112.5	4.7	100	0.0286
V8	112.5	4.9	100	0.0313

the emissivity of freeze-dried milk. For the emissivity of the vacuum chamber wall, the value of 0.96 for flat black lacquer as listed by Rohsenow and Choi (1961) was used. The subscript o indicates the outside surface of the specimen and the subscript c indicates the chamber wall. The specimen calculated surface temperature, T_o, was determined using this expression.

The thermal conductivity of freeze-dried milk was determined using both the calculated and measured surface temperatures of the milk and the conduction heat transfer equation for a hollow cylinder:

$$k = \frac{q \ln (r_o/r_i)}{2\pi L (T_i - T_o)} \qquad (2)$$

where k is the thermal conductivity and the subscript i indicates the inside surface of the specimen.

The results of the steady-state tests are shown in Table 1 and indicate that the different freezing rates employed in these tests had no apparent effect on the thermal conductivity. Although no effects were expected because of the moderate freezing rates employed, extremely fast or slow freezing rates may have an effect on the thermal conductivity. The thermal conductivity did vary with the pressure of the gas in the pores and with the temperature of the specimen.

The increase in the thermal conductivity (for these low pressures) with the gas pressure was previously reported and explained by reasoning that the gas within the pores transfers more heat as its density increases. The variation of thermal conductivity with temperature at low pressures could not be found in the literature. The increase in the thermal conductivity with the moderate temperature range used in these tests is possibly due to an increase in the thermal conductivity of the gas in the pores as well as that of the dried milk.

It can also be seen from Table 1 that the values of thermal conductivity calculated using the measured surface temperature are in reasonable agreement with that calculated using the calculated surface temperature. The difference between the two values of thermal conductivity can be attributed to the assumed property values used in the equation to calculate the surface temperature and the difficulty in measuring the surface temperature.

Transient

With the transient method a simple model based on a quasi-steady-state analysis is used to evaluate thermal conductivities from actual drying data. Values of thermal conductivity obtained in this manner are referred to as effective thermal conductivity values. Unlike the values obtained from a steady-state method using a guarded hot plate or tube, the effective values include the effects of the pressure, temperature and moisture variations which occur across the drying region during the drying process. Sandall (1966) compared the values for turkey breast meat and determined that the effective values were higher than those obtained by the guarded hot plate method.

The effective values of thermal conductivity shown in Table 2 were calculated from drying data using a simple model. The simple model was similar to that described by Harper and Tappel (1957) and Sandall et al. (1967) and was developed for a cylindrical specimen instead of a slab. The drying rate equation for the cylindrical model was that derived by Gentzler (1970) and is:

$$t = \frac{\rho_F \Delta H_F}{k (T_o - T_F)} \cdot \left[\frac{r_F^2}{2} \ln (r_F/r_o) - \frac{r_F^2}{4} + \frac{r_o^2}{4} \right] \qquad (3)$$

where t is drying time, ρ_F is the density of frozen water expressed in lb_m of frozen water per cubic foot of specimen, ΔH_F is the heat of sublimation, k is the thermal conductivity of the dried region, T_o and T_F are the temperatures at the outside radius, r_o, of the specimen, and at the frozen interface radius, r_F, of the specimen, respectively.

To evaluate thermal conductivity in this manner, the values for the parameters, t, ρ_F, ΔH_F, T_o, T_F, r_o and interface position r_F must be substituted into Equation (3). The value for the interface position r_F was determined from the measurement of the amount of water removed, ρ_F, and specimen size. Because the specimen surface temperature did not instantly jump to a constant value at the start of drying, only drying time data obtained during the removal of from 50–75% of the water was used. During this period of time, T_o and T_F were essentially constant. The mass average value for ΔH_F of 1488 Btu/lb_m was used (Gentzler and Schmidt, 1971).

The effective thermal conductivity values shown in Table 2 follow the same trend with pressure as those in Table 1, i.e., the value of thermal conductivity increases with pressure. Also it is readily apparent that the effective values are significantly greater than those measured by the steady-state method–Table 1. Unlike the value of thermal conductivity shown in Table 1 which are for *no* pressure variations across the specimen and very small temperature variations, the effective values in Table 2 are *integrated values* which account for variations that occur across the specimen during drying such as:

1. Large temperature difference present in the specimen (higher temperatures-higher values of thermal conductivity);
2. Pressure differential across specimen (higher interior pressures–higher values of thermal conductivity);
3. Water vapors instead of air in the pores; and
4. Moisture gradient across the drying region of the specimen (described by Bralsford, 1967).

The *effective* values of thermal conductivity should be used when simple drying rate equations, similar to Equation 3, are employed to determine the drying time of the freeze-drying process. Otherwise, if the steady-state values of thermal conductivity are used, the mathematical model describing the drying must account for mass transfer and variations in pressure, temperature and moisture across the specimen.

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HYDROGEN PEROXIDE OXIDATION AND COAGULATION OF EGG WHITE

INTRODUCTION

LOW CONCENTRATIONS of hydrogen peroxide are used for processing of egg products. H_2O_2 reduces the time-temperature requirements for the pasteurization of liquid egg white (Lloyd and Harriman, 1957) and is an oxygen source for the enzymatic desugarization of egg products (Baldwin, 1956). Previous reports have indicated that H_2O_2 will oxidize, coagulate or modify egg white performance. Dhar and Raghavan (1947), using an iron catalyst at high temperatures, destructively oxidized egg white with H_2O_2. Fernandez and de Mirasiorra (1950) utilized a H_2O_2-egg white gel in studying the antigenic properties of denatured proteins. Cunningham and Cotterill (1962) observed that low levels of H_2O_2 improved the functional properties of egg white in angel cakes.

Coagulation or gelation is the most visible effect of high concentrations of H_2O_2 on egg white. Little is known of the conditions for coagulation by H_2O_2 or the changes that occur when egg white is treated with high levels of H_2O_2. This investigation determined some factors affecting the coagulation of egg white by H_2O_2 and some protein modifications that occur.

MATERIALS & METHODS

Egg white

The egg white (EW) was prepared as described by Cotterill (1968). Characteristics were normal for fresh, yolk-free EW, i.e., pH 8.6, 12.5% solids and a normal electrophoretic pattern. The pH adjustments were made by adding either 1M HCl or 1M NaOH, with constant stirring and continuous monitoring with a pH meter.

Heat-treated egg white

Heat treatments of 3 ml of EW were done in 9 x 130 mm glass tubes in a water bath at various temperatures for 10 min after the EW reached within 0.2°C of the bath. Subsequently, the tubes were immediately cooled in ice water.

Oxidized egg white

Oxidized EW was prepared by reacting 10 ml native EW with 2.5 ml of various concentrations of H_2O_2 for 2 hr at room temperature. The reaction was stopped prior to coagulation by the dilutions used in subsequent analyses.

Hydrogen peroxide

Original H_2O_2 was approximately 30% and contained no stabilizers. It was stored in polyethylene bottles at 4°C.

Coagulation

Coagulation was initiated using EW and H_2O_2 solutions in the volume ratio 4:1 (i.e., 10 ml EW and 2.5 ml H_2O_2 solution). The reaction was normally carried out at room temperature (27°C) in beakers covered loosely with aluminum foil. Coagulation was judged complete if the solution was not fluid when the container was inclined.

Electrophoresis

Electrophoretic analyses were done in an E-C Model 470 vertical cell (E-C Apparatus Corp., Philadelphia, Pa.), utilizing a 7% gel solution containing 2M urea. The gel solution was made by dissolving 70g Cyanogum 41, 120g urea, and 1.0 ml N,N,N',N'-tetramethylethylenediamine to 1 liter with a pH 9.2 Tris-Na_2EDTA-H_3BO_3 buffer [10g Tris(hydroxymethyl) aminomethane, 1g $Na_2EDTA \cdot 2H_2O$ and 0.38g boric acid in 1 liter water]. Ammonium persulfate (0.1g per 100 ml gel solution) was used to catalyze gel formation. A pH 8.4 Tris-$Na_2EDTA \cdot H_3BO_3$ buffer (43.1g Tris, 3.7g $Na_2EDTA \cdot 2H_2O$ and 22g H_3BO_3 in 4 liters H_2O) was used in the electrode compartment.

The egg white samples were diluted 1:1 with the pH 9.2 Tris buffer containing 16% sucrose to increase density. After 0.05 ml of this diluted material was placed in each slot, a constant voltage of 300v was applied for 2.5 hr. Gels were stained with Amido Black 10B (0.25% in 5:5:1, methanol water, acetic acid) for 5 min. Destaining was accomplished by washing the gel several times in the same solvent system. Tracings were made on a Photovolt Densicord Model 542 Densitometer (Photovolt Corp., New York, N.Y.) after slicing the destained gel.

Lysozyme activity

Lysozyme activity was determined by measuring the rate of clearing of a suspension of *Micrococcus lysodeikticus*. The method used was a modification of the procedure of Parry et al. (1965). The reaction mixture consisted of 2 ml EW solution (1 ml oxidized EW diluted to 500 ml with pH 6.2 phosphate buffer) and 2 ml of a 50 mg% suspension of *M. lysodeikticus* (in buffer). The two reagents were mixed in a cuvette and the transmittance read against a buffer blank every 15 sec for 1 min at 540 nm.

Iron-binding capacity

The iron-binding capacity of oxidized EW was determined using a modification of the method outlined by Fraenkel-Conrat and Feeney (1950). The reaction mixture was 1.0 ml EW solution, 1.0 ml deionized distilled water, 1.0 ml 0.3M phosphate buffer containing 0.5% $NaHCO_3$ (pH 7.6), 0.5 ml 0.1M sodium citrate (pH 7.6) and 2.0 ml 0.25 mM ferrous ammonium sulfate. The color of the conalbumin-iron complex was developed for 1 hr at room temperature and the absorbance read against an iron-free reference solution at 450 nm.

Sulfhydryl groups

The method of Gandhi et al. (1968a) was used to determine the relative sulfhydryl groups of oxidized EW. This colorimetric procedure utilized 5,5'-dithiobis-(2-nitrobenzoic acid).

Amino acid analyses

Amino acid analyses were carried out both on native and oxidized EW. The samples were hydrolyzed by refluxing with 20% HCl for 24 hr then analyzed in a Beckman Model 116 Automatic Analyzer (Beckman Instruments, Inc., Fullerton, Calif.) using standardized procedures and reagents (Benson et al., 1967; Anonymous, 1966).

RESULTS

Concentration of H_2O_2 and egg white solids

The coagulation times of EW solutions containing various amounts of H_2O_2 (determined both at room temperature at

Table 1—Relative lysozyme activity and iron-binding capacity of oxidized egg white

% H_2O_2	Relative lysozyme activity	Relative iron-binding capacity
0	100	100
1.2	98	103
2.4	95	98
3.6	96	97
4.8	101	–
6.0	95	–

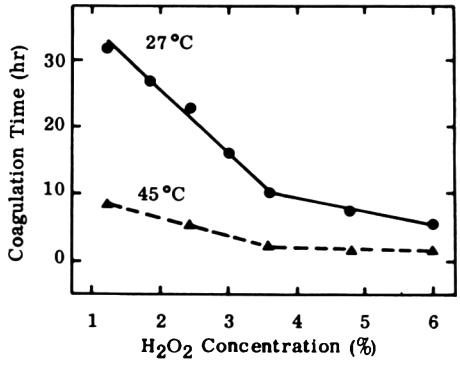

Fig. 1—Coagulation time of egg white containing various amounts of hydrogen peroxide.

27°C and in a convection oven at 45°C) are shown in Figure 1. The lowest level, 1.2%, represents the actual minimum concentration of H_2O_2 that would produce coagulation within 5 days at room temperature. The highest level, 6.0%, was chosen for further study to avoid excessive dilution of the EW solids in the final solution.

Coagulation times for diluted EW containing 6% H_2O_2 are presented in Figure 2. Diluted EW having a solids level less than 7% did not coagulate within 5 days.

pH

The coagulation times of pH-adjusted EW were determined using a final concentration of 6% H_2O_2. No attempt was made to correct for the differences in solids content that resulted from the pH adjustment. Coagulation time related to pH is shown in Figure 3. The shortest coagulation times were for the extreme pH values (4 and 11) and the longest time observed was 24.5 hr for pH 6.

Heat treatment

The coagulation times of heat-treated EW were studied using a 6% H_2O_2 concentration. Figure 4 depicts the results. No deviation was found for temperatures up to and including 50°C. Heating at 50–60°C reduced coagulation time. Heat treatment was not attempted above 60°C because the EW coagulated prior to adding H_2O_2.

Electrophoresis

Protein denaturation was observed by electrophoresis. Figure 5 shows densitometer traces of oxidized EW compared to native albumen. The separate curves all represent the same original amount of protein. For native EW, the peaks are as follows: (1) immobile-protein still in the original slot–possibly ovomucin; (2) conalbumin; (3) globulin G_2; (4) globulin G_3; (5, 6 and 7) ovalbumins.

The sample of oxidized EW containing 1.5% H_2O_2 had slightly reduced areas for most of the proteins. The migration of globulin G_3 was slightly decreased in this sample. EW reacted with 3% H_2O_2 had further reduced peak areas and a general spreading of the two globulin peaks was observed. The sample containing 6% H_2O_2 was drastically altered. All peaks were greatly reduced and spread over a much wider area. Migration of a great amount of protein was affected, as a large peak appeared at 4–9 cm.

Lysozyme activity

Determination of lysozyme activities were made on samples of oxidized EW having H_2O_2 concentrations up to 6%. These data are presented in Table 1. No reduction in enzymatic activity was found.

Iron-binding capacity

Table 1 also presents the results of the analysis of relative iron-binding capacity. Again, no signficant change was found for oxidized EW. Samples containing levels of H_2O_2 greater than 3.6% were too turbid at the end of the 2 hr oxidation period for measurement by this method of analysis.

Sulfhydryl groups

The relative -SH groups of oxidized EW were measured as another indicator of protein modification. Figure 6 presents the results of these measurements. The number of sulfhydryl groups was little affected by concentration of H_2O_2 below 1.2%. Higher levels of H_2O_2 eliminated essentially all -SH groups. The 1.2% level of H_2O_2 is significant because it is also the minimum amount needed to produce coagulation (See Fig. 1).

Amino acid composition

A summary of the amino acid analyses is presented in Table 2. Only five amino acids were affected to any great extent.

Table 2–Amino acid composition of native and oxidized egg white

Amino acid	Amount found (g/100g EW) Native EW	Oxidized EW (6% H_2O_2)
Aspartic acid	1.30	1.40
Threonine	0.60	0.65
Serine	0.87	0.93
Glutamic acid	1.77	1.77
Proline	0.40	0.42
Glycine	0.41	0.43
Alanine	0.75	0.80
Cystine	0.32	Tr
Valine	1.02	1.07
Methionine	0.53	Tr
Isoleucine	0.68	0.74
Leucine	1.12	1.16
Tyrosine	0.49	-0-
Phenylalanine	0.89	0.22
Lysine	0.76	0.88
Histidine	0.26	0.16
Arginine	0.69	0.72

The sulfur-containing acids almost completely disappeared, aromatic acids tyrosine and phenylalanine were greatly reduced and there was a 40% loss of histidine. An additional five peaks appeared on the chromatogram, but their identities were not established. The remainder of the amino acids were not affected by oxidation with 6% H_2O_2 for 2 hr.

DISCUSSION

AN EXPLANATION can be given for the effects of the different factors (concentration of H_2O_2 and EW solids, pH and heat treatment) on the coagulation times. The increase in coagulation times due to decreasing either the amount of H_2O_2 or the level of EW solids is probably a dilution effect. The additional water

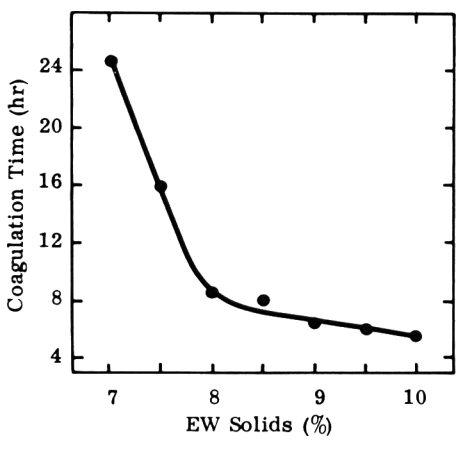

Fig. 2–Coagulation time of diulated egg white containing 6% hydrogen peroxide.

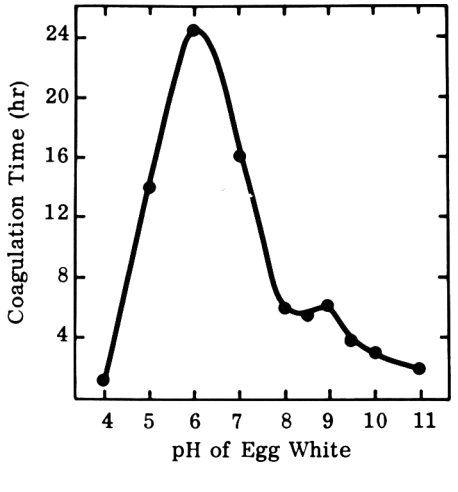

Fig. 3–Coagulation time of pH-adjusted egg white containing 6% hydrogen peroxide.

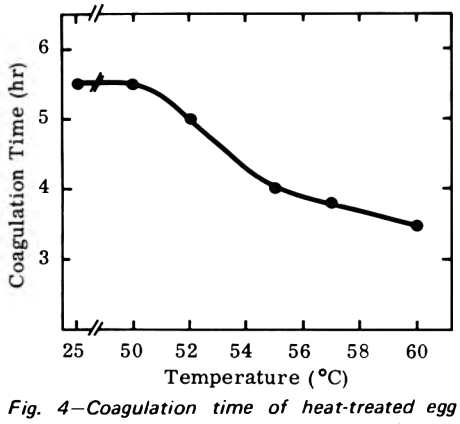

Fig. 4–Coagulation time of heat-treated egg white (10 min at various temperatures) containing 6% hydrogen peroxide.

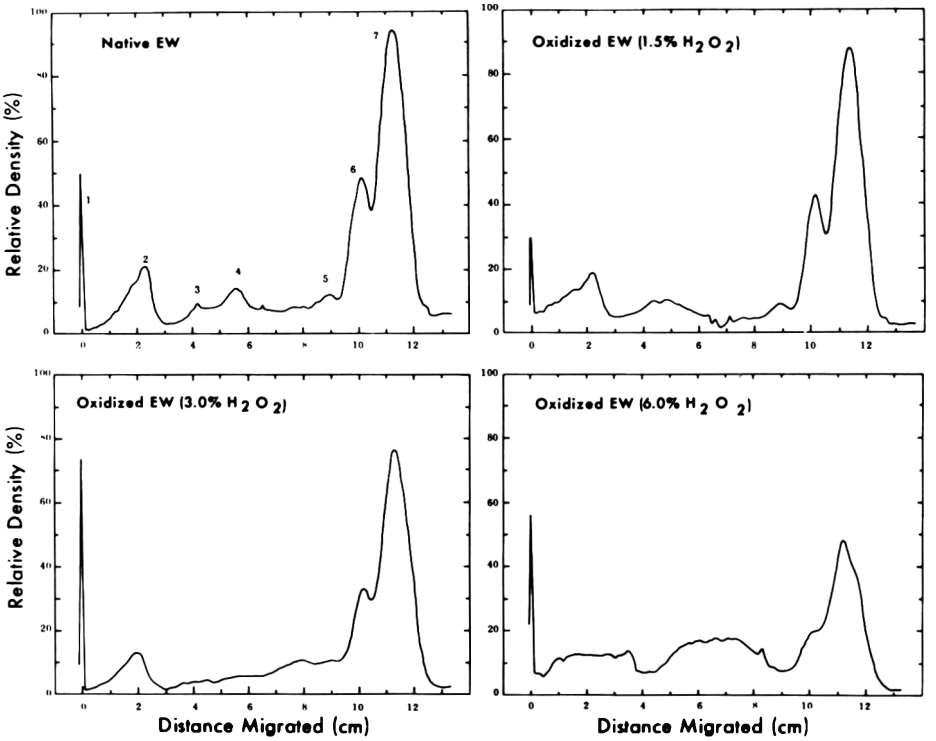

Fig. 5–Electrophoretograms of native and oxidized egg white.

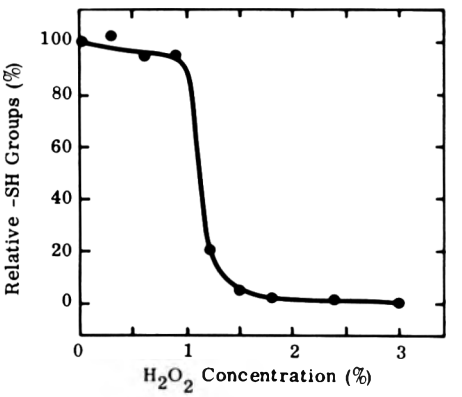

Fig. 6–Relative sulfhydryl groups of egg white after treatment with various amounts of hydrogen peroxide.

molecules present in the solution are a barrier between the protein and the oxidant. This increase in coagulation time due to decreased protein availability was also noted in previous studies on alkaline and acidic EW coagulation (Cunningham and Cotterill, 1962, 1964). Results of both the pH and heat treatment studies indicate that partially denatured proteins are more sensitive to oxidation by H_2O_2 than are native proteins. This concept was suggested by the data of Hachimori et al. (1964) who studied the ease of oxidation of tryptophan residues in a variety of native and denatured proteins. The decrease in coagulation times observed at the pH extremes is probably due to this prior denaturation. The increased coagulation times in the pH 5–7 region may be caused by the partial insolubilization of the EW proteins at or near their isoelectric points. A protein near its isoelectric point would tend to agglomerate, thereby protecting itself from oxidation by H_2O_2.

The evidence suggests a mechanism of coagulation that involves the oxidation by H_2O_2 of certain amino acid groups to highly polar species and resultant coagulation by polymerization of the proteins through extensive hydrogen bonding and/or by electrostatic interactions. The decrease in the number of free sulfhydryls implicates these groups in the coagulation mechanism. Coagulation occurred only at levels of H_2O_2 which greatly decreased the number of sulfhydryls. It is speculated that oxidized sulfhydryl groups would tend to form disulfide bonds and this, in turn, would lead to cross-linking and gelation. The formation of disulfide bonds needs only a mild oxidizer, such as iodine (Kushner and Frenkel', 1965). Oxidation of a sulfhydryl with H_2O_2 would be more likely to lead to a sulfonic acid (Roberts and Caserio, 1967). The amino acid data further indicate an oxidative mechanism. Methionine, which contains a sulfide group, is easily oxidized to the sulfoxide or sulfone (Roberts and Caserio, 1967). The other residues showing significant decreases–tyrosine, phenylalanine and histidine–are all ring compounds. These compounds are also fairly sensitive to oxidation. Tryptophan, lost due to acid hydrolysis, would decrease similar to the other amino acids (Hachimori et al., 1964).

The biological aspects of the oxidation are interesting because neither the activity of lysozyme nor the iron-binding capacity of conalbumin were affected. As discussed previously, the sulfhydryl groups appear to play an important role in the coagulation mechanism. However, Canfield (1963) reported that lysozyme contained four disulfide bonds, but no free sulfhydryls. Conalbumin was also reported to have no cysteine (Lewis et al., 1950). Loss of sulfhydryls should have no effect on the biological properties of either of these two proteins. However, the data indicated the disappearance of other amino acids. The amino acids in these two proteins may be protected from oxidation by their location in the polypeptide chain, or the other proteins in EW may be selectively attacked. Modification of EW with other reagents usually results in a loss of activity. Gandhi et al. (1968a, b) reported a loss in the iron-binding capacity and a drastic decrease in lysozyme activity when EW was treated with potassium persulfate or 3,3-dimethylglutaric anhydride.

The concentrations of H_2O_2 used in these experiments were much higher than those normally employed for processing egg products. Hence, these results may or may not be indicative of changes caused by low concentrations of H_2O_2.

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CALLOSE FORMATION BY BRUISING AND HEATING OF TOMATOES AND ITS PRESENCE IN PROCESSED PRODUCTS

INTRODUCTION

A MOST DESIRABLE quality in fresh and processed tomatoes is firmness. In tomato juice the consistency of the product is an important factor when considering commercial value. A major contribution to the firmness of fruits and vegetables and to the consistency of tomato juice is believed to be made by pectins. However, recent investigations indicate that other constituents of tomatoes contribute significantly to consistency.

Viscosity of canned tomato juice has been shown to vary with heat treatment of the fresh fruit during extraction (Kertesz and Locanti, 1944).

Whittenberger and Nutting (1957) showed that the flesh cell is the principal structural element in tomato juice, and that the cell wall was identified as the structure most closely related to viscosity. In 1958 further investigation by these researchers revealed that tomato cell walls, less than 6% of the total solids, were shown to be the juice component essential for high viscosity. The cell walls were boiled in sulfuric acid and sodium hydroxide and then extracted with ethyl alcohol and ether. This supposedly removed all substances from the cell walls except cellulose which was found to be the single substance most closely related to consistency.

Dekazos and Worley (1967) showed that bruise induced firming of sour cherries was accompanied by the formation of considerable callose on the cell walls. This report marked the introduction of a new polysaccharide as a contributing factor in textural characteristics of foods. The foregoing work, plus certain important characteristics of callose, an amorphous polymer of glucose residues in β-1-3 linkages (Kessler, 1958), led the author to suspect that this compound might be responsible for the viscosity of tomato juice.

Callose is widespread throughout the plant kingdom (Eschrich, 1956) and is formed in certain types of cells in response to injury (Eschrich, 1956; Currier, 1957), ultrasound (Currier and Webster, 1964), and high temperature (Lerch, 1960; Webster and Currier, 1968). In addition, callose is insoluble in water, ethanol and either hot acid or base (Currier and Strugger, 1956; Currier, 1957) and has strong affinity for water (Kessler, 1958).

The aim of the work reported here was to study the effect of bruising, hand or mechanical harvesting and elevated temperature on callose formation in tomatoes. Possible relationship of callose formation to texture and consistency is attempted.

MATERIALS & METHODS

FRUIT OBTAINED from the Vegetable Research Plots and Greenhouse of the Plant Industry Station at Beltsville, Md. was carefully hand-picked with stems attached to avoid bruising. Uniformity of maturity was determined by visual observation of color and size. Samples were transported to the laboratory in cotton-lined containers. Special care was taken to avoid any injury to the fruit while enroute to the laboratory. The fresh unbruised tomatoes, mature-green and ripe (full red color and firm), as well as heated fruit were examined immediately upon reaching the laboratory. Fruit was dropped three times from an 18-in. height onto a flat, glass surface. These bruised tomatoes were noticeably softer to the touch but their skins were not broken.

Bruised, green and ripe, tomatoes were placed in incubators for the desired length of time and temperature. Since the tomato is bulky, temperature equilibrium should not be extremely rapid. Tomatoes removed from the incubator were allowed to remain at room temperature for 1 hr prior to sectioning for staining.

Commercially hand harvested, ripe fruit was used to obtain tomato juice. Twenty tomatoes were washed, stemmed and preheated by placing in an incubator of 50°C ± 0.2 for 2 hr. The fruit was then quartered, coarse crushed and filtered through a 200-mesh sieve to remove skins, seeds and vascular tissues. Samples of the extracted juice were removed by disposable pipettes and placed on concavity slides. Staining solution was then added. After 10–20 min, each sample was examined with a fluorescence microscope for callose formation.

Tomatoes from the Vegetable Research Plots of the Plant Science Research Division of ARS were obtained before and after mechanical harvesting (Hart Carter machine). Fresh, unbruised fruit was carefully hand-picked with stems intact from the same vines from which the bruised (mechanically harvested) samples were selected. Tomatoes selected from machine harvested fruit were considered to be uniformly bruised by harvesting and representative of commercially harvested fruit. All tomatoes were carefully transported to the laboratory and divided into two uniform groups. One group was placed on the laboratory bench for 24 hr at room

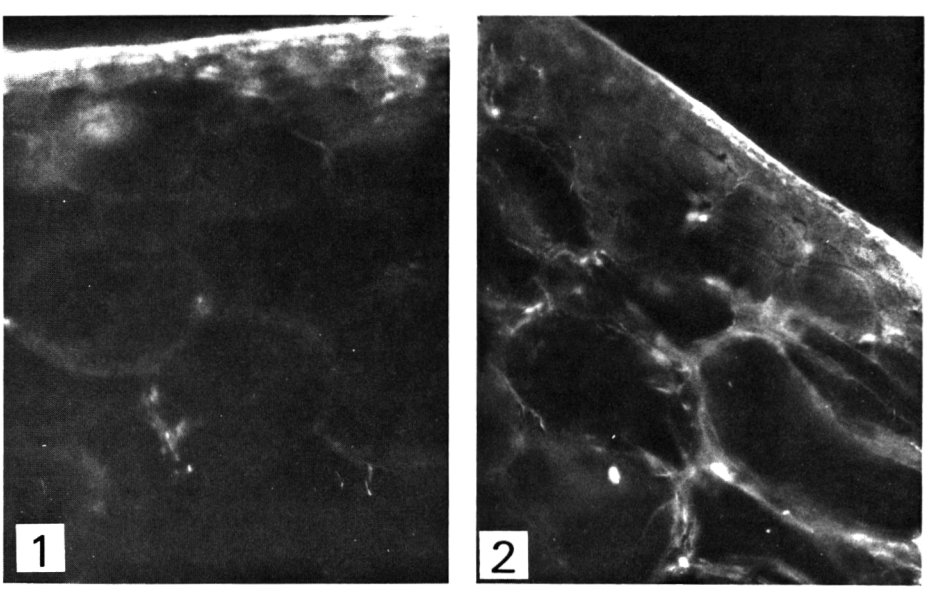


Fig. 1–2–No callose formation in unbruised and unheated tomatoes: (1) Mature green tomato (106X); (2) Ripe-red tomato (86X).

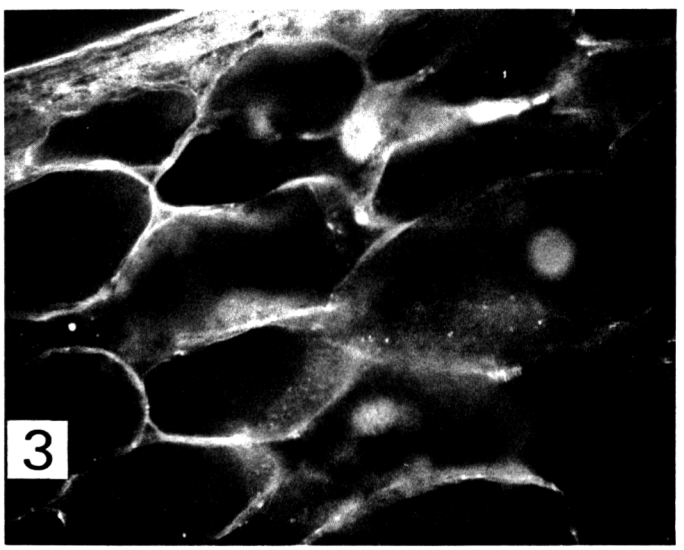
temperature before examination while the other similar group (firmness, red color and size) was placed in an incubator at 45°C for 1 hr and sectioned an hour after removal. Unbruised tomatoes were also sectioned and stained for examination.

Commercially processed whole tomatoes were obtained from the Williamsburg Can Co., Williamsburg, Md. The tomato cultivar "Heinz 1350" had been hand-picked approximately 24 hr prior to unloading at the plant. These tomatoes were followed through the normal processing cycle: wash, scald, peel, fill, seal, continuous cooker, cool and storage. Representative, commercial samples of canned whole tomatoes were taken to the laboratory, sectioned, stained and examined.

Callose was determined by the aniline blue reaction viewed under fluorescence microscopy as described by Dekazos and Worley (1967). The specificity of this staining procedure has been discussed earlier (Currier, 1957; Eschrich and Currier, 1964). Thin freehand sections from fresh tissue were used. Sections were stained with water-soluble aniline blue for 10–20 min and examined in the same medium. Primary fluorescence (no staining) was determined for each tissue. After aniline blue staining, all structures which appeared bright yellow in UV light, but didn't exhibit autofluorescence were considered to be callose.

RESULTS & DISCUSSION

TOMATOES are subjected to some degree of bruising during harvesting, whether by hand or machine. Hand picking of tomatoes for fresh market and especially for processing has given way to mechanical harvesting in recent



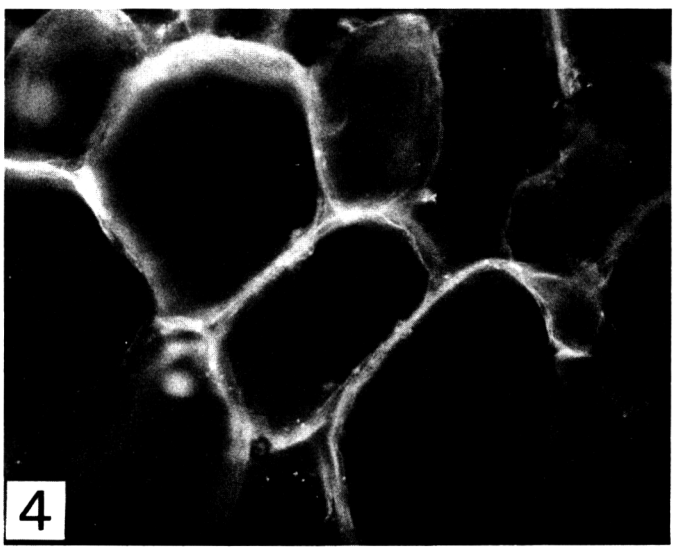
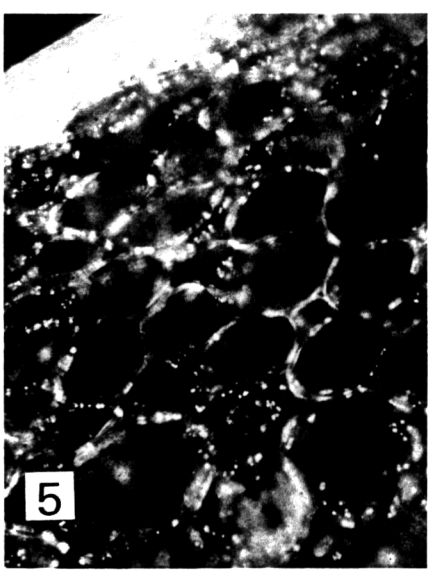
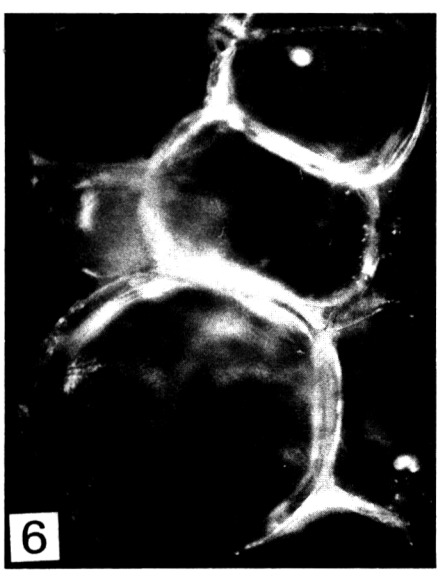


Fig. 3–4—Heat-induced callose in unbruised fresh, ripe-red tomatoes heated in an oven at 50°C for 1 hr. Aniline blue fluorochrome: (3) Callose covering the entire cell wall surface of the skin and of the underlying parenchyma cells (100X); (4) Heavy callose deposits in the walls of parenchymatous cells of the mesocarp (208X).





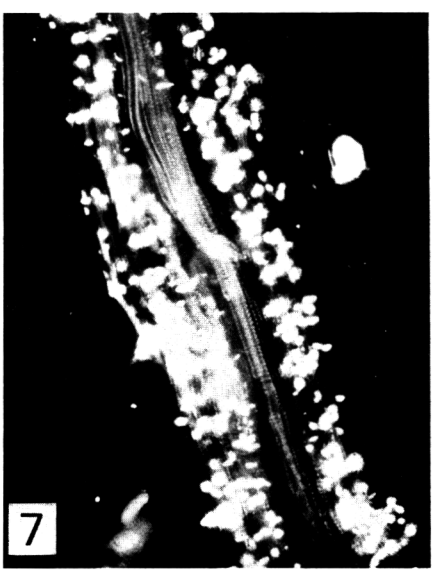


Fig. 5–7—The callose staining reaction in mature-green bruised tomatoes. All light areas represent a yellow fluorescence of varying intensity: (5) All stages of growing depositions are seen, from dots of pit callose, to the large plate-like extensions along the wall as well as numerous callose dots on the surface of the protoplast in the parenchyma cells. Tomatoes at 45°C for 30 min (73X); (6) Callose covering most of the cell wall surface of a fresh section of tomatoes heated at 45°C for 1 hr (198X); (7) Heavy sieve tube callose in the phloem of a vascular strand. Tomatoes heated at 35°C for 24 hr. A callosed druse lies in the upper right (79X).

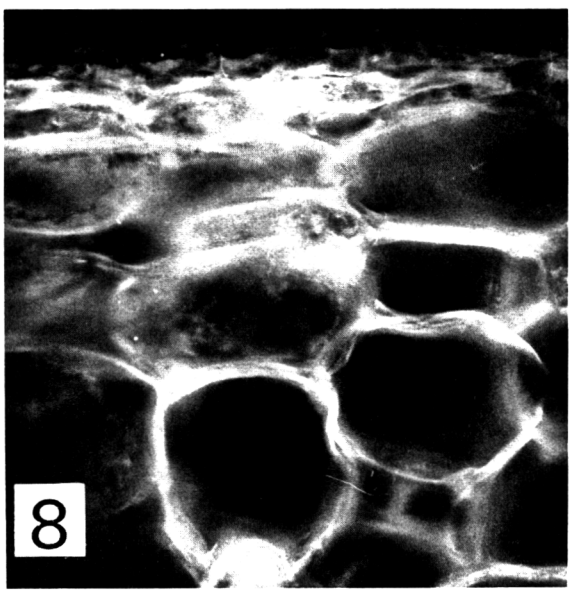
years due to labor shortage, demand of increased efficiency, etc. Rough handling of tomatoes during harvesting, hand or mechanical, causes initial damage. At best, the tomatoes will be bruised again when transferred from the grower's container to the processor's tank.

The effect of high temperatures on the firmness of tomatoes has been reported by Hall (1964). He reported that during the early stages of ripening, tomato fruit held up to 24 hr at 108° and 110°F was firmer than control fruit held continuously at 68°F.

Artificially bruised red tomatoes incubated at 35°C, 45°C, 50°C and 60°C were found to become noticeably firmer to the touch than the original fresh fruit for all temperatures except 60°C. The firmest fruit was obtained from fruit held at 45°C and 50°C; however, even at these temperatures bruised tomatoes which were excessively damaged did not become firm.

Considering the foregoing, the question arose as to the effect of bruising, heating, or bruising and heating on callose formation in tomatoes.

Until quite recently, callose in plant tissues has been identified either by its characteristics, after staining with certain dyes, or by its solubility properties. It should be emphasized that in all instances in this study, callose was identified only by virtue of its location and its staining behavior.



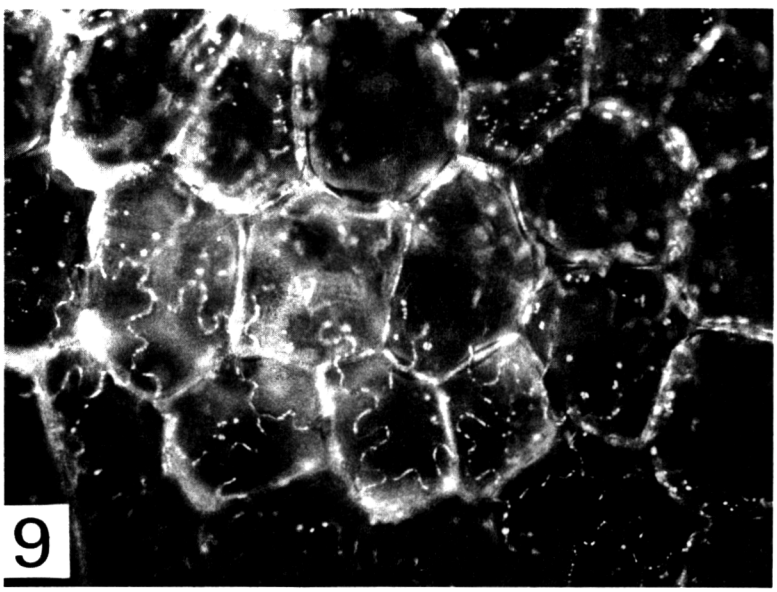
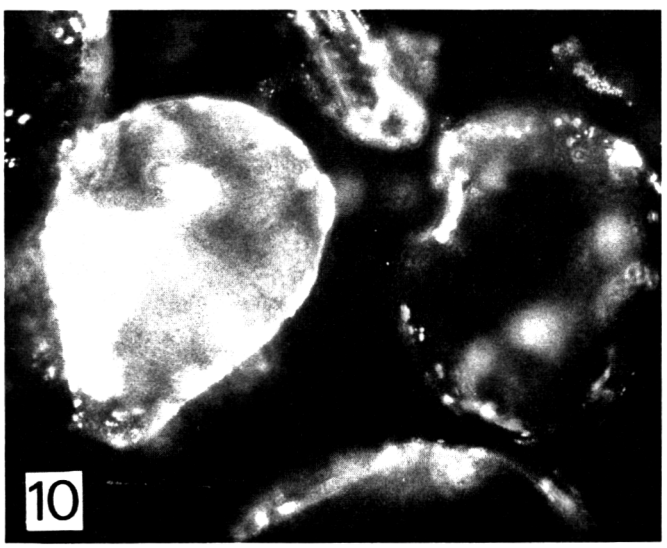


Fig. 8–9–Wound and heat-induced callose in fresh, bruised ripe-red tomatoes heated in an oven at 45°C for 1 hr. Aniline blue fluorochrome: (8) Callose covering the entire cell wall surface of the epidermis, subepidermis collenchyma and of the underlying parenchyma cells (110X); (9) Heavy callose deposits in the walls of the parenchymatous cells of the placenta (110X).



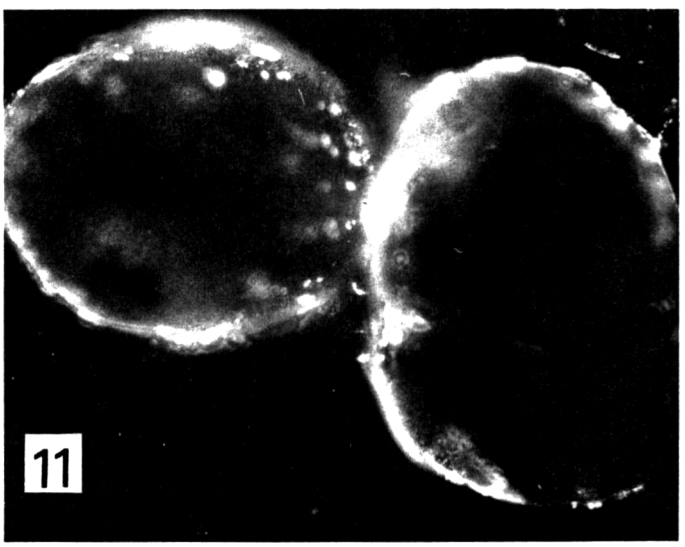


Fig. 10–11–Heat-induced callose in the cells of tomato juice from preheated (50°C) ripe tomatoes. Aniline blue fluorochrome: (10) Callose in the walls and cytoplasm of separated flesh cells and cell wall fragments. Note that one cell is practically completely fluorescent (232X); (11) Heavy callose covering the entire cell wall surface. Several callose dots can be seen in the cytoplasm (244X).

Callose deposits of tomato tissue induced by bruising or bruising and heating always gave a brilliant yellow against an almost black background. The degree of fluorescence is considered to be directly related to the amount of callose present.

Unbruised and unheated tomatoes

Stained sections of either fresh green or ripe tomatoes for this study showed no fluorescent areas, indicating no callose formation. The exceptions were the cuticle and vessel rings which gave primary fluorescence. Their color is more greenish yellow in UV light than the golden yellow of the stained callose. Phloem fibers fluoresced dull yellow. The red of the chloroplasts predominated the section.

The stained sections of unbruised and unheated mature-green (Fig. 1) and red (Fig. 2) tomatoes were practically callose-free. It should be noted that untreated sections frequently exhibit some callose formation in varying amounts in the epidermal layer. These formations are presumed to be the result of injury that occurred either before or during the sectioning of the tomato.

Heated and unbruised tomatoes

Unbruised and heated, 50°C for 1 hr, red tomatoes exhibited large amounts of callose synthesis in stained sections across the pericarp (Fig. 3, 4). Formation of parenchyma callose in response to heat was recorded in various tomato tissue regions. The temperature dependence of callose formation in tomato was similar to that in cotton as observed by Webster and Currier (1968). They reported that maximum phloem callose formation was induced at 50°C and that it took about a week to return to normal.

Artificially bruised tomatoes

The stained sections of bruised mature-green tomatoes heated at 45°C for 30 min exhibited all stages of callose depositions from pit to plate as well as callose dots on the surface of the protoplast (Fig. 5). It has been reported that under some conditions callose is formed within the cytoplasm (Lerch, 1960). When the heating time was extended to about 1 hr, callose of parenchyma cells (Fig. 6) appeared to increase in intensity.

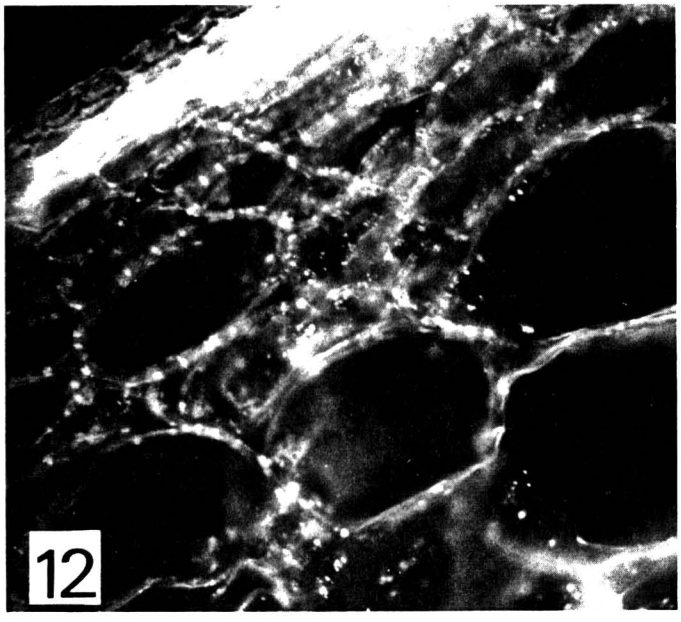
Massive callose synthesis due to bruising and heat (35°C) stimuli was detected in the sieve tubes in the phloem of a vascular strand (Fig. 7). There was always some phloem callose (thin plates) in control samples. Sieve tube callose in the phloem of a vascular strand was greater in heated tomatoes than in tomatoes kept at room temperature. Schumacher (1930) reported that sieve elements of injured phloem often formed great masses of callose. Webster and Currier (1968) showed that brief localized heating of cotton cotyledonary petioles (plant intact) increased phloem callose. They reported that amounts of phloem callose returned to normal within 1 day after heating.

A pattern of bright yellow fluorescent cell walls (Fig. 8) was exhibited by the stained sections of bruised and heated (45°C for 1 hr) red tomatoes. In some cases, fluorescence was observed as pit callose spreading into plate callose. At other times the fluorescent areas appeared as several forms of callose in the parenchymatous cells of the placenta (Fig. 9).

Tomato juice from commercially harvested tomatoes

Tomato juice from preheated (50°C) ripe tomatoes showed callose deposits in the walls and cytoplasm of intact separated flesh and broken cells and cell wall fragments (Fig. 10): one cell was almost completely fluorescent. At other times the fluorescent areas appeared as irregular thickenings of the cell wall with several callose dots in the cytoplasm (Fig. 11). Webster and Currier (1968) stated that callose formation was maximum at about 50°C, hence the obvious abundance of callose in the tomato juice samples.

Consistency development in tomato juice following heat treatment has been reported earlier. Whittenberger and Nutting (1957) identified the cell wall as the structure most closely related to consistency of tomato juice. In 1958 they reported removal of all substances except cellulose and concluded that cellulose was the single substance closely



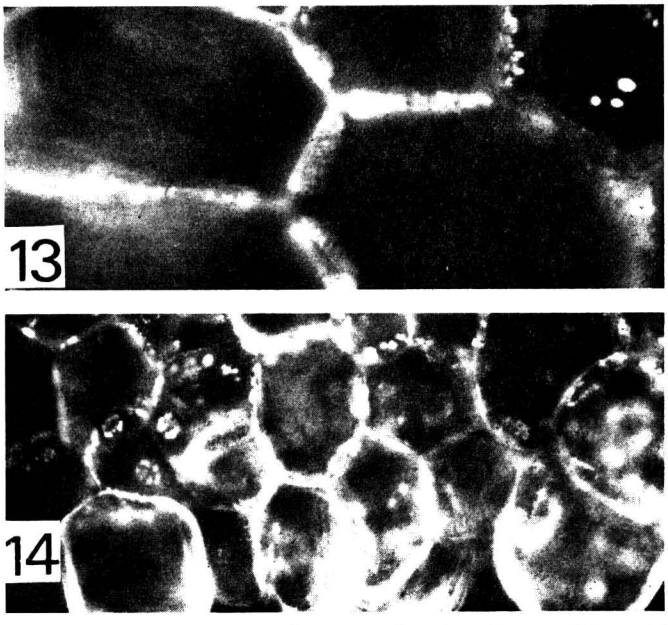
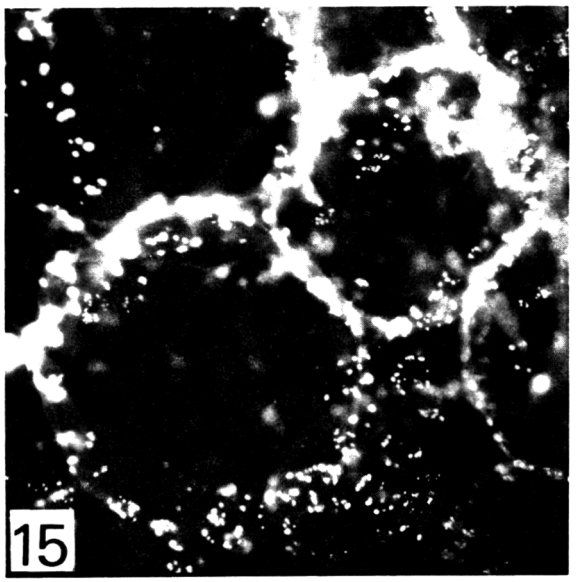


Fig. 12–14—Deposits of callose in mechanically harvested, ripe tomatoes. Aniline blue fluorochrome: (12) Callose of various forms and intensities covers the entire cell wall surface. Note autofluorescent thin cuticle, weak callosed cuticular and non-cutilarized layers, heavy callosed epidermis and subepidermis collenchyma and mainly pit callose in the parenchyma. Tomatoes kept at room temperature for 24 hr (120X); (13) Heavy callose in the walls of the parenchyma cells. Tomatoes heated at 45°C for 1 hr (218X); (14) Callose covers the entire cell wall surface of parenchymatous cells of the placenta. Tomatoes heated at 45°C for 1 hr (88X).



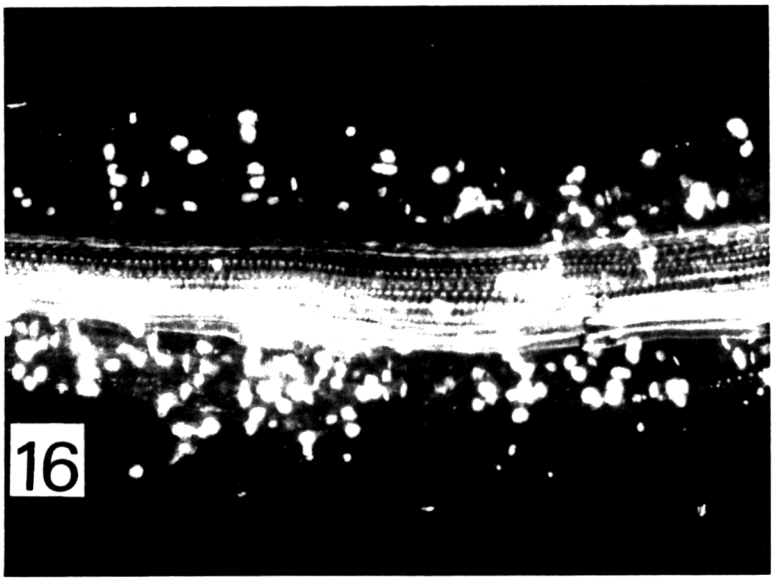


Fig. 15–16—Callose structures in processed whole tomatoes. Aniline blue fluorochrome: (15) Heavy pit callose and patches in the walls of parenchyma cells. Note the formation of callose dots on the surface of the protoplast (247X); (16) Massive callosing of sieve plates and lateral pit callose in the phloem of a vascular strand (115X).

related to consistency. Furthermore, they showed that cellulose walls swell, bind quantities of water and promote high viscosity. They also noted that tomato cellulose suspensions exhibited some properties which are not commonly associated with cellulose suspensions. Their research revealed that tomato cellulose, although fibrilar in appearance, is essentially amorphous. However, due to its solubility properties, callose, if present, would not have been removed from their cell walls. In addition, callose has a strong affinity for water. Kessler (1958) attributed the strong affinity of callose for water to an extreme amorphous structure, and he cites comparable values of density for callose and cellulose at 1.62 and 1.61 respectively. Therefore, the above noted anomalous observations on tomato cellulose suspension properties can be explained by the presence of callose—a substance not previously reported as being present in tomato juice. Callose can no longer be neglected; it is an important cell wall component (Currier, 1957).

Mechanically harvested tomatoes

Bruising by mechanical harvesting appeared to stimulate synthesis of stainable callose substance of various forms and intensities covering the cell wall surface (Fig. 12). The pit callose was evident as yellow dots or dashes in parenchyma cells and large amounts appeared to cover the skin. Callose in mechanically harvested tomatoes placed at room temperature (22°C) was due to injury rather than to temperature. McNairn (1967) found that callose in plants heated at 25°C was no greater than in unheated plants. Deposition of callose in response to injury has been noted in a variety of living cells and may be rapid (Eschrich, 1956; Currier, 1957). Mechanically harvested tomatoes heated at 45°C for about 1 hr (Fig. 13, 14) produced large amounts of parenchyma callose covering the entire cell wall.

Commercially processed whole tomatoes

Examination of commercially processed whole tomatoes revealed the existence of callose structure in parenchymatous tissues as pit callose, heavy pit callose, patches and dots associated with the cell walls (Fig. 15); and in the phloem as a sieve plate callose (Fig. 16). The detection of the abundance of parenchyma callose in canned whole tomatoes reconfirmed its stability to heat once the substance is formed. During canning processes, the tomatoes are passed through the continuous cooker at 212°F for a specified time. Currier (1957) reported that callose once formed is stable to heat and chemical fixation. Dunning (1959) found callose to be stable in boiling water. One would therefore expect to find callose, if formed during harvest and processing, in the finished product.

Callose found in bruised and/or heated tomato tissues is consistent with the existing views of callose formation. This work has firmly established that amounts of callose increase in experimentally bruised and heated tomato tissues. Subjecting tomatoes to different temperature, however, brings about wide differences in the callose response. Callose was found in the skin (exocarp) composed of the epidermis and subepidermis collencyma; in the parenchymatous tissue of the flesh (mesocarp, endocarp, the partitions and the placentae), and on sieve plates in the phloem of a vascular strand.

The presence of large quantities of callose in canned whole tomatoes supports the idea that once formed, the substance is stable to heat.

Perhaps callose is the plant polysaccharide that is the major contributor to the consistency of tomato products and to the texture in tomato fruit.

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

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IMPROVEMENT OF THE TEXTURE OF DEHYDRATED CELERY BY GLYCEROL TREATMENT

INTRODUCTION

DEHYDRATION is a reliable process for the preservation and reduction of weight of foods. The Armed Forces are expanding their usage of freeze-dried foods for these reasons. The high-water content plant products (celery, tomatoes, lettuce, etc.) primarily used for salads, do not withstand the freeze-drying process. They rehydrate to a mushy, unacceptable product due mostly to tissue damage during freezing. The purpose of this study was to develop a method of dehydrating celery so as to retain turgidity upon reconstitution. A process was sought which would reduce tissue damage and yield a product with good eating qualities.

Literature review

Dehydration, as well as freeze-drying techniques used in previous studies on celery, have failed to yield a product which, after rehydration, retains its textural qualities (Neubert et al., 1968; Schwimmer, 1969; Sullivan and Cording, 1969; Wilson, 1965). Upon examination of these processes, it became apparent that freezing and drying cause an irreversible damage to the celery tissue. Meryman (1966) points to the paradoxical situation that most labile biochemicals, even living organisms, can be stabilized by freeze-drying, but this success is not duplicated with food. Damage may occur during freezing, drying, storage and reconstitution. Such damage, which is cumulative, becomes apparent when the rehydrated product is eaten. Blast freezing to −20°F., ordinarily used to freeze foods before freeze drying, causes severe damage. However, the freezing rate and the final temperature are important factors in minimizing damage to cells (Joslyn, 1966; Mazur, 1963; Mohr and Stein, 1969). Finkle (1971) believes that the large ice crystals which are formed at the intermediate sub-zero temperatures are responsible for tissue damage. The general belief is that in plant tissue intracellular ice crystals develop at slower freezing rates. This type of freezing dehydrates the cells, thereby enlarging the intracellular spaces. Ice crystal enlargement to many times the size of individual cells, disrupts cell membranes and middle lamellae, etc., causing textural changes (Finkle, 1971; Joslyn, 1966; Mazur, 1970; Meryman, 1966; Moore et al., 1969). Levitt (1966) hypothesized that freezing damage is due to the closeness of macromolecules caused by water loss during freezing. This compaction favors the formation of disulfide bonds which distort the product upon rehydration.

Freezing and desiccation damage are analogous in that both are attributed to water loss from vital positions in the cell (Finkle, 1971; Parker, 1969). While the importance of bound water, disulfide bonding, etc., are considered significant, there is less literature pertaining to mechanical damage in desiccation. The requirements for protection, however, seem to be very similar, if not the same.

Most researchers agree that the best way to prevent freezing and desiccation damage is through the use of chemical additives. Manipulation of freezing rates and final temperatures may be important (Finkle, 1971; Moore et al., 1969), but are not satisfactory solutions by themselves. Most researchers agree that a useful protective agent must: preserve biochemical integrity of membranes, prevent water from freezing (formation of hydrogen bonds), permeate the cell membrane freely, prevent shrinkage below a minimum size, be a solvent for electrolytes, and be nontoxic (Finkle, 1971; Heber, 1968; Mazur, 1970; Meryman, 1966; Williams, 1969). These properties would also be effective in preventing damage according to other theories (Levitt, 1966; Parker, 1969). This was refined even further by the requirement that the additive must be edible and minimize any change in the organoleptic properties. Of the chemicals reviewed for use, glycerol seemed to be the most promising. It satisfied more of the above requirements than any other additive.

EXPERIMENTAL

Preparation of dehydrated celery

Fresh California celery was locally procured and the outer stalks sliced into ¼ in. cross-cut pieces. Equal quantities were soaked for 18 hr

Table 1—Experimental data on pretreated dehydrated celery

% Glycerol	Wt of celery after soak[a]	% Moisture after soak	After dehydration	Rehyd ratio[b]	% Yield[c]
100	–	40.1	4.0	3.1	54.8
90	89.5	–	–	2.7	54.0
80	104.0	46.2	4.1	2.9	47.0
70	115.5	36.0	–	2.8	63.5
60	129.0	32.0	3.8	3.0	61.2
50	153.3	52.0	–	2.8	62.1
40	184.2	63.0	3.5	3.1	67.3
30	172.6	67.5	–	3.4	59.7
20	189.0	76.8	3.7	4.0	55.2
10	180.5	85.6	–	6.2	48.1
0	300.0	95.5	1.7	12.1	35.2

[a]Original fresh celery, wt 300g
[b]Rehydrated wt/dry wt (inc glycerol)
[c]Rehydrated wt/original wt × 100

Table 2—Texture of freeze-dried vs. air-dried glycerated celery (technological panel analysis)[a]

% Glycerol treatment	Air dried	Freeze dried
0%	4.3	2.0
10%	5.2	5.0
20%	5.9	4.4
40%	6.1	5.4
60%	6.1	5.8
80%	5.9	5.4
Fresh celery (control)	7.5	7.5
LSD	0.8	1.3

[a]1—Extremely poor; 2—Very poor; 3—Poor; 4—Below fair, above poor; 5—Fair; 6—Below good, above fair; 7—Good; 8—Very good; 9—Excellent

in aqueous glycerol solutions of 0–100% (by volume) at 10% intervals. An excess of the glycerol solutions (2:1 by volume) was used for equilibration. This glycerol treatment is somewhat similar to osmotic dehydration as explained by Ponting et al. (1966).

Equal quantities representing each variable were freeze dried following commercial practices. It should be understood that glycerol treated samples underwent low temperature evaporative drying rather than true freeze-drying as the glycerol prevented freezing in most cases. At the same time, similar quantities were air dried in a bin drier with blowing air at 110°F for 16 hr. For rehydration, the samples were put in excess water (approximately 10 to 1 by weight of water to celery) and stored overnight at 40°F followed by two changes into fresh water. Weights and percentages of moisture (determined by weight loss after 16 hr at 70°C in a vacuum oven) were recorded before and after the various treatments to determine the direct effects of the processes. Percent glycerol in the rehydrated product was determined by the Perodate method (AOAC, 1970).

Sensory evaluation

Sensory evaluation panels of 15 members (food technologists) were used to determine the odor, flavor, texture, color and appearance qualities of the various rehydrated samples: ratings were on a qualified scale from 1 to 9 (extremely poor to excellent, Pilgrim and Peryam, 1958). No attempt was made in this study to correlate these tests with acceptability.

Mechanical measurements

The Allo-Kramer shear press has been used in this (Kapsalis et al., 1970a; Rahman et al., 1969) and other (Sullivan and Cording, 1969) laboratories to measure the texture of foods expressed as a maximum cutting-extrusion force. Freeze-dried celery was found to be spongy and tough (in sensory terms) upon rehydration, whereas fresh celery is crisp due to turgidity of the tissue. Preliminary studies indicated that the Kramer shear press did not adequately differentiate between the toughness and crispness that the technological panels indicated existed.

The Instron Universal Testing Apparatus (Kapsalis et al., 1970b) was used because it was capable of differentiating the textural changes. Fifteen replicates of each treatment were tested.

Celery slices ¼ in. thick were compressed to the rupture point, using a cylindrical, flat-surfaced anvil 57 mm in diameter moving at a speed of 1 cm/min. The force of compression was recorded on a L/N ¼ sec recorder, the chart running at a constant speed of 30 cm/min. Two parameters were determined for these curves: (1) Toughness, defined as the work per

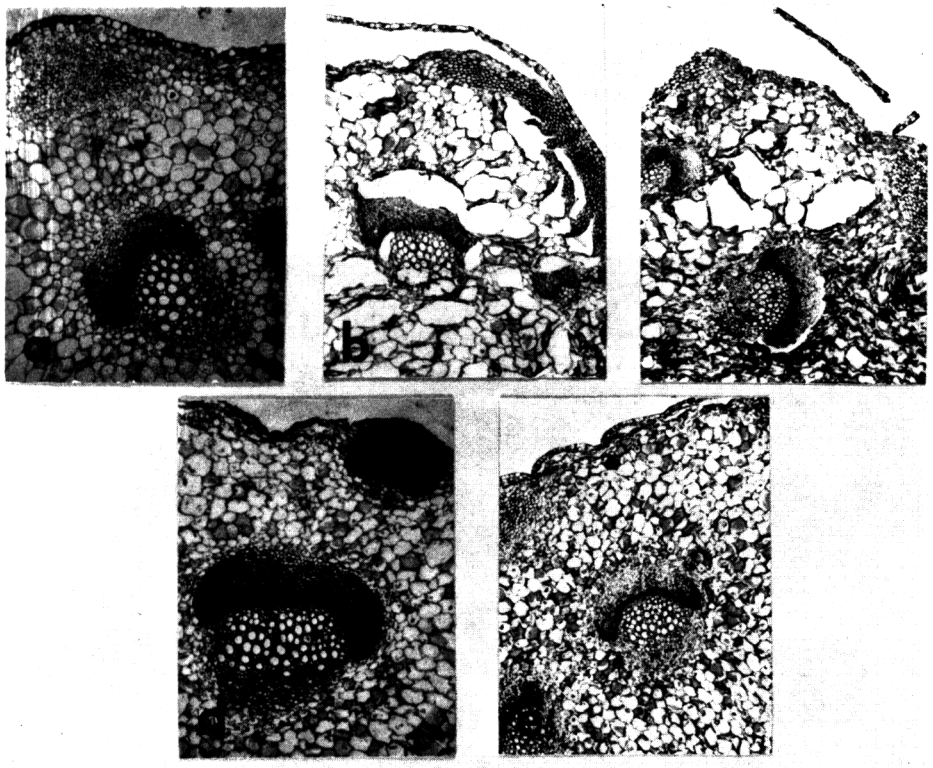

Fig. 1—Effects of glycerol on celery tissue: (a) Fresh celery; (b) Freeze-dried, rehydrated celery; (c) Air-dried, rehydrated celery; (d) 60% glycerated, freeze-dried, rehydrated celery; (e) 60% glycerated, air-dried, rehydrated celery. Magnification ≈ 25X.

Table 3—Mechanical evaluation of celery texture

Treatment[a] (% Glycerol)	Apparent modulus of elasticity (kg/cm²)	Toughness (kg/cm²)[b]
0% Fresh	59.8	1.91
0% FD	0.94	0.0043
0% AD	0.77	0.0078
20% AD	20.5	0.77
40% AD	19.3	0.51
60% AD	20.9	0.74
60% FD	12.8	0.40
80% AD	19.5	0.62
LSD	4.68	0.14
r	0.79	0.80

[a]AD = Air dried; FD = Freeze dried
[b]kg cm/cm³ = kg/cm²

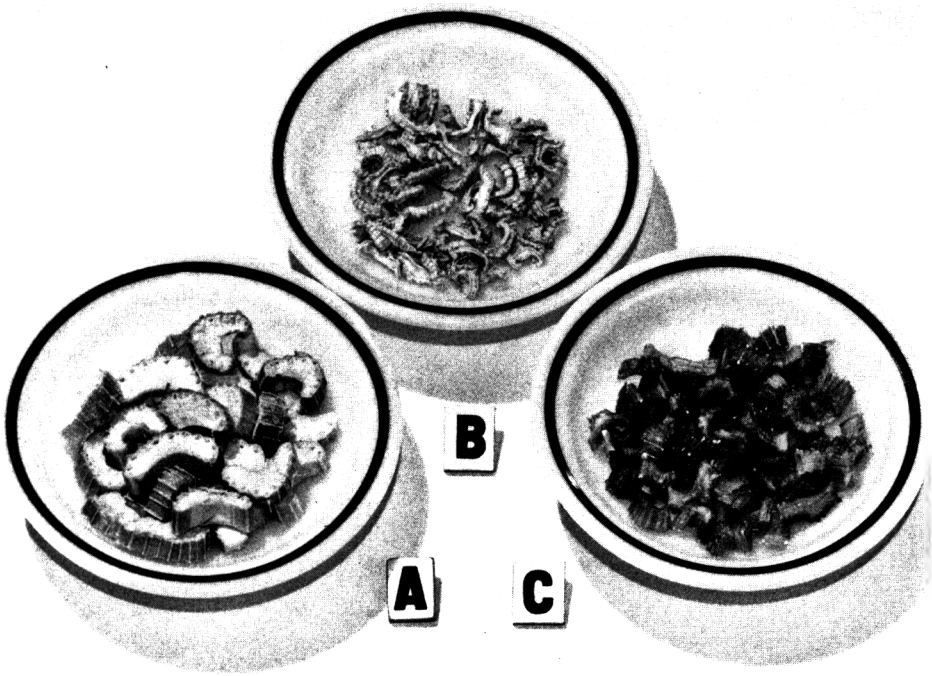

Fig. 2—Dehydrated celery: (a) Freeze dried; (b) Air dried; (c) Glycerated, air dried.

unit volume necessary to compress the sample to its rupture point (Kapsalis et al., 1970b) and (2) Apparent modulus of elasticity, which is the firmness or rigidity of the product as indicated by the slope of the initial straight-line portion of the curve.

Upon inspection and as shown by sensory panel results, the conventional freeze-drying process had no advantage over air drying. Consequently, to keep the testing within reasonable limits, some samples were deleted from the mechanical tests. Fresh, freeze-dried, air-dried, 20, 40, 60 and 80% glycerol-air dried and 60% glycerol-freeze dried samples were tested.

Statistical analysis

Analysis of variance and least significant difference (LSD) were calculated for the toughness, apparent modulus of elasticity and panel ratings. The correlation coefficients between each of the two mechanical test methods and the subjective tests for textural properties were also calculated.

Histological studies

In order to determine the site of damage, as well as the effects of additives on the tissue, histological studies were also conducted. Attempts were made to obtain uniform samples by using sections taken from the middle of outer stalks. The method included killing all samples in Nawashin Craf (chromic-acid type) fixative (Jensen, 1962; Sass, 1958), dehydrating with an ethanol-butanol series and embedding in tissuemat. Sections of all samples were cut at 21μ on a lipshaw rotary microtome and stained in haematoxylin-safranin (Johansen, 1940). Photomicrographs were taken of all slides using a R and L type microscope fitted with a Polaroid camera.

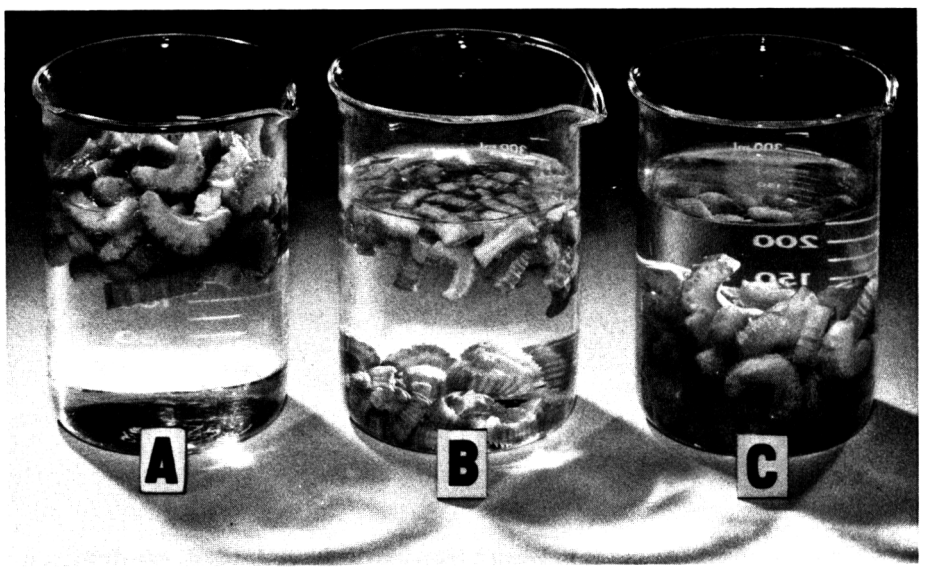

Fig. 3—Dehydrated celery, rehydrated: (a) Freeze dried; (b) Air dried; (c) Glycerated, air dried.

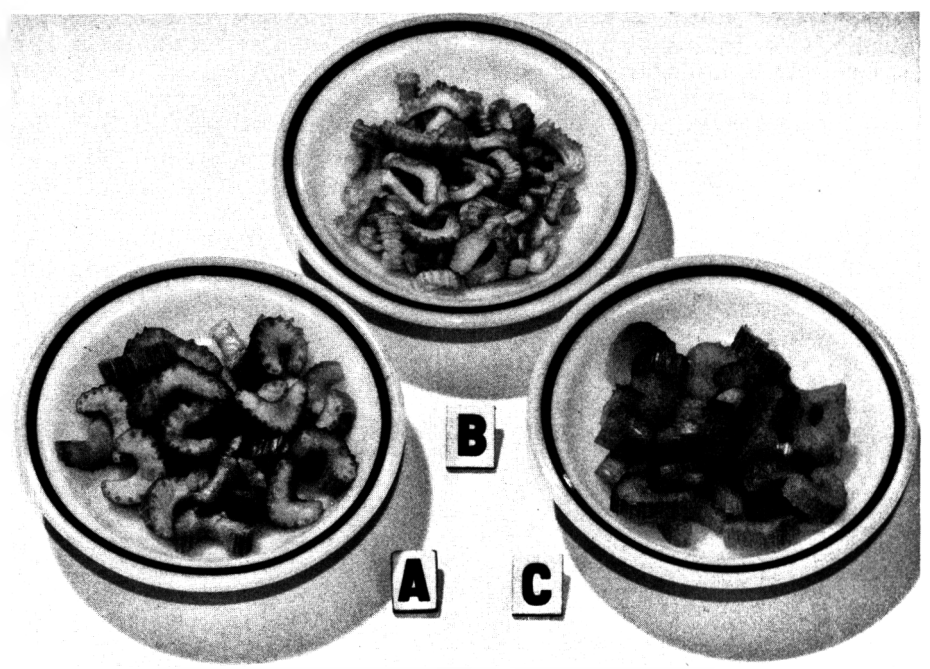

Fig. 4—Dehydrated celery, rehydrated: (a) Freeze dried; (b) Air dried; (c) Glycerated, air dried.

RESULTS & DISCUSSION

Effects on moisture

As seen in Table 1, celery soaked in glycerol showed a noticeable loss of moisture. Higher moisture loss was exhibited in celery soaked in glycerol of higher concentrations. Additional dehydration reduced the samples to about 4% moisture. The rehydration ratio of celery dehydrated after soaking in glycerol solutions ranging between 30–60% was approximately 3:1, with an average yield of 63% from the fresh product. However, the rehydration ratio was significantly increased in celery with concentrations of glycerol less that 20%. Rehydration time, as in many air-dried products, is rather long compared with their freeze-dried counterparts; here, 18 hr (overnight) was used for convenience. Initial tests indicated that it can be reduced to approximately 2 hr when several changes in warm water (100°F) are used. The glycerol residue in the rehydrated product depends upon the method of rehydration. For example, it was approximately 1% for an overnight rehydration and 6% for the 2 hr rehydration.

Sensory evaluation of texture

Results shown in Table 2 indicate significant difference in texture (as measured by a technological panel) between the dehydrated treated and untreated celery regardless of the method of dehydration.

Instron tests

Both the toughness and the apparent modulus of elasticity as determined with the Instron, indicated the magnitude of textural differences among the samples (Table 3). The toughness of fresh celery had a value of 1.91 Kg/cm^2, while that of freeze-dried and air-dried were very poor, 0.0043 and 0.0078 kg/cm^2, respectively. However, the glycerated samples averaged about 0.6 kg/cm^2 which is much closer to fresh celery than are the untreated samples. This property, when compared with the technological panel ratings, showed a significant correlation with an r value of 0.80. The apparent modulus of elasticity (Ea) also showed significant correlation with the panel ratings (r=0.79). The Ea of fresh celery was 59.8 kg/cm^2 whereas that of freeze-dried and air-dried unglycerated celery was 0.94 and 0.77, respectively. The glycerol treatments definitely

improved the texture as indicated by an average value of 21.5.

The results obtained from the Instron Universal Testing Apparatus and their correlation with the sensory evaluations of texture point out the applicability of this apparatus to predicting the textural properties of fresh, freeze-dried or treated celery.

Histological investigations

The effects of the various treatments on the tissue and cell structure of the rehydrated celery are shown in Figure 1.

Photomicrographs of fresh, untreated celery (Fig. 1a) show polyhedron-shaped cells with organized nuclei, and the collenchyma, parenchyma, epidermis and vascular bundles all intact. Figures 1b and 1c show the effect of freeze-drying and air-drying, respectively, where large crevices are visible and tissue is signficantly disrupted. The epidermis has separated and large crevices appear in the collenchyma, parenchyma and vascular bundles. 60% glycerol treatment with a subsequent freeze-drying-like process (Fig. 1d) and air-drying (Fig. 1e) protects the tissues as evidenced by absence of major tissue damage and disruption of the cell walls. Appearance is similar to fresh celery, although the epidermis and some of the cell walls seem to be partially distorted.

Figure 2 shows the differences among the samples in the dehydrated state. The freeze-dried samples (2a) hold their original shape but are very fragile; the air-dried (2b) are highly shriveled and hard; the glycerated air-dried (2c) are partly shriveled but soft and flexible and take on a darker (green) color.

While rehydrating (Fig. 3), the samples show obvious differences. The freeze-dried celery (3a) floats as it takes up water like a sponge, with a lot of air still present within the tissue. The air-dried celery (3b) rehydrates poorly and remains partly shriveled, some air also remaining in the tissue. The glycerated, air-dried celery (3c) remains in phase with the water, has little or no air in the tissues and returns to its original shape.

The fully rehydrated samples are seen in Figure 4. Therefore, it is concluded that fresh celery treated with glycerol can be successfully air dried to approximately 4% moisture and subsequently rehydrated to have textural characteristics that approach fresh celery.

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FERROMAGNETIC PARTICLES IN FOODS

INTRODUCTION

THE PRODUCTION and processing of much of our food involves considerable grinding, cutting and pulverizing with metal surfaces and some adventitious contamination with metal particles might be expected. In a survey of 147 food manufacturing firms (Selby, 1954) it was reported that the contaminants most frequently found in raw materials and finished products were metal particles, predominantly iron. Among the more common causes of metallic contamination are wear of machinery, particularly during grinding operations, worn plating, unsuitable joints, clips from belts and loose nuts and bolts (Chatt, 1964). Even apparently soft materials may exhibit abrasive properties when worked at high speeds as in grinding and pumping operations (Selby, 1956). Some of the contamination due to normal grinding operations is largely unavoidable while abrasion such as that due to the scraping of a bent stirrer or beater blades against the sides of a mixing vessel is unnecessary and can be eliminated.

Much use has been made of metal detectors, screens and magnetic devices to detect and remove from food small nuts and bolts and fragments over 1 mm long but very little attention has been devoted to determining the extent of contamination with particles less than 1 mm in length. Methods have been dveloped to separate these small particles from samples of foods along with other "heavy filth" (AOAC, 1970) but little effort has been spent on further isolating these particles to determine their size, weight and number.

The following study was made to determine the amount of metal particles that could be withdrawn by a magnet from a number of foods which are normally subjected to considerable metallic abrasion during their processing.

EXPERIMENTAL

10g OF FLOUR or finely divided food (coarse materials were powdered with a mortar and pestle) were placed in a 100 ml beaker with 50 ml of chloroform:methanol (2:1, v/v). The mixture was stirred for 1 min with a small test tube (10 mm ID × 75 mm long) containing a stirring magnet (6 mm × 30 mm). Ferromagnetic particles adhered to the outside of the tube adjacent to the magnet. Small food particles occasionally adhered to the metal particles but were removed by transferring all particles on the tube to a 50 ml Erlenmeyer flask containing 40 ml of Soluene (solubilizer, Packard Instrument Co.). This was done by swirling the tube in the Soluene while removing the magnet. The magnet was removed quickly to avoid pulling metal particles up the outside of the tube. The contents of the beaker were again stirred with the tube and magnet to recover any particles missed on the first stirring. These were also transferred to the Soluene and the procedure repeated until no further particles were recovered. Usually two or three stirrings were sufficient. The tube and magnet were then used to recover the clean metal particles from the

Table 1—Metal particles recovered with a magnet from 10-g samples of foods

Food	No. of samples	No. containing particles	Length of particles (mm) 0.001 to 0.015	0.015 to 0.05	0.05 to 0.15	0.15 to 0.50	0.50 to 1.50	Total no. of particles	Total wt. of particles (μg)
1. Whole wheat flour[a]	3	3	57	173	115	30	3	378	47
2. White wheat flour[a]	5	3	218	373	106	5	0	702	75
3. Buckwheat flour	2	0							
4. Rye flour	1	0							
5. Bran cereal	1	0							
6. Bran cereal[a]	3	3	29	389	231	10	1	660	104
7. Oat cereal	3	1	0	2	0	1	0	3	–
8. Wheat cereal	4	3	70	166	3	0	0	239	–
9. Corn cereal	2	0							
10. Barley, rice and rye cereal	3	2	0	3	1	0	0	4	–
11. Cold cereal[a]	6	3	100	26	7	1	0	134	–
12. Wheat germ	2	0							
13. Rice	2	0							
14. Biscuits, wafers, mixes	4	3	0	1	1	0	0	2	–
15. Melba toast, bread sticks	2	2	1	34	12	2	0	49	5.5
16. Macaroni, spaghetti, tapioca	3	1	0	1	1	0	0	2	–
17. Coffee (ground)	2	0							
18. Cocoa	2	2	60	96	9	1	1	167	69
19. Chocolate	3	3	1532	98	9	0	0	1639	2.7
20. Coconut	1	0							
21. Chili powder	1	1	208	142	11	1	0	362	–
22. Sugar and cinnamon mix	1	1	0	6	0	0	0	6	–
23. Corn oil	1	1	8	0	0	2	0	10	–
24. Apple sauce	1	1	0	3	0	0	0	3	–
25. Chicken soup (dry mix)	1	1	2	0	0	0	0	2	–
26. Baby food-strained beef	2	0							

[a]Enriched with an iron supplement; all samples in No. 6 were labelled "enriched with Fe pyrophosphate."

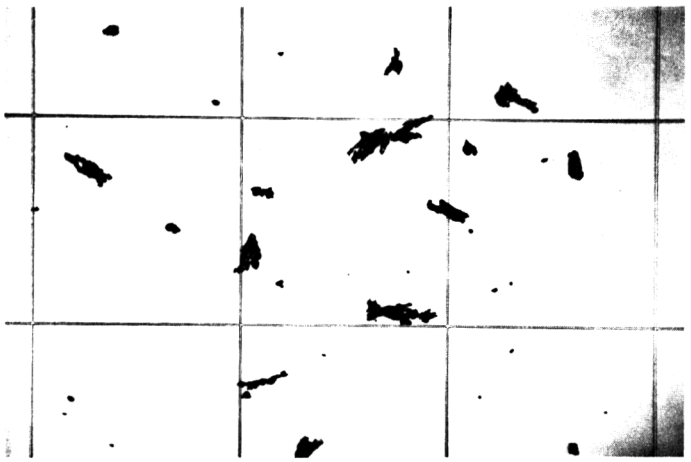

Fig. 1—Photomicrograph of metal particles isolated from a sample of enriched whole wheat flour on a micrometer disc with 1 mm squares.

Soluene (may be reused). The Soluene remaining on the tube was washed off with a fine stream of methanol from a wash bottle and when the methanol evaporated the particles were released into a small glass funnel held over a micrometer disc (1 mm squares). Any particles adhering lightly to the glass funnel were loosened by gently tapping the funnel with a spatula. The micrometer disc was then placed on the stage of a light microscope and an ocular micrometer was used at 10× and 40× to count and measure the particles.

After counting, in cases where the samples contained enough particles to permit weighing, they were transferred with the funnel to a tared aluminum combustion boat (4 × 4 × 12 mm) and weighed on a microbalance.

Recovery checks were made by adding known numbers of metal particles of predetermined size to 10g samples of particle-free flour. The particles, ranging in size from 0.001–1.5 mm were obtained by filing a soft steel bar. There was a slight tendency for particles to become magnetized when in contact with the magnet so that two or more could cling together and appear as a single particle. This was overcome by keeping the magnet in close proximity to the particles only as long as required and by careful microscopic examination of the particles.

RESULTS & DISCUSSION

RECOVERIES from 150–200 μg samples of metal filings consisting of 200–300 particles averaged 85.7 ± 1.4% by weight. Recovery of particles over 0.015 mm in length always exceeded 90% but with particles less than 0.015 mm it ranged from 16–44%. The smaller particles evidently became attached to particles of food and could not be separated as readily as the larger ones. The technique is therefore quite sensitive for detecting and counting particles larger than 0.015 mm but figures on those below this size should only be used to indicate if they are present in significant numbers. The technique will not detect copper, brass, zinc, glass or other nonmagnetic particles.

The data in Table 1 give the number of ferromagnetic particles, partitioned according to size, that were found in 34 out of 61 samples of food. Four samples contained particles ranging from 0.5–1.5 mm in length, 16 from 0.15–0.5 mm and the remainder from 0.001–0.15 mm. Large numbers of particles were consistently found in "enriched" cereal products, (Fig. 1) indicating that powdered iron was being used as a supplement. However, in some of these samples the metal particles approached 1.5 mm in length and since Canadian Food and Drug Regulations (Canada Dept. of National Health and Welfare, 1971) specify that flour must pass through a 100 mesh sieve (0.15 mm openings) it would appear that either the powdered iron supplements were not sufficiently finely divided or the large particles resulted from the milling process.

Iron-supplemented flour may also have been responsible for the presence of small numbers of particles in some baked products such as melba toast and bread sticks but there were some foods such as chili powder and chocolate which contained large numbers of particles and in which one would not normally expect iron supplementation. No explanation is available for the iron particles in the chili powder but it is well known that the processing of chocolate involves much abrasive contact of metal against metal.

Even without iron supplementation, the milling process of some cereal products would at some times appear to contribute considerable numbers of metal particles to the product. One sample of wheat cereal was labelled to be "made from the heart of the wheat . . . . with no added concentrates" but still contained 774 particles in 10g, all less than 0.10 mm in length. The other wheat cereals in this group contained negligible numbers of metal particles. Another cold cereal, labelled to contain iron pyrophosphate contained a total of 370 particles in 10g while another cold cereal similarly labelled by the same company contained only 23 particles. This would indicate that either the first cereal was picking up more iron particles during the manufacturing process than the second or that it was supplemented with iron powder instead of iron pyrophosphate or an impure grade of iron pyrophosphate.

The appearance of small numbers of metal particles in soft foods such as corn oil and apple sauce, although of little consequence, indicates that the production processes may not be free of abrasive action.

It has been shown that iron supplied as particles greater than 0.01 mm in size is not as well absorbed as that in those smaller than 0.01 mm (Hoglund and Reizenstein, 1969). This would indicate that iron particles may not be completely dissolved by gastric juice in the stomach and may pass through the intestine as solid particles.

Very little information is available as to whether iron or nickel particles less than 1.5 mm in length can penetrate the walls of the digestive tract of man. Asbestos fibres up to 0.023 mm in length have been found to pass through the walls of the digestive tract, enter the blood and travel to all the major organs of the body (Cunningham and Pontefract, 1971). Asbestos fibres, however, are more needle shaped than metal particles and are perhaps more likely to pierce the intestinal wall but the degree to which metal particles may penetrate the intestinal mucosa still remains to be investigated.

It may be concluded from the present study that the addition to our food of ferromagnetic particles of less than 1.5 mm in length, both intentional and unintentional, can be readily detected by the techniques described above. Although the significance of these particles in our diet is still unknown the technique will allow us to monitor foods for evidence of excessive contamination and to check on the type and size of powdered iron additives.

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JFS BASIC SCIENCE

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ISOLATION AND CHARACTERIZATION OF THE NATIVE, THERMALLY INACTIVATED AND REGENERATED HORSE RADISH PEROXIDASE ISOZYMES

INTRODUCTION

THE PHENOMENON of regeneration of thermally inactivated enzymes was noted as early as 1901. Early publications are adequately reviewed by Schwimmer (1944). Schwimmer reported from his work on turnip and cabbage juice that both the precipitate formed upon heating and the supernatant therefrom were essential for regeneration, which can amount to 35% of the original activity.

Much work has been done on the inactivation and regeneration of peroxidase in high-temperature short-time (HTST) processed canned foods (Guyer and Holmquist, 1954; Esselen, 1950; Farkas et al., 1956; Zoueil and Esselen, 1959; Vetter et al., 1959; Yamamoto et al., 1962; Resende et al., 1969).

Yamamoto et al. (1962) in studying the inactivation of peroxidase in sweet corn at 191°F found the curve to consist of an initial steep straight line, an intermediate curved portion and a final straight line with a shallow slope. Similar results were obtained by Resende et al. (1969) from the inactivation study of spinach peroxidase at 138°F and by Wilder (1962) from the inactivation of purified HRP at 280°F.

Regeneration of the inactivated peroxidase was attributed to a reversible denaturation of protein. Esselen (1950) and Vetter et al. (1959) explained the regeneration phenomenon as the ability of the enzyme molecule to regain its helical shape and form the enzyme substrate complex. Joffe and Ball (1962), in studying the kinetics and energetics of thermal inactivation and regeneration of a peroxidase system, concluded that the change in the tertiary structure of the protein moiety of the enzyme molecule during inactivation involves more than hydrogen bond and disulfide rupture.

The multiplicity of HRP has been demonstrated since 1940 (Theorell). Shannon et al. (1966) isolated seven isozymes from commercial preparations of HRP. Kasinsky and Hackett (1968) were able to duplicate Shannon's results except that they were not able to isolate a substantial amount of the more acidic HRP isozymes. Klapper and Hackett (1965) obtained five isozymes from the same commercial preparations of HRP as was used by Shannon.

The purpose of this study was to gain a better understanding of the changes involved in thermal inactivation and subsequent regeneration of a highly purified peroxidase isozyme in a model system.

EXPERIMENTAL

Materials

Horse radish *(Armoracis rusticana)* peroxidase (HRP) was purchased from Worthington Biochemical Corp., Freehold, N.J. This commerical product, Worthington peroxidase D (code: HPOD) is a lyophilized preparation from horse radish root and is stable for over a year when kept dry and cold. Ribonuclease A, chymotrypsinogen A and aldolase were products of Worthington Biochemical Corp. These enzymes are highly purified materials for using as the standards for molecular weight determinations.

Both CM-(carboxyl methyl) and DEAE-(diethyl amino ethyl) cellulose were medium mesh, 0.89 meq/g capacity, and were purchased from Sigma Chemical Co., St. Louis, MO. Special care was taken to remove small particles by repeated decantation in order to obtain cellulose column with high flow rates. Recrystallization of o-dianisidine was done to remove impurities. Other reagents used were reagent grades.

Methods

Enzyme assay. The method developed by Worthington Biochemical Corp. was used (Worthington, 1968). The rate of decomposition of hydrogen peroxide by peroxidase with o-dianisidine as hydrogen donor was determined by measuring the rate of color development at 460 nm.

The specific activity of the peroxidase of isozymes was expressed as micromoles of product/mg protein/min. Protein was determined by biuret method (AOAC, 1960).

Isolation of HRP isozymes. The isolation procedure was performed essentially according to that described by Shannon et al. (1966). A solution of 20 mg enzyme/ml was made by dissolving 100 mg HRP in acetate buffer (pH 4.4, 0.005M). The enzyme solution was then applied to a 2.5 cm × 20 cm column of CM-cellulose which had been equilibrated with the same buffer. A linear gradient elution system was applied according to Shannon et al. (1966). The fraction which did not adsorb to the CM-cellulose column was dialyzed against 0.005M Tris (Tri hydroxymethyl amino methane) buffer, pH 8.4, and was transferred to a DEAE-cellulose column previously equilibrated with the same buffer. Elution was done according to Shannon et al. (1966). The emerging effluent was monitored at 276 nm for estimation of protein content and at 401 nm for estimation of heme. Peroxidase activity of each fraction was tested qualitatively. Effluent fractions collected under a single peak of the elution profile were pooled and purified by repeated chromatography until elution patterns indicated that each fraction was discrete from other peroxidase fractions.

Thermal treatment. A pyrex tube 20 cm long × 3mm OD and wall thickness of 0.6 mm was filled with 0.5 ml of enzyme solution by a capillary-tipped dropper. One end of the tube was sealed in an oxygen-gas flame before filling and the other end of the tube was sealed after the filling process.

Heating of the tubes was carried out in a constant temperature glycerol bath (± 0.6°F). In order to eliminate the differences in buffer concentration existing among the eluants obtained from gradient elution, the pooled eluant was dialyzed against acetate buffer at the optimum pH of each isozyme before thermal treatment. The concentration of isozyme was 5×10^{-10} M, and the concentration of buffer was 0.005M.

Spectral studies. The absorption spectra were

Table 1—Chemical and physical properties of native, thermally treated and regenerated HRP isozymes

Isozymes	Specific activities μM product/mg protein /min	pH optima	RZ values	Molar absorption index at 401 nm × 10^5	Sedimentation coefficient s	Diffusion coefficient F	Thermal resistance z values[a] °F
A-1	2.4	5.8	3.74	1.38	3.42	6.15	42
Thermally treated A-1 (280°F, 1 min)	–	–	2.31	–	–	4.23	–
Regenerated A-1 (70°F, 2 days)	–	–	3.58	–	–	–	–
A-2	2.2	5.7	3.50	1.36	–	–	–
A-3	1.9	5.7	3.25	1.25	–	–	–
B	0.2	5.0	2.52	0.67	–	–	–
C	0.4	5.0	2.48	0.58	3.56	–	66
Thermally treated C (280°F, 1 min)	–	–	1.82	–	–	–	–
D	0.3	5.0	2.49	0.63	–	–	–

[a]Since temperatures were not corrected by the time lag of the thermal treatment, the z values reported are not true values.

recorded using a Beckman DBG recording spectrophotometer at a scan rate of 50 nm/min. RZ (Reinheitzahl) values (absorbance ratio A401/A276) were calculated from the absorbances measured at 401 nm and 276 nm.

The reduced pyridine hemochromogens of the isozymes were prepared by the technique of Morrison and Horie (1965) with slight modification. Aliquots of 0.05 ml of 1N NaOH and 0.2 ml of pyridine were added to 0.6 ml of the isozyme solution. A few crystals of dithionite were added to complete the reduction and the absorbance was then measured.

Sedimentation studies. A Beckman/Spinco Model E analytical ultracentrifuge equipped with a Schlieren optical system was used for sedimentation studies. Before analysis, the enzyme preparations were dialyzed against 0.005M Tris buffer overnight, at the optimum pH of the individual isozyme. A 12 mm double sector cell (filled Epon centerpiece) was used for the sedimentation runs at 59,780 rpm. Ten exposures at 16-min intervals were made in all sedimentation experiments. Data obtained from the photographic plates by means of a two-dimensional micro-comparator were used for the calculations of the sedimentation coefficients according to Schachman (1957). Diffusion coefficients were determined from experiments performed in a double sector, synthetic boundary cell (filled Epon centerpiece), capillary type, at 5,750 rpm. Calculations of diffusion coefficients were carried out using data obtained from magnifications projected into millimeter graph paper. Areas were calculated by mechanical planimetry. Calculations were performed according to the height-area method (Svensson, 1951). The diffusion coefficients were corrected for the density and viscosity of the medium to those of water at 20°C. The results are given in Fick units, F(F = 10^{-7} cm^2 sec^{-1}). The technique of approach to sedimentation equilibrium according to Archibald (1947) was employed for the molecular weight determination of one of the isozymes.

Sephadex gel chromatography for molecular weight determinations. Four protein standards, aldolase, HRP isozyme A-1, chymotrypsinogen A, and ribonuclease A were used. The K_{avg} values of these protein were determined by the following equation using a 2.5 cm × 25 cm column of Sephadex G-200 (Leach and O'Shea et al., 1965).

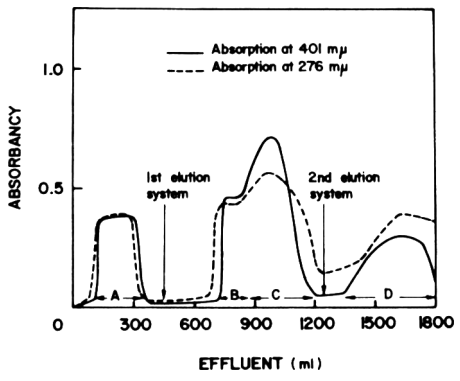

Fig. 1—Chromatography of HRP on a column of CM-cellulose, 2.5 × 20 cm. 500 mg of enzyme, A_{400}/A_{280} = 1.03 was applied to the column. Linear gradient elutions were used. First elution system, 0.005M acetate, pH 4.4 and 0.1 M acetate, pH 4.4, 600 ml each; second elution system, 0.1M acetate, pH 4.4, and 0.25M acetate, pH 4.9, 400 ml each.

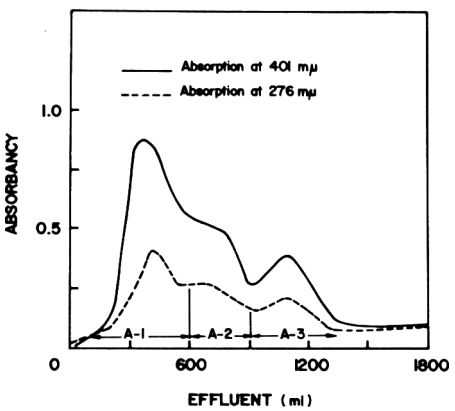

Fig. 2—Chromatography of fraction A on a column of DEAE-cellulose, 2.5 cm × 20 cm. Elution system, 800 ml of 0.05M Tris buffer, pH 8.4, and 800 ml of 0.005M Tris buffer, pH 8.4, containing 0.1M NaCl.

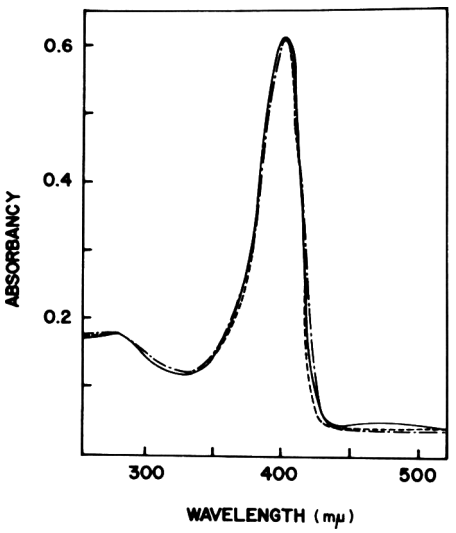

Fig. 3—Absorption spectra of horse radish peroxidase isozymes A-1, A-2 and A-3.

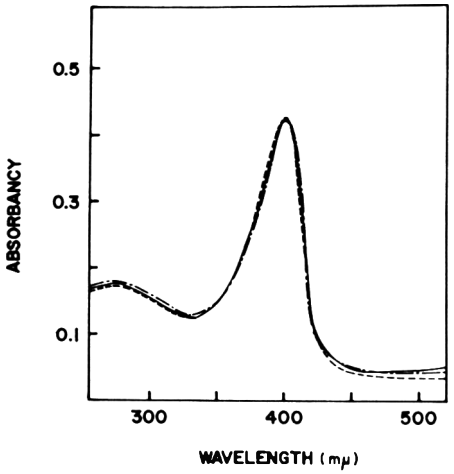

Fig. 4—Absorption spectra of horse radish peroxidase isozymes B, C and D.

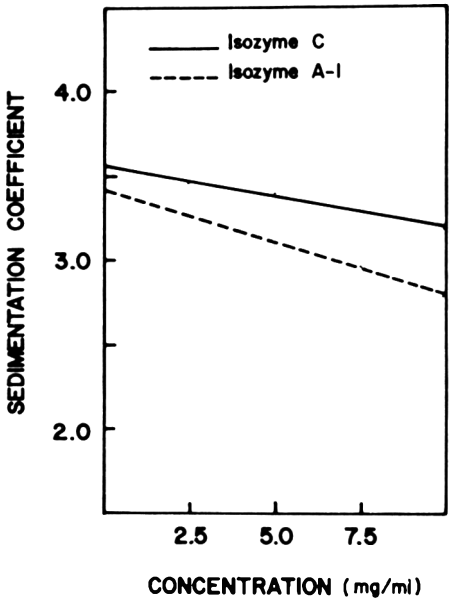

Fig. 5—Concentration dependence of the sedimentation coefficient, $S_{20,w}$, of horse radish peroxidase isozymes A-1 and C.

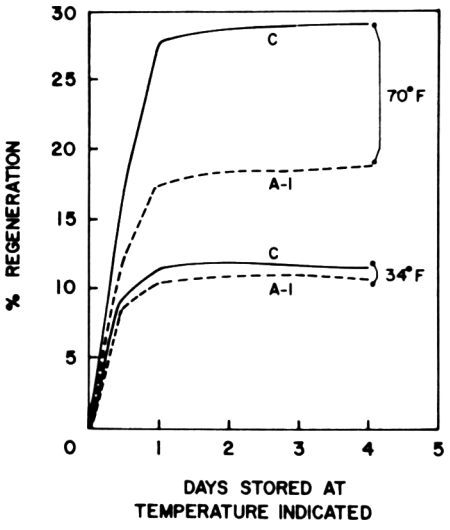

Fig. 6—Regenerations of heat inactivated horse radish peroxidase isozymes A-1 and C stored at the temperature indicated.

$$K_{avg} = \frac{V_e - V_o}{V_t - V_o}$$

where, V_e = elution volume for the protein; V_o = elution volume for blue dextran 2000; and V_t = total bed volume.

The K_{avg} value for each protein standard was then plotted on a semilogarithmic paper to obtain the selectivity curve. After the selectivity curve was constructed, native peroxidase isozymes, thermally treated isozymes and the regenerated isozymes were chromatographed on the same column, and the K_{avg} value of each eluted fraction was determined. The molecular weights of the fractions were then calculated from the selectivity curve. Acetate buffer, 0.005M, at the optimum pH of each isozyme, was used as elution solvent. The flow rate of the column was 15 ml/hr.

RESULTS

Isolation of HRP isozymes

Six isozymes, namely, isozymes A-1, A-2, A-3, B, C and D were obtained from CM- and DEAE-cellulose column chromatography (Fig. 1 and 2).

Isozymes A-1, A-2 and A-3 were purified by three-times rechromatography on DEAE-cellulose column, and isozymes B, C and D were purified by five-times rechromatography on CM-cellulose column.

Chemical and physical properties of native HRP isozymes

Specific activities, pH optima, RZ values, molar absorption indices at 401 nm, sedimentation coefficients and diffusion coefficients of the isolated isozymes are shown on Table 1.

The absorption spectra of isozymes A-1, A-2 and A-3 were almost identical, and so were those of isozymes B, C and D. (Fig. 3 and 4). As shown in Table 1 the six isozymes were divided into two sub-groups with each group having similar specific activities, pH optima and RZ values. Consequently, only isozymes A-1 and C, each representing its group, were further studied.

Sedimentation coefficients. The sedimenting boundary of both isozymes A-1 and C in ultracentrifuge migrated as a single peak. Analysis of the gaussian symmetry of the peaks according to Hall and Ogston (1956) at a protein concentration of 10 mg/ml showed that the protein was homogeneous in sedimentation.

The concentration dependence of the sedimentation coefficients of HRP isozymes A-1 and C was demonstrated in Figure 5. The lines in Figure 5, calculated by means of the method of least squares, can be fitted by the following equations:

$$s_{20,w} = (3.42 - 0.0909c)\text{...Isozyme A-1}$$

$$s_{20,w} = (3.56 - 0.0625c)\text{...Isozyme C}$$

where, c is the concentration of isozymes in mg/ml, $s_{20,w}$ the sedimentation coefficient in pure water at 20°C. By extrapolation to infinite dilution, $s_{20,w}$ values are 3.42 for isozyme A-1 and 3.56 for isozyme C.

Molecular weight. The approach to equilibrium method of Archibald (1947) was used to determine the molecular weight of HRP A-1. Molecular weights were calculated to be 39,800 and 39,600 at the bottom of the cell and at the meniscus, respectively. Molecular weight was also calculated according to Svedberg's formula (Svedberg and Pederson, 1940).

$$M = \frac{RTs}{D(1-\bar{v}\rho)}$$

where, $(1-\bar{v}\rho)$ is the buoyancy term, which is calculated to be 0.33 for isozyme A-1 by plotting density versus protein concentration. The diffusion coefficient, D, of isozyme A-1 was found to be concentration independent and was calculated to be 6.15F ($F = 10^{-7}$ cm^2 sec^{-1}). Consequently, the molecular weight of isozyme A-1 was calculated to be 41,000 according to Svedberg's equation.

Molecular shape. A frictional ratio, f/f_o, equal to 1.365 for HRP isozyme A-1 was calculated using the following formula (Svedberg and Pederson, 1940):

$$\frac{f}{f_o} = \left| \frac{1-\bar{v}\rho}{(D_{20,w})^2 \times s_{20,w}} \right|^{1/3} \times 10^{-8}$$

where f is the frictional constant of the enzyme molecule, and f_o is the frictional constant of a spherical molecule with an equal molecular weight.

Thermal resistance of HRP isozymes A-1 and C. HRP isozyme C was more heat resistant than isozyme A-1. HRP isozyme C had an inactivation z value (temperature difference required for the thermal destruction curve to traverse one log cycle, temperature range from

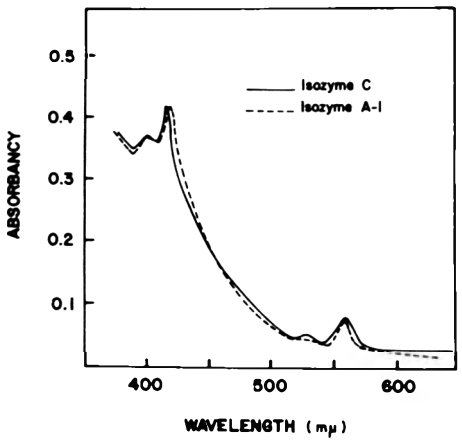

Fig. 7—Absorption spectra of the dithionite-reduced pyridine hemochromogen of isozymes A-1 and C of horse radish peroxidase.

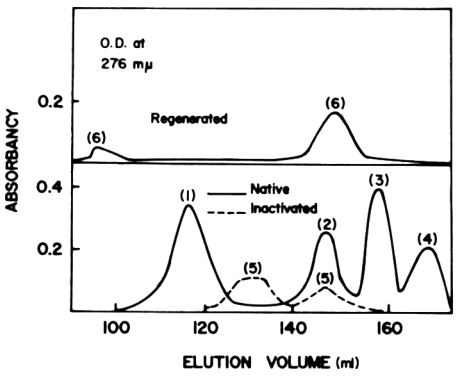

Fig. 8—Elution profile of aldolase (1); HRP isozyme A-1 (2); chymotrypsinogen A (3); ribonuclease A (4); thermally treated (280°F, 1 min) (5); and regenerated HRP isozyme A-1 (2 days storage at 70°F) on Sephadex G-200 column (6); column size 2.5 × 25 cm; elution solution, 0.005M acetate buffer, pH 5.8; 1-ml sample contains 20 mg each of the protein used.

180–280°F) of 66°F, where that of isozyme A-1 was 42°F. Regeneration of HRP activity reached a maximum of 28.5% for isozyme C and 17% for isozyme A-1 after approximately 30 hr of storage at 70°F (Fig. 6); whereas at 34°F both isozymes showed about 12% regeneration under the same conditions.

Chemical and physical properties of thermally treated HRP isozymes

Spectral studies. In the thermally treated (280°F, 1 min) HRP isozymes, the RZ values were decreased (Table 1) corresponding to a decreased Soret band absorption and an increased protein absorption. During regeneration RZ values were increased to resume part of the original values.

Dithionite-reduced pyridine hemochromogen of HRP isozymes A-1 and C are almost identical (Fig. 7). The spectra of the dithionite reduced pyridine hemochromogen of the inactivated HRP isozymes A-1 and C are the same as that of the native enzymes.

Sedimentation studies. Sedimentation velocity experiment of the thermally inactivated HRP isozymes A-1 and C showed a peak with a shoulder on the right. A sedimentation coefficient based on the moving of the right shoulder in the centrifugal field was calculated to be 4.52s for HRP isozyme A-1. The s values of the main peaks were 3.39 and 3.52 which are very close to the values of the native HRP isozymes A-1 and C, respectively. After regeneration (2 days at 70°F) the right shoulders of the inactivated samples disappeared and instead heavier materials appeared which moved rapidly in the centrifugal field.

Diffusion studies in the ultracentrifuge showed that the inactivated HRP isozyme A-1 had a decreased diffusion coefficient (4.23F) as compared to that of the native isozyme (6.15F). If one assumes that change in diffusion coefficient is due to a change in molecular shape only, one can calculate the frictional coefficient of the inactivated isozyme molecules according to the "two state" theory of Brandts and Hunt (1967). If one further assumes that there is total inactivation, and no partial inactivation, the diffusion coefficient of the denatured isozyme was calculated to be 3.84F by solving the following equation:

$$4.23 = 83\%\ D + 17\%\ N$$

where D is the diffusion coefficient of the denatured protein, N is that of the native protein, namely 6.15F and 83% is the percentage of the activity lost in the thermal treatment of isozyme A-1 (280°F, 1 min). Since the partial specific volume of most native proteins are around 0.73, one can further assume that the bouyancy term, $(1 - \bar{v}\rho)$, for the inactivated isozymes remains unchanged during the inactivation. Consequently, frictional ratio, f/f_o, and the average molecular weight of the heavier component shown in the sedimentation studies are calculated to be 1.72 and 87,000, respectively.

Sephadex gel chromatography. Using aldolase, HRP isozyme A-1, chymotrypsinogen A and ribonuclease A as standards on a Sephadex G-200 column, the following equation was obtained by fitting the selectivity curve according to the least square method:

$$\log(\text{mol wt}) = -0.3434K_{avg} + \log(86{,}000)$$

The elution curve of the thermally treated HRP isozyme A-1 (Fig. 8) shows molecular species with molecular weight larger than that of the native isozyme. From the elution volumes of the heavier fractions, their molecular weights were estimated to range from 75,000–130,000 for isozyme A-1.

When regenerated HRP isozymes A-1 and C were chromatographed on the same column, materials heavier than that found in the inactivated sampel were evidenced by their even smaller elution volumes (Fig. 8). The material with the smallest elution volume was estimated to have a molecular weight larger than 450,000.

DISCUSSION

A HIGH DEGREE of purification of the isozymes isolated was evidenced by the following data. Firstly, rechromatographic separation of each isozyme yielded a single protein peak, which superimposes on heme content and peroxidase activity. Secondly, HRP isozymes A-1 and C behaved homogeneously on ultracentrifugation. Thirdly, the RZ values of the purified HRP isozymes increased either two- or three-fold over that of the crude enzyme. The observation that the pyridine hemochromogen spectra of HRP isozymes A-1 and C are very similar, and are typcial of proto-hemin IX, provides evidence that the hemin of all the isozymes is the same, and is proto-hemin IX as is the case with most of the oxidoreductases. The fact that thermal inactivation did not change the spectra of dithionite-reduced pyridine hemochromogen of the isozymes suggested that during thermal inactivation the heme moiety of the molecule remained unchanged.

The adsorption spectra of the regenerated HRP isozymes A-1 and C tend to resume the spectra of the native isozymes as indicated by the change in RZ values. This suggests that thermal inactivation involved reversible changes.

The average of the molecular weights of HRP isozyme A-1 determined by the Archibald (1947) method and Svedberg's equation is 40,350. Sedimentation studies showed that inactivation of HRP isozymes involved the aggregation of HRP molecules into oligomers with average molecular weight of 87,000. In the studies of the shapes of the molecules, protein molecules are usually regarded as approximate ellipsoid of revolution. According to Perrin (Cohn and Edsall, 1950), a frictional ratio of 1.365 corresponds to a molecule which is a flattened ellipsoid with the axial ratio, b/a equal to 7.8, while inactivated HRP isozyme A-1 has a frictional ratio of 1.72 which corresponds to a flattened ellipsoidal molecule with an axial ratio of 18. The increase in the molecular axial ratio could be due to the unfolding of the HRP molecules, and the stacking of the unfolded molecules. However, it has to be noted that the frictional coefficients are not only affected by shape but also

the degrees of hydration of the molecules.

Sephadex gel chromatography showed that the molecular weights of the heavier components in the inactivated isozyme were between 75,000–130,000. Based on the molecular weight and shape of the heavier components existing in the inactivated isozymes they were considered to be either dimers or trimers of the native isozymes. In the regenerated isozyme, a fraction corresponding to a molecular weight as large as 450,000 was eluted from the column. This is consistent with the sedimentation studies, where a very fast moving boundary was formed in the regenerated isozyme. The formation of this heavy material is very possibly through further aggregation of the dimer or trimer found in the inactivated isozyme. The majority of the inactivated isozyme possibly resumes the molecular size and shape of the native isozyme as shown by the increase in peak size of native isozyme in the elution profile of the regenerated sample on Sephadex G-200 (Fig. 8). However, molecular weight determination through Sephadex gel chromatography depended not only on the sizes of the molecules but also on their shapes. From ultracentrifugation studies, it was shown that the shape of the molecules of HRP changed drastically. Hence, the molecular weight calculated from Sephadex gel chromatography, and the conclusion drawn from it can only be an approximation.

Since during regeneration of HRP activity, there is only 17% of the original activity of isozyme A-1 and 28.5% of isozyme C being resumed it indicates that the aggregation during inactivation and subsequent dissociation during regeneration only contribute to part of the activity changes of the enzyme. Consequently, one would suggest that there are other subtle conformational changes which result in detectable changes in molecular sizes and shapes but not in comparable changes of enzyme activities.

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FLAVOR QUALITY AND STABILITY OF POTATO FLAKES
Volatile Components Associated with Storage Changes

INTRODUCTION

DEHYDRATED POTATO products undergo two distinct types of flavor change during storage: flavor defects arising from reducing sugar-amino acid interactions and flavor defects associated with lipid oxidation. The relative extent of these changes depends on product composition, processing conditions, the presence of additives, product moisture content and storage conditions (Burr, 1966).

Potato flakes, a form of dehydrated mashed potato, have a shelf life of only 6 months in air at room temperature, even when stabilized by the incorporation of sulfur dioxide and antioxidants and by drying to an optimal moisture content (Cording et al., 1961; Strolle and Cording, 1965). Preliminary experiments conducted at the Eastern Marketing & Nutrition Research Div. (EMN) and reports from commercial producers of potato flakes (Sapers, 1970, unpublished data) have indicated that the shelf life of this product is usually limited by the development of hay-like off-flavors, attributed to oxidation, rather than to nonenzymatic browning. Potato flake instability is considered by the processing industry to be a significant problem.

Research was undertaken at EMN to establish the causes of flavor defects in potato flakes in terms of specific volatile compounds and their precursors, to relate flavor defects to specific raw material, processing, packaging and storage variables, and to develop procedures to improve product flavor and storage stability. Changes in volatile components associated with the development of off-flavors during storage are reported herein.

EXPERIMENTAL

Materials and storage conditions

Potato flakes were prepared from recently harvested (Fall, 1970) Pontiac potatoes (specific gravity 1.080–1.085) at our Red River Valley Potato Processing Laboratory (RRVPPL), East Grand Forks, Minn., using the USDA process (Cording et al., 1957). Two products were prepared: one contained no added antioxidants; the other contained Tenox 4 (20% BHA, 20% BHT, 60% corn oil), which was added to the cooked mashed potatoes, before drying, at the rate of 1.5 ml/50 lb (approximately .028%, moisture-free basis). Upon receipt at the laboratory, these products were found to contain 6.4 and 8.1% moisture, and 1000 and 1660 ppm SO_2 (moisture-free basis), respectively. Both products were packaged in No. 10 cans under air and under nitrogen (less than 2% oxygen) and were stored at 0° and 73°F.

A commercial potato flake product which had been prepared from recently harvested (Fall,1970) Kennebec potatoes by a conventional process similar to that used at RRVPPL (with added antioxidants) and shipped to us in bulk was repacked in No. 10 cans under air and under nitrogen and was also stored at 0° and 73°F. This product contained 4.7% moisture and 330 ppm SO_2 (moisture-free basis).

Sensory evaluation

Samples were removed from storage at intervals for evaluation by a 15-member trained panel which had previously been familiarized with the flavor of fresh and stored potato flakes. The samples were reconstituted without added milk, butter, or salt by mixing 60g of flakes with 1 cup of boiling water and 1/3 cup of cold water. An additional 1/2 cup of hot water was added to obtain the desired texture. Samples were served in aluminum foil dishes and examined under green light. Panelists were asked to compare the flavor of coded samples, including a hidden standard, with a standard which had been stored under nitrogen at 0°F using a five-point rating scale ranging from five, "same as standard," to one, "extreme off-flavor." The significance of differences reported by the panel was determined using Duncan's multiple range test (LeClerg, 1957).

Headspace vapor analyses

Headspace vapor analyses for lower boiling volatile components were performed on all dehydrated potato products using a modification of the procedure of Sapers et al. (1970). A ground glass joint was substituted for the rubber stopper used with the original flask-stopper assembly. Vapor samples were analyzed with a Hewlett-Packard Model 7624A Gas Chromatograph using dual 12 ft, 1/8 in. stainless steel columns containing 15% Carbowax 20M on 80/100 acid washed Chromosorb W, a helium flow rate of 15 ml/min, and flame ionization detectors. The injection port and detector temperatures were 200°C and the column temperature was programmed as follows: 60°–70°C at 1°C/min, 70°–100°C at 2°C/min, 15 min isothermal at 100°C. Retention time and peak area measurements were made using a Hewlett-Packard Model 3370A Electronic Digital Integrator.

Preparation and analysis of potato volatile concentrates

Concentrates of higher boiling potato volatiles were prepared from all dehydrated potato

Table 1—Flavor changes in potato flakes during storage

Storage conditions			Product flavor[a]					
			RRVPPL[b] flakes				Commercial flakes	
			Without antioxidants		With antioxidants			
Time (Mo.)	Temp (°F)	Atm	Score	Description	Score	Description	Score	Description
3	73	Air	4.19	Like std	4.13	Like std	3.71[c]	Hay
	73	N_2	4.19	Like std	4.44	Like std	4.32	Like std
	0	N_2	4.44	Like std	4.25	Like std	4.79	Like std
6	73	Air	3.07[d]	Hay	4.71	Like std	3.59[d]	Hay
	73	N_2	4.53	Like std	4.71	Like std	4.53	Like std
	0	N_2	4.67	Like std	4.71	Like std	4.65	Like std
9	73	Air	–	–	3.78[c]	Hay	–	–
	73	N_2	–	–	4.50	Like std	–	–
	0	N_2	–	–	4.57	Like std	–	–

[a] All samples, including a hidden standard, compared with a standard stored under nitrogen at 0°F.
[b] Red River Valley Potato Processing Lab.
[c] Significant at .05
[d] Significant at .01

[1] Operated cooperatively by the USDA Eastern Marketing & Nutrition Research Div., ARS, the Minnesota Agricultural Experiment Station, the North Dakota Agricultural Experiment Station and the Red River Valley Potato Growers' Association.

products using a modification of the procedure described by Sapers et al. (1971). Ground 280-g samples of potato flakes were dispersed in 4 liters of distilled water, preheated to approximately 95°C, to form a slurry which was steam distilled at atmospheric pressure as previously described. Distillates, adjusted to pH 8 by the addition of saturated $NaHCO_3$ solution, were saturated with Na_2SO_4 and then extracted with freshly distilled diethyl ether. Ether extracts were dried over anhydrous Na_2SO_4 and concentrated to approximately 0.5g by refluxing under nitrogen, a portion of condensing solvent being continuously removed by the nitrogen stream. Potato volatile concentrates prepared by this procedure were stored at −18°C for no more than 3 days before being analyzed. 10 μl of an internal standard solution (1.76 μg ethyl butyrate/μl diethyl ether) were added to each concentrate immediately prior to its analysis.

A 2-μl aliquot of each potato volatile concentrate was analyzed using the chromatographic apparatus employed for headspace vapor analysis. The injection port and detector temperatures were 250°C, and the column temperature was programmed as follows: 4 min at 60°, 60°–80° at 1°/min, 80°–120° at 4°/min, 6 min at 120°, 120°–160° at 2°/min, 20 min at 160°C. The helium flow rate was 17 ml/min.

All samples were analyzed in duplicate. Component concentrations were expressed as mean peak area ratios, obtained by calculating the ratio of the component peak area to the area of the internal standard (ethylbutyrate) peak for the same analysis, and then averaging the ratios for replicate analyses.

The aroma of components separated by this procedure was determined by diverting half of the GC column effluent through a TC detector heated to 250°C and by smelling the gas stream at the detector exit port during the course of the analysis.

Mass spectral analyses

GC-MS analyses were carried out on headspace vapor and volatile concentrate samples using a Varian 1740 Gas Chromtograph interfaced with a jet separator to a du Pont 21-492 Mass Spectrometer. Spectra were obtained using a scan rate of 100 sec/decade, an ionization voltage of 70 ev, inlet temperatures of 150° and 200°C and source temperatures of 200° and 220°C for headspace vapor and volatile concentrates, respectively. Chromatographic columns and conditions were identical to those described previously.

All GS-MS identifications reported herein were based on analyses of experimentally produced dehydrated potato pieces rather than with potato flakes since the latter yielded insufficient volatile material. The potato pieces were processed from Maine Katahdin and North Dakota Manona tubers using the process described by Turkot et al., 1966. No antioxidants were used; both products contained approximately 1000 ppm SO_2. The dehydrated Katahdin pieces were held at 100°F under N_2 until judged by the trained taste panel to have a moderate scorched off-flavor level and were then used as a source of volatile browning products. The dehydrated Manona pieces, which were more easily oxidized and less subject to nonenzymatic browning than the Katahdin pieces, were stored in air at 73°F until judged by the panel to have a moderate oxidized off-flavor level and were then used as a source of volatile oxidation products.

Gas chromatographic comparisons of potato pieces and flakes stored under comparable conditions demonstrated that the headspace vapor and volatile concentrate chromatograms for both products were qualitatively similar. Volatile components which were increased in one product by storage in air or under nitrogen at elevated temperatures showed a similar response in the other product. The odor of such components (observed at the detector exit port) was found to be the same with both products. On the basis of these observations, the GC-MS data obtained with dehydrated potato pieces was applied to potato flakes.

RESULTS & DISCUSSION

Flavor changes during storage

The flavor scores of potato flake samples stored for 3, 6 and 9 months in air at 73°F and nitrogen at 73° and 0°F are summarized in Table 1. Flakes produced at our Red River Valley Potato Processing Lab. which contained BHA and BHT showed no flavor change during 6 months storage in air at 73°F; a slight off-flavor was noted by the panel after 9 months storage in air at 73°F.

Flakes which did not contain added antioxidants developed a moderate hay-like off-flavor during 6 months storage in air at 73°F. Samples of commercial flakes developed a slight to moderate hay-like flavor during 3 months in air at 73°F.

The hay-like off-flavor noted with air-packed flakes was similar to the flavor defect found in oxidized potato granules, characterized by Buttery (1961), and differed from the rancid off-flavor previously encountered in oxidized explosion puffed potatoes (Sapers et al., 1970).

No flavor changes were noted in nitro-

Table 2–Volatile products of sugar-amino acid reactions in the headspace vapor of potato flakes stored in nitrogen at 73° and 0°F

		Mean peak area ratio					
		2-Methylpropanal and Acetone[b] (Peak 9)			2- and 3-Methylbutanal[b] (Peak 14)		
Product	Storage Temp (°F)	0 Mo	3 Mo	6 Mo	0 Mo	3 Mo	6 Mo
Commercial	73	0.166	0.185	0.205	0.098	0.119	0.128
	0	–	.158	.151	–	.096	.070
RRVPPL[a] (without antioxidants)	73	.281	.362	.282	.203	.200	.209
	0	–	.265	.376	–	.196	.230
RRVPPL[a] (with antioxidants)	73	.364	.587	.385	.233	.500	.263
	0	–	.391	.646	–	.304	.464

[a]Red River Valley Potato Processing Lab.
[b]Identified by mass spectrometry and retention time

Table 3–Volatile products of sugar-amino acid reactions in volatile concentrates prepared from potato flakes stored in nitrogen at 73° and 0°F

		Mean peak area ratio								
		Furfural[b] (Peak 40)			Benzaldehyde[b] (Peak 45)			Phenylacetaldehyde[b] (Peak 51)		
Product	Storage Temp (°F)	0 Mo	3 Mo	6 Mo	0 Mo	3 Mo	6 Mo	0 Mo	3 Mo	6 Mo
Commercial	73	0.58	0.76	0.78	0.51	1.22	1.40	1.76	4.20	5.03
	0	–	.65	.61	–	.81	.97	–	3.35	4.11
RRVPPL[a] (without antioxidants)	73	.92	1.28	1.22	.72	1.11	1.17	5.67	7.14	7.98
	0	–	1.44	1.05	–	.79	.95	–	7.82	7.51
RRVPPL[a] (with antioxidants)	73	–	2.16	1.82	.91	1.08	1.57	5.90	12.8	11.1
	0	–	1.92	1.79	–	.79	1.11	–	12.4	13.4

[a]Red River Valley Potato Processing Lab.
[b]Identified by mass spectroscopy and retention time

gen-packed potato flakes stored at 73° and 0°F.

Effect of storage temperature on potato flake volatiles

Headspace vapor analyses performed on nitrogen packed RRVPPL and commercial potato flakes (Table 2) demonstrated that 6 months storage at 73°F resulted only in small increases in low boiling Strecker degradation aldehydes previously associated with "browning" off-flavors in explosion puffed dehydrated potatoes (Sapers et al., 1970) and potato granules (Buttery and Teranishi, 1963).

Analyses of volatile concentrates prepared from nitrogen packed flakes (Table 3) revealed small differences between samples stored at 73° and 0°F in levels of higher boiling compounds previously associated with off-flavors in puffed potatoes (Sapers et al., 1971). Alkylpyrazines were not evident on chromatograms and could not be detected by aroma in GLC effluents during the analysis of flake volatile concentrates. The absence of this class of compounds in flakes is probably a major factor accounting for the lack of off-flavors after storage in nitrogen.

Furfural levels showed little or no change during storage but were substantially higher in RRVPPL flakes, especially those containing antioxidants, than in the commercial flakes. This may be due to differences in the extent of heat damage during processing which would result in different levels of volatile browning products. Phenylacetaldehyde, a major component of potato flake volatile concentrates, showed a similar trend as did the lower boiling Strecker degradation aldehydes determined in the headspace vapor. Increases in levels of phenylacetaldehyde, which occurred principally during the first 3 months of storage at both 73° and 0°F, may have been due to the further reaction of browning intermediates formed during processing. Benzaldehyde levels increased in all nitrogen-packed products stored at 73°F and to a lesser extent at 0°F. Greater increases in this compound occurred in air-packed products during storage at 73°F (see Table 5). Differences between the air- and nitrogen-packed samples in levels of furfural and the Strecker degradation aldehydes were small and variable.

The absence of off-flavors in nitrogen-packed potato flakes stored at 73° and the minimal formation of "browning" volatiles in these samples during storage indicate that the shelf life of potato flakes is not normally limited by flavor defects resulting from reducing sugar-amino acid reactions. This conclusion does not apply, of course, to products which are abused during processing or storage, i.e., by exposure to excessively high temperatures. Such products might be unacceptable when fresh or after brief storage due to the presence of high levels of volatiles derived from the browning reaction. One must also consider the possibility that these volatiles, while not apparently objectionable at moderate levels, might modify the flavor contribution of compounds derived from lipid oxidation and other reactions in stored potato flakes.

Effect of storage in air on potato flake volatiles

Headspace vapor analyses of air-packed potato flake samples which developed a hay-like off-flavor during storage at 73°F (Table 4 and Fig. 1) revealed increases in n-hexanal and other compounds previously associated with lipid oxidation in various foods, for example, potato granules (Buttery, 1961) and soybean milk (Wilkens and Lin, 1970). Corresponding nitrogen-packed samples which lacked the off-flavor showed much smaller increases in these components, probably indicating the breakdown of hydroperoxides formed prior to storage and/or autoxidation involving residual headspace

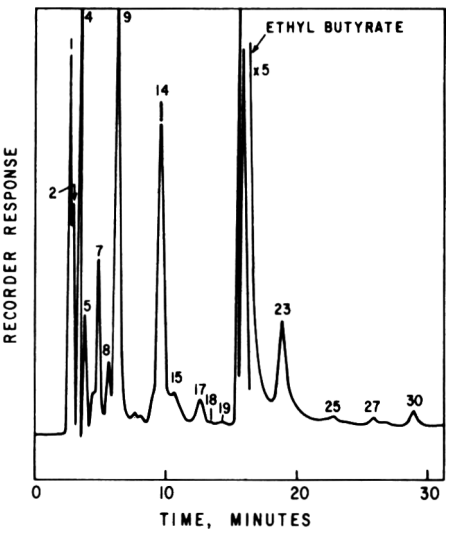

Fig. 1—Chromatogram of headspace vapor of commercial potato flakes stored 6 months in air at 73°F.

oxygen. The presence of antioxidants in RRVPPL flakes greatly reduced the extent of these changes during 6 months storage in air at 73°F, as one might predict from the flavor stability of this product; analytical results for 6 and 9 months were similar although flavor changes were noted after 9 months storage.

Analyses of volatile concentrates prepared form air-packed RRVPPL (no added antioxidants) and commercial flake samples after storage at 73°F (Table 5 and Fig. 2) showed substantial increases in a number of components indicative of lipid oxidation. These included n-hexanal, 2-pentenal (tentative), n-heptanal and 2-hexenal reported by Buttery (1961) in oxidized potato granules, and 2-heptanone, 2-pentylfuran and benzaldehyde reported by Buttery et al. (1970) in fresh potato. Unknown components 24 and 47

Table 4—Effect of storage in air at 73°F on the headspace vapor composition of RRVPPL[a] and commercial potato flakes

Peak No.	Identity	Method of identification[b]	Mean peak area ratio: RRVPPL[a] flakes, Without antioxidants, 0 Mo	3 Mo	6 Mo	RRVPPL[a] flakes, With antioxidants, 0 Mo	3 Mo	6 Mo	Commercial flakes, 0 Mo	3 Mo	6 Mo
1	Unknown	–	0.018	0.043	0.087	0.022	0.046	0.051	0.045	0.086	0.080
2	Pentane	MS, RT	.007	.048	.073	.015	.031	.027	.029	.073	.044
8	n-Propanal	MS, RT	.002	.008	.014	.002	.002	.002	.009	.018	.015
17	n-Pentanal	MS, RT	T[d]	.002	.013	T	T	.004	T	.008	.010
23	n-Hexanal	MS, RT	.012	.045	.129	.010	.016	.034	.025	.048	.084
30	2-Hexenal[c]	RT	.001	T	.005	.001	T	.001	.009	.006	.009

[a] Red River Valley Potato Processing Lab.
[b] MS = mass spectrometry; RT = retention time
[c] Tentative
[d] T = Trace

Table 5—Effect of storage in air at 73° F on the composition of volatile concentrates prepared from RRVPPL[a] and commercial potato flakes

Peak No.	Identity	Method of Identification[b]	Mean peak area ratio								
			RRVPPL[a] flakes						Commercial flakes		
			Without antioxidants			With antioxidants					
			0 Mo	3 Mo	6 Mo	0 Mo	3 Mo	6 Mo	0 Mo	3 Mo	6 Mo
16	n-Hexanal	MS, RT	0.46	1.19	4.86	0.31	0.39	0.45	0.61	1.72	2.64
19	2-Pentenal[c]	MS	.06	.10	.30	.04	.05	.04	.04	.28	.38
21	n-Heptanal	MS, RT	.11	.17	.23	.12	.12	.12	.18	.35	.40
	2-Heptanone	MS, RT									
23	2-Pentylfuran	MS, RT	.01	.08	.05	T	.01	.06	.05	.21	.20
	2-Hexenal	MS, RT									
24	Unknown	–	T[d]	T	.40	T	T	.06	.02	.05	.33
45	Benzaldehyde	MS, RT	.72	1.24	2.67	.91	1.56	2.79	.51	2.33	3.65
47	Unknown	–	T	.07	.41	T	.03	.05	.20	.40	.71

[a]Red River Valley Potato Processing Lab.
[b]MS = mass spectrometry; RT = retention time
[c]Tentative
[d]T = Trace

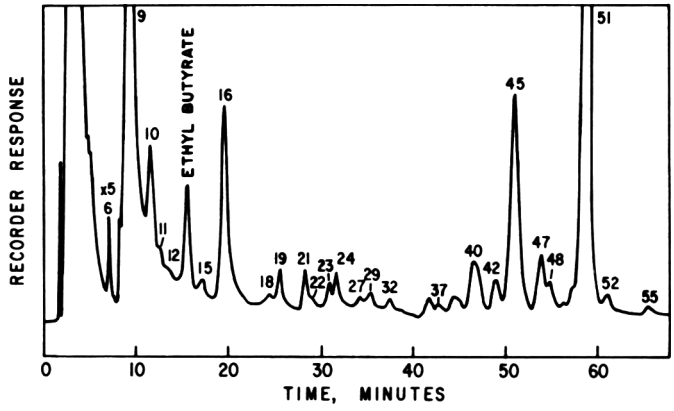

Fig. 2—Chromatogram of potato volatile concentrate prepared from commercial potato flakes stored 6 months in air at 73° F.

also increased during storage in air. Mass spectral data obtained with the latter peak [m/e 43 (100), 39 (80), 41 (60), 95 (34), 53 (28), 81 (26), 51 (20), 79 (16), 77 (14), 55 (14), 109 (4), 124 (2)] indicate that it may be a furan. Much smaller increases in the previously mentioned components (except benzaldehyde) were seen in air-packed RRVPPL flakes containing added antioxidants, even after 9 months storage at 73°F; analyses at this time were similar to those obtained at 6 months. Little or no change was seen in volatile concentrates prepared from all nitrogen-packed flake samples after storage.

GLC effluents were examined during the analysis of RRVPPL flakes (no added antioxidants) which had been stored for 6 months in air and nitrogen at 73°F to determine the odors of volatile concentrate components. While no single component had an odor identical to the hay-like off-flavor, the odors of a number of components were suggestive of oxidized off-flavors and/or were more intense in the air-packed sample than in the nitrogen-packed sample. These included aldehydic odors from peaks 10 (n-pentanal), 16 (n-hexanal), 19 (2-pentenal), 21 (n-heptanal and 2-heptanone), 23 (2-pentylfuran and 2-hexenal) and unknown component 37; a sulfury odor from peak 24; a benzaldehyde odor from peak 45 (benzaldehyde); and a green-bean odor from peak 47. The hay-like off-flavor probably represents a blend of these and perhaps other flavor notes contributed by volatile components of oxidized potato flakes.

Additional examinations of GLC effluents from analyses carried out at a column temperature of 180°C instead of the standard program revealed the presence of a violet-like odor suggestive of β-ionone in both air- and nitrogen-packed samples at a time coincident with the retention time of this compound. However, quantitative comparisons were precluded by the absence of a measurable peak at the retention time of β-ionone with these and all other oxidized flake samples. This is not surprising in view of the extremely low odor threshold of β-ionone (0.007 ppb) reported by Buttery et al. (1971). The presence of this compound would be indicative of carotenoid oxidation (Ayers et al., 1964).

The conspicuous increase in benzaldehyde in air-packed flake samples during storage is noteworthy. This compound has been identified in many food products besides potatoes and may arise from a number of sources including sugar caramelization (Hodge, 1967), phenylalanine-sugar reactions (Ramshaw and Dunstone, 1969), the oxidation or thermal degradation of compounds derived from lignin (Kazeniac and Hall, 1970) and the thermal oxidation of lipids (Kawada et al., 1967). Antioxidants apparently do not inhibit its formation.

It can be concluded from these data that the hay-like off-flavor in aged potato flakes results from oxidation and may be due at least in part to the flavor contributions of the products of lipid oxidation and other components described herein. The shelf life of this product is limited by these oxidative changes, but may vary widely, even in the presence of antioxidants. Research on the cause of such variation is in progress and will be the subject of future publications.

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EFFECTS OF ENVIRONMENTAL FACTORS ON THE OXIDATION OF POTATO CHIPS

INTRODUCTION

POTATO CHIPS are a deep-fat-fried snack food containing a high percentage of oil; in common with other fat-containing dry foods, they frequently deteriorate due to oxidative rancidity. Loss of acceptability often occurs because of production of objectionable off-flavor compounds resulting from reactions involving oxygen absorption. Knowledge of the factors which affect the rate of oxygen uptake of food products is important for process and product development, packaging and storage.

The effect of oxygen and moisture content on the quality of dry foods has been investigated by many authors, including Tamsma et al. (1961, 1967) and Tamsma and Pallansch (1964) who made storage stability studies of whole milk powder under different oxygen concentrations. Their results are particularly significant since they show that for certain products even oxygen concentrations lower than 1% can have a marked effect on the quality of the product.

Tuomy et al. (1969) and Tuomy and Hinnergardt (1968a, b) made an extensive study of the effect of headspace oxygen concentration on the oxygen uptake and organoleptic properties of freeze-dried combination foods. They obtained good correlations between the amount of oxygen absorbed and the panel rating for flavor. Approximate analysis of their data indicates that the rate of oxygen uptake is a strong function of oxygen concentration in the range of 0–5% for these combination foods.

More recently, the effect of water activity or equilibrium relative humidity on the rate of oxygen uptake of dry foods and model systems was studied by Labuza et al. (1969, 1971) and Heidelbaugh et al. (1971). The results indicate that usually the rate of oxidation is high at very low water activity and decreases with water activity until it reaches a minimum. At higher water activities (usually larger than 0.5, corresponding to the so-called intermediate moisture foods) the rate of oxidation increased with water activity.

McWatters et al. (1971) determined the peroxide value of roasted peanut kernels as a function of time at different temperatures. In most cases the peroxide value increased during the initial six months and then decreased to a level lower than the starting value. Samples packed under vacuum showed significantly smaller peroxide values than those packaged at atmospheric oxygen concentration. Unfortunately, the water activity of the samples also increased during the experiments. The authors also concluded that high moisture content (slightly above monolayer value) resulted in high levels of peroxides.

Cavaletto et al. (1966), Dela Cruz et al. (1966), and Cavaletto and Yamamoto (1968, 1971) studied the factors affecting macadamia nut stability. They found that the stability of the product decreased with increasing moisture content. The lowest moisture content (1.4%) resulted in the most stable product. Light had no effect on the stability of the product. Similar results were observed for raw and roasted kernels. The authors found that the stability of the nuts could be increased by adding antioxidants to the frying oil and by vacuum packaging.

Another factor which can affect significantly the rate of oxygen uptake of food products is light. Smits et al. (1970) and Radtke et al. (1970) made a comprehensive theoretical and experimental study of this factor for edible oils.

Fuller et al. (1971) studied the effect of frying oils and light on the storage stability of potato chips. They found that fluorescent light accelerated the quality deterioration significantly. Addition of antioxidant (propyl gallate) to the frying oil did not increase the storage stability of the chips.

Table 1–Effect of equilibrium relative humidity (RH) on the rate of oxygen uptake of potato chips (from run 1)[a]

RH (%)	Rate $\left(\frac{\mu l\ O_2\ STP}{g \cdot hr}\right)$
0.1	0.15
11.0	0.13
32.0	0.07
40.0	0.06
62.0	0.19
75.0	1.60

[a]Initial headspace concentration, 12.2% O_2; concentration at which rate was determined 10% O_2; temperature, 37°C; extent of oxidation, 57 $\mu l\ O_2$ STP/g

Berger (1971) found that accelerated tests were useful for predicting storage life of several products during the initial slow period of oxidation.

We have undertaken a study with the aim of predicting the storage behavior of oxidation-susceptible dry foods on the basis of laboratory evaluation of kinetics of deteriorative reactions and of data for oxygen and water transfer into containers. An initial phase of the investigation dealt with a survey of various types of foods with respect to their oxidation behavior (Quast and Karel, 1971). Potato chips were then selected for further study

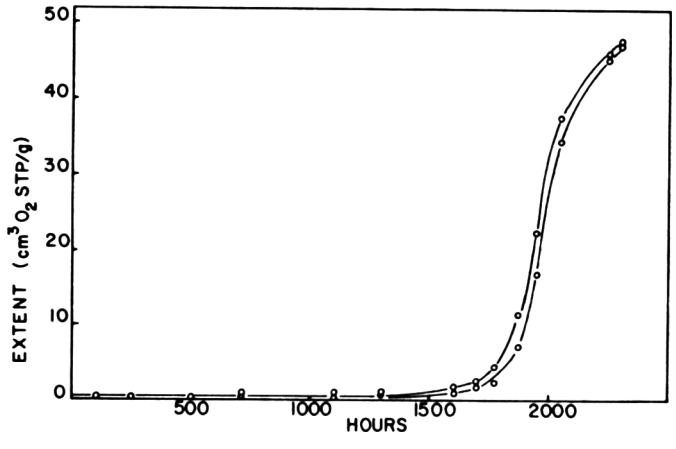

Fig. 1–Oxidation of potato chips at 37°C up to a very high extent.

as representative of a moisture- and oxygen-sensitive group of products.

This paper presents the first phase of our investigation and is concerned with the influence of environmental factors on rates of oxidation of the products. Subsequent papers will deal with the development of mathematical models for storage behavior and with evaluation of these models.

EXPERIMENTAL

Samples

Potato chips were obtained from the John E. Cain Co., Cambridge, Mass., on the same day they were produced. Two experimental runs were made in which the samples had the following characteristics:

Run 1:

Oil used for frying:	Sunflower oil
Peroxide value:	0.55 meq/kg chips
Fat content:	34%
Density:	1.21g/cm³
Moisture content:	0.90g/100g solids

Run 2:

Oil used for frying:	Cottonseed oil
Peroxide value:	0.40 meq/kg chips
Fat content:	39%
Density:	1.165g/cm³
Moisture content:	1.30g/100g solids

The peroxide values were determined according to the "Official Methods" of the American Oil Chemists' Society after fat extraction (Karel and Labuza, 1969). The results were always expressed as meq/kg of dry product. Each equivalent of peroxide, as calculated from the titration, corresponds to half a mole of oxygen taken up by the product if all of the oxygen is incorporated into hydroperoxides. Consequently, a peroxide value of one corresponds stoichiometrically to an oxygen uptake of 11.2 μl O_2 STP/g (or 0.5 μmole O_2/g).

Oxygen uptake by measuring headspace concentration

In these experiments, 20.0-g samples of crushed potato chips were transferred to wide-mouth glass bottles of a total volume of 72 cm³. These flasks were provided with rubber stoppers such as are used in packaging of pharmaceuticals. The equilibrium relative humidity (RH) of the samples and the headspace oxygen concentration could be adjusted to a desired level. The change of headspace oxygen concentration was determined with the aid of an oxygen probe with a tip 10 mm long which could be inserted through the 6 mm thick rubber stopper (Quast and Karel, 1972). The net headspace volume was calculated by subtracting the volume occupied by the sample. From the headspace oxygen concentration, measured at regular intervals, the extent of oxidation as well as the rate of oxidation at any time could be calculated.

Oxygen uptake by Warburg technique

This method has been fully described by Umbreit et al. (1964). The flasks used had volumes from 15–60 ml, and samples of 2–5g were used. Two types of fluids were employed for the manometers. For long-term experiments at constant oxygen concentration and water activity, mercury was used. The use of mercury results in low sensitivity, and readings were usually taken once every 5–10 days. The advantage of mercury is that the levels never exceed the physical limits of the manometer even during very large changes in atmospheric pressure.

For the accurate determination of rates over small time intervals, Apiezon oil (Associated Electrical Industries, Ltd., distributed by Shell Oil Corp.) was used in the manometers.

A special technique was developed to allow changing the oxygen concentration (PO2) inside the flasks: Triplicate manometer flasks containing samples were transferred to desiccators containing saturated solutions of given equilibrium RH. Adequate equilibration was obtained after 2 days at 37°C under high vacuum. Then the flasks were connected to their manometers and the rate of oxygen uptake at atmospheric oxygen concentration and at certain RH was determined over a period of 1–2 days.

Next the oxygen concentration inside the flasks was adjusted to some other value by passing a stream (20–50 cm³/min) of an oxygen-nitrogen mixture of known composition through the system. First, this gas was bubbled through a wash bottle which contained a saturated solution of the same RH as a given sample and which was at the same temperature. This prevented addition or removal of moisture from the samples. Next, the gas stream was connected to the opened sidearm of the manometer flask. The valve on top of the manometer was opened to allow the gas to flow through. In this manner the gas previously present in the system was flushed out and after some time the oxygen concentration inside the flasks was equal to that of the standard gas mixture. To establish when the system had been sufficiently flushed, the oxygen concentration of the gas leaving the Warburg manometer was monitored continuously with an oxygen probe. Then the manometer valve and the sidearm valve were closed and, after thermal and diffusional equilibrium were established, the rate of oxygen uptake was determined at the new oxygen concentration over a short period.

After this the oxygen concentration was changed to some other value in a similar manner. After determining the rates at all the desired oxygen concentrations, the procedure was repeated through new cycles of oxygen concentrations until the critical extent of oxidation was reached. In this manner the water activity of a given sample stayed constant during the whole process while the oxygen concentrations were changed frequently and the extent of oxidation increased.

Since the amount of calculations is formidable, only the manometric readings were recorded, and the rates together with their standard deviations were calculated by a computer program using least-squares data-fitting.

RESULTS & DISCUSSION

Preliminary experiments

In order to establish the significant range of oxygen uptake in potato chips, a preliminary experiment was made over a long time and with extensive oxidation. After approximately 1200 μl O_2 STP/g were absorbed, the rate increased very rapidly to a level roughly 100 times the rate observed in the previous period (see Fig. 1). The rapid increase of the rate coincides with a very marked development of rancid odor.

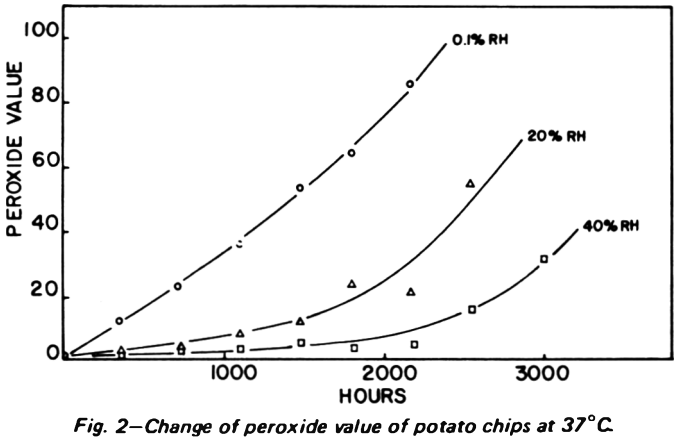

Fig. 2—Change of peroxide value of potato chips at 37°C.

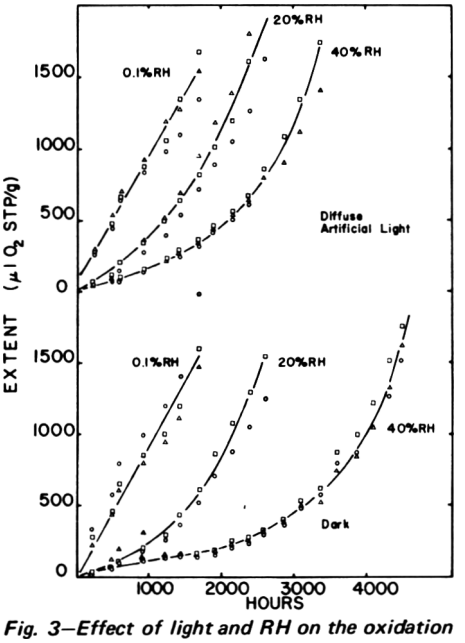

Fig. 3—Effect of light and RH on the oxidation of potato chips at 37°C.

Even though rancid odor appears before the critical extent of 1200 μl O_2 STP/g is reached, it was assumed that this is the maximum allowable extent. At this point the product is of very low quality from the point of view of flavor. In later investigations it was found that the period of rapid oxidation was reached at an extent of 1200 to 1500 μl O_2 STP/g, independent of water activity, light, temperature and oil in which the chips were fried.

The effect of equilibrium RH on the rate of oxidation is shown in Table 1. It can be seen that the rate is lowest around 40% RH. At high RH the rate increases considerably, possibly due to increase in mobility of the reactants. The product kept at 75% RH also showed strong nonenzymatic browning.

Furthermore, the product with a water activity of 0.40 (RH=40%) was found to be unacceptable because of loss of crispness. Kaghan (1968) indicated that potato chips are considered commercially unacceptable at a moisture content of 3% (RH=32%). Consequently subsequent work concentrated on RH below 40%.

The pattern of oxidation of the potato chips over time followed the usual autoxidation kinetics (Fig. 2 and 3), in which most of the oxygen absorbed in early stages of oxidation is present in the hydroperoxides (Labuza et al., 1969). If all of the absorbed oxygen is present in hydroperoxides, the EXT/PV ratio is equal to 11.2. Larger values indicate significant oxygen content in other oxidation products. In experiments in which peroxide value as well as oxygen absorption were studied under identical conditions, the ratios of oxygen absorbed to peroxide value were close to the theoretical value (Table 2). The subsequent discussion of individual environmental factors is based primarily on oxygen uptake, which will hereafter be referred to as "extent" (μl O_2 STP/g). The rate of oxygen uptake will be given in units of μl O_2 STP/g·hr.

Table 2–Effect of light on oxygen uptake of potato chips at 23°C, 0.1% RH (from run 2)

Time (days)	Oxygen conc (% O_2) inside flasks	PV[a]	EXT[b]	EXT/PV
	Dark: flasks wrapped in aluminum foil			
0	21	0.4	∿0	–
15	19.0	–	–	–
30	17.4	–	–	–
45	17.0	–	–	–
60	–	20.6	–	–
75	12.6	–	–	–
92	10.5	23.1	270	11.7
116	7.5	30.8	350	11.4
	Room light: no sunlight, strong artificial light			
0	21	0.4	∿0	–
15	18.5	–	–	–
30	15.4	–	–	–
45	12.0	–	–	–
60	–	25.1	–	–
75	7.5	–	417	25.4
92	4.8	16.4	497	13.2
116	1.7	37.5		
	Sunlight: flasks near window, receiving some sunlight during the day			
0	21	0.4	∿0	–
15	16	–	–	–
30	8	–	–	–
45	0.7	–	–	–
60	0.0	38.9	540	13.9
75	–	–	–	–
92	0.0	38.8	540	13.9
116	–	38.6	540	14.0

[a]PV = peroxide value
[b]EXT = extent

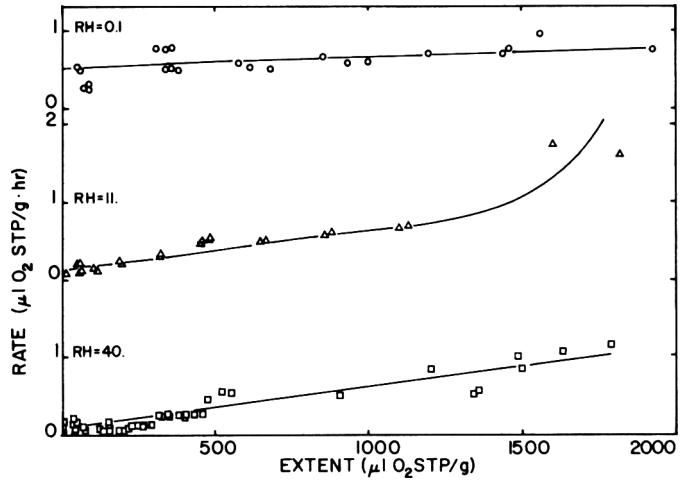

Fig. 4–Rate of oxidation of potato chips at 37°C as a function of extent at PO2=0.0055 atm.

Effect of light

The effect of light on the rate of oxygen uptake is shown in Figure 3 and Table 2. From the figure one can see that even diffuse artificial room light can have a significant effect on the rate of oxygen uptake. This effect becomes more important at higher water activity.

Table 2 shows results obtained by flask experiments at 23°C where the peroxide values were also determined. Similar results were also obtained at 37°C. In addition, the very strong accelerating effect of occasional sunlight is shown. At higher water activities an even more significant effect is to be expected, according to Figure 3. In practice, it is difficult to assess accurately the effect of light on packaged foods since it depends on the area exposed to the light.

Effect of temperature

The rate of oxygen uptake was determined at three temperatures at an RH of 0.1%. From an Arrhenius plot of the rates, we found the activation energy for oxygen uptake of potato chips to be 10 kcal/g-mole at low RH. This is a relatively low value as compared with 19 kcal/g-mole calculated from the results of Berger (1971). Thus, the effect of temperature on the rate of oxidation of potato chips is not very strong. Consequently, relatively high temperatures have to be used in accelerated stability tests if significant acceleration is desired. It is possible,

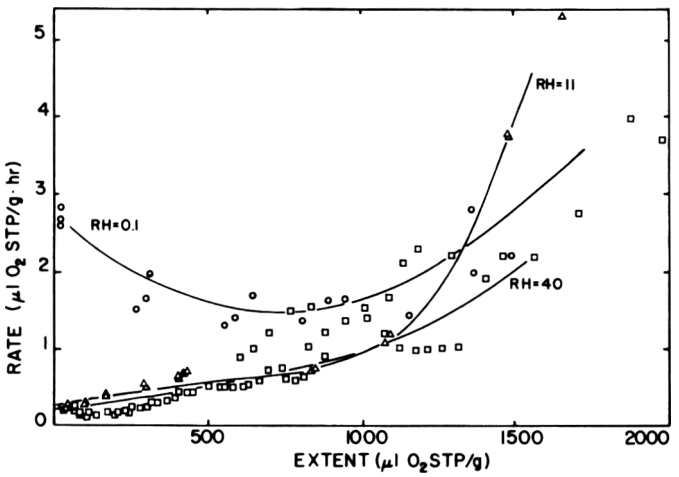

Fig. 5—Rate of oxidation of potato chips at 37°C as a function of extent at PO2=0.21 atm.

however, that at very high temperatures the mechanism of the reaction changes, thus limiting the application of accelerated tests.

Effect of extent of oxidation

The rates of oxygen uptake were determined in triplicate over small time intervals at the following equilibrium relative humidities: 0.1, 11, 20, 32 and 40%. The oxygen concentrations were cycled as described previously over the following concentrations (PO2): 0.0055, 0.035, 0.072, 0.122 and 0.21 atm of partial pressure. The rate determinations were continued with increasing extent until a maximum extent of about 2000 μl O_2 STP/g was reached. In this manner, rate determinations were made in triplicate at five different values of RH, six values of PO2 (oxygen partial pressure, including PO2=0.0), and between five and 15 different values of extent, depending on the relative humidity. About 1000 experimental rates at the combinations of the three independent variables were obtained.

A limited number of the experimental results at some of the values of RH and PO2 is shown in Figures 4 and 5. It can be seen in all cases that the rate of oxygen uptake is approximately a linear function of the extent but the slope of the straight line portion of plots is a function of the other variables. At PO2= 0.21 the straight line approximation is good only up to an extent of 1200–1500, while at low oxygen concentration this approximation is valid for slightly higher extents. The initial decrease of the rate at low water activity could be due to a very small increase of the water activity during the experiment.

Effect of equilibrium relative humidity (RH)

Due to the experimental procedure in which the rates were obtained, it was not possible to determine them at regular and pre-established intervals. Therefore, the rates at five extents (0, 300, 600, 900 and 1200) at each of the values of the other independent variables (RH and PO2) were taken from the plots, such as shown in Figures 4 and 5. This technique allows crossplotting the experimental results. Figure 6 shows how the rate of oxygen uptake depends on the RH at selected values of the other independent variables over the whole range of interest.

It can be seen that in early stages of oxidation and at low humidities the rate is a very strong function of RH. As the reaction progresses to the critical extent, however, the rate becomes almost independent of the equilibrium RH. Also, at high oxygen concentration the rate is a stronger function of RH.

Effect of oxygen concentration

The experimental results can also be crossplotted as a function of PO2 at selected values of the other two independent variables, as shown in Figure 7. It can be seen that in all cases the rate is a very strong function of oxygen concentration when the concentration is low. The shape of the curves is similar for all values of the other independent variables except for RH=0.1, where the curve is slightly different. This could indicate a different mechanism of reaction at this low water activity. It must also be mentioned, however, that the error in the determinations at this water activity is likely to be larger due to the high sensitivity of the system to very small changes in moisture content.

All the environmental factors studied had a strong effect on the rate of oxygen absorption of potato chips. Similar effects are to be expected for a large number of snack foods. Under typical commercial storage conditions the light intensity and temperature can be quite variable. Completely opaque packaging materials should be used to eliminate the effect of light on oxidation rates.

In a typical flexible package containing a food product, the equilibrium RH and the headspace oxygen concentration change during storage. This change is a

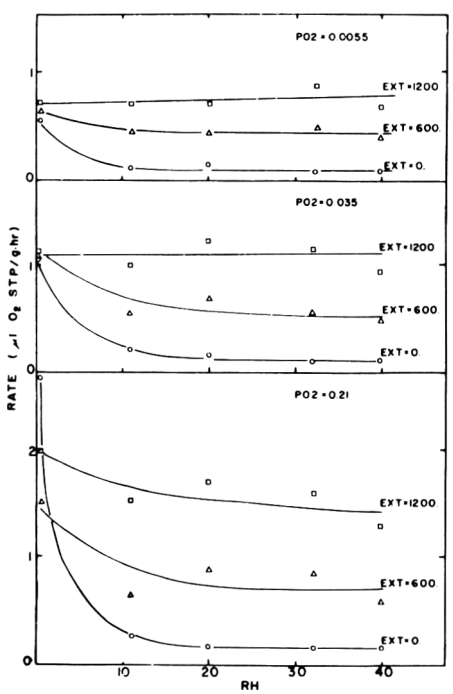

Fig. 6—Rate of oxidation of potato chips at 37°C as a function of equilibrium relative humidity.

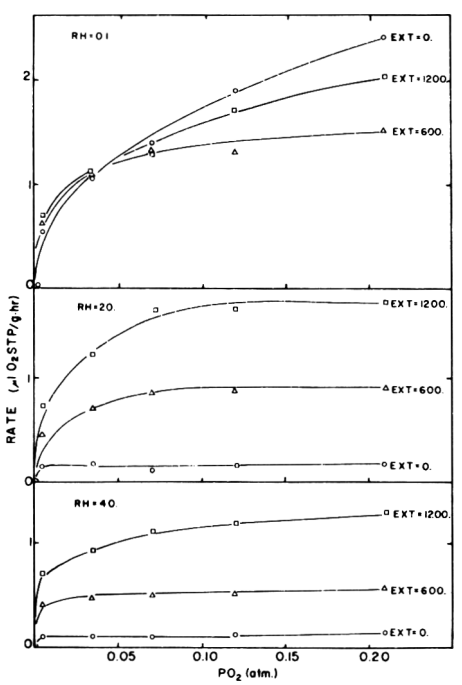

Fig. 7—Rate of oxidation of potato chips at 37°C as a function of oxygen partial pressure.

function of package parameters and of the rate at which oxygen is absorbed by the product. In future work on this subject we will explore the possibilities of using the experimental results for the development of mathematical models for storage life prediction.

CONCLUSIONS

CONSIDERING the spoilage of potato chips due to oxidative rancidity, there are several approaches for improving the storage stability of this product. Since the bulk density of this product is typically $0.056 g/cm^3$, it has a very large headspace volume per unit weight of product. If the product is packaged at atmospheric oxygen concentration, then the headspace oxygen is enough to cause oxygen uptake in excess of 3000 μl O_2 STP/g. Consequently, inert gas packaging would result in a very significant increase of the storage life of potato chips, provided the headspace oxygen concentrations attained were below 1% and the package permeability to oxygen was chosen accordingly. The package should also be designed to avoid light penetration.

From the results shown, it can also be concluded that an excessively dry product is not desirable for good storage stability. The activation energy for oxygen uptake of potato chips is not very high compared with that of other mechanisms of food quality deterioration.

The peroxide value appears to be a fairly reliable index of the extent in early stages of oxidative deterioration of the potato chips used in this study.

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THE CAROTENOIDS OF THE AVOCADO PEAR
Persea americana, Nabal Variety

INTRODUCTION

THE AVOCADO (*Persea americana*) is a fruit with an unusually high oil content (between 10–30%) and a relatively high concentration of chlorophyll under the peel.

In the last decade the commercial importance of the avocado has greatly increased. The fruit is no longer rare and is consumed on a large scale both in America and Europe. In Israel many varieties are successfully cultivated both for export and the local market.

Data in the literature about the carotenoid content of the fruit are dated and contradictory. The so-called high content of vitamin A reported in tables concerned with the nutritive value of the fruit expressed in IU (Weatherby et al., 1929; Platt, 1962; McCance and Widdowson, 1960), cannot be interpreted unequivocally, i.e., it is difficult to determine whether the data are concerned with free vitamin A, provitamins such as α- or β-carotene, or with total carotenoids. A detailed investigation of the carotenoids present in the avocado fruit has not been carried out.

In the earliest investigation, Lassen et al. (1944) reported that a crude carotene fraction extracted from avocado oil was composed of α- and β-carotene plus another unidentified carotene. Three unknown pigments were isolated and the transmission curves determined. The crude carotene fraction amounted to 2.36 μg/g of oil while β-carotene amounted to 0.54 μg/g. They suggest this carotene content is sufficient to account for all vitamin A activity. Schwob (1951) reported a carotene content of 60–70 μg percent of fresh matter. Franzke and Henning (1956) suggested that avocado oil is similar to olive oil and the fruit contains β-carotene. In 1963, François and Gauthier investigated the vitamin A content of a commercial avocado oil. They suggest that the ripe fruit contains vitamin A but that it rapidly disappears from the freshly extracted oil, while β-carotene is stable. A content of 11.4 IU vitamin A/g is reported–calculated from the total carotenoid content.

It therefore seemed of importance to reinvestigate the carotenoid content of avocado fruit using the newer chromatographic methods.

Table 1–Characterization of carotenoids from avocado fruit Persea americana, Nabal variety

Identification	Abs max[a] (nm)	Epoxide test (HCl treatment) Hypsochromic Color	Hypsochromic shift (nm)	Carbonyl test reduced pigment Abs max (nm)
Carotenes				
α-Carotene	420,443,472	–	–	–
β-Carotene	424,449,474	–	–	–
ξ-Carotene	380,400,424	–	–	–
γ-Carotene	436,460,486	–	–	–
Monols				
OH-α-carotene	418,443,472	–	–	–
Cryptoxanthin	424,450,475	–	–	–
Unknown 388	372,388,410	blue	bathochrom	–
Unknown 420	390,420,445	–	–	–
Unknown 390	370,390,415	–	–	–
α-Citraurin	442	–	–	388,413,438
Diols and polyols				
Lutein	420,443,472	–	–	–
cis-Lutein	330,418,440,465	–	–	–
Isolutein	412,437,466	green	18	–
cis-Isolutein	326,410,435,464	green	17	–
Violaxanthin	413,438,468	green-blue	40	–
Carbonyl 446	446	–	–	395,415,436
Chrysanthemaxanthin a	395,419,447	blue	–	–
Chrysanthemaxanthin b	395,420,447	blue	–	–
Chrysanthemaxanthin c	393,416,443	blue	–	–
Luteoxanthin	392,420,448	blue	20	–
Unknown 370	350,370,393	blue-violet	–	–
Trollichrome	398,420,448	blue	–	–
Neoxanthin-like	415,438,467	blue	18	–
Unknown 437 (Trollein?)	418,437,467	–	–	–
Neoxanthin	413,436,467	green	16	–
Unknown 437	414,438,468	–	–	–

[a]Carotenes and monols determined in petroleum ether; diols and polyols in ethanol.

EXPERIMENTAL

Extraction

The mesocarp of fully ripe avocado (batches about 2 kg each), was homogenized with acetone in an Ultra Turrax homogenizer (Typ T.P. 18/2N) and extracted in a Büchner filter until colorless. The pigments were transferred to ether by addition of aqueous NaCl solution. The ether was evaporated in a rotatory evaporator.

Saponification

The oil was weighed and the saponification carried out as for vitamin A determination in margarine according to Strohecker and Henning (1963).

Removal of sterols and acetylenic and olefinic alcohols

The nonsaponifiable matter was dissolved in a minimum volume of hot methanol and kept overnight at –4°C. After 24 hr the bulk of the sterols precipitated and were removed by filtration. For the removal of acetylenic and olefinic alcohols (Kashman et al., 1970), the residue was dissolved in enough petroleum ether to allow them to come out of solution. The solution was kept at 4°C. In this way loss of carotenoids was avoided. Sometimes this operation was repeated.

Chromatography

Identification of pigments and chemical tests, as well as the quantitative determinations were carried out as described by Gross et al. (1971). The chromatographic analysis was difficult because of contamination of the samples with various lipidic substances. The chromatographic separation of vitamin A from carote-

noids and its determination was carried out as described by Gross and Budowski (1966). Standard carotenoids were isolated from citrus fruits as well as from spinach and carrots.

RESULTS & DISCUSSION

TABLE 1 represents the qualitative analyses of the carotenoids of the avocado fruit *Persea americana*, Nabal variety presented in order of increasing adsorption affinity.

The colorless hydrocarbons phytoene and phytofluene were absent. In addition to the usual α- and β-carotenes it was possible to identify ζ-carotene, albeit in very small concentration. Generally this pigment accompanies phytoene and phytofluene, being the third intermediate in the carotenes biogenesis that occurs by stepwise dehydrogenation. γ-Carotene, the last carotene of the group could be detected only in fully ripe fruits. No epoxidic carotene derivatives were detected.

In the monol fraction, in addition to OH-α-carotene and cryptoxanthin some minor pigments with more unusual properties could be separated. Close to cryptoxanthin was a yellow lemon pigment with absorption maxima in the shorter region of the spectrum (372,388,410 nm). Upon acid treatment a bathochromic shift to γ max: 415 nm was observed, indicative of an allylic hydroxy-group. The blue color obtained changed to red after several minutes. The next pigment is unknown and has been also observed in citrus (Yokoyama and White, 1966; Gross et al., 1971). The following unknown pigment exhibited three maxima in the short region 370,390,415 nm having the same spectrum as sinensiaxanthin, but a negative epoxide test and a weaker polarity. The carbonyl pigment with a broad maximum at 442 nm gave a hypsochromic shift upon reduction with $NaBH_4$; fine structure appeared with maxima at 388,413,438 nm. In accord with its properties it was tentatively identified as α-citraurin, the analog of β-citraurin with the formula 3-hydroxy-α-apo-8′-carotenal. This is the first time that an α-apocarotenal has been detected as occurring naturally. Its existence is a proof for the type of degradation at one end of the molecule (Wiss and Thommen, 1963). Thus it is the natural degradation product of lutein. As it was present in small quantities its structure could not be investigated.

In the diol fraction lutein appeared in two isomeric forms, trans and cis, accompanied by the epoxyderivative isolutein, that also appeared in two isomeric forms. Its furanoxide, chrysanthemaxanthin, was present in three isomeric forms. Chrysanthemaxanthin was cochromatographed with the furanoxide obtained from the avocado isolutein. A set of four isomers appeared on TLC and the three isomeric chrysanthemaxanthins had the RF's as the last more polar isomers. Violaxanthin was followed by an unknown carbonyl 446 that upon $NaBH_4$ treatment gave about the same reduction spectrum as α-citraurin. Luteoxanthin was followed by an unusual pigment that was pink on silica gel adsorbent, with blue fluorescence in UV light and maxima in the near UV region at 350,370,393 nm. HCl treatment produced a blue-violet color which indicated a furanoxide, sinensiachrome-like (Curl and Bailey, 1956). In the following polar pigment group a trollichrome-like pigment had properties identical to those of citrus trollichrome. Of the two neoxanthin pigments the more polar was similar to spinach neoxanthin. The main, less polar pigment was situated between spinach antheraxanthin and neoxanthin. Two other pigments, with the same spectra as neoxanthin, did not react in the HCl test. The more prevalent was as polar as the trollein(?) pigment detected by Ricketts (1967), but with the maxima at some shorter wavelengths. Having the same spectrum as neoxanthin it must have the same chromophore. The trollein formula has not as yet been elucidated, but it may be an allenic derivative of α-carotene triol, with the formula 3,3′,5′-trihydroxy-6′,7′-dehydro-α-carotene. Such a pigment is not discussed in Weedon's recent review about allenic carotenoids (Weedon, 1969), but the suggested formula would be in accord with its properties—the same chromophore as neoxanthin—having the α-ionone ring instead of the 5,6-epoxidic violaxanthin ring. Its structure will be further investigated.

Table 2—Quantitative composition of carotenoids from avocado fruit Persea americana, Nabal variety

Composition	Percent of total carotenoids[a]
Carotenes	
α-Carotene	0.9
β-Carotene	4.0
ζ-Carotene	0.5
Monols	
OH-α-carotene	1.2
Cryptoxanthin	5.2
α-Citraurin	0.7
Diols and polyols	
Lutein	25.0
Isolutein	9.0
Violaxanthin	4.0
Chrysanthemaxanthin	20.4
Luteoxanthin	2.1
Trollichrome	9.7
Neoxanthin-like	7.3
Unknown 437 (Trollein?)	8.1
Neoxanthin	0.5
Unknown 437	0.5

[a]Expressed as β-carotene. Isomeric forms of the same pigment were determined together. Minor pigments were not recorded.

The quantitative composition of carotenoids from avocado fruit is given in Table 2. The concentration of total carotenoids varied between 10–14 μg/g fresh matter of mesocarp (edible portion). The oil content was about 10%. The approximate composition is about 12% carotenes and monols versus 88% diols and polyols and their epoxides.

In the carotene fraction β-carotene predominates; in the monol fraction cryptoxanthin; and in the diol fraction lutein is the main pigment, together with its furanoxide which is present in about the same percentage. These two pigments represent about half the total carotenoids. Isolutein, the epoxide of lutein and neoxanthin are also present in quite high amounts as are the tentatively identified trollichrome and trollein.

In summary, the major discernible pigment pattern of avocado fruit is β-carotene, cryptoxanthin, unusually large amounts of lutein and chrysanthemaxanthin, large amounts of isolutein and polyols. This pattern does not agree with any other pattern given by Goodwin (1970) for fruits and is closer to the pigment pattern of the chloroplasts (Strain, 1966). This characteristic will be useful in taxonomic studies.

Vitamin A could not be detected either as retinene or as retinol, although vitamin aldehyde was detected by Winterstein et al. (1960) in green vegetables, grass and fruit of *Rosa canina*. We assumed that vitamin A alcohol would be present in its esterified form in a fruit with so high a content of lipids. But the vitamin fraction of the control sample, which should contain the aldehyde and the alcohol as well, was totally devoid of vitamin A while recovery of standard vitamin A added was 100%. The so-called high content of vitamin A of the fruit was not confirmed.

The vitamin A potency of the provitamins A present in the fruit was calculated according to Tiews (1963) and was found to be approximative 150 IU vitamin A/100g fresh fruit. [For OH-α-carotene whose structure was not definitely established and that can have the OH group or at the α-ionone ring (α-cryptoxanthin), or at the β-ionone ring (zeinoxanthin), (Liaaen-Jensen and Jensen, 1965), we assumed that the OH-α-carotene is α-cryptoxanthin with an unsubstituted β-ionone ring in the molecule and that it has the respective vitamin A potency.] The further carotenoid investigation of other varieties of avocado, which is now being carried out in this laboratory, as well as the elucidation of the unknown structures, will permit a better characterization of the fruit and will be useful in chemotaxonomy.

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DEGRADATION OF ANTHOCYANINS AT LIMITED WATER CONCENTRATION

INTRODUCTION

DEGRADATIVE REACTIONS in low-moisture foods have been reviewed by Labuza et al. (1970). Lipid oxidation rates decrease with increasing water activity (A_w). Carotenoid pigments show a similar relationship to A_w; Chou and Breene (1972) demonstrated that β-carotene in a freeze-dried model system was more stable at high A_w. LaJollo et al. (1971) found that chlorophyll degradation in a freeze-dried model system and in freeze-dried blanched puree increased with increasing A_w. In a study of browning in a model system containing sucrose, Karel and Labuza (1968) found that even at low water activities hydrolysis of sucrose can occur giving rise to reducing sugars which have a potential for browning. From this and other studies Labuza et al. (1970) concluded that water has a dominant influence on the rate of browning in systems which contain carbonyls. Browning usually increases with water content to a maximum (intermediate-moisture range) in most food systems. Such a relationship was observed in dehydrated orange juice by Karel and Nickerson (1964). Enzymic reactions in low-moisture foods are dependent on A_w. Acker (1969) in his review of this subject stated that a water sorption isotherm can be used to predict enzymic reactions.

While a number of investigators (Daravingas and Cain, 1968; Decareau et al., 1956; Hamdy et al., 1961; Keith and Powers, 1965; Markakis et al., 1957; Meschter, 1953; Starr and Francis, 1968) have studied the effect of pH, metal ions, organic acids, oxygen, hydrogen peroxide, sugars and sugar breakdown products (furfural and hydroxymethylfurfural) on anthocyanin degradation, no work has been reported on the relationship between A_w and anthocyanin stability. Markakis et al. (1957), observing that anthocyanins were stable when stored in dry crystalline form or on dried paper chromatograms, suggested that water was involved in decolorization reactions.

Enzymic decolorization of anthocyanins has been documented for a number of plant materials and has had commercial application in the preserve and wine industry. Huang (1955) using a fungal enzyme preparation (anthocyanase) studied the decolorization of cyanidin-3-glucoside.

The purpose of this investigation was to study the relationship between relative humidity (RH) and anthocyanin degradation. Freeze-dried strawberry puree was stored at RH's of 100, 75, 57, 32, 11 and $\simeq < 1\%$ under air or nitrogen at 37°C in the dark. In an effort to ascertain the role of enzymes in anthocyanin degradation, blanched strawberry puree was also studied. The rate of anthocyanin degradation was measured and attempts to characterize the reddish-brown insoluble pigments were made.

EXPERIMENTAL

Materials

Strawberries (*Fragaria ananassa* Duch. variety Northwest) were obtained from Oregon State University's North Willamette Experiment Station. The fruit was washed, individually quick frozen (IQF) at −35°C, packed in polyethylene bags and stored at −24°C. A representative sample had a pH of 3.6, contained 0.77% acid (calculated as citric) and had a soluble solids content of 9.7%. IQF whole fruit and puree (thawed IQF berries, pureed in a Waring Blendor and frozen in ca. ½-in thick sheets) were freeze dried in a commercial unit at 49°C and at pressures from 300–900μ. The final product (10% of original weight of berries) was stored under nitrogen at −24°C in sealed 603 × 700 tin cans containing silica gel desiccant.

In studying the effect of enzyme inactivation 200g of puree were divided into two 100-g portions; one sample was heated to 75°C and held for 10 min in a water bath and the other was not heated. Both samples were then freeze-dried.

Pigment determination

Anthocyanins were assayed using the procedure of Swain and Hillis (1959). Modifications included using 0.1% HCl in methanol as the extracting solvent and measuring the absorbance at 510 nm. Concentration was expressed as μM of pelargonidin 3-glucoside per g powder. Calculations were based on a molar absorptivity of 36,600 (Wrolstad et al., 1970).

The methanol-insoluble reddish-brown pigments present in the residue were assayed by the procedure of Sistrunk and Cash (1970). A modification was that the fruit had been extracted with acidic methanol rather than 80% ethanol.

Thin-layer chromatography (TLC)

Pigments present in the acidic methanol extract used in the total anthocyanin assay were further purified using Polyclar AT (Wrolstad and Putnam, 1969; Wrolstad et al., 1970), concentrated and subjected to cellulose TLC. Both one- and two-dimensional TLC were carried out. A solvent system of glacial acetic acid-water-HCl (15:82:3) was used for one-dimensional TLC. In two-dimensional work the solvent system used was: first direction, n-butanol-HCl-water (5:2:1); second direction, water-HCl-formic acid (8:4:1) (Nybom, 1968).

Moisture determination

A gas-liquid chromatographic procedure based upon the reaction of water with 2,2-dimethoxypropane (DMP) to yield acetone and methanol was used to measure moisture content (Mary, 1969); 2-pentanone was used as an internal standard.

An Aerograph 1520 gas chromatograph (hydrogen flame ionization detector) equipped with a 71/2 ft × 1/8 in. stainless steel column packed with 30% tetrahydroxyethylethlenediamine on Chromosorb W was used to separate the reaction product mixture. Operating conditions were: oven temperature, 75°C (isothermal); injection block temperature, 80°C; detector temperature, 160°C; nitrogen (carrier gas) flow rate, 15 ml/min; hydrogen, 20 ml/min; air,

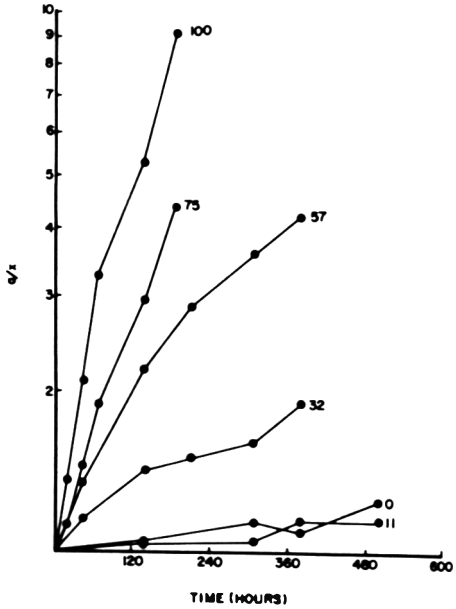

Fig. 1—Reaction rate curves for the degradation of anthocyanin pigments in freeze-dried strawberry puree at various RH's and 37°C (a = 100%; x = % pigment retained).

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Table 1–Comparison of pigment and water content in freeze-dried strawberry puree stored at various relative humidities at 37°C

% Relative humidity	Time (hr)	μM pigment/ g puree	μM × 10^{-3} water/ g puree	μM water/ μM pigment
0	0	13.1	1.2	91
	141	12.5	1.3	100
	309	11.5	1.7	150
	381	11.9	1.7	140
	501	10.4	1.7	160
	719	10.3	1.2	120
11	0	12.8	1.3	100
	141	12.3	1.2	100
	309	12.1	1.2	100
	381	11.2	1.2	110
	501	10.9	1.3	120
	719	10.3	1.2	120
32	0	13.0	1.9	150
	45	11.1	3.7	330
	141	9.1	3.7	410
	213	8.7	3.8	440
	309	8.0	3.6	450
	381	6.9	3.6	520
57	0	12.6	3.2	250
	45	9.3	7.2	770
	141	5.8	7.4	1,300
	213	4.5	7.2	1,600
	309	3.6	7.2	2,000
	381	3.1	7.2	2,300
75	0	12.3	3.2	260
	21	10.9	7.7	710
	45	8.5	9.1	1,100
	69	6.5	10.4	1,600
	141	4.2	12.2	2,900
	189	2.8	11.7	4,200
100	0	11.8	7.2	610
	21	8.6	14.7	1,700
	45	5.7	18.6	3,300
	69	3.7	19.9	5,400
	141	2.3	26.2	11,000
	189	1.3	27.8	21,000

275 ml/min. Peak areas were calculated by triangulation according to the procedure of Kingston (1964).

Increase in moisture content in the degradation experiments was measured gravimetrically.

Gel filtration

Anthocyanins for gel filtration analyses were isolated by extracting 2g of freeze-dried strawberry puree powder with 10 ml methanol and filtering through two layers of Whatman No. 1 filter paper. Studies were also made utilizing the acidic methanol extract prepared for total pigment determination (1g freeze-dried powder extracted with 200 ml of 0.1% HCl in methanol). 3 ml of the anthocyanin extract were applied to a 2.5 × 25 cm solvent-resistant column filled with Sephadex LH-20 which had been swollen in 0.01% HCl in methanol overnight at 2°C. Flow rates of 2.1–2.5 ml/min of the elution solvent, 0.01% HCl in methanol, were maintained. The absorbance of the effluent was monitored at 510 nm by a Beckman DB spectrophotometer equipped with a Flow Cell Assembly. After passing through the spectrophotometer the effluent was collected in 10 ml quantities in a fraction collector (ISCO – Model 272) coupled with an ISCO Model 400 Volumeter.

Degradation studies

Freeze-dried strawberry puree was ground using a mortar and pestle into a coarse powder. Approximately 1-g samples were transferred to weighed open-top vials (2.5 × 6.5 cm) and stored in a large desiccator containing silica gel in the dark in vacuo. After 16 hr the sample vials were reweighed and the weights recorded. Six vials were placed in each of six desiccators containing the following materials to give the desired relative humidities (Rockland, 1960): 0%, silica gel desiccant; 11%, saturated LiCl solution; 32%, $MgCl_2$; 57%; NaBr; 75%, NaCl; and 100%, distilled water. The chambers were then evacuated and the samples allowed to equilibrate with the constant humidity of the chambers. "Original" moisture and total anthocyanin content readings were determined after 2 hr. Samples were left in the humidification chambers and moisture uptake was measured gravimetrically. Experiments were performed in the dark at 37°C.

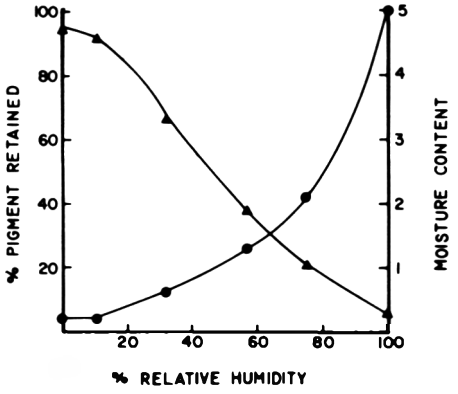

Fig. 2–Comparison of water sorption isotherm (–●–) with pigment retention (–▲–) of strawberry puree after 180 hr at 37°C (moisture content = g H_2O per 10g puree).

In studying the effect of headspace air and nitrogen, 500 ml filter flasks were used for relative humidity chambers as they better facilitated flushing the chamber with gas. These experiments were performed at an RH of 100%.

RESULTS & DISCUSSION

THE PRONOUNCED effect of relative humidity (RH) on the rate of anthocyanin degradation is shown in Figure 1. Preliminary experiments yielded similar results but are not included as experimental conditions were not precisely the same. The data are for one experiment and do not represent averaged values. No difference in rate is evident between 0 and 11% RH; large differences are observed between 11 and 32% and between 32 and 57%. These results are similar to those reported for other deteriorative reactions, e.g., nonenzymatic browning (Karel and Labuza, 1968), chlorophyll degradation (LaJollo et al., 1971) and enzymatic degradation (Acker, 1969).

None of the samples show first order kinetics throughout the entire storage time. Reasonably straight lines, however, can be drawn for the 75 and 100% RH samples for the first 69 hr. Several workers (Markakis et al., 1957; Keith and Powers, 1965; Tinsley and Bockian, 1960; Wrolstad et al., 1970; Daravingas and Cain, 1968) have established that degradation of anthocyanin solutions follows first order kinetics.

In our studies, it is evident that water availability is essential for anthocyanin breakdown. Water itself may be required for pigment degradation; Markakis et al. (1957) postulated two hydrolytic mechanisms, one being hydrolysis of the glycosidic bond to yield the unstable aglycone and the other involving opening of the pyrilium ring to form a substituted chalcone. Alternatively, degradation may require the pigment being in solution.

The molar ratio of water to pigment was calculated for samples stored at

different relative humidities for certain time periods (Table 1). Cross reference with Figure 1 reveals that a low degradation rate occurred when the molar ratio was below 150. An intermediate rate occurred with a ratio between 150 and 700 and a rapid rate was associated with ratios greater than 700. These values could coincide with the theoretical division of the sorption isotherm into three regions (monolayer, multilayer and capillary condensation).

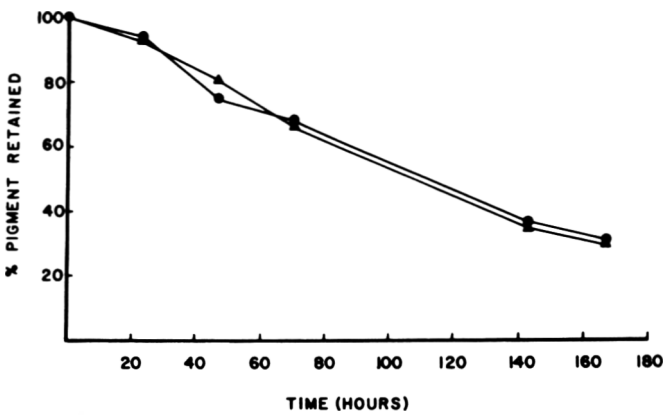

Fig. 3—Effect of enzyme inactivation (heat treatment) on pigment retention of freeze-dried strawberry puree at 100% RH and 37°C (—▲—, unheated; —●—, held at 75°C for 10 min).

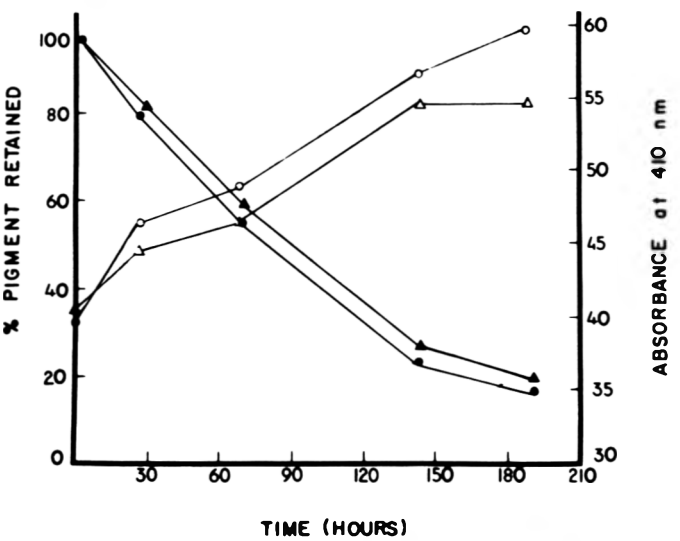

Fig. 4—Effect of air and nitrogen on the degradation of anthocyanins and formation of brown pigments in freeze-dried strawberry puree at 100% RH and 37°C (anthocyanins: —▲— nitrogen, —●— air; brown pigments: —△— nitrogen, —○— air).

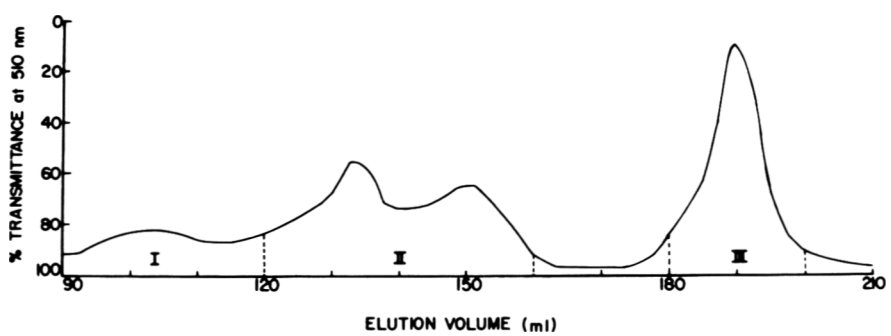

Fig. 5—Gel filtration curve of a methanol extract of freeze-dried strawberry puree on Sephadex LH-20 in 0.01% HCl in methanol.

These findings suggest that a sorption isotherm could be used to assess storage stability of anthocyanin pigments in low moisture systems. The inverse relationship between pigment retention and moisture content is evident from Figure 2 which shows the sorption isotherm along with pigment retention. Karel and Nickerson (1964) obtained a similarly shaped isotherm for dehydrated orange juice. By using a more sensitive gravimetric procedure giving accurate measurements at low water activities, they did obtain isotherms which were sigmoidal.

The activity of enzymes which require water either as a medium for reaction or as a reactant show the same dependence on water activity (Acker, 1970) as does pigment degradation in this study. Therefore, the possibility exists that enzymic hydrolysis of the pigment might be occurring instead of, or in addition to, chemical hydrolysis. Huang (1955) proposed that anthocyanase acted like a β-glucosidase. The aglycone resulting from hydrolysis precipitated from solution and degraded to various colorless entities, e.g., 2-carbinol or chalcone. Scheiner (1961) stated the mechanism of enzymic decoloration of cherries included enzymic oxidation of o-dihydroxyphenols to quinones and subsequent reaction between the quinone and anthocyanin forming a colorless compound. A comparison of pigment degradation between freeze-dried puree which had been heated to inactivate all naturally occurring enzymes and unblanched material was carried out. Figure 3 shows that the difference in degradation rate is very small. The two plots are similarly shaped indicating this degradation is not enzymic.

Oxygen was found to have little effect on anthocyanin degradation. The pigment degradation rates in air and nitrogen atmospheres at a RH of 100% are shown in Figure 4. Degradation is always greater in air, the rate difference being small but relatively constant. The rate of browning was influenced to a greater degree by oxygen. Reddish-brown material was present in the residue remaining after acidic methanol extraction of the puree and was observed to increase with storage time. The material was soluble in aqueous 5% NaOH. The absorbance at 410 nm of this extract was used to assay browning and found to be higher for samples stored in air than in nitrogen.

Attempts to characterize the red-brown precipitate by gel filtration were made. Neutralization of the alkali extract with 10% HCl precipitated the pigment. Methanol partially solubilized the material. Gel filtration resolved the methanol extract into 3 bands (Fig. 5). The effluent

was pooled into three fractions and concentrated. Poor resolution of the concentrated fractions on cellulose TLC suggested that some interfering material was present in the concentrates. Purification of the pigment with insoluble polyvinylpyrrolidone (PVP) resulted in all fractions having very nearly the same R_f. The three fractions and pelargonidin-3-glucoside were subjected to two-dimensional thin-layer chromatography. Each fraction was developed by themselves and also in combination with pelargonidin-3-glucoside as an internal standard. Chromatographic behavior of the three fractions was identical to pelargonidin-3-glucoside indicating they were composed of the pigment. Possibly the bands were artifacts, each band being pelargonidin-3-glucoside adsorbed to the gel in different degrees. Alternatively they could have been polymers of different molecular weights or complexes whose structure was destroyed in the PVP purification process.

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THE EFFECT OF GAMMA IRRADIATION ON THE ANTITHIAMINE ACTIVITY OF SKIPJACK TUNA

INTRODUCTION

PREVIOUS STUDIES from this laboratory have shown that Skipjack tuna (*Katsuwonus pelamis*) possesses antithiamine activity which is not enzymatic (Hilker and Peter, 1966, 1968; Tang and Hilker, 1970). These studies have indicated that the antithiamine activity resides in a protein-nonprotein complex with the nonprotein moiety responsible for the antithiamine activity. Kündig and Somogyi (1967) isolated a thermostable antithiamine factor from carp viscera which was identified as hemin or a related compound. Heme proteins, including hemoglobin and catalase, also showed antithiamine activity which is associated with the heme component (Kündig et al., 1970).

Irradiation has been reported to reduce or destroy "thiaminase" activity in alewife (Krzeczkowski, 1968) and clams (Bert et al., 1970). The purpose of the present investigation was to determine the effect of gamma radiation on the antithiamine activity of Skipjack tuna and protein fractions of the water soluble extracts.

EXPERIMENTAL

THE SKIPJACK TUNA used in this study were caught in Hawaiian waters by commercial fishermen. The fish were refrigerated on the boat and marketed within 2–3 days. The evicerated fish were purchased at Honolulu markets, the skin, bone and dark muscle (blood channel) removed and sliced into 2-in. wide sections. The sections were placed in air-tight glass jars and treated immediately in the Hawaii Research Irradiator with Co^{60} as the source of gamma rays for various periods of time depending upon the irradiation dosage desired. Each dosage treatment group was accompanied by a control group. The control jars were placed in the irradiation room beside the pool area in order to eliminate time and temperature factors.

In order to study the effect of irradiation under anaerobic conditions, slices of fish in glass jars were placed in a glove bag (Instruments for Research & Industry) and flushed with N_2 gas six times. The lids were placed on the jars and tightened in the bag. They were removed from the bag and irradiated immediately along with similar samples prepared in air. Controls included jars prepared in air and also in N_2 gas.

After irradiation the fish samples were homogenized in distilled water (20g/100 ml) in a Waring Blendor then centrifuged at 10,000 × G for 20 min. The supernatent solution was filtered through Whatman No. 2 filter paper and the antithiamine activity (ATA) determined immediately as previously described (Tang and Hilker, 1970). The dry weights of the total soluble solids in the fish extract used for ATA determinations were determined by placing beakers with known volumes of the extract in an oven at 70°C for 5 hr then in a vacuum oven overnight until constant weight was achieved.

The isolation of protein fractions by $(NH_4)_2SO_4$ precipitation was carried out by a procedure similar to that employed by Dollar et al. (1959), for the isolation of myoglobin. The light muscle from Skipjack tuna was homogenized with distilled water (1:2) for 3 min in a Waring Blendor. The homogenate was centrifuged at 10,400 × G for 10 min at 4°C and the supernatent passed through several layers of cheesecloth, the pH brought to 6.8 with 1N NaOH and the requisite amount of $(NH_4)_2SO_4$ added slowly with stirring at 4°C to give 30% saturation. The system was centrifuged (27,300 × G, 15 min 0°C) and the precipitate collected. The precipitation and centrifugation were repeated with increments of 10% saturation until 80% saturation was reached. Each fraction was then dissolved in 50 ml distilled water, dialyzed against distilled water until free of $SO_4^=$, centrifuged (36,000 × G, 20 min 0°C) and the supernatant collected. The supernatant solutions from each level of $(NH_4)_2SO_4$ saturation were analyzed for ATA. Fractionations with $(NH_4)_2SO_4$ were made on fish samples treated as follows: Fresh, fractionated immediately after purchase; Irradiated, 4 Mrad; and Control, kept in irradiation room during irradiation period.

Solutions of hemin (50μg/ml, Sigma Chemical Co.), hemoglobin (bovine 250 μg/ml, Calbiochem) and myoglobin (equine heart 250 μg/ml Calbiochem) in phosphate buffer at pH 7 were irradiated (1 Mrad) and tested for ATA.

Table 1—The effect of gamma irradiation on ATA of tuna fish in air atmosphere[a]

Mrad	ATA (% of control)	Dry wt (% of control)
1	95.3 ± 18.1	98.1 ± 7.7
2	139.0 ± 35.3	88.9 ± 2.9
3	158.3 ± 26.5	87.9 ± 5.7
4	172.1 ± 15.8	86.8 ± 8.0

[a]Values are an average of three experiments in duplicate.

RESULTS

IN THE FIRST experiment (Table 1) the ATA of fish samples irradiated in air at dosages of 2–4 Mrad, but not 1 Mrad appeared to be increased over that of the controls. Dry weights of the samples irradiated at 2, 3 and 4 Mrad were decreased. Since insufficient samples were tested to provide statistical significance, further studies were made to verify these findings.

The second experiment was conducted to test the effect of a lower irradiation dosage (0.5 Mrad) and also the effect of irradiation under N_2 atmosphere. Under air atmosphere (Table 2) the same pattern of increased ATA at higher dosage was found although the increase was less marked than in the first experiment. The increase was statistically significant ($p < .05$) only at the 4 Mrad dosage. Under N_2 atmosphere irradiation at 4 Mrad resulted in a statistically significant decrease in activity. The dry weight of the soluble solids of the air irradiated samples followed a reverse pattern to that of the ATA with the highest dosage having lowest dry weight. There were no significant differences among the dry weights of N_2 irradiated samples.

In order to determine whether irradiation at 4 Mrad would cause changes in

Table 2—The effect of gamma irradiation on ATA under air and N_2 atmosphere[a]

Mrad	ATA (% of control)		Dry weight (% of control)	
	Air	N_2	Air	N_2
0.5	83.6 ± 12.1	82.0 ± 11.7	101.0 ± 4.2	98.5 ± 2.9
1.0	91.3 ± 9.8	91.2 ± 17.5	99.7 ± 4.6	98.2 ± 4.1
2.0	118.6 ± 10.9	107.1 ± 6.6	94.9 ± 3.7	99.2 ± 3.0
4.0	125.9 ± 14.2*	68.1 ± 6.6*	86.0 ± 3.6*	90.7 ± 2.5

[a]Values are an average of six experiments in duplicate.
*Significant difference from controls ($p < .05$)

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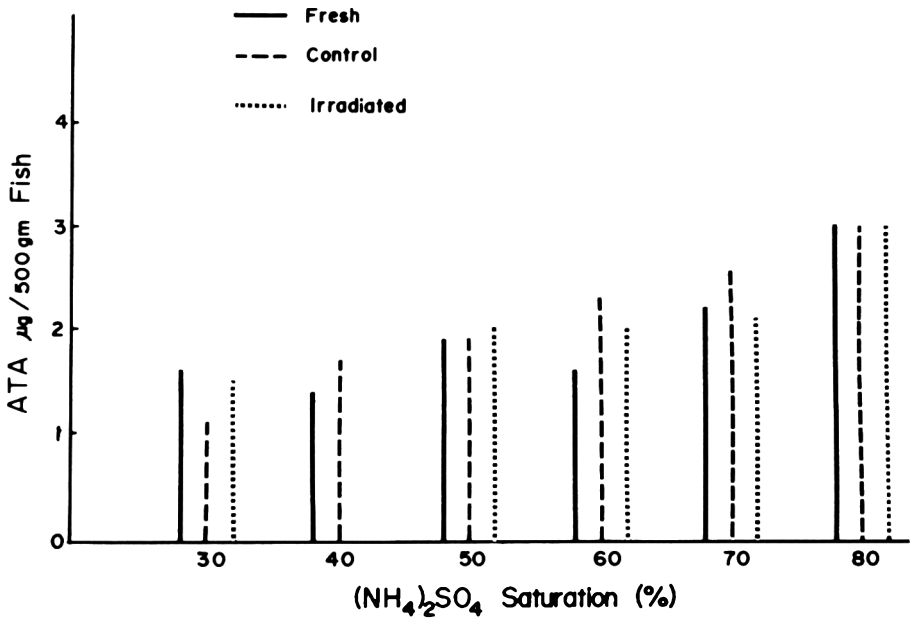

Fig. 1–Antithiamine activity of $(NH_4)_2$ fractions at various saturation levels. Values are an average of three experiments.

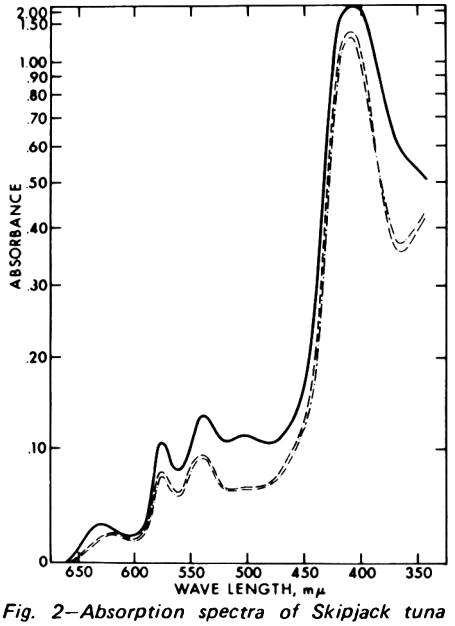

Fig. 2–Absorption spectra of Skipjack tuna extracts, nonirradiated, irradiated under air and irradiated under N_2 atmosphere. (Nonirradiated —; air atmosphere – – –; N_2 atms – · –).

protein which would affect the ATA, the water soluble extracts from fresh, control and irradiated (4 Mrad) fish were subjected to fractional precipitation with $(NH_4)_2SO_4$ from 30% to 80% saturation, and the ATA tested on these fractions after removing the $SO_4^=$ by dialysis. These results are shown in Figure 1. Although ATA was present in all fractions of fresh and control samples, major activity was found in the 70% and 80% fractions which contained the heme proteins. There was no difference in the activity among the three treatments which indicated that the irradiation had not affected the ATA of the isolated proteins. However, a most interesting finding was the complete absence of ATA in the 40% saturation fraction after irradiation. This occurred in each of the three fish tested.

It was observed that the water extract of the irradiated fish was light yellow rather than the pink color of the nonirradiated samples suggesting that heme protein in the fish muscle had been changed. The spectra of extracts of the irradiated and nonirradiated fish sections are shown in Figure 2. All absorption peaks were lower for the irradiated samples than for the nonirradiated samples. The presence of an absorption peak at 615 mµ and the reduction of the 635 mµ and 500 mµ absorption for treatments under air and N_2 is similar to the spectra of a green pigment in irradiated extracts of beef muscle (Ginger and Schweigert, 1956).

Solutions of hemin, myoglobin and hemoglobin were essentially colorless after irradiation at 1 Mrad. No ATA was present after irradiation.

DISCUSSION

SINCE THE calculation of ATA is based on the amount of thiamine destroyed (as determined by the thiochrome procedure) per mg total dry solids in the fish extract, changes in ATA could result from an increase or decrease in the amount of ATA-inactive material in the dry solids as well as the amount of ATA-active material present. Thus the increase in ATA accompanied by decreased dry weight of the fish samples irradiated in air suggests that irradiation had caused ATA-inactive protein to become insoluble while the ATA-active protein had remained soluble. Denaturation and formation of insoluble protein aggregates has been shown to be caused by irradiation (Kumta and Tappel, 1961). Since these changes in dry weight did not occur in the samples irradiated under N_2 gas it would appear that aerobic conditions are required for protein denaturation. The decrease in ATA in the sample irradiated at 4 Mrad in N_2 gas was possibly a direct effect on ATA-active protein.

The results of the study on the ATA of fractions precipitated by $(NH_4)_2SO_4$ show that activity is associated with protein in all fractions but that the highest ATA occurred in the 70% and 80% $(NH_4)_2SO_4$ saturation fractions which are predominately heme protein, myoglobin and hemoglobin. Irradiation did not affect the ATA of heme proteins or the 30%, 50%, or 60% fractions but had possibly changed the solubility characteristics of the 40% fraction. These data support our contention that the principal effects of irradiation on the ATA of tuna fish protein involves denaturation of ATA-inactive protein.

The spectra of the extracts of irradiated and nonirradiated fish sections indicate that a pigment was formed similar to that of irradiated beef muscle (Ginger and Schweigert, 1956). Further work by Fox et al., 1958 indicated that this compound was sulfmyoglobin. Although changes in the spectra of heme proteins resulted from irradiation of the fish samples, apparently this did not affect the ATA as shown by the studies on the $(NH_4)_2SO_4$ fractions. The complete destruction of both color and ATA of the solutions of hemin, myoglobin and hemoglobin by irradiation showed that these compounds are less stable in aqueous solutions than the same compounds present in fish tissue.

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APPROACHES TO THE UTILIZATION OF FISH FOR THE PREPARATION OF PROTEIN ISOLATES

Isolation and Properties of Myofibrillar and Sarcoplasmic Fish Proteins

INTRODUCTION

FOR THE PAST 8 yr, extensive research has been done on the preparation and utilization of fish protein concentrate (FPC) (Library of Congress, 1970). In the United States, part of this effort has resulted in the design, construction and operation at Aberdeen, Wash. of an experimental and demonstration plant with the capacity of processing 50 tons of raw fish per day (Ernst, 1971). The FPC currently being produced is a light-tan-colored product practically free of flavor and odor. As it is now produced, FPC is essentially without functional properties except that of nutrition. Indeed, it was developed as a nutritive additive for incorporation into protein-deficient diets.

Concurrently with the latter stages of development of the FPC program, studies have been carried out on the feasibility of using fish muscle as a raw material for the production of fish protein isolates having functional properties that meet various requirements of the protein market. The desirability of using fish as a raw material for the preparation of functional protein isolates becomes evident when one considers that fish is a relatively inexpensive source of high quality protein. For example, the current price of Pacific hake *(Merluccius productus)* ranges from \$20–\$30 per ton. At a muscle protein content of approximately 15%, the cost of the protein on a 100% basis would range from \$0.067–\$0.10 per pound. Given the right properties, fish muscle proteins at a finished cost price comparable to functional proteins of animal origin could share in a 2 billion pound potential market for functional proteins described by Hammonds and Call (1970).

Some consideration has already been given to the use of fish for preparing functional protein isolates. Tannenbaum et al. (1970) have reported on their investigation of using FPC as a starting material for the preparation of protein isolates. In that work, FPC was partially hydrolyzed with NaOH and recovered as an iso-electric protein. An alkaline hydrolysis process is also being investigated at Oregon State University (Anglemier, 1971). Although partial alkaline hydrolysis may prove useful in the preparation of functional protein isolates, this procedure can lower the nutritional quality through racemization (Tannenbaum, 1970) and the possible conversion of cysteine to lanthionine (Lindley and Philips, 1945). In addition, the economics of using a once-processed product as a raw material source might lessen the economic advantage that unprocessed raw fish would mainly enjoy. This latter factor, of course, cannot be fully evaluated unless functional and organoleptic properties of the isolates are fully ascertained. For example, the price of sodium caseinate can vary several-fold depending on its functional properties along (Guardia, 1970).

Recently Meinke et al. (1972) discussed some of the factors that influence the production of protein isolates from fish. In this work, data on the solubility characteristics and the recovery of isolates from whole raw fish and frozen fish at various pH's, salt concentrations and temperatures were presented. No attempts, however, were made to separate the basic protein components of fish muscle, and factors that influence the functional and organoleptic characteristics of isolates when subjected to various processing procedures were not reported. The work at our laboratory deals in these latter areas and was divided into two parts:

1. To separate the two major protein fractions of fish muscle (myofibrillar and sarcoplasmic) and to determine some of the physical, chemical and organoleptic properties of each before and after drying.
2. To evaluate the use of some proteolytic enzymes as an aid in modifying the physical and chemical characteristics of the separated protein fractions.

MATERIALS & METHODS

Fish

In the work presented here, only Pacific Ocean rockfish *(Sebastes sp.)* were used because of their year-round availability. In addition, the muscle lipids of these fish are extremely labile to oxidation and it was felt that the knowledge gained working with these fish would be applicable to others considered for similar use.

Separation of myofibrillar and sarcoplasmic proteins from nonprotein constituents

Fish muscle freed from skin and bone was ground through a Hobart grinder equipped with a 1/8 in. plate. The separation of the protein fractions from the nonprotein fractions was performed as schematized in Figure 1. The ground tissue was slowly mixed for 10 min in 0.1M NaCl (4:1 ratio of solution to fish). The solids consisting mostly of myofibrillar protein were separated from the sarcoplasmic and lipid portions by centrifugation in a bowl-type centrifuge (11 in. diam at 2500 rpm) for 10 min. The myofibrillar fraction was resuspended once in the 0.1M salt concentration (2:1 ratio of solution to fish) and once in tap water (2:1 ratio of solution to fish) and recentrifuged to further reduce the sarcoplasmic and lipid fraction associated with the myofibrillar fraction. The sarcoplasmic proteins in the aqueous extract were centrifuged to remove small amounts of suspended oils, dialyzed and freeze dried. They were also recovered as phosphate complexes (Spinelli and Koury, 1970) prior to drying.

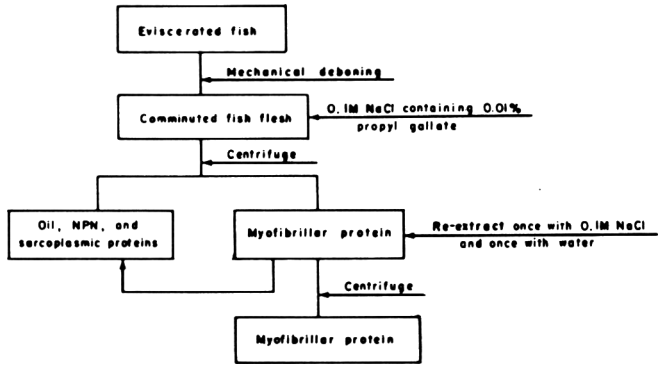

Fig. 1—Aqueous separation of protein and nonprotein constituents of fish tissue.

Drying

Within 2 hr after preparation, the protein fractions were spray dried or were frozen and freeze dried. Freeze drying was done in a Thermovac Model FDC IND 32F at a shelf temperature of 25° ± 5°C. Spray drying was done with a Nichols/Niro Spray Dryer Model IV using nozzle atomization. Inlet temperature 280°C, outlet temperature 100–105°C. When freeze dried, the protein fractions were reduced in particle size by dry grinding in a Waring Blendor. Analyses were made on material passing through a 100 mesh stainless steel screen.

Extraction of proteins with isopropanol

The dried protein fractions were mixed in a beaker with azeotropic isopropanol at a ratio of 1 part protein to 10 parts alcohol. Extractions at elevated temperatures were done in a water bath. Residence time in the alcohol was 5 min after the desired temperature was reached.

Vacuum packing

Dried proteins were placed in No. 301 C–enameled cans and sealed in a Rooney can sealer after holding a vacuum of 635 mm of Hg for 1 min.

Emulsifying capacity

0.5g of protein material was mixed in 100 ml of water and emusifying capacity was measured by a modification of the electrical resistance method of Webb et al. (1970). In our work, a 1-liter jacketed Waring Blendor cup was equipped with electrodes mounted at the base of the cup. Blender speed was 16000 rpm and the temperature 40° ± 2°C.

Total lipids

The method of Bligh and Dyer (1959) was used.

Phosphorus

Approximately 0.2g of oil was digested in a mixture of 3.5 cc H_2SO_4, 5 cc HNO_3 and 0.5 cc $HClO_4$. The digests were brought to 100 cc with distilled water and phosphorus was determined by the method of Fisk and Subbarow (Hawk et al., 1949).

Protein

The method of Lowry et al. (1951) was employed.

Salt solubility

The method of Dyer et al. (1950) was used. Other references to protein solubility were calculated on the basis of 100% solubility of the protein.

Malonaldehyde

The dried proteins (0.5–1.0g) were mixed in a distilling flask with 97.5 ml of distilled H_2O and 2.5 ml of 4N HCl. The flask was heated in a mantle and 50 ml of distillate collected. Analyses for thiobarbituric acid reactive substances were made by the method of Tarladgis et al. (1960) on 5 cc of distillate. All absorbance values are given on the basis of a 1-g sample.

RESULTS & DISCUSSION

Composition of fish

Commercially caught fish can come in all sizes and different stages of maturity. The tissue constituents of a catch of a single species of fish can therefore not only vary in composition depending on maturity but also can vary depending on where and when the fish are caught.

Basically, the protein components of fish after evisceration, skinning and deboning consist of myofibrillar and sarcoplasmic proteins in a ratio of about 70% myofibrillar to 30% sarcoplasmic (Dassow et al., 1970) (Fig. 2). Muscle tissue also contains highly unsaturated lipids, phospholipids and considerable amounts of nitrogenous compounds (NPN) ranging from peptides, amino acids, free amines, betaines, trimethylamine oxide and urea.

Removal of sarcoplasmic proteins and nonprotein constituents from myofibrillar proteins

The amount of sarcoplasmic protein removed after each extraction is shown in Figure 3. After three extractions, approximately 95% of the sarcoplasmic protein is removed. Estimation of protein removal was made by subtracting the amount of protein found in the extracting solution from the original total protein content of the comminuted tissue. The small amount of protein found in the extracting solution after the 4th extraction was probably due to some solubilization of actomyosin. The amount of nonprotein constituents that was removed after each extraction followed the same pattern as that found for sarcoplasmic proteins.

Lipid reduction and distribution

Typical analyses on residual lipids and the phosphorus contents of the lipids after each aqueous extraction are shown in Figure 4. In the experiment shown, the total lipid content of the tissue was reduced by 70% after three extractions: 40% was removed in the first extraction, 20% in the second and 10% in the third. A fourth extraction did not significantly reduce the lipid content. An increase in the ratio of phosphorus to total residual lipids after the first extraction of the myofibrillar fraction shows that the lipids removed were mostly triglycerides. The subsequent decrease in phosphorus content of the lipids remaining on the myofibrillar protein with corresponding increases in the phosphorus content of the lipids associated with the sarcoplasmic fraction shows that these extractions progressively remove an increasing amount of phospholipids with respect to total lipids. However, after three extractions, the phospholipid content of the lipids remaining on the myofibrillar fraction showed only a slight decrease and the gross composition of the lipids remaining with this fraction is practically the same as that originally present in the tissue.

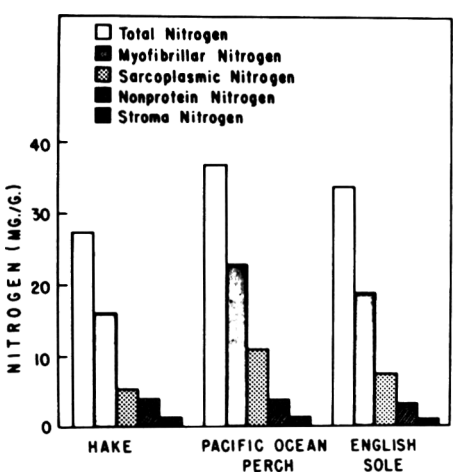

Fig. 2—Nitrogenous constituents in three species of fish.

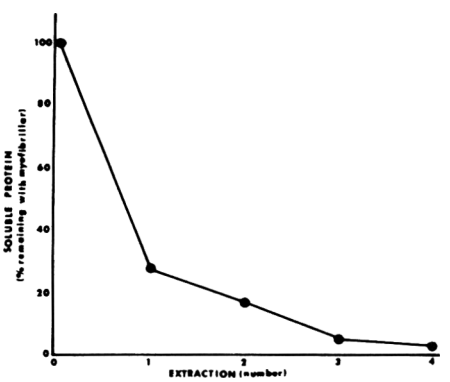

Fig. 3—Amount of sarcoplasmic protein remaining with myofibrillar proteins after each stage of aqueous extraction.

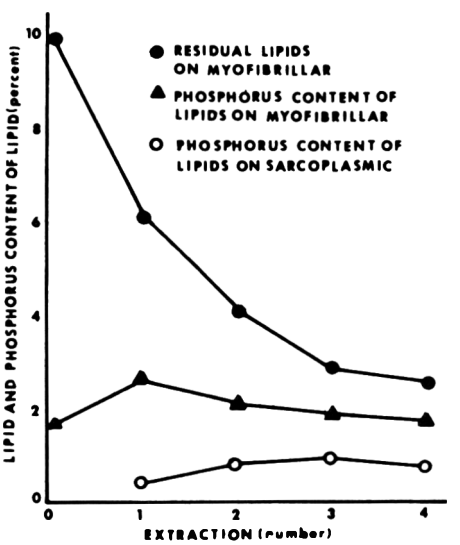

Fig. 4—Total lipids and the phosphorus content of lipid fractions remaining on the sarcoplasmic and myofibrillar proteins after each stage of aqueous extraction.

Properties of sarcoplasmic protein

The freeze-dried sarcoplasmic proteins were light tan in color and had a slight rancid odor and taste that became more intense during storage in air at room temperatures. Attempts to stabilize this fraction against oxidative deterioration with antioxidants such as BHT, BHA, propyl gallate and α-tocopherol were ineffective. The use of purines and purine derivatives (Matsushita et al., 1963) and amino acids (Castell et al., 1966) were ineffective as was the use of ribose as suggested by Andrews (1969). Ascorbic acid was effective in retarding oxidation on dialyzed and dried sarcoplasmic proteins but only for short periods (less than 7 days) of time. Determinations of the comparative rates of oxidative deterioration in the dried sarcoplasmic and myofibrillar fraction showed that sarcoplasmic fraction was far more labile than the myofibrillar fraction. Figure 5 shows the comparative amounts of malonaldehyde found in the sarcoplasmic and myofibrillar fractions after four stages of extraction with 0.1M NaCl. In these experiments, the salt was dialyzed from the proteins and both fractions were freeze dried. After 24 hr, comparative TBA determinations were made on each fraction. TBA values ranged from 10–15 times higher (adjusted to the same lipid content) in the sarcoplasmic fraction. These findings substantiate the views of Castell and Bishop (1969) that the sarcoplasmic fraction contains pro-oxidant materials such as hemoglobin and catalase. This fraction may also bind more labile lipids than does the myofibrillar fraction. In this latter respect, however, it should be noted that the sarcoplasmic fraction actually contained less residual phospholipid than did the myofibrillar fraction (Fig. 4).

Solubility characteristics of dried sarcoplasmic proteins. In native form, the sarcoplasmic proteins are essentially water-soluble. Freeze drying these proteins irreversibly denatured about 80% of the proteins with respect to water solubility. Precipitating the proteins with 60% (20°C) isopropanol followed by vacuum drying at 60°C results in similar denaturation. Precipitation with hot isopropanol (70°C) with similar drying conditions results in an almost completely water-insoluble protein.

Effect of IPA extraction on organoleptic characteristics. Three successive extractions of each dried protein fraction with azeotropic isopropanol (at 20°, 50° and 70°C) revealed that flavor- and odor-producing substances could be removed from the myofibrillar fraction at the higher extraction temperatures. With the sarcoplasmic fraction, however, an odor- and taste-free product could not be obtained. Somewhat better results were obtained when the IPA extractions were made on the phosphate-complexed form of the sarcoplasmic proteins, but even here slight off flavors were noted in the dried product.

Results from these experiments indicated that factors contributing to organoleptic stability of fish protein isolates resided largely with the sarcoplasmic fraction. Further efforts in preparing isolates were directed to only the myofibrillar fraction.

Properties of myofibrillar protein

Solubility characteristics. Myofibrillar protein prepared as described has low water solubility near neutrality (Fig. 6) but is highly soluble, forming very viscous solutions at pH's above 10.0 and below 3.5. These properties show some alteration after freeze drying. There is a significant decrease in solubility at pH values below 4 and the isoelectric point shifts from 5 to 4.5. The dried proteins have a comparatively higher solubility between pH 4.5 and 8.0 and a lower solubility at pH values ranging from 9–11.

Effect of carbohydrate on salt solubility. Perhaps one of the most important functional properties of myofibrillar protein is its ability to form water-protein-lipid emulsions. Fukuzawa et al. (1961) studied the lipid-emulsifying capacity of myofibrillar protein in processed meats and related this function to its solubility in neutral 5% salt (NaCl). Our work showed that the salt solubility of freshly prepared myofibrillar protein ranged from 70–80% and that freeze drying immediately reduced this solubility to about 15%, indicating severe denaturation of the proteins. Yasui and Hashimoto (1966), however, showed that sugars can exert a protective effect on the native properties of myosin even after freezing and freeze drying. Love (1962) showed that related compounds such as glycerol also protect fish protein against denaturation during frozen storage.

In view of these observations, myofibrillar proteins were co-dried with sugars and partially hydrolyzed starches to determine the effect of these compounds on the salt-soluble properties of the dried proteins.

The protein was prepared as previously described and freeze dried in the presence of 0–25% sugars or partially degraded starches. No attempt was made to adjust the pH in these experiments. The pH of

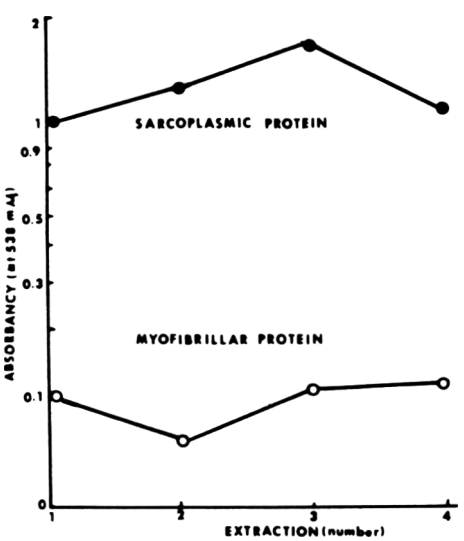

Fig. 5–Comparative amounts of malonaldehyde found in sarcoplasmic and myofibrillar proteins 24 hr after freeze drying.

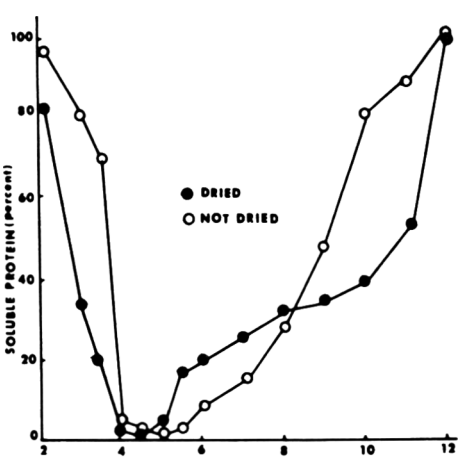

Fig. 6–Solubility of myofibrillar protein before and after freeze drying at pH values ranging from 2–12.

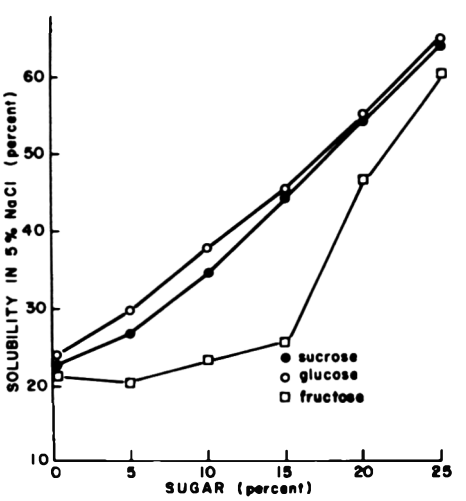

Fig. 7–Relation of salt solubility to the amounts of free sugars co-dried (freeze dried) with myofibrillar proteins.

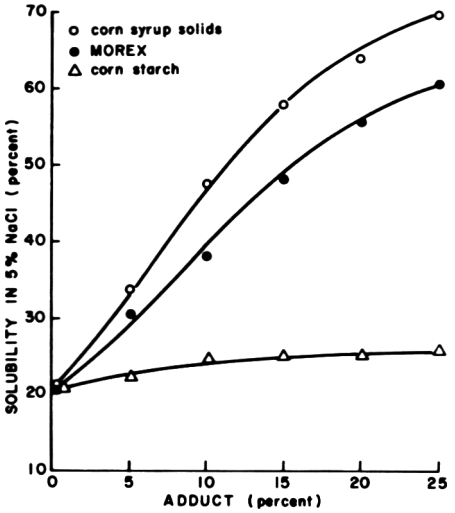

Fig. 8–Relation of salt solubility to the amounts of corn starch and partially degraded starches co-dried (freeze dried) with myofibrillar proteins.

Table 1–Malonaldehyde formation in freeze-dried myofibrillar protein prepared in the absence and presence of propyl gallate (0.01% based on weight of wet muscle) and stored in air and vacuum atmospheres

Treatment	Malonaldehyde	
	Initial value (Absorbancy at 538 μM)	After 6 months of storage (Absorbancy at 538 μM)
Air packed[a]	0.066	0.114
Air packed[b]	0.037	0.114
Vacuum packed[a]	0.062	0.060
Vacuum packed[b]	0.018	0.012

[a]No propyl gallate
[b]With propyl gallate

the system was related to the pH of the prepared proteins which ranged from 6.5–6.7. The adducts used in these experiments included sucrose, glucose, fructose, corn starch, corn syrup solids (42DE) and Morex (8DE). After drying to a moisture content of 4%, the solubility of the protein in 5% salt was measured. For subsequent testing, the products were stored in screw-cap bottles. Results of this experiment are shown in Figures 7 and 8. In the presence of free sugars (Fig. 7) glucose and sucrose, the salt solubility of the protein is almost directly related to the sugar content. Glucose exerted slightly more protection than sucrose at lower concentration. The effect of fructose is interestingly different from glucose and sucrose. At concentrations from 0–15%, fructose exerts practically no effect on the solubility of the protein. Only after the fructose concentration exceeds 15% does the effect on salt solubility of the protein become apparent. It is also interesting that at the lower concentrations glucose offers slightly more protection to the protein than does sucrose. This difference may be due to the fructose moiety of the sucrose.

The effect of corn starch and partially degraded starch is shown in Figure 8. Corn syrup solids with a dextrose equivalent (DE) of 42 were superior to all other additives tested, the protein reaching about 70% solubility at the 25% level, about the same degree of solubility the myofibrillar protein possessed before drying. Morex was about 15% less effective than corn syrup solids at all levels of addition. Corn starch exhibited no effect. The protection afforded by the carbohydrate treatment was found to be of short duration. After 3 wk storage at room temperatures, salt solubility significantly began to decrease. After 6 wk, about 70–80% of the original solubility in 5% salt was lost. Based on work that is currently in progress, there is evidence to indicate that the decrease in salt solubility during storage is related to the lipid residues associated with the myofibrillar protein.

Effect of propyl gallate and atmosphere. Freeze-dried samples of myofibrillar protein were white in color and remained organoleptically stable for about 7–14 days of storage at room temperatures in air-permeable containers. Longer storage periods resulted in the development of off odor and flavor–these flavors being typified as rancid or "fishy." Vacuum packing aided in maintaining the original organoleptic integrity of the product by retarding lipid alteration. It did not, however, prevent some organoleptic deterioration from occurring during storage. The best results were obtained when propyl gallate (PG) (0.01% based on weight of wet muscle) was added during the aqueous-salt extraction.

Table 1 shows the difference in the amount of malonaldehyde formed during 6 months of storage of freeze-dried myofibrillar protein prepared in the presence and absence of PG and stored under air and vacuum. The air-packed samples prepared in the presence of PG had lower initial TBA values. However, during the 6-month storage period, oxidation as measured by TBA values reached the same levels in both treated and untreated samples. The combination of treating with PG followed by vacuum packing showed the best results both from the standpoint of malonaldehyde formation and organoleptic testing. The vacuum-packed samples were organoleptically superior to the air-packed samples. There was evidence of flavor intensification in the vacuum-packed samples, however, after the 6-month storage period showing that while TBA values are indicative of oxidative changes, they do not reflect all of the organoleptic changes that occur in the system. The higher initial TBA values in the samples prepared with no propyl gallate show that there is a considerable amount of lipid alteration initiated during the extraction procedure. This observation was recorded in several experiments and with other species of fish such as Pacific herring (*Clupea pallasi*) and anchovy (*Engraulis mordax*).

Effect of solvent extraction. Although it is possible to prepare organoleptically stable myofibrillar protein isolates by extracting with hot polar solvents such as isopropanol, the isolates have drastically reduced functional properties. For example, freshly prepared freeze-dried myofibrillar protein will emulsify approximately 185g oil/g protein. Extracting the myofibrillar protein with IPA (at 20°C) prior to freeze drying reduces the emulsifying capacity to 136g oil/g protein. Extractions carried out at 50°C reduce the emulsifying capacity to less than 90g oil/g protein and at 70°C all emulsifying capacity is destroyed. In addition, extractions at temperatures lower than 50°C do not effectively remove all of the lipids, particularly the phospholipids, and these preparations suffer organoleptic deterioration after about 1 month of storage at room temperatures. Extraction of the dry myofibrillar preparations with nonpolar solvents such as hexane (either hot or cold) did not remove sufficient phospholipids to insure against organoleptic deterioration during storage. Subsequent work showed that if myofibrillar protein was modified by partial hydrolyses with a proteolytic enzyme and recovered as a phosphate complex, the lipids could be removed by extracting the wet complex with IPA resulting in small losses of functional properties. Data supporting these observations are presented in the second paper of this series.

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APPROACHES TO THE UTILIZATION OF FISH FOR THE PREPARATION OF PROTEIN ISOLATES
Enzymic Modifications of Myofibrillar Fish Proteins

INTRODUCTION

SPINELLI ET AL. (1972) previously reported that fish tissue could be separated by aqueous procedures into two major protein fractions of which the myofibrillar fraction showed the greater promise for further processing into functional protein isolates. The work showed that dried myofibrillar proteins prepared by an aqueous fractionation system lost important functional properties and developed off flavors and odors during storage.

We concluded that the sensitivity of fish proteins to physical and chemical alterations precluded their use as functional ingredients in foods unless they could be modified so that desirable functional characteristics could be preserved during processing and subsequent storage.

The use of proteolytic enzymes to produce soluble protein hydrolysates from fish proteins has been studied by several groups. Cheftel et al. (1971) describe the use of proteolytic enzymes to solubilize fish protein concentrate. The use of raw fish as a substrate for proteolytic enzyme hydrolysis was studied by Sen et al. (1962) and Sripathy et al. (1964). Some of the problems associated with such procedures are the prolonged times required for the complete solubilization of the proteins and the development of bitter flavors due to the formation of peptides.

The possibility that some of these difficulties could be avoided by the use of a system in which the degree of enzyme hydrolysis was controlled was suggested by the work of Fujimaki et al. (1970), who studied the relationship between the degree of hydrolysis of soybean protein and the development of bitterness, and by the work of Rutman (1971), who developed a process for preparing a soluble fish protein concentrate from eviscerated and deboned fish.

In the following work, a study was made on the feasibility of modifying myofibrillar fish proteins by partially hydrolyzing them with proteolytic enzymes. Our specific objective was to establish a basic procedure by which the partially hydrolyzed proteins could be isolated and processed into functional and organoleptically stable preparations.

MATERIALS & METHODS

Hydrolysis

Substrate preparation. Myofibrillar protein prepared from rockfish fillets as described by Spinelli et al. (1972) was used as substrate.

Enzyme. Rhozyme P-11 was obtained from Rohm and Haas, Special Products Dept. This enzyme preparation is standardized to contain 10,000 casein solubilization units per gram.

Determination of hydrolysis rates. A slurry of myofibrillar protein and water containing 3.5% protein was prepared and the pH was adjusted to 6.5 The slurry was then heated to 30°C. Rhozyme P-11 was added at enzyme-to-protein ratios varying from 1:25 to 1:100. The samples were incubated at 30°C and aliquots were removed periodically. They were mixed with an equal volume of 10% trichloroacetic acid to stop the reaction. The samples were allowed to stand for 30 min and were then filtered. The nonprotein nitrogen content of the filtrate was determined by the method of Lowry et al. (1951).

Hydrolysis conditions. Unless stated otherwise, hydrolysates were prepared using slurries of myofibrillar protein and water containing 3.5% protein. The enzyme-to-protein ratios were 1:75. Hydrolysis was conducted for 1 hr at 30°C, pH 6.5–6.7. No pH adjustments were made as it was found that the pH dropped only about 0.2 of a unit under these hydrolysis conditions.

Recovery of enzymically modified myofibrillar protein

Isolectric precipitation. After completion of the hydrolysis period, the pH of the hydrolysate was lowered to 4.5 by the addition of 1N H_2SO_4. The isoelectric point was found to be between 4.0–5.0. The precipitated protein was collected by centrifugation.

Recovery of protein as a phosphate complex. After completion of the hydrolysis period, a solution of 5% sodium hexametaphosphate (HP) (based on weight of protein) was added to the hydrolysate. The pH of the mixture was then lowered to precipitate the protein-phosphate complex. Care had to be taken during the acidification. The solution containing the protein-phosphate complex was very viscous. If mixing was not adequate, areas of high acid concentration formed. Under these conditions, the complex formed gelatinous masses instead of a fine precipitate.

To avoid this problem, the acid was added in two stages. The pH was first lowered to pH 5.5 by slowly adding dilute acid (0.1N H_2SO_4). At this pH the complex began to precipitate, reducing the viscosity of the solution. Further pH adjustments could then be made with stronger acid (1.0N H_2SO_4). The precipitated protein-phosphate complex was then collected by centrifugation.

Effect of HP concentration and type of acidulent on protein recovery. HP was added to samples of hydrolysate at concentrations of

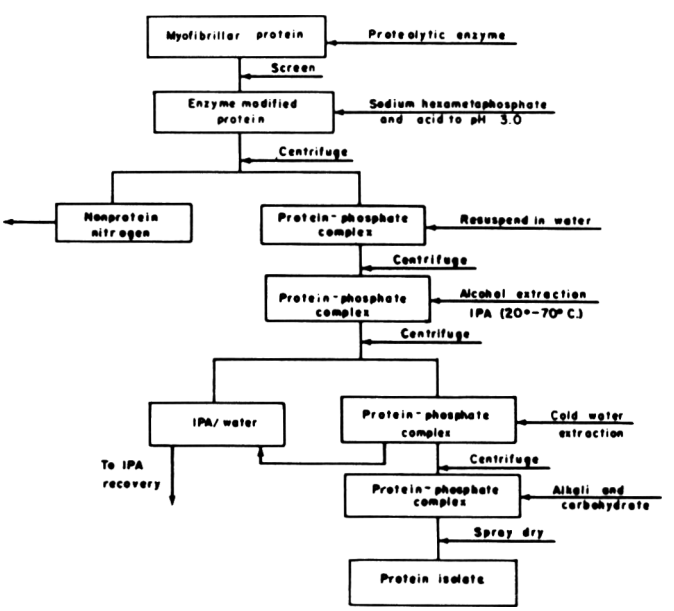

Fig. 1—Preparation of enzymically modified myofibrillar proteins.

0–10% (based on the weight of protein). H_2SO_4, HCl, H_3PO_4, and lactic acid were used to acidify the solutions to pH 3.8.

The precipitated complexes were then centrifuged for 10 min at 2500 rpm. Aliquots of the supernatant were analyzed for protein and nonprotein nitrogen by the method of Lowry et al. (1951). The difference between these two values was used as an indication of the amount of protein left in solution. The solids content of the precipitate was also determined by drying in an air oven at 103°C for 24 hr.

Lipid extraction

Azeotropic isopropanol extraction. The residual lipids were removed from the wet acidic protein-phosphate complex by extracting it three times with azeotropic isopropanol (AzIPA). A ratio of 1 part AzIPA to 1 part wet complex was used. Extraction temperatures used were 20°, 50°, 60° and 70°C. The complex was held at the desired temperature for 5 min during each extraction.

Estimation of residual lipids. 5g of dry protein material were weighed into a cellulose extraction thimble and refluxed in a Goldfish extractor for 24 hr with methylene chloride.

Isopropanol removal. After AzIPA extraction, residual alcohol was removed by extracting the complex four times with water at 20°C. Ratios of 1 part water to 1 part complex were used for each extraction.

IPA determinations. Residual IPA was determined by the method of Smith and Brown (1969).

Drying

Spray drying. For spray drying, a Nichols/Niro Spray Dryer Model IV with nozzle atomization was used. Inlet temperature 280°C, outlet temperature 100–105°C.

Freeze drying. For freeze drying, a Thermovac Model FDC-IND-32F was used. Shelf temperatures were 25° ± 5°C.

Preparation of enzymically modified myofibrillar protein (EMMP)

Samples of EMMP were prepared as schematized in Figure 1. Sufficient myofibrillar protein and water were mixed to form a slurry with a protein concentration of 3.5%. The slurry was heated in a water bath to 30°C and Rhozyme P-11 was added at a ratio of 1 part enzyme to 75 parts protein. The sample was then incubated for 1 hr at 30°C. The pH during hydrolysis was 6.5–6.7. At the end of the incubation period, a solution containing 5% HP (based on weight of protein) was added. The pH of the mixture was then lowered to 3.0 with H_2SO_4 in two stages as described previously.

The insoluble protein-phosphate complex was collected by centrifugation and washed twice by suspending it in water. Residual lipids were then removed by extracting the wet complex three times with AzIPA at 50°C. After AzIPA extraction, residual alcohol was removed by washing the complex four times with water. Ratios of 1 part extracting solution to 1 part wet complex were used throughout. The protein-phosphate complex was then neutralized (pH 7.0) with 1.0N NaOH. If desired, carbohydrates were added at this stage. The complex was then spray dried or freeze dried.

Determination of functional properties

Emulsifying capacity. Described in the first paper of this series (Spinelli et al., 1972).

Emulsifying stability. A 1.0g samples of protein was suspended in 75 ml of 0.1M, pH 7.0 citrate-phosphate buffer. The protein suspension and 25 ml of oil were placed in a Waring Blendor and mixed at 16,000 rpm for 2 min. The emulsion was then poured into a graduated cylinder and allowed to stand at room temperature until emulsion breakdown, indicated by a phase separation, was observed. The time between emulsion formation and phase separation was recorded.

Solubility in water. 1g of protein was dispersed in 100 ml of water and stirred for 1 hr. The pH of the solution was 7.0. The suspension was then filtered through Whatman No. 41 filter paper to remove insoluble material. The protein content of the filtrate was determined by the method of Lowry et al. (1951). The results were expressed as follows:

$$\% \text{ soluble protein} = \frac{\text{g protein in filtrate}}{\text{g protein in sample}} \times 100$$

RESULTS & DISCUSSION

Process parameters

Hydrolysis. Hale (1969) determined the relative activities of commercially available proteolytic enzymes using washed, freeze-dried fish protein as substrate. Several of these enzymes were evaluated and found to be suitable for partially hydrolyzing myofibrillar protein. For the purpose of this study, however, Rohm and Haas Rhozyme P-11 was used throughout.

The rate of myofibrillar hydrolysis at various enzyme-to-substrate ratios was determined. The results are shown in Figure 2. On the basis of these data, we selected enzyme-to-substrate ratios of 1:75 for use in preparing partially hydrolyzed myofibrillar protein. At higher ratios of enzyme to substrate, the hydrolysis rates were considered too rapid for adequate control, a condition that could lead to extensive formation of nonprotein nitrogen. This could result in decreased yields of protein.

Recovery of protein as a phosphate complex. A formidable obstacle in preparing and then recovering a partially hydrolyzed (by enzyme) protein from solution is that of terminating the reaction at any given time. In our work, heat could not be used for terminating the reaction because of the effects of heat denaturation on the proteins. Isoelectric precipitation was not satisfactory for two reasons. First, yields were not quantitative at the isoelectric point, and second, at pH values in the region of the isoelectric point, enzymic activity remained high, and proteins passed back into solution. The activity of Rhozyme P-11 did not diminish rapidly until the pH was lowered to below 3.5.

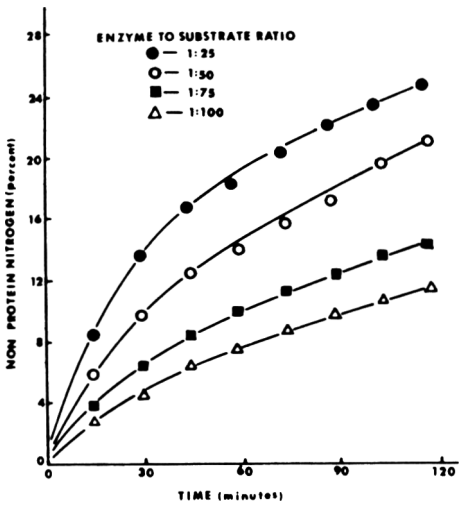

Fig. 2–Rate of myofibrillar protein hydrolysis at 30°C, pH 6.5, using Rhozyme P-11 at enzyme-to-substrate ratios varying from 1:25 to 1:100.

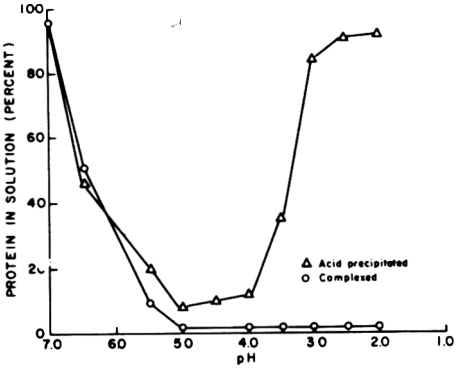

Fig. 3–Recovery of enzymically modified myofibrillar protein by phosphate complexing and isoelectric precipitation.

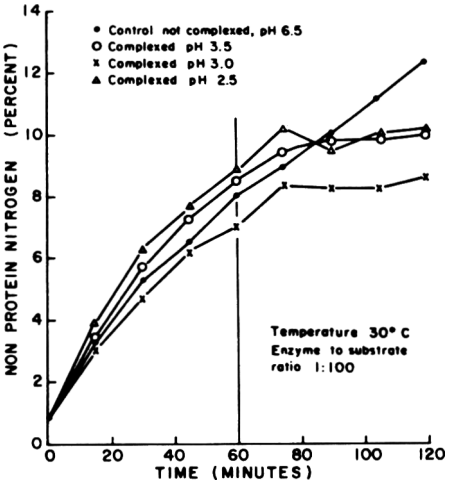

Fig. 4–Enzyme activity of Rhozyme P-11 after complexing myofibrillar protein at pH 2.5, 3.0 and 3.5. Complexing was done 60 min after starting the hydrolysis.

Utilizing the reaction between HP and protein in an acidic medium to form an insoluble protein-phosphate complex provided both the means of terminating the reaction and that of quantitative recovery of the proteins. In addition, the complexed proteins could be washed free of occluded nonprotein nitrogen components with no loss of protein nitrogen. These points are shown in Figures 3 and 4. Figure 3 shows that about 90% of the EMMP can be recovered by isoelectric precipitation, i.e., in the pH region of 4.0–5.0. At pH values below 4.0, recoveries of EMMP become poorer because proteins pass back into solution. On the other hand, by complexing the proteins (Fig. 3) quantitative recoveries of proteins could be obtained at pH values ranging from 2.0–5.0. Thus, by adjusting the pH of the reaction mixture to a pH in the region of 2.5–3.5, the proteins could be recovered in the relative absence of enzymic activity (Fig. 4).

Effect of HP concentration and type of acidulent on protein recovery. Protein-phosphate complexes were prepared using various concentrations of HP and using various acidulents. The results of these experiments (Fig. 5) show that when using H_2SO_4, 95% of the protein was recovered using 3% HP and 100% was recovered using 5% HP. When HCl, H_3PO_4, or lactic acid was used, quantitative recoveries were not obtained until 6% HP was added. The physical characteristics of the complex, as reflected by the solids content of the centrifuged complexes, are given in Figure 6. Maximum solids concentrations of 12.5% were obtained with H_2SO_4 and 3% HP. With HCl, H_3PO_4, and lactic acid, 10% HP was required to achieve approximately the same solids concentration. With lactic acid, gelatinous precipitates were obtained at HP concentration between 1% and 4%. This indicates that at the same hydrogen ion concentration, the formation and physical characteristics of the protein-phosphate complex are influenced by the anionic component of the acid.

Lipid extraction and solvent removal. It is generally accepted that lipid residues in FPC must be lower than 0.5% to prevent lipid alteration during storage. Dambergs (1969) in 10-yr storage tests found, however, that in order to insure complete stability in FPC prepared from cod, lipid residues had to be less than 0.05% in preparations containing more than 4% water-soluble materials. Although not specifically identified, these water-soluble materials were undoubtedly components found in the sarcoplasmic fraction, since myofibrillar proteins are insoluble in AzIPA. Without solvent extraction, protein-phosphate complexes contain approximately 2% lipid on a dry weight basis. Our work on storage stability definitely shows that in order to maintain the organoleptic quality expected of a commercially acceptable protein isolate, the lipid content should be under 0.2%. Samples of EMMP extracted at 50°C with AzIPA and containing lipid residues averaging 0.15% began to show some flavor reversion after 2 months of storage. Samples of EMMP extracted at 70°C show lipid residues ranging from 0.05–0.120%. Organoleptic assessment of these products is in progress.

We found that removal of lipids from the complex by solvent extraction is preferably done with AzIPA from the wet state. The removal of lipids from dry EMMP with IPA or hexane did not yield a consistently acceptable product, probably due to lipid-protein binding that had occurred during drying.

Solvent removal of lipids from wet protein does, however, present problems, the major one being denaturation of the proteins and the difficulty associated with removal of the solvent from the product. For example, in the preparation of FPC (Ernst, 1971), final desolventization of the FPC to meet current 250 ppm IPA residue standards, requires steam stripping after air or vacuum drying. Steam stripping is a severe treatment and attempts to remove residual IPA from EMMP in this way destroyed its functional properties.

We found that solvent removal could be accomplished with minimal effects to the functional properties of the EMMP by first extracting the wet protein-phosphate complex with AzIPA and then re-extracting the residual IPA with water. As shown in Figure 7, the IPA content of the complex was reduced from 60% to 3% by four aqueous extractions.

The difficulty of removing the residual IPA is also shown. Regardless of the initial quantity of IPA in the complexed protein, approximately 0.5% remained associated with the protein after it was freeze-dried. Spray drying the water-extracted complex reduced the IPA content to less than 50 ppm.

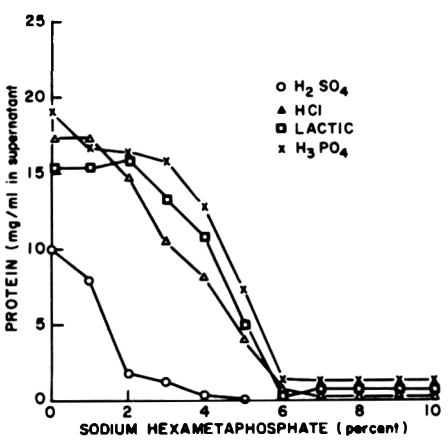

Fig. 5—Recovery of enzymically modified myofibrillar protein using four different acids for pH adjustment.

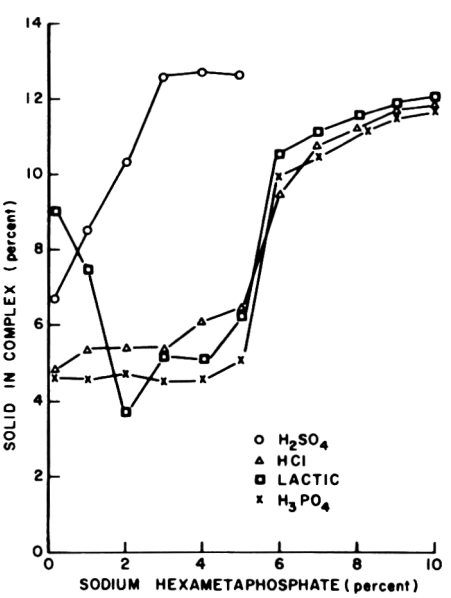

Fig. 6—Solids content of enzymically modified proteins recovered at various concentrations of sodium hexametaphosphate using four different acids for pH adjustment.

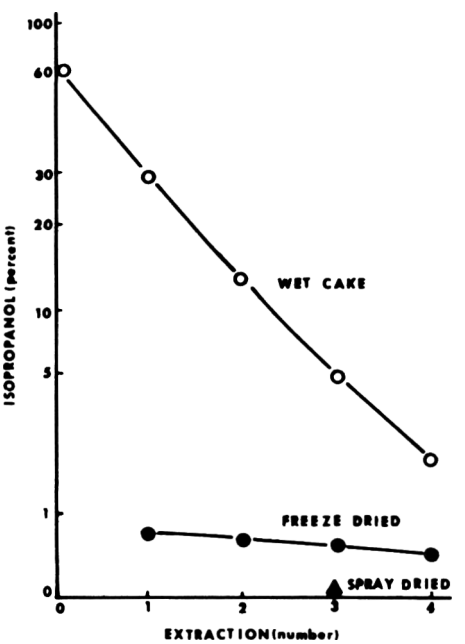

Fig. 7—IPA reduction in complexed wet cake after four stages of aqueous extraction, and the corresponding IPA left in the product after freeze drying. Also shown is the amount of IPA in the spray-dried (dried after the third aqueous state) product.

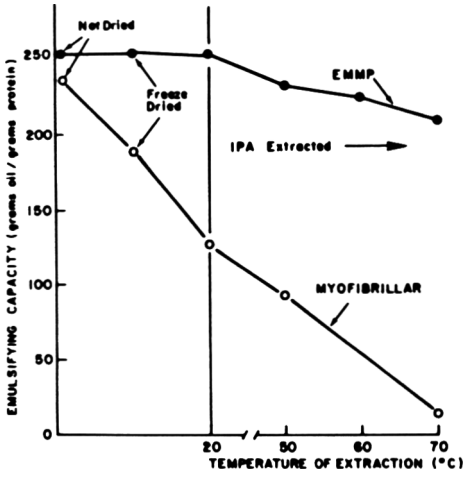

Fig. 8–Effect of freeze drying and extraction with AzIPA at various temperatures on the emulsifying capacity of EMMP and myofibrillar fish proteins.

Table 1–Emulsion stability and emulsifying capacity of enzymically modified myofibrillar proteins and sodium caseinate

Hydrolyzed (min)	Emulsion stability (min)	Emulsifying capacity (g oil/g protein)
0	30	145
15	60	225
30	120	225
60	90	231
90	90	224
Na caseinate	20	140

Properties of EMMP

Emulsifying characteristics. During this phase of our study, criteria for assessing the functional properties of EMMP were limited. Our main index for functionality was the ability of the protein to emulsify lipids. We chose emulsifying capacity because of the importance of this property in the food industry. Also preliminary data showed that a decrease in emulsifying capacity during storage generally correlated with changes in organoleptic quality and loss of solubility. We recognize that the ability to emulsify lipids is not necessarily a criterion for the ability of a protein to perform other important functions.

Table 1 shows the relationship between the degree of hydrolysis and the emulsifying capacity and emulsion stability of the resulting EMMP. With these preparations, residual lipids were not extracted prior to drying. For comparative purposes, the emulsifying capacity and stability of sodium caseinate was also determined.

EMMP isolated after 30 min of hydrolysis made the most stable emulsions. Stability decreased in samples isolated after 60 min of hydrolysis. No further decreases were noted in samples hydrolyzed for 90 min. The emulsifying capacity of EMMP did not show a similar correlation. Without hydrolysis, the emulsifying capacity of the samples was 145g oil/g protein. This value increased to 225g oil/g protein in samples hydrolyzed for 15 min. No further increases were obtained from longer hydrolysis times.

Extraction of the wet complex with AzIPA at 50°, 60° and 70°C reduced the emulsifying capacity of the complex by 13%, 14% and 19%, respectively (Fig. 8). This reduction is considerably less than that experienced with unmodified myofibrillar protein where AzIPA extraction at 50°C reduced the emulsifying capacity by over 50% and extraction at 70°C completely destroyed this property.

Composition and protein efficiency ratio (PER)

When prepared using the conditions described in Materials & Methods, the EMMP is white in color, has a bland flavor, and is nonhygroscopic. The proximate composition (dry basis) of a typical preparation is as follows: protein, 93.5%; lipid, 0.15%; phosphate, 1.4%. The PER of a freeze-dried sample of EMMP was 3.1 compared to a value of 3.0 for a standard reference casein.

Solubility and dispersion characteristics

Freeze-dried EMMP is 20% soluble in both water and 5% salt. About 220g of oil are emulsified per g of protein. The solubility and emulsifying capacity of spray-dried EMMP are about 5% lower than those of freeze-dried samples. EMMP is easily dispersed in water. The resulting dispersions are colloidal in nature, forming very light sediments on standing.

Preliminary studies on the stability of the EMMP showed that in some samples both loss of emulsifying capacity and flavor reversion began to occur after 2 months of storage at room temperature.

It may well be that the concentration of lipid residues in the EMMP samples is not low enough. In one series of tests, the emulsifying capacity of EMMP that had not been solvent-extracted (3.0% lipid) decreased 60% after 2 months of storage at ambient temperature. The emulsifying capacity of a corresponding EMMP sample that had been extracted with AzIPA at 50°C (0.2% lipid) decreased by only 15% during the same period.

Medwadowski et al. (1967) found in their studies with fish protein concentrate that small amounts of lipids remain tenaciously associated with the product, even after prolonged extraction with organic solvents. It is likely that even small quantities of lipid are sufficient to cause alterations in proteins by free radical reactions as proposed by Roubal and Tappel (1966) and Roubal (1971). The relation of lipid residues and their effect on the properties of EMMP needs further study.

CONCLUSIONS

THE FOREGOING work shows that fish protein isolates possessing important functional properties can be prepared from myofibrillar proteins that are partially hydrolyzed with a proteolytic enzyme. The partially hydrolyzed proteins are recovered and processed in the relative absence of enzymic activity by complexing them with a condensed phosphate such as sodium hexametaphosphate. Most of the residual lipids associated with the complexed protein can be removed by extracting the wet acidic complex with isopropanol prior to neutralizing and drying. Extracting the wet complex protein with AzIPA at temperatures ranging from 20°–70°C does not seriously impair the functional properties of the proteins.

The preparation of isolates with long-term storage stability has yet to be demonstrated. It is suspected that small quantities of residual lipids are implicated in the product alterations that occur during storage.

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POST-MORTEM BIOCHEMICAL CHANGES IN THE MUSCLE OF GULF SHRIMP, Penaeus aztecus

INTRODUCTION

SHRIMP from the Gulf of Mexico is one of the most important seafoods landed by fishermen in the United States. In spite of its great popularity as a food, relatively little attention has been given to the post-mortem biochemistry of shrimp from this area. Tarr and Comer (1965) investigated the nucleotides, sugars and homarine in pink shrimp (*Pandalus borealis*) caught in Canada; Japanese workers (Arai, 1966; Nakajima, 1961) studied adenine nucleotide degradation in the prawn. Dingle et al., (1968) reported on adenosine triphosphate and related decomposition products in the American lobster; Porter (1968) made similar measurements in the king crab and Bailey et al. (1956) determined the relationships between glycogen, lactic acid or pH and sensory quality in Gulf shrimp.

At the time this study was undertaken, no research had been reported on the changes in the adenine nucleotide derivatives in the muscle of nonstressed, Gulf of Mexico shrimp, measured at close intervals from the point of death through an extended storage period.

Consequently, this study was conducted to make qualitative and quantitative determinations of adenosine triphosphate and its degradation products to hypoxanthine in rested shrimp muscle stored at 0°C. Thin-layer chromatography was used for separation of the nucleotides with subsequent elution and spectrophotometric analysis for quantitative determinations. As a supplement, glycogen, lactic acid and orthophosphate were measured and pH changes were determined. These data were used to compare post-mortem degradative pathways in shrimp with those in other marine and terrestrial animals, to provide a meaningful biochemical definition of rigor mortis in shrimp muscle, and to ascertain the possibility of some of these degradation products being used as quality indices in low-temperature-stored shrimp.

EXPERIMENTAL

THE TWO PRINCIPAL varieties of shrimp that inhabit Louisiana's salt water lakes and bayous are the brown (*Penaeus aztecus*) and the white (*Penaeus setiferus*) shrimp. The brown shrimp migrate into inland waters about early May with the white shrimp appearing in October. Unfortunately, premature cold weather as well as tropical weather disturbances in the Gulf affect the quantity of white shrimp found in inland fishing waters. Because of the uncertainty of acquiring white shrimp throughout the shrimping season, only brown shrimp were used in this study.

Collecting and handling of samples

In many studies concerning post-mortem biochemical characteristics of fish and shellfish muscles, the samples were obtained from commercial fishing sources and the animals were permitted to undergo severe exhaustion before death. Inasmuch as this experiment concerned several compounds that would undergo marked ante-mortem degradation during animal stress, commercial shrimping methods for collecting the samples were not used in this study. The shrimp were originally caught in a bayou several miles inland from the coast of Louisiana and transported alive on the trawler to shrimp boxes where they were held for later distribution as fish bait. The shrimp collected for analyses were held 2–4 days during which time they were fed.

The shrimp were removed from the boxes and killed immediately by removing their heads with a scissors. This method of slaughter was found to be quickest and minimized stress. The shrimp which were to be analyzed at zero time post-mortem were immediately dropped into liquid nitrogen. Those to be analyzed following various storage periods were placed into polyethylene bags and packed in crushed ice. Collection began in early May, near the beginning of the shrimping season, with four collections being made throughout the summer at approximately 4-wk intervals.

Each collection of shrimp was analyzed for nucleotides, orthophosphate, glycogen, lactic acid and pH. Two samples of 30 shrimp each were homogenized for each analysis. The shrimp were analyzed according to the following schedule of storage time: 0, 6, 12, 24, 48, 72, 96, 168 and 240 hr. The storage condition was ice-pack.

Nucleotide analysis

In preparing the samples for analysis, two samples of 30 shrimp each were quickly frozen in liquid nitrogen and were then ground to a fine powder in a cold-room at −20°C. Homogeneous aliquots of the two powdered samples were used for each analysis.

Extraction was conducted in a refrigerated room at approximately 3°C. Two 30g samples of the frozen shrimp were rapidly weighed and placed into 120 ml of ice-cold 0.6N perchloric acid and homogenized at high speed for 1 min in a Virtis 45 homogenizer at 0°C. The homogenate was filtered and the filtrate was immediately adjusted to a pH of 6.5 with potassium hydroxide and allowed to stand at 3°C for 30 min for potassium perchlorate to precipitate. The supernatant was filtered again.

1 vol of prewashed polyethyleneimine cellulose and 6.5 vol of water were homogenized for 20 sec and the slurry poured into the thin-layer applicator that was used to quickly coat clean glass plates with 0.5 mm-thick layers and allowed to dry.

The samples were applied in quadruplicate with 20 μℓ pipettes on a starting line 3.0 cm from the lower edge of the plate. A standard mixture, containing adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (INO) and hypoxanthine (Hx), was applied to a point on the starting line with a 10 μℓ pipette. The standard mixture contained 10 μm of each compound per 10 μℓ.

Ascending chromatography was carried out in closed tanks according to the procedure prescribed by Randerath and Randerath (1965). The plate was first developed in distilled water and dried in a current of warm air, then developed in a discontinuous stepwise procedure comprised of three transfers without intermediate drying with lithium chloride at strength of 0.2M, 1.0M and 1.6M.

The spots were located by viewing under ultraviolet light and identified by comparison with the standard. Each spot was circled and subsequently scraped from the plate into tubes containing 2 ml of 2N HCl. The tubes were allowed to stand for 18 hr, then centrifuged. The supernatant was measured spectrophotometrically for absorbance of ultraviolet light. ATP, ADP, AMP and IMP were measured at

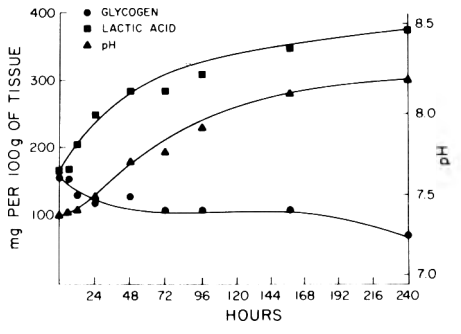

Fig. 1—Changes in glycogen, lactic acid and pH concentrations in shrimp muscle stored at 0°C.

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259 nm wavelength and inosine and hypoxanthine were measured at 249 nm.

Glycogen, lactic acid, pH and orthophosphate analysis

Glycogen was determined with the frozen, powdered shrimp using combined procedures of Carroll et al. (1956) and Montogomery (1961). Lactic acid was determined using the procedure of Barker and Summerson (1941). For pH determination, 10g of the powdered shrimp was placed in 90 ml of 0.005M sodium iodoacetate solution and blended. The pH was measured at 23°C with an expanded scale pH meter. Orthophosphate was determined using the procedure of Mozersky et al. (1966).

RESULTS

THE DEGRADATION of ATP in the tail muscle of unexercised brown shrimp (*Panaeus aztecus*) from the Gulf of Mexico followed the route: ATP to ADP to AMP to IMP to Ino to Hx since only inosine monophosphate was found on the thin-layer chromatograms. If adenosine was an intermediate in the degradation pathway, its presence should have been observed. This pathway has been found in terrestrial animals, marine vertebrates and in several marine invertebrates including the king crab and lobster.

This is not in agreement with Japanese workers on several species of marine invertebrates and prawns (Arai, 1966) where it was found that ATP degradation favored the following pathway: ATP to ADP to AMP to Adenosine (Ad) to Ino to Hx. However, the former decomposition pathway is in agreement with Tarr and Comer (1965) who reported the mechanism for ATP degradation in pink shrimp.

The progression of changes in shrimp muscle at 0°C, as revealed by quantitative analysis of the nucleotides at various time intervals postmortem, is illustrated in Figure 1.

The initial concentration of ATP in shrimp muscle, 6.1 (μm) indicated that it probably was in a nonstressed condition. This value is close to those reported for rested cod muscle by Jones and Murray (1961) and Fraser et al. (1961) who reported 5.43 and 5.2 μm/g, respectively. Porter (1968) found 3.8 μm of ATP per g of crab muscle and Nakajima et al. (1961) reported 3–5 μm/g for prawns. The decrease in ATP was relatively slow. At 6 hr it was 5.0 and at 12 hr, 4.7. Even at 96 hr it was present in detectable amounts. It was impossible to correlate this change with physical rigor since the shrimp tails remained tender and soft during the entire storage period of 10 days and did not exhibit any of the characteristics commonly associated with rigor mortis. If rigor is to be defined in Gulf shrimp, some definition based on chemical composition or a change in chemical composition will have to be utilized.

Adenosine diphosphate was present in the relaxed shrimp muscle in relatively low concentrations initially, 1.7 μm/g, and decreased rapidly postmortem. The level of AMP remained virtually constant near 0.65 μm/g of tissue during the entire storage period.

The initial levels of IMP were quite high and after four days increased to 5.3 μm/g of tissue. After the fourth day the level gradually decreased to 2.9 μm at 10 days storage. The increase of IMP up to 4 days was contrary to that found in cod muscle by Fraser et al. (1967) who found a continuous decrease in relaxed cod muscle, but was similar to other fish species investigated by Kassemsarn et al. (1963). Tarr and Comer (1965) found relatively high levels of IMP in fresh pink shrimp.

Inosine was first detected in the muscle at approximately the same time ATP had decreased to near half of its original concentration, which was 3.0 μm/g at approximately 24 hr. Inosine accumulation in shrimp appears to be closely related to ATP disappearance.

Hypoxanthine was first detected in low quantities, 0.4 μm/g, after 48 hr. It showed a consistent increase throughout the 10-day period increasing to 4.5 μm/g. The data indicate that hypoxanthine is a degradation product of ATP and that after an extended period of low-temperature storage, hypoxanthine accumulates in substantial quantities in shrimp muscle. Had the experimental storage period extended longer, the hypoxanthine values would have possibly increased more. These high hypoxanthine values were revealed somewhat earlier than at the 16 days reported for trawled gutted cod by Jones (1965); however, Spinelli (1967) found hypoxanthine present in halibut after 3 days of ice-storage.

As hypoxanthine is the purine residue from inosine degradation, it would be expected that the hypoxanthine increase would be associated with an inosine decrease; however, this was not the case. It is possible that an extension of the storage period would show a decrease in inosine while hypoxanthine continued to increase. Similar results showing increases in inosine monophosphate, inosine and hypoxanthine have been reported by Dingle and Hines (1971) in halibut fillets from "zero" time to 192 hr of storage. This unexpected relationship between these compounds is a break in the degradation pattern usually found.

Concomitant with glycogen degradation was an increase in lactic acid content in shrimp muscle as shown in Figure 2. Glycogen decreased from 160 mg per 100g of tissue to 70 mg by the tenth day of storage. During this time, lactic acid increased from 160 mg per 100g of tissue to 370 mg. These values are low when compared to the concentrations usually found in unexercised mammels. However, glycogen and lactic acid in marine animals are generally lower than those in warm blooded animals. The values presented here for shrimp are somewhat higher than corresponding values reported for cod by Fraser et al. (1965) reported a concomitant increase in glycogen and lactic acid for the first 4 days in iced shrimp with maximum values of 135 and 140 mg per 100g of tissue, respectively.

The pH increased from 7.4 to 8.2 in 0 to 10 days. This is in agreement with the findings of Bailey et al. (1965). With advanced bacterial spoilage, increases in pH are usually observed in fish and shellfish; however, in this study pH measurements were made as early as 6 hr postmortem and no decrease was measured. An explanation for this may be associated with a release of low molecular weight bases in the tissue soon after death throughout analysis and microbiological action. Crustacean muscle also contains higher concentrations of nonprotein nitrogenous compounds than fish or mammals.

Another unusual characteristic of shrimp muscle was the decrease in orthophosphate during post-mortem storage (Table 1). This was also reported by Bailey et al. (1956) in their studies with

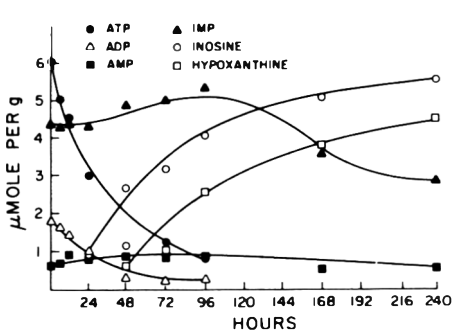

Fig. 2—Nucleotide degradation in shrimp muscle stored at 0° C.

Table 1—Average values and standard deviation for orthophosphate in post-mortem shrimp muscle stored at 0° C[a]

Storage time (hr)	Orthophosphate (μg/100g tissue)
0	3078 ± 7.8
6	3000 ± 6.6
12	2900 ± 5.9
24	2100 ± 4.2
48	1675 ± 4.4
72	1500 ± 9.9
96	1360 ± 11.1
168	794 ± 9.8
240	725 ± 8.8

[a]Data represent averages for four collections of shrimp.

ice-stored shrimp. In fish and rabbit muscle, inorganic phosphate increased in the muscle with the dephosphorylation of sugar phosphates and nucleotides. The loss in shrimp may have been due to leaching during storage.

DISCUSSION

THE SHRIMP used in this study were presumably in good post-nutritive condition and not under stress at the time of death. The data represent four collections made throughout the shrimping season, large sample sizes for each analysis (60 shrimp), close observations of early post-mortem changes (measurements at 6, 12 and 24 hr), careful control of all experimental conditions affecting the samples and improved analytical procedures, particularly in the case of the nucleotides. Consequently, the data should be a representative and useful elucidation of some important post-mortem biochemical changes in nonstress Gulf shrimp stored at 0°C.

The nucleotide degradation pathway appeared to be the same as that for fish and mammals. Initial levels of ATP were similar in the nonstressed shrimp muscle to those found in unexercised marine and land vertebrates. The initial level of IMP in shrimp, as in other marine species, was considerably higher than that found in mammalian muscle. It is quite probable that IMP makes an important contribution to the characteristic flavor of fresh shrimp. Hypoxanthine, the purine residue of ATP degradation, accumulated in large quantities after a period of time post-mortem.

It is possible that hypoxanthine could be used as an indicator of the length of time shrimp have been held in storage or the way in which they were treated postmortem. No attempt was made in this study to establish a relationship between hypoxanthine concentration and sensory quality. Under commercial conditions, 10-day old iced shrimp show incipient to advanced bacterial spoilage. Perhaps a hypoxanthine value of 4–8 μm/g of muscle may be indicative of incipient spoilage in shrimp. This should be determined with commercially caught shrimp.

Part of the reason for flavor deterioration in ice-stored shrimp could be related to the production of inosine and hypoxanthine, which have been reported (Kazeniac, 1961) as imparting a bitter taste to seafoods, plus the concurrent loss of IMP which is an important flavor complement in seafoods.

Glycogen levels in shrimp were somewhat lower than those for fish. Detectable levels of glycogen may not be expected in commercially-caught shrimp which are severely exhausted at the time of death. The glycogen decrease–lactic acid increase patterns for shrimp are similar to those of other marine and land animals; however, the absence of an initial post-mortem drop in pH is unique.

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STUDIES ON NUCLEOTIDE METABOLISM IN PORCINE LONGISSIMUS MUSCLE POSTMORTEM

INTRODUCTION

STIFFENING of muscle postmortem is related to a decrease in ATP content (Erdos, 1943; Bate-Smith and Bendall, 1947; Bendall, 1951; Marsh, 1952, 1954; Lawrie, 1953). The formation of 5'-AMP results from removal of the two terminal phosphate groups of ATP by the combined catalytic action of ATPase and myokinase. 5'-AMP is rapidly deaminated to inosinic acid (5'-IMP) by the catalytic action of the AMP deaminase as described by Schmidt (1928). Further degradation of 5'-IMP into inosine and hypoxanthine occurs during prolonged storage.

Bendall and Davey (1957) showed that the pre-rigor nucleotide pattern in rabbit muscles consisted of ATP, ADP with a trace of IMP, and a mixture of DPN and TPN. Howard et al. (1960) studied nucleotide breakdown in beef tissue and found that with progressive aging the hypoxanthine levels rose to 1.5–2.0 μmoles/g meat. Lee and Webster (1963) studied the effect of temperature, freezing, frozen storage, thawing and pH on the rate of hypoxanthine production of beef tissue and found that an increase in temperature, and a higher ultimate pH, or thawing of frozen samples stimulated the rate of hypoxanthine production. No increase was noted on freezing or during frozen storage.

Another interesting feature of nucleotide metabolism in muscle is the indication that nucleotides contribute to typical flavor of meat (Jones 1961; Wagner et al. 1963; Kuninaka et al. 1964; Shimazono, 1964). 5'-IMP and 5'-GMP are the main flavor enhancers. There is only a small amount of 5'-GMP in meats. However, 5'-IMP is the major nucleotide in postmortem muscle.

The objective of this work was to establish quantitative information about the concentration of the nucleotides and other related compounds at various times in postmortem muscle of the pig. The work was conducted on muscle that underwent either a fast or slow change postmortem. Additionally, some specific enzyme reactions related to the pathway of nucleotide catabolism were studied.

MATERIALS & METHODS

PIG MUSCLE was obtained from two "stress-susceptible" Poland China and two "stress-resistant" Chester White pigs. Stress-susceptibility of these pigs was determined by the rigor mortis pattern which was determined by following extensibility changes (Schmidt et al. 1968). The pigs were exsanguinated and a pre-rigor sample of longissimus muscle (lumbar) was excised within 5 min. After pre-rigor samples were excised, the carcasses underwent the normal processing procedure. Samples at 3 hr, 24 hr, 72 hr and 144 hr postmortem were obtained from dressed carcasses which had been stored at 4°C. All samples were frozen and stored in liquid nitrogen until analyzed.

Extraction and analysis of the nucleotide mixture

Muscle nucleotides and related compounds were extracted by a method similar to that of Hurlbert et al. (1954). 10g of muscle was pulverized in an aluminum blender cup with added liquid nitrogen (Borchert and Briskey, 1965). The powder was extracted with 20 ml of cold 0.6N perchloric acid. The precipitated protein was centrifuged at 1200 × G for 10 min at 4°C and extracted again with 20 ml of cold 0.2N perchloric acid. Each extraction was carried out by occasional stirring with a glass rod for 30 min at room temperature. The two supernatant fluids were combined and carefully neutralized to pH 6 by addition of 5N KOH and by using a pH meter. The solution was chilled until it was nearly frozen and then filtered through No. 1 Whatman filter paper at 2–4°C in order to remove the $KClO_4$.

The neutralized extracts were analyzed by chromatography on Dowex 1-formate by a procedure similar to that described by Hurlbert et al. (1954). A 10 ml-portion of the neutralized extract was placed on a 24 cm × 1.4 cm Dowex 1-10x formate column and washed into the column with 100 ml of water. 5-ml fractions were collected using a linear gradient (Rosett et al. 1970) of 1,000 ml of 0–2.0M ammonium formate (pH 5). The column was run at room temperature at an average flow rate of 24 ml per hr. The distribution of nucleotides was observed by reading the optical density of each fraction at 260 nm with a Gilford 240 spectrophotometer. A standard nucleotide chromatogram was obtained with known nucleotides. The fractions washed from the column with water were pooled and lyophilized to reduce their volume. A paper chromatographic technique (Fink et al. 1963) was used to identify the nucleotides and bases. The solvent was n-butanol, glacial acetic acid and water in the ratio 50:25:25 v/v/v.

The concentration of the nucleotides and other compounds were calculated on the basis of their maximum absorbance, max $258 = 12.3 \times 10^3$ at pH 6 for inosine, 5'-IMP, 5'-IDP, max $257 = 15.0 \times 10^3$ at pH 2 for 5'-AMP, 5'-ADP, 5'-ATP (Burton, 1969; Dunn and Hall, 1968).

Deaminase activity determination

The method of differential spectrophotometry described by Kalckar (1947) was used to determine AMP, adenosine and adenine deaminase activities. Potassium succinate 0.1M, pH 6.5 (Stone, 1970) was used as an extraction buffer. Extracts for enzyme assay were prepared from each muscle sample by homogenization of 1g of muscle powder with 7 ml of cold extraction buffer with a polytron (Brinkmann, type 10 OD) tissue homogenizer at a rheostat setting of 2 for 2 min. The muscle slurry was then centrifuged at 12,000 × G for 30 min. The supernatant was filtered through two layers of cheesecloth. The substrates were (a) 15 mg of 5'-AMP per liter in 0.1 potassium succinate pH 6.5, (b) 12 mg of adenosine per liter in 0.05M phosphate pH 7.5 and (c) 8 mg of adenine per liter in 0.5M phosphate pH 7.0 for measuring AMP, adenosine and adenine deaminase activities, respectively. The reaction was carried out in a 3 ml quartz cuvette (1 cm light path) containing 0.1 ml of extracted enzyme and 3.0 ml of the appropriate substrate. The rate of decrease in absorbance at 265 mμ and 25°C was determined using a Gilford 240 Automatic Recording Spectrophotometer. The initial slope of the absorbance change versus time was converted to μmoles of substrate deaminated per min per g of muscle by multiplying by reaction volume (liters) and dividing by the difference between the molar absorbancies of the substrate and products at 265 mμ and by the weight (g) of moist tissue represented. Differences in molar absorbance between substrate and product used was 8860 for AMP and IMP (Smiley et al., 1967), 7900 for adenosine and inosine (Hoagland and Fisher, 1967) and 5100 for adenine and hypoxanthine (Hepple et al., 1957).

5'-nucleotidase activity determination

The method used was described by Hepple and Hilmoe (1955). A total volume of incubation mixture of 1.4 ml was made from 0.1 ml of 1.0M glycine-NaOH buffer, pH 8.5, 0.1 ml of 0.1M $MgCl_2$, 1 ml of 3 mM 5'-AMP, and 0.2 ml of enzyme which was prepared from one part of muscle and seven parts of 0.1M Na succinate buffer, pH 6.5. The final pH was 6.5. The mixture was incubated for 15 min at 37°C, the reaction was stopped by the addition of trichloroacetic acid in a final concentration of 5%. Following centrifugation, an aliquot was analyzed for inorganic phosphate by the method of Fiske and Subbarow (1925). A unit of activity corresponds to the liberation of 1 μmole of P

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Table 1—Postmortem changes in the concentrations[a] of nucleotides and related compounds in porcine longissimus muscle

Breeds	Postmortem (time hr)	Inosine + hypoxanthine	DPN⁺ + DPNH	5'-IMP	5'-IDP	5'-ADP	5'-ATP
	0 hr	–	0.62 ± 0.06	2.11 ± 0.16	–	1.09 ± 0.06	4.13 ± 0.16
	3 hr	1.65[b] ± 0.03[c]	0.10 ± 0.07	5.48 ± 0.05	0.59 ± 0.05	0.38 ± 0.08	0.16 ± 0.01
Poland China	24 hr	2.12 ± 0.04	0.10	5.17 ± 0.01	0.54 ± 0.01	0.63 ± 0.05	0.10
n = 2	72 hr	2.75 ± 0.11	0.10	5.03 ± 0.03	0.69 ± 0.15	0.54 ± 0.01	0.10
	144 hr	2.73 ± 0.16	0.10	4.47 ± 0.43	0.46 ± 0.07	0.36 ± 0.05	0.10
	0 hr	–	0.66 ± 0.11	0.78 ± 0.25	–	0.90 ± 0.03	6.04 ± 0.01
	3 hr	1.26 ± 0.13	0.49 ± 0.14	5.67 ± 0.39	0.42 ± 0.09	0.33 ± 0.03	0.38 ± 0.02
Chester White	24 hr	1.72 ± 0.02	0.30 ± 0.14	5.44 ± 0.46	0.69 ± 0.08	0.31 ± 0.08	0.21 ± 0.02
n = 2	72 hr	2.07 ± 0.10	0.17 ± 0.02	5.20 ± 0.42	0.79 ± 0.09	0.37 ± 0.05	0.23 ± 0.01
	144 hr	2.90 ± 0.08	0.15 ± 0.04	4.59 ± 0.53	0.57 ± 0.01	0.42 ± 0.08	0.11 ± 0.06

[a] μmoles/g of wet tissue
[b] Mean value
[c] Standard error of the mean

per hour, and specific activity is defined as units per milligram of protein. Protein was determined by the biuret reaction (Layne, 1957).

RESULTS

THE CHROMATOGRAMS of various postmortem muscle samples are shown in Figures 1 and 2. Four major identified nucleotides DPN⁺+DPNH, IMP with a trace of AMP, ADP and ATP) were found in the pre-rigor muscles. The striking difference between stress-susceptible Poland China and stress-resistant Chester White pigs was that stress-susceptible Poland China pigs had a much lower ATP and higher IMP compared to stress-resistant Chester White pigs at 0 hr. The time for completion of rigor mortis (extensibility loss) in longissimus muscle ranged between 0.5 and 1.0 hr for stress-susceptible Poland China animals and between 3 and 4 hr for stress-resistant Chester White animals.

Dramatic changes in the chromatograms were noted at 3 hr postmortem. These changes can be summarized as follows:

(1) The optical density of the total acid extractable nucleotide decreased over 35% at 260 mμ as shown in Figures 1 and 2.
(2) Over 90% of ATP and 60% of ADP had been depleted at this stage.
(3) A large peak of IMP appeared (more than 5 μmoles/g of muscle).
(4) A large peak of inosine + hypoxanthine appeared in the water washable fractions.
(5) A small amount of IDP appeared.
(6) The level of nucleotides DPN⁺+DPNH decreased rapidly, particularly in muscle from Poland China pigs.

The nucleotide concentrations showed little change after 3 hr postmortem (Table 1). However, inosine + hypoxanthine peaks increased gradually while DPN⁺+DPNH, IMP, ADP and ATP peaks decreased gradually as the postmortem period lengthened.

The activities of AMP and adenosine deaminases are given in Table 2. Muscle

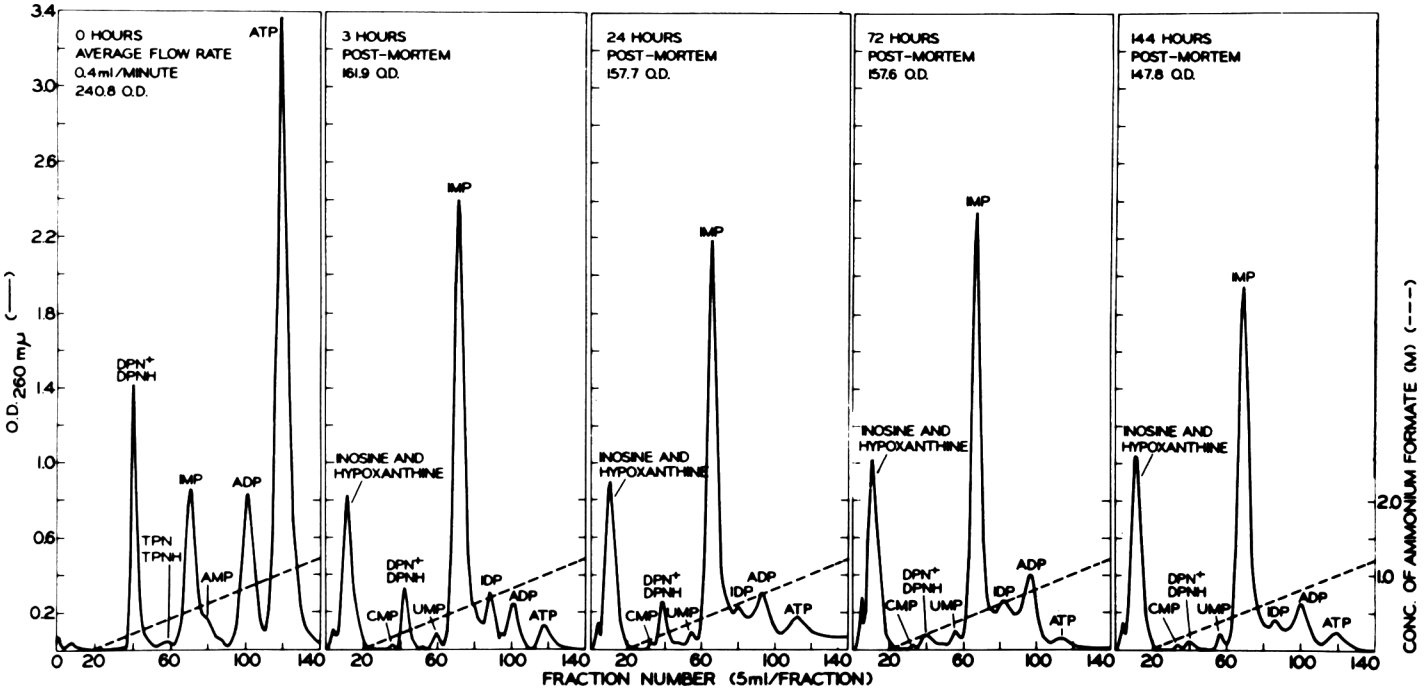

Fig. 1—Postmortem changes in nucleotides and related compounds in longissimus muscle from Poland China pigs.

Table 2—Postmortem changes in AMP and adenosine deaminase activities in porcine longissimus muscle[a]

Postmortem time (hr)	AMP deaminase		Adenosine deaminase	
	Poland China n = 2	Chester White n = 2	Poland China n = 2	Chester White n = 2
0	1.83[b] ± 0.16[c]	1.04 ± 0.23	0.08 ± 0.01	0.09 ± 0.00
3	–	0.83 ± 0.30	0.09 ± 0.01	0.11 ± 0.02
24	–	0.42 ± 0.12	0.09 ± 0.02	0.12 ± 0.01
72	–	0.27 ± 0.02	0.10 ± 0.01	0.11 ± 0.01
144	–	0.38 ± 0.02	0.11 ± 0.01	0.11 ± 0.01

[a] μmoles of substrate deaminated/min/g of wet tissue
[b] Mean value
[c] Standard error of the mean

from Poland China pigs had a higher AMP deaminase activity than that from Chester White pigs at 0 hr postmortem. There was no AMP deaminase detectable in Poland China pigs after 3 hr postmortem. In Chester White pigs, the enzyme activity remained although it decreased gradually with time. Adenosine deaminase activity was detected in both breeds of pigs, and there appeared to be a slight increase in activity after the 0 hr time period. Adenine deaminase activity was not detected in these muscle samples. The 5′-nucleotidase activities are shown in Table 3. Increased activity of this enzyme was also observed after 0 hr postmortem.

DISCUSSION

BENDALL AND DAVEY (1957) demonstrated with an ion-exchange chromatographic technique and a discontinuous gradient system that the pre-rigor nucleotide pattern from rabbit muscle is very simple and consists of three main components. The three major components are ATP, ADP with trace of IMP contamination and a mixture of DPN and TPN. In our studies, we also used an ion-exchange chromatographic technique. However, we applied a linear gradient system which provided a better resolution among the nucleotide peaks, and we were able to calculate the amount of each nucleotide present in the muscle. We did not examine thin-layer chromatographic techniques in this study; even though such methodology is more rapid, we believe that the ion-exchange technique yields more quantitative data.

The main defect of our column was that AMP and IMP could not be separated completely. Both components tended to come out in the same peak. Fortunately, there is only a small amount of 5′-AMP present in meat (0.082–0.216 μmoles/g) according to Kastenschmidt et al. (1968). Therefore, this defect has little effect in the experimental results. In our studies, we computed the amount of 5′-IMP which included 5′-AMP in muscle.

The only nucleotide which was not eluted by the gradient was 5′-GTP. This nucleotide is present in muscle in only small amounts. The concentrations of individual nucleotide calculated from individual peaks at their maximum absorbance were approximately the same as other workers had found with different

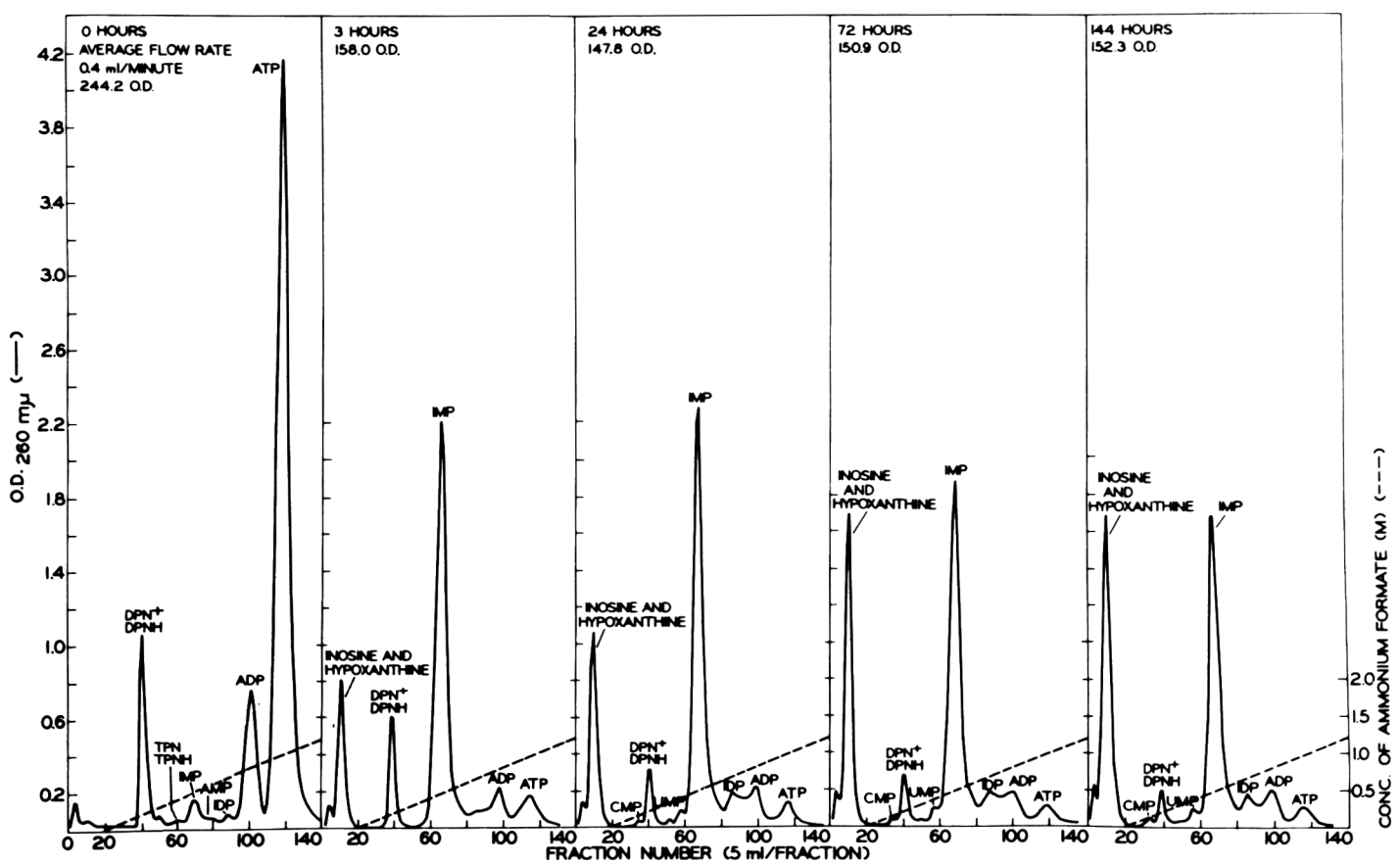

Fig. 2—Postmortem changes in nucleotides and related compounds in longissimus muscle from Chester White pigs.

Table 3—Postmortem changes in the specific activity of 5'-nucleotidase[a] in porcine longissimus muscle

Postmortem time (hr)	Poland China n = 2	Chester White n = 2
0	0.017[b] ± 0.002[c]	0.017 ± 0.005
3	0.029 ± 0.005	0.035 ± 0.005
24	0.027 ± 0.002	0.035 ± 0.005
72	0.034 ± 0.001	0.041 ± 0.006
144	0.034 ± 0.001	0.037 ± 0.002

[a]Specific activity is defined as units per milligram of protein. (A unit of activity corresponds to the liberation of 1 μmole of P per hr)
[b]Mean value
[c]Standard error of the mean

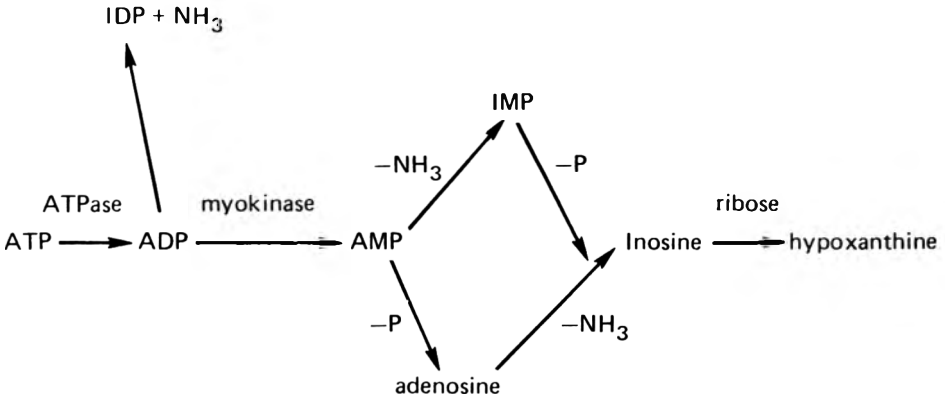

Fig. 3—Outline of different ATP degradation steps which may occur postmortem.

techniques. For example ATP ranged from 4.63–5.39 μmoles/g, ADP ranged from 0.514–0.919 micromoles/g, and NAD ranged from 0.578–0.769 micromoles/g in 0 hr muscle samples (Kastenschmidt et al. 1968). IMP was about 5–6 μmoles/g when the ATP had virtually all disappeared (Newbold, 1966).

The O.D. of total acid extractable nucleotide decreased approximately 35% between 0 and 3 hr postmortem. A further minor decrease occurred in the 3 hr to 144 hr period. This decline in total optical density appears to be due to the lower extinction coefficients of inosine versus adenine nucleotides and also to the decrease in DPN^++DPNH. The reason for this loss is not known. Figure 3 outlines the different ATP degradation steps which may occur postmortem. One conversion (AMP to inosine) theoretically can proceed either via IMP or adenosine (Webster, 1953, Needham, 1942).

No adenosine was detected in our Dowex-1 effluents, in agreement with the work of Kerr and Seraydarian (1945). This might suggest that the major pathway in the conversion of AMP to inosine is through IMP; however, it might also indicate that the adenosine deaminase is much more active than the 5'-nucleotidase and thus prevents the accumulation of adenosine. The present results do not distinguish between these alternatives.

Paper chromatography of the inosine plus hypoxanthine fractions indicated that inosine was the major component (about 90%). This was true for the 144 hr samples as well as those collected earlier. Thus the inosine to hypoxanthine conversion appears to be a rather slow process.

Of four enzymes studied, only AMP deaminase activity decreased after 3 hr postmortem. In fact, Poland China pigs lost all activity at this stage. This is probably due to the fact that "stress-susceptible" pigs attain a low pH (5.5) in less than 3 hr postmortem. On the other hand the pH drop in the muscles of "stress-resistant" pigs is much more gradual, with the temperature of the carcass declining significantly before a pH near 5.5 is obtained.

The activity of 5'-nucleotidase and adenosine deaminase of stress-susceptible and stress-resistant pigs increased at 3 hr postmortem and remained high throughout the prolonged storage periods. The reason for these increases was not determined, although it may be due to a more effective disruption of the subcellular compartments in postmortem muscle.

There is no significant difference between stress-susceptible and stress-resistant pigs in the production of 5'-IMP at various stages postmortem. Therefore, we conclude that the nucleotide related flavor of these two meat products should not differ.

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RETENTION OF 2-PROPANOL AT LOW CONCENTRATION BY FREEZE DRYING CARBOHYDRATE SOLUTIONS

INTRODUCTION

IN THE PAST few years there has been much interest in the retention of flavor compounds during the freeze-drying process. The current literature in this area has been recently reviewed by King (1970). Currently, there are three mechanisms which have been presented in the literature: sorption of volatile on the dry layer of the freeze-drying material (Rey and Bastien, 1962); moisture content dependent selective diffusion of water vs. volatile (Menting and Hoogstad, 1967a,b; Thijssen and Rulkens, 1968; Chandrasekaran and King, 1971); and volatile containing structures called microregions formed during the freezing and subsequently stabilized in the freeze-dried material (Flink and Karel, 1969; 1970a). The latter two reflect the difference of the macroscopic (selective diffusion) and the microscopic (microregion) description of the mechanism of the volatile loss.

The effect of processing conditions on flavor retention has been presented by Sauvageot et al. (1969) for orange and raspberry juices, and Flink and Karel (1970b) for carbohydrate-based model systems. Flink and Karel explained the observed behavior on the basis of their previously presented mechanism. Their work was conducted at the relatively high volatile concentration of 7,500 ppm. While this volatile concentration may be applicable for desolventizing situations or dry aroma concentrate production, it is very much higher than those likely to be encountered in natural food systems, even considering the possibility of preliminary concentration steps. The results presented by Sauvageot et al. (1969) for the natural fruit juices are in qualitative agreement with those of Flink and Karel (1970b) for the model system. However, to better evaluate the mechanism the same model system and process parameters as utilized in the previous work should be evaluated. That is the substance of the study reported on here.

MATERIALS & METHODS

Sample preparation

The model system consisted of a nonvolatile solute [either maltose, or Dextran-10 (mol wt $\cong 10^4$)] present at a concentration of 20% (w/v), a volatile solute (2-propanol) present at either 100 or 200 ppm with the remainder being water. The initial solution was prepared by mixing aqueous carbohydrate and volatile solutions of suitable concentrations to give the desired final concentration. Individual samples were pipetted from this mixture. The samples were frozen and freeze dried for 48 hr (Virtis Model 10-MRTR). Control samples were held in sealed flasks at a temperature just above 0°C.

Propanol analysis

The dry samples were dissolved in distilled water and then aliquots of the solution were analyzed gas chromatographically. A flame ionization chromatograph (F & M Model 1609) was used with a Porapak Q column (Waters Associates). Chromatographic conditions were optimized to give quantitative results in the shortest possible time (Hollis, 1966). Previous experience has shown that typical accuracies of 5–10% of the retention value can be expected (Flink, 1970).

Sample freezing rate

Samples were subjected to fast or slow freezing. Fast freezing was achieved by immersion of the sample flask in a liquid nitrogen bath in which freezing is complete in less than 1 min. Slow freezing resulted from placing the stoppered flasks in a −20°F freezer. The freezing time was estimated to be about 1 hr.

Sample thickness

The sample thickness was varied by placing 5 ml of sample in Erlenmeyer flasks of 25, 50 or 125 ml capacity (giving thickness of 1.0, 0.5 or 0.3 cm respectively).

Drying temperature effects

Freeze drying was conducted with the heating platens set for either no heat input or 175°F. Drying time was held at 48 hr for both temperatures to insure dryness. The chamber pressure registered below 100μ for most of the drying cycle (thermocouple gauge).

RESULTS & DISCUSSION

OF THE VARIOUS process parameters investigated, the freezing rate gave the most sizable effect on the retention of 2-propanol. This is shown in Table 1 for both maltose and Dextran-10, at the two initial 2-propanol concentrations. As can be seen, freezing at −20°F gave sizably higher retentions than the liquid nitrogen immersion. The improvement in retention with the slow freezing was much greater

Table 1—The effect of freezing rate on retention of 2-propanol by freeze-dried maltose or Dextran-10[a]

Carbohydrate	Initial conc (ppm)	% retention of 2-propanol: Fast freezing	Slow freezing
Maltose	100	10	84
Dextran	100	43	88
Maltose	200	8	87
Dextran	200	63	82
Maltose[b]	7,500	71	87

[a] 1 cm thick samples freeze dried at room temperature
[b] Flink and Karel (1970b)

Table 2—Effect of thickness on retention of 2-propanol by maltose or Dextran-10

Carbohydrate	Freezing rate[b]	% Retention[a] Thickness (cm): 1.0	0.5	0.3
Maltose	fast	10.4	16	18
Maltose	slow	84	88	92
Dextran-10	fast	43	56	72
Dextran-10	slow	88	97	98
Dextran-10[c]	fast	–	14	21

[a] Initial 2-propanol concentration = 100 ppm
[b] Fast freezing = immersion in liquid nitrogen; Slow freezing = holding in −20°F freezer; Freeze-drying platens at room temperature (70°F)
[c] Flink and Karel (1970b)

Table 3—The effect of platen temperature on retention of 2-propanol by freeze drying maltose or Dextran-10[a]

Carbohydrate	Initial conc (ppm)	Freezing rate[b] (ppm)	% Retention Room temp	% Retention 175°F
Maltose	100	fast	16	11
Maltose	100	slow	89	93
Maltose	200	fast	10	10
Maltose	200	slow	97	90
Dextran	100	fast	56	50
Dextran	100	slow	97	68
Dextran	200	fast	79	81
Dextran	200	slow	80	93

[a]Sample thickness = 0.5 cm
[b]Fast = liquid nitrogen immersion; Slow = holding in −20° F freezing

for the maltose samples than the Dextran-10, giving up to an 11-fold increase. This behavior is in qualitative agreement with the results of Sauvageot et al. (1969) and Flink and Karel (1970b). The relative improvement in retention for maltose and 2-propanol is much higher at the lower 2-propanol concentrations than was reported by Flink and Karel (1970b), though this appears to be due to the lower percentage retention with liquid nitrogen freezing at the lower volatile concentration.

The influence of sample thickness on the retention of 2-propanol is presented in Table 2. It can be seen that as the sample is made thinner, the retention of the 2-propanol increases. The effect of thickness is more pronounced for quickly frozen samples, though this probably reflects the high retention levels for all the slowly frozen samples. These results also agree with those of Sauvageot et al. (1969) for raspberry juice and Flink and Karel (1970b) for model systems.

Table 3 presents data comparing the retention of 2-propanol at two platen temperatures. These results are somewhat mixed, samples of the same solid showing varying trends, probably reflecting an influence of other processing parameters. Slowly frozen samples show a larger effect of platen temperature than the rapidly frozen samples; the slowly frozen Dextran-10 samples are more responsive to platen temperature change than maltose samples. While retention variation for the two initial volatile concentrations for Dextran-10 and maltose samples are somewhat unusual, these results are somewhat in agreement with the finding of Sauvageot et al. (1969) that the frozen layer temperature showed varied effects on retention for different compounds and conditions. Flink and Karel (1970b) also showed that retention behavior varied for different solid compounds. The temperature extremes reported on here are wider than those utilized by Flink and Karel and the duration of drying was much longer. While it is not possible to claim complete agreement with the results of Sauvageot et al. (1969) and Flink and Karel (1970b) with respect to the effect of platen temperature on 2-propanol retention, it appears that for rapidly frozen samples there is a qualitative agreement that small effects of a variable nature result.

It can be seen, that the process variable of overriding significance is the sample freezing rate. For samples that were slowly frozen, all other process parameters exerted very little influence. For rapidly frozen samples, the thickness would be of importance, with thinner samples giving higher retention.

The fact that the results of Flink and Karel (1970b) obtained at high volatile concentrations (7500 ppm) are in agreement with results presented here obtained at low volatile concentrations (100 and 200 ppm), indicate that the retention model developed on the basis of the earlier results may be applicable to retention occuring at the low volatile concentrations present in natural foods.

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PROPERTIES OF A FUNGAL LACTASE

INTRODUCTION

LACTASE is the common name for many β-galactosidases, E.C.3.2.1.23, the enzymes which hydrolyze β-galactosidic bonds such as the one present in lactose. In the latter case, glucose and galactose are the end products of the reaction, but higher oligosaccharides may also be formed due to transgalactosidic activities (Aronson, 1952).

Lactases have been identified in many tissues and cells of vegetable or animal origin. In microorganisms they have been found in various bacterial species (Cohn, 1957; Tacquet et al., 1966; Rohlfing and Crawford, 1966a, b; Anema, 1964), mycobacteria (Citti et al., 1965), *Neurospora crassa* (Lester and Byers, 1965), *Aspergillus oryzae* (Neuberg and Rosenthal, 1924), and *Saccharomyces fragilis* (Caputto et al., 1948; Szabo and Davies, 1964).

Lactase, mainly the *S. fragilis* enzyme, has been suggested for many practical uses, all based on the conversion of lactose into a mixture of glucose and galactose. Lactose is less soluble than its hydrolytic products, glucose and galactose. Examples are the addition of lactose or milk products in bread making (Pomeranz et al., 1962; Pomeranz and Miller, 1963), increasing the milk solids in ice cream (Sampey and Neubeck, 1955), decreasing the thickening during storage of frozen concentrated milk products (Stimpson, 1954), improving animal feeds (Stimpson, 1957).

This paper reports on the rather different properties of a fungal lactase obtained from a mutant strain of *Aspergillus foetidus*.

MATERIALS & METHODS

THE CRUDE ENZYME concentrate was obtained from the Microbiological Pilot Plant of Miles Lab., Inc. as an experimental product. The product had been produced from a fermentation of a selected strain of *Aspergillus foetidus*. The filtrate of the fermentation was treated as described by Sternberg (1970) to precipitate the enzyme. The redissolved enzyme was concentrated to 19% solids and stored at 4°C. Analytical grade reagents and distilled water were used throughout the work. Other materials used were: Sephadex G75 (lot 7571), Sephadex G100 (lot 226), Sephadex G200 (lot 3249), calibration kit #OPA, Pharmacia; DEAE Cellulose DE52 Microgranular, CM Cellulose CM-32 Microgranular, Whatman; bovine serum albumin (lot 16), Pentex, Inc.; lactose, maltose, Fischer; p-nitrophenyl-β-D-thiogalactoside, Calbiochem; other disaccharides and galactosides, Nutritional Biochemicals Corp.

The Amicon 402 cell and American Standards 207 membrane were used for ultrafiltration and diafiltration.

Assay of β-galactosidase

The chromogen o-nitrophenyl-β-D-galactoside (ONPG) was used routinely for determining β-galactosidase. 0.5 ml of suitably-diluted enzyme solution was blown into 2.0 ml 0.25% ONPG in 0.1M sodium acetate, pH 4.0, prewarmed to 60°C. At 15 min, 1.0 ml was transferred from the reaction to 1.0 ml 10% w/v sodium carbonate (anhydrous), 8.0 ml water added and the absorbance of 420 nm determined (Hestrin et al., 1955). The absorbance was corrected for substrate blank and, with crude preparations, for enzyme blank. One unit of activity was defined as the amount of enzyme which will catalyze the hydrolysis of 1μM of ONPG per min at pH 4.0 and 60°C.

For a large number of samples such as screening fractions from column chromatography, a "drop assay" modification was used. Two drops of ONPG substrate were added to one drop of column fraction. After 15 min at 60°C., 1.0 ml 10% sodium carbonate was added to the reaction. Water was added to dilute the o-nitrophenol to permit determining the absorbance at 420 nm.

During purification of β-galactosidase, enzyme activity was also determined using lactose as substrate. 1 ml enzyme solution (0.34–1.36 units per ml) was blown into 9.0 ml 11.1% lactose in 0.1M sodium acetate, pH 4.0, prewarmed to 60°C. After 15 min, 1.0 ml M hydrochloric acid was added and the reaction tube allowed to cool. After standing 3 hr or refrigerated overnight to permit mutarotation, 1.0 ml M sodium hydroxide was added to neutralize the acid and the glucose released was determined by Technicon Auto Analyzer using glucose oxidase and peroxidase (Barton, 1966). One unit of activity was defined as the amount of enzyme which will catalyze the hydrolysis of 1μM of lactose per min at pH 4.0 and 60°C.

α-Galactosidase was not determined quantitatively. To observe the degree of separation of the two galactosidases, a drop assay similar to the β-galactosidase drop assay was used. The substrate was p-nitrophenyl-α-D-galactoside and absorbance at 400 nm was determined.

Protein concentration was determined with the Folin-Ciocalteu reagent (Lowry et al., 1951). Bovine serum albumin was used as protein standard.

Purification of β-galactosidase

Ammonium sulfate precipitation. Ammonium sulfate was ground to a powder and 39.0g added per 100 ml (60% saturation) crude concentrate at 24°C. The mixture was centrifuged after standing for 2 hr. To 100 ml supernate 5g crude lactose and 22.7g powdered ammonium sulfate were added (90% saturation). After standing overnight at 4°C, the precipitated enzyme was obtained by centrifugation. The precipitate was dissolved in a minimum amount of water and diafiltered with 0.05M sodium acetate, pH 5.0.

Sephadex G75 gel filtration. A 7.5 by 51 cm column of Sephadex G75 was prepared in 0.05M sodium acetate, pH 5.0. Ultrafiltered concentrate (150 ml) was layered on the column and eluted with 0.05M sodium acetate, pH 5.0; 25 ml fractions were collected. Active fractions were pooled and concentrated by ultrafiltration.

Chromatography on DEAE-cellulose. DEAE-cellulose was cycled with HCl and NaOH (Whatman Technical Bulletin, IE2), washed with 0.05M acetic acid to about pH 4 and then suspended in 0.05M acetic acid and titrated to pH 5.0 with NaOH. A column, 4.8 cm × 27 cm (about 500 ml) was poured and equilibrated by passing a liter of 0.05M sodium acetate, pH 5.0, through the column. The concentrated G75 eluate was diafiltered with 0.05M sodium acetate, pH 5.0, and 250 ml containing 2–4g protein applied to the column. A linear increasing gradient prepared from 2.0 liters 0.2M sodium chloride in 0.05M sodium acetate, pH 5, and 2.0 liters 0.05M sodium acetate, pH 5.0, was applied after most of the breakthrough protein was eluted. Fractions containing enzyme were pooled, concentrated by ultrafiltration and diafiltered. The DEAE-cellulose was regenerated by cycling before reuse.

Carboxymethyl cellulose chromatography. The CM-cellulose was cycled with NaOH and 0.5M acetic acid, washed with 0.1M acetic acid to about pH 3, suspended in 0.1M acetic acid and titrated to pH 3.7 with NaOH. A 2.5 cm × 35 cm column was poured and 0.1M sodium acetate, pH 3.7, passed through the column. Concentrated DEAE-cellulose eluate was dialyzed against water and titrated to pH 3.7 with 2.0M acetic acid; 214 mg protein was applied. Lactase was eluted with a linear increasing gradient prepared from 1.0 liter 0.4M sodium chloride in 0.1M sodium acetate, pH 4.5, and 1.0 liter 0.1M sodium acetate, pH 3.7.

Gel filtration, Sephadex G100. A 2.5 cm × 38.4 cm column of Sephadex G100 was prepared in 0.1M sodium phosphate, pH 6.5. 20 mg lyophilized CM-cellulose eluate was dissolved in 2.0 ml buffer, filtered, sucrose added to increase the density and 0.3 ml (3.0 mg) layered on the column. The eluate was collected in 0.91 ml fractions.

Optimum temperature and pH

The optimum temperature for hydrolysis of lactose and ONPG was determined at pH 4.0 and pH 6.0. The polythermostat permitted determinations at sixteen temperatures simultaneously (Palumbo et al., 1967). For lactose hydrolysis, the method was the same as the assay procedure except 4.5 ml substrate and 0.5 ml enzyme solutions were used. The procedure

Table 1–Purification of fungal lactase

Step	Yield	Specific activity u/mg
Filtrate	100%	0.20
1. Ammonium sulfate, 0–60%	3.4%	–
, supernate	96.6%	0.79
2. Sephadex G75	100%	1.20
3. DEAE-cellulose	84.6%	50.0
4. CM-cellulose	74.3%	149.

was modified for ONPG hydrolysis. To 1.0 ml 0.25% ONPG, 3.5 ml 0.1M sodium acetate (both solutions pH 4.0 or pH 6.0) was added. After 15 min to allow temperature stabilization, 0.5 ml enzyme solution was added. At 15 minutes, 1.0 ml. 10% sodium carbonate was added and 4.0 ml water added for determining absorbance. McIlvaine's citric acid-phosphate buffer was used for determining the optimum pH for β-galactosidase at 37°C and 60°C (McIlvaine, 1921).

Lactose solutions (11.1%) were prepared in 0.1M citric acid and in 0.2M sodium monohydrogen phosphate. For pH 2.2–4.5, the lactose/citric acid solution was titrated with the lactose/phosphate solution. The order was reversed for pH 5.0–7.0. For ONPG hydrolysis, 3.5 ml McIlvaine's buffer (pH 2.2–7.0) was added to 0.5 ml 0.5% ONPG in water. After 15 min for warming to temperature, 0.5 ml enzyme solution was added and 15 min later, 1.0 ml 10% sodium carbonate. The tubes were diluted with 4.5 ml water before determining the absorbance. The enzyme had been purified through the DEAE-cellulose stage, 87.7 units/mg protein.

pH Stability

The crude enzyme (0.16 units/mg protein) and enzyme purified through the DEAE-cellulose stage (50 units/mg protein) were diluted with buffers of 0.05M citric/0.05M phosphoric acids titrated to pH 1–9 with HCl or NaOH. Preparations from the crude enzyme had a protein concentration of 1.56 mg/ml and from the purified enzyme of 0.005 mg/ml (equal enzyme concentration). The solutions were incubated for 1 hr at 37°C and cooled in an ice bath before assay. The pH of the incubated solutions was determined after assay.

Michaelis-Menten kinetics

ONPG. The absorbance at 420 nm of a 1.0 mM ONPG solution in 0.1M acetate, pH 4.0, 60°C was 0.004 and of a similarly prepared 1.0 mM o-nitrophenol solution was 0.206. The large difference in absorbance between substrate and product permitted determining reaction velocity from recordings of the change in absorbance. To 3.0 ml substrate in 0.1M acetate, pH 4.0, prewarmed to 60°C was added 0.2 ml enzyme solution containing 0.25 units (purified through the DEAE-cellulose stage). The change in absorbance vs. substrate blank was recorded. The slope of the initial straight portion of the recording was used to calculate velocity. The data were plotted by the Lineweaver-Burk method (Lineweaver and Burk, 1934) and the 1/v intercept and slope were determined by method of least squares.

Lactose. To 5.0 ml lactose solution in 0.1M acetate, pH 4.0, prewarmed to 60°C was added 0.2 ml enzyme solution (0.25 units, purified through the DEAE-cellulose stage). After 1–5 min, enzyme action was stopped by addition of 1.0 ml 1M HCl. The glucose released was determined by automated glucose oxidase assay after adding 1.0 ml 1M NaOH. The data were plotted to obtain the reaction velocities and 1/v intercept and slope of the Lineweaver-Burk plot determined by the method of least squares.

Activators and inhibitors

Test materials were prepared in 0.01M acetate, pH 4.0. Enzyme purified through the CM-cellulose stage was diluted to 0.74 units/ml and 0.2 ml enzyme solution transferred to 1.0 ml test material. After 30 min at room temperature, 0.5 ml treated enzyme was transferred to 1.0 ml ONPG substrate at 60°C. At 15 min, 1.0 ml 10% sodium carbonate and 7.5 ml water were added.

Substrate specificity

After passage through a Sephadex G100 column, the enzyme preparation hydrolyzed ONPG but not p-nitrophenyl-α-D-galactoside. 0.5 ml enzyme (1.89 units) was added to 2.0 ml 10 mM test substrate in 0.1M sodium acetate, pH 4.0, 60°C. 0.5 ml M HCl was added at 15 min to stop the reaction and 0.5 ml M NaOH added after cooling to neutralize the acid for sugar determination. The hydrolysis of substrates containing glucose was estimated by the automated glucose oxidase assay. The hydrolysis of lactose, melibiose, lactobionic acid and lactulose was assayed by an automated method based on the copper reduction-neucuproine method of Dygert et al., (1965).

Action on whey and milk

Sweet whey (Country Line Cheese Co., Leo, Ind., preserved with 90 ppm hydrogen peroxide) and acid whey (Burger Dairy Co., New Paris, Ind.) were adjusted to the desired pH with M HCl or NaOH. Raw and pasteurized milk were obtained from the Burger Dairy Co. Crude enzyme concentrate had been precipitated with alcohol and dried. Solutions were prepared from the dried powder to contain 10.2 units/ml and 1.02 units/ml. 1 ml enzyme solution was added to 50 ml whey or milk and

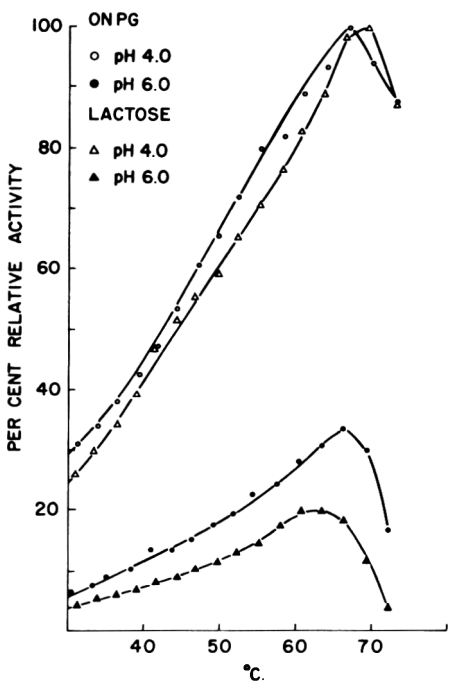

Fig. 1–Relative activity of fungal lactase toward ONPG and lactose at pH 4.0 and 6.0 vs. temperature.

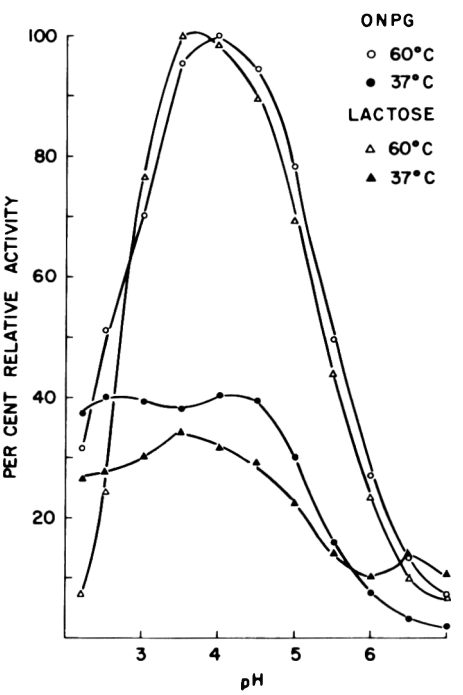

Fig. 2–Relative activity of fungal lactase in hydrolyzing ONPG and lactose at 37°C and 60°C vs. pH.

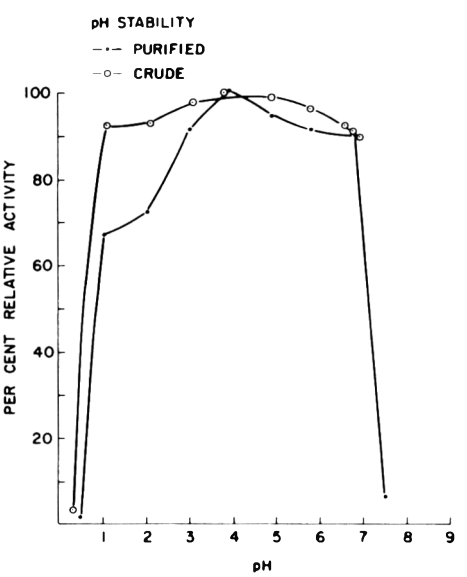

Fig. 3–Stability at 37°C for 1 hr at various pH values of crude and purified preparations of fungal lactase. The protein concentration was 1.56 mg/ml for the crude preparation and 0.005 mg/ml for the purified preparation (equal enzyme concentration).

Table 2–Substrate analogs and hydrolysis products

Material[a]	Relative activity[b]
p-Nitrophenyl-β-thiogalactoside	38
γ-D-Galactonolactone	34
Galactose	63
Glucose	103

[a]Preincubation concentration was 20.8 mM; concentration during assay was 6.93 mM for test material, 5.54 mM for ONPG.
[b]Activity of enzyme preincubated in 0.01M acetate, pH 4.0, taken to be 100.

Table 3–Metal ions and sulfhydryl reagents

Material[a]	Relative activity[b]
KCl	99.5
$CaCl_2$	99
$MgCl_2$	99
$MnCl_2$	100
$Zn(C_2H_3O_2)_2$	99.5
EDTA	97.1
o-Phenanthroline	97.6
8-Oxyquinoline	97.6
Phenylmercuric acetate	99.4
$Hg(C_2H_3O_2)_2$	0
Cysteine	100.2

[a]Preincubation concentration was 1.04 mM; concentration during assay was 0.47 mM.
[b]The activity of the enzyme preincubated in 0.01M acetate, pH 4.0, was assumed to be 100.

Table 4–Substrate specificity

Substrate	Hydrolysis
Glucose Oxidase	
Lactose (gal-β-1,4-gluc)	12.6%
Melibiose (gal-α-1,4-gluc)	0
Gentiobiose (gluc-β-1,6-gluc)	0.98%
Cellobiose (gluc-α-1,4-gluc)	0.98%
Maltose (gluc-α-1,4-gluc)	0
Neocuproine	
Lactose	19.2%
Melibiose	0.86%
Lactobionic acid, calcium salt (gal-β-1,4-gluconate)	1.02%
Lactulose (gal-β-1,4-fruct)	20.4%

incubated in constant temperature baths as indicated. Enzyme action was stopped by heating in a boiling water bath for 15 min. The treated solutions were centrifuged before determining the glucose released with the automated glucose oxidase assay.

The initial levels of lactose were determined by the Brobst and Lott (1966) modification of the Sweeley et. al. (1963) method for quantitative gas-liquid chromatography of sugars. A known amount of erythritol was included in all determinations as an internal standard for calculating the lactose present. Since detector response is not identical for all sugars, a correction factor was obtained using known quantities of lactose. The variation due to this assay was ±3% of the mean value based on a determination run in triplicate.

Bacterial counts were determined on the whey samples containing no enzyme and containing 20 units of enzyme per 100 ml whey. After the 72-hr incubation period, 0.1 ml was examined by the pour-plate method using Standard Methods Agar (Baltimore Biological Lab.) fortified to contain an additional 1% agar.

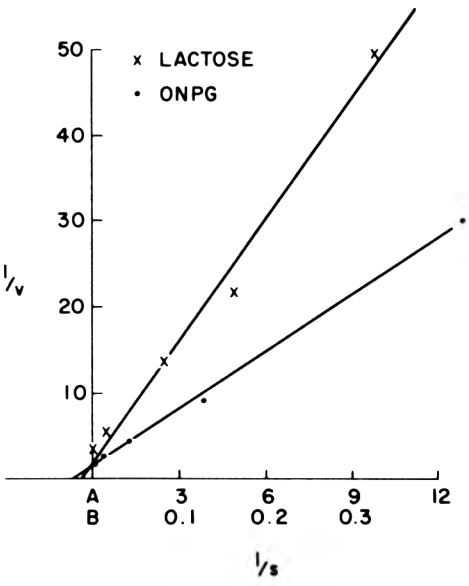

Fig. 4–Lineweaver-Burk plot of initial velocity vs. substrate concentration. The units for the axes are: abscissa A–reciprocal mM ONPG; abscissa B–reciprocal mM lactose; and ordinate–reciprocal μmoles formed per min of o-nitrophenol (ONPG as substrate) or glucose (lactose hydrolysis).

Molecular weight estimation

A Sephadex G200 column with dimensions 39.3 cm height × 2.5 cm diam (193 ml bed volume) was prepared and equilibrated with 0.05M sodium phosphate, pH 6.5, containing 0.1M sodium chloride and 50 ppm phenylmercuric acetate for preservative. The column was calibrated according to the instructions in the Pharmacia Calibration Kit Instruction Manual using the Pharmacia Calibration Kit OFA and crystalline bovine serum albumin, fraction V. The eluate was collected in 0.92 ml fractions. The elution of Blue Dextran 2000 was monitored by adding 2.0 ml water to each fraction and obtaining the absorbance at 280 nm. For the proteins used in calibrating the column, the Lowry method for protein determination was used. The drop assay was used for β-galactosidase.

RESULTS

Enzyme purification

Gel filtration on Sephadex G75 gave little or no purification measured by specific activity; however, a great deal of smaller molecular weight colored bodies was separated from the active portion. Removal of the colored bodies increased the capacity of the ion-exchange column. With repeated chromatography on DEAE-cellulose, a specific activity of 116 units/mg protein was obtained. This high-purity preparation did not hydrolyze p-nitrophenyl-α-D-glucoside or phenyl-β-D-glucoside, but did hydrolyze p-nitrophenyl-α-D-galactoside. A drop assay of the column fractions showed the α- and β-galactosidase peaks were nearly coincident.

Chromatography on CM-cellulose separated the two enzyme peaks though there was still some overlap of the peaks. Using lactose as substrate, peaks of lactose and ONPG hydrolysis were coincident in both DEAE- and CM-cellulose chromatography and only one β-galactosidase peak was observed with each procedure. For characterization of β-galactosidase, further purification was not attempted. To obtain material for determining substrate specificity, gel filtration on Sephadex G100 separated the α- from the β-galactosidase. The recovery of enzyme and specific activity at each stage of purification are given in Table 1. The overall recovery was 60.7%.

Optimum temperature and pH

The temperature for maximum hydrolysis of lactose and ONPG was determined at pH 4.0 and pH 6.0 (Fig. 1). With ONPG, the optimum temperature, 66–67°C, is identical for ph 4.0 and 6.0. The relative hydrolysis of lactose is markedly different from the ONPG curve. At pH 6.0, the optimum temperature is 61–64°C and at pH 4.0, 67–70°C.

Maximum lactose hydrolysis occurs at pH 3.5–4.0 with the temperature at 37°C or 60°C (Fig. 2). At 60°C, maximum ONPG hydrolysis is observed at pH 3.5–4.5. The peak of ONPG hydrolysis is broadened at 37°C to pH 2.2–4.5.

pH stability

The enzyme lost no activity when held at ph 5.0 for 1 hr at 37°C (Fig. 3). The enzyme retained 90% or more of its activity from pH 4–8. The crude enzyme at the same enzyme concentration as the purified preparation but at 312 times the protein concentration retained 90% or more of its activity from pH 2.2–8.

Michaelis-Menten kinetics

The hydrolysis of ONPG was determined using 0.083–8.31 mM concentrations. Lactose concentrations were 3.08–308 mM. Least squares analysis was used to determine the intercept and slope of the double reciprocal plot (Fig. 4). The Michaelis-Menten constant is 1.613 mM for ONPG hydrolysis and 68.8 mM for lactose hydrolysis. The maximum velocity is 440 μmoles × min^{-1} × mg $prot^{-1}$ for ONPG and 295 μmoles × min^{-1} × mg $prot^{-1}$ for lactose.

Activators and inhibitors

The effect on enzyme activity by compounds related to lactose hydrolysis

Table 5–Lactose in milk and whey

Material	Lactose	Equivalent glucose
Raw milk	4.2%	2.2%
Pasteurized milk	3.5%	1.8%
Sweet whey	3.1%	1.7%
Acid whey	3.7%	1.9%

is presented in Table 2. The substrate analog, p-nitrophenyl-β-thiogalactoside, and α-galactonolactone strongly inhibit ONPG hydrolysis. Galactose reduced enzyme activity 37% whereas glucose had no effect on the enzyme.

The effects of metal ions, chelating agents and sulfhydryl compounds are given in Table 3. Neither the addition of metal ion nor metal ion removal by chelating agent markedly affected the enzyme. Mercuric ion completely inhibited the enzyme indicating an important sulfhydryl group.

Substrate specificity

The substrates were chosen to be representative of the classes: α- and β-galactosides and α- and β-glucosides. The degree of substrate hydrolysis is given in Table 4; the method used to detect hydrolysis is indicated.

Lactose and lactulose are readily hydrolyzed, but α-galactosides and α- and β-glucosides are not attacked. The apparent 1% hydrolysis of melibiose is due to assay sensitivity; after purification the presence of α-galactosidase was determined to be absent with the chromagen p-nitrophenyl-α-galactoside–a simper and highly sensitive assay.

Gel filtration of the enzyme indicated a molecular weight of 126,000 ± 2,000.

Lactose hydrolysis in milk and whey

The lactose content and equivalent glucose concentration of the milk and whey are listed in Table 5. The lactose concentrations are low compared to literature values. Nickerson (1965) states cow's milk has been reported to contain 4.4–5.2% lactose.

The milk was incubated with enzyme for 3 hr at 40°C and at 60°C. The extent of lactose hydrolysis was calculated using the quantity of glucose produced by enzyme corrected for glucose in an identical sample without enzyme and the equivalent glucose content of the milk. The degree of hydrolysis observed using raw milk, raw milk containing 300 ppm hydrogen peroxide and pasteurized milk was nearly identical. The average values obtained from the three milk preparations are given below. At two units enzyme per 100 ml milk, 0.2% lactose hydrolysis was obtained at 40°C and 0.8% hydrolysis at 60°C. With ten-fold higher enzyme concentration, 1.1% lactose hydrolysis took place at 40°C and 3.8% at 60°C. The activity of fungal lactase is greatly reduced at the pH of milk (pH 6.5).

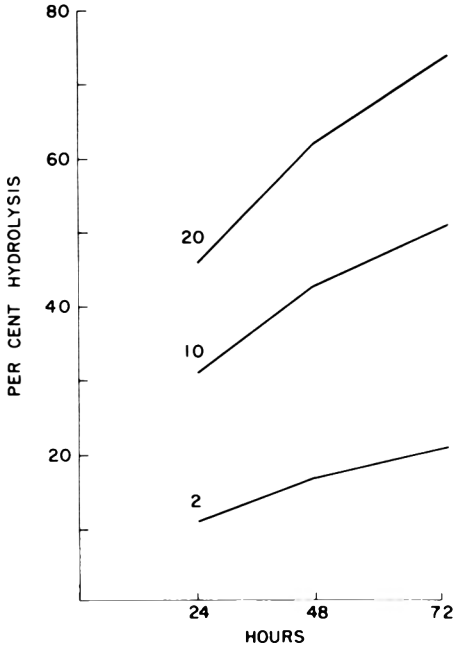

Fig. 5–Hydrolysis of lactose in whey. A concentrated enzyme solution was added to give enzyme levels of 2 units, 10 units and 20 units enzyme per ml whey. The points are averages using sweet and acid wheys adjusted to pH 3.5 and 4.4.

The whey was held at 60°C during the enzyme treatment. The bacteria count determination at 72 hr showed no bacterial colonies using 0.1 ml of the incubated materials. The glucose in the samples containing enzyme was corrected for the glucose in identically treated whey aliquots containing no enzyme. The hydrolysis of lactose during the 72-hr period is presented in Figure 5 for enzyme levels of 2, 10 and 20 units per 100 ml whey. Sweet and acid whey titrated to pH 3.5 and 4.4 were used. Since the degree of lactose hydrolysis did not differ greatly among the four whey-pH combinations, the values are averages of the four determinations. In 72 hr at 60°C, fungal lactase at 2, 10 and 20 units per 100 ml catalyzed 20.8%, 50.6% and 74.6% lactose hydrolysis. The hydrogen peroxide (90 ppm in the sweet whey) did not affect the hydrolysis of lactose. Bacterial growth was prevented by the high temperature and low pH.

DISCUSSION

LACTASE has been found in several microorganisms (*vide supra*). The enzymes from *Aspergillus* and from *Saccharomyces* appear to be the most useful for industrial exploitation because of ease of producing the enzymes, properties of the enzymes and acceptance of *Aspergillus* enzymes and of *Saccharomyces* in processing of foods.

The combination of high temperature and low pH required for optimum activity of the fungal lactase minimizes or eliminates microbial spoilage during processing. Plate counts of the whey samples containing the greatest amount of enzyme and thus highest glucose level indicated less than ten bacteria per milliliter at 72 hr. The fungal enzyme has considerably less activity at pH near neutrality and thus is not suitable for treating milk. The highest activity of yeast lactase was reported by Wendorff and Amundson (1971) to be at pH 6.5 and 37°C. The yeast enzyme is optimally active on milk at a temperature which would not contribute an off-flavor to the milk. The fungal lactase thus complements rather than supplants the *Saccharomyces* enzyme.

Fungal lactase has no metal ion requirement for activity or stability. Dialysis vs. distilled water, used routinely during purification, resulted in no discernible enzyme loss. The enzyme was inhibited by mercuric ion suggesting a sulfhydryl group is required for acitivity or enzyme integrity.

Galactose inhibited the enzyme whereas glucose did not. This is the reverse of the results of Wendorff and Amundson who found the yeast enzyme to be inhibited only 7% by galactose and 60% by glucose. Under conditions for industrial use of the enzyme (Fig. 5), the fungal enzyme catalyzed up to 74% hydrolysis of lactose in whey in 72 hr.

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PERCEPTUAL ATTRIBUTES OF THE TASTE OF SUGARS

INTRODUCTION

IN TASTE PSYCHOPHYSICS the quality of sweetness is often regarded as a single taste dimension, along with saltiness, sourness and bitterness. Despite their apparent singularity, however, sweet substances differ in "flavor" as can be seen by a simple comparison of sodium saccharin, glucose and sucrose. Saccharin at moderate and high levels produces an undesirable "off-taste," usually bitter, which inheres in the saccharin molecule itself, and is not traced to any measurable impurity (Helgren et al., 1955). Glucose produces a moderate burning or bitter side taste at the back of the mouth (Cameron, 1947), whereas sucrose provides a uniquely acceptable sweet taste, free from the considerable side-tastes that pervade other sweeteners.

The present study was designed to measure the perceptual differences between sugar flavors, and to assess the importance of dimensions besides sweetness as factors that differentiate sugars from each other. Sugars are ideal test materials with which to uncover the more subtle variations in the sweet taste, because virtually all sugars thus far tested psychophysically with the exception of mannose (Moskowitz, 1971) and gentiobiose are primarily sweet, and are not characterized by an overwhelming bitter side taste. [See Cameron (1947) and Moskowitz (1971) for extended bibliographies on sweetness.] The method of multidimensional scaling, which was proposed in a modified version by Shepard (1966) to accommodate nonmetric versions of interpoint distances, provides a simple means by which different perceptual attributes can be uncovered from similarity or dissimilarity judgments for pairs of sugars. The multidimensional analysis is used here in order to generate a sweetness "flavor space" in which distances between points in the space reflect flavor differences between sugars.

EXPERIMENTAL

Stimuli

Based upon the results of earlier psychophysical studies with direct magnitude estimation of sweetness (Moskowitz, 1971), four levels of glucose solution were selected as standards: 0.25M, 0.5M, 1.0M and 2.0M. Matching levels of other sugars were then selected on the basis of equal-sweetness matches, according to sweetness functions reported by Moskowitz (1971). These sugars and their concentrations are listed in Table 1. All of the sugars were reagent-grade quality and the water solvent was distilled and deionized (Hydro Service Supply Co., Inc.). The stimulus solutions were prepared 1 wk prior to the experiment, and were frozen at −10°C until the night before use, when they were thawed, and vigorously remixed. In this way, the solutions were maintained free of molds.

Subjects

Ss were 12 unpaid volunteers from the test platoon of enlisted men at Natick Laboratories. Four of the Ss had had previous experience with the method of magnitude estimation in evaluating taste intensity. Untrained Ss were shown cards with circles of different sizes and asked to assign numbers in proportion to apparent area. Afterwards, all Ss were introduced to the concept of "flavor difference" via the following procedure. They were shown different objects in the room and different colors, and asked to assign magnitude estimates to the degree of apparent dissimilarity of pairs of forms and pairs of colors. When each S showed that he understood the task, he was permitted to begin the experiment.

Procedure

On each of five days a different series of stimuli were used. On any one day Ss were presented with a series of stimulus pairs on a lazy susan. The solutions were contained in small 3/4-oz paper souffle cups, containing 12–15 ml of solution. In each pair, one stimulus was a reference sugar (either one of the four glucose references, or one of the four fructose references judged to be equally sweet as shown in Table 1). In a single session, S sampled up to 18 pairs of solutions, and required up to 30 min to complete the session. In Experiments 1–4, two different sugar sets were run in the same session (viz. glycerol and sorbitol, arabinose and xylose, maltose and lactose, galactose and sorbose), and each concentration was compared both to its glucose referent of equal sweetness, and to its fructose referent of equal sweetness. In Experiment 5, fructose was compared directly to glucose, and sucrose was compared to its two referents. In each experiment except Experiment 5, two replicate judgments were obtained for each sample. In Experiment 5 four replicate judgments were obtained for each glucose-fructose pair. In all experiments the order of concentrations within a pair was randomized as well as the order of pairs. Rinsing with tap water was required between pairs, but not within a pair. However, S had to wait at least 10 sec between samples.

Analysis

The median magnitude estimates of "flavor difference" were computed after the size of numbers used by each S was equated to a standard modulus via the process of *modulus normalization* (Moskowitz, 1970). Briefly, the estimate of each S to a common stimulus across all experiments (viz., 1.8M glycerol and 1.0M glucose) was multiplied by a constant so that it came to 10. All the remaining judgments of that S in the session were also multiplied by the normalizing factor. Each S required a separate multiplier in every experiment.

The median data provided estimates of selected "interpoint distances" corresponding to subjective dissimilarity or difference. The computer program MDSCALE, developed by Kruskal and Carmone was used to find a geometrical configuration so that the distances between points reflected their subjective difference. The program MDSCALE sought this best-fitting configuration with a 4,3,2 and 1 dimensional space, where points corresponded to sugars. The analysis was "nonmetric" because the computer program sought a configuration of points so that only the rank order of interpoint distances (rather than their arithmetical value) was preserved. The "badness-of-fit" was minimized by reducing the so-called "stress"

Table 1—Concentrations used in flavor difference experiments (Molarity)

Sugar[a]	Level 1	Level 2	Level 3	Level 4
Glucose	0.25	0.50	1.00	2.00
Fructose	0.12	0.26	0.62	3.50
Glycerol	0.43	0.88	1.80	3.20
Xylose	0.32	0.68	1.40	2.50
Arabinose	0.30	0.65	1.40	2.70
Galactose	0.26	0.72	1.50	–
Sorbose	0.18	0.45	1.15	2.50
Sorbitol	0.37	0.78	1.51	2.80
Sucrose	0.08	0.20	0.45	0.90
Maltose	0.25	0.50	1.00	1.90
Lactose	0.16	0.35	0.60	–

[a]Reference Pair: Level 3 of glycerol and Level 3 of glucose (for modulus normalization)

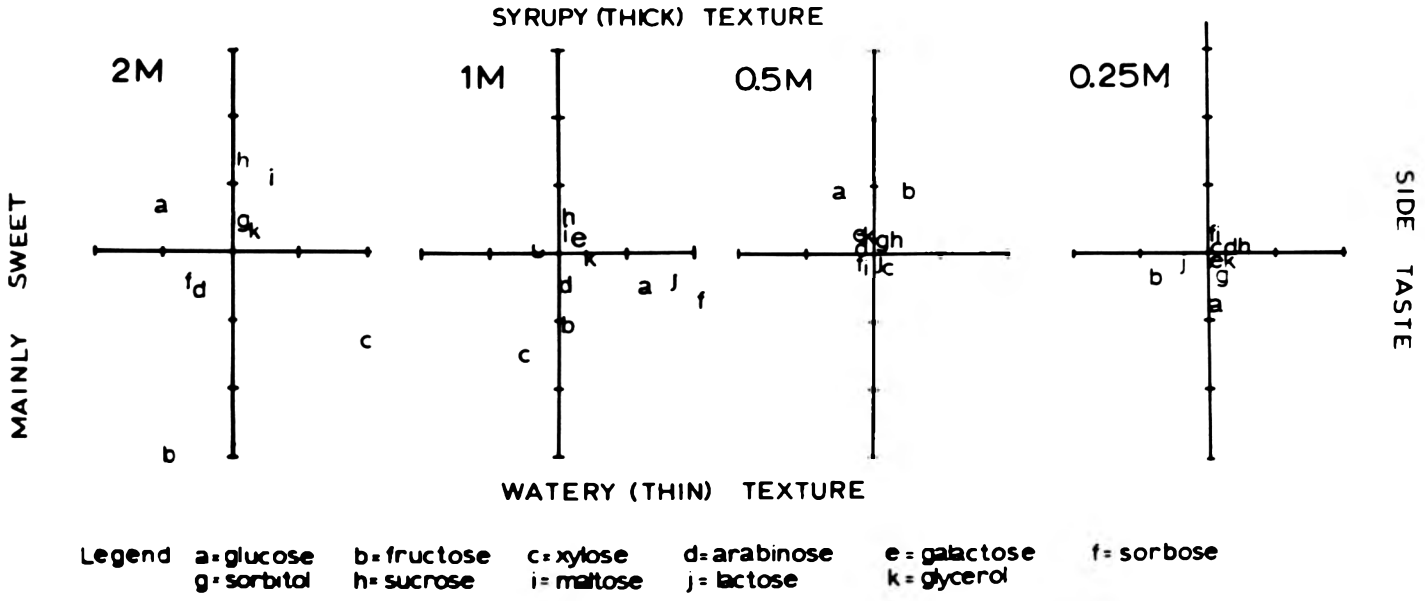

Fig. 1—The two-dimensional space reflecting the perceptual differences among sugars. Distances between points indicate perceptual differences between sugars based upon nonmetric, multidimensional scaling results. A two dimensional space, comprising the attributes of viscosity and 'side-tastes' adequately accounts for the distances. The map comprises four layers, one for each level of perceived sweetness (equal to that of 0.25M, 0.5M, 1.0M and 2.0M glucose). Each marker represents 1 unit and the map is unique up to a linear transformation. The names of the sugars used and their locations are coded alphabetically by single letters.

value, and the relation between stress value and dimensionality was a curvilinear, inverse function. As more dimemsions were added, stress decreased in the following way: 1 dimension stress = 0.1524; 2 dimension stress = 0.0086; 3 dimension stress = 0.0091; 4 dimension stress = 0.0093. Because of considerations of parsimony, a two-dimensional configuration of points was selected to economically represent the space of sugar flavor.

RESULTS

FIGURE 1 presents the two dimensional representation of points that correspond to sugars. Figure 1 is divided into four segments, which each corresponded to the four sweetness levels, equal to the sweetnesses of 0.25M, 0.5M, 1.0M and 2.0M glucose. The entire set of empirical distances (total = 72 interpoint distances) were analyzed together to obtain Figure 1, but the configuration was separated into the four parts shown here.

Based upon the author's tasting of the samples, two appropriate labels may be given to the attributes uncovered by multidimensional analysis: (a) presence vs. absence of side tastes and (b) viscous vs. watery texture. The representation of sugars is slightly confounded by the variability of some points, so that a high concentration of xylose at a sweetness of 2.0M glucose is on one side of the space, towards the region of pronounced side taste, whereas it is primarily sweet at a lower intensity equal to 1.0M. As a general representation, however, the two-dimensional space appears adequate to describe most of the sugars.

Table 2—Distribution of sugars in two-dimensional space (side taste, viscosity)

Level	Standard deviation (glucose-fructose) Side taste	Viscosity	Standard deviation (others)[a] Side taste	Viscosity
1	0.61	0.31	0.17	0.03
2	0.10	0.71	0.11	0.09
3	0.80	0.37	0.75	0.82
4	0.33	2.68	0.85	0.93

[a]Except glucose, fructose, galactose, lactose

A major finding of this study is that the perceptual differences between moderately and minimally sweet sugars are relatively small, so that the space that they generate is relatively compact. The differences are much greater at the high concentrations, and consequently the space is enlarged. Table 2 presents the standard deviations of the coordinates for all sugars except lactose and galactose. As the sugars increase in sweetness Table 2 shows that the standard deviation of their location coordinates rises on both dimensions, at first slowly, but then drastically with increases in sweetness between 0.5 and 1.0M glucose. The scatter of points is approximately the same for both dimensions.

The coordinates for glucose and fructose were analyzed separately because their placement in the two dimensional space had to satisfy many more interpoint distance constraints than did the remaining sugars (see Table 2). As a consequence, the highest concentration of fructose is placed in an anomalous position at its highest sweetness level (coordinates = 0.700, −3.084 on the two axes, respectively). Such a placement may have been caused by slight inconsistencies among the interpoint distances, so that the anomalous placement is actually "best" in that it minimizes the "badness-of-fit" (stress statistic). Neither lactose nor galactose was sufficiently sweet to match the taste of 2.0M glucose, and these sugars were also eliminated from the computation of variability for the placement of points. Thus, the standard deviation on each axis is comparable across intensities, since the same sugars are represented at each intensity level.

DISCUSSION

Ss APPEAR CAPABLE of changing their "focus" in sensory evaluation, so that the flavor differences between like-tasting

substances are enhanced by attention to other salient dimensions. For sugars, these dimensions appear to be the attribute of viscosity-fluidity, which is not a taste attribute, and the attribute of "side-tastes." It is quite possible that were the viscosity to be balanced (e.g., by adding sodium carboxy methyl cellulose, a "tasteless" thickener), the perceptual differences among the sugars might diminish considerably, since only one attribute, "side-tastes," would remain. It is also quite possible, however, that the subject possesses a hierarchy of salient attributes, so that equalization for viscosity, and perhaps for "side-tastes" might reveal yet a third and possibly a fourth dimension along which subjects differentiated sugar flavor.

It is instructive to consider the use of evidence presented in Figure 1 as a quantitative indicator about the degree to which sugars can be interchanged in a product. Were all processing properties to remain the same across many sugars, low sweetness products would probably be less sensitive to random interchanges of sugars than high sweetness ones. At the very sweet levels sucrose and sorbitol, as well as glycerol and maltose might be more interchangeable (in this data) than, say, sorbose and arabinose. It should be noted, however, that once attention is paid to a smaller set of sugars for product development the nature of the configuration, as shown in Figure 1 can radically change. Differences between glycerol and sucrose will increase when S pays close attention to them, whereas the differences might be much smaller when glycerol and sucrose are embedded in a much larger set of stimulus sugars.

In order to make the multidimensional scaling of flavor a more useful tool to the food technologist, flavor spaces such as that obtained here, ought to be obtained for specific products, and with appropriate and potentially useful sugars. By that approach, it may well be possible to quantitatively assess the sensory effects to be expected when sugars are substituted for each other in products. The method also promises potential usefulness for the quantification of other taste materials, as well as odors and flavors, which stimulate both taste and smell.

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JFS RESEARCH NOTES

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A Research Note
A RAPID SALT-CURING TECHNIQUE

INTRODUCTION

THIS IS A description of a technique for salt-curing fish in less than 8 hr. It was developed as the basis of a commercial process for turning seasonal abundances of species such as whiting (*Merluccius bilinearis*) into a shelf-stable fishery product. The process would make use of fish of low value to produce a suitable substitute for salt cod, for which there is still a strong demand in many parts of the world at a time when larger percentages of the cod supply are being diverted to the fresh and frozen markets.

A quick salting process has been reported (Del Valle and Nickerson, 1968; Del Valle and Gonzalez-Inigo, 1968) where ground fish is mixed with salt, pressed to release fluid and to form into cakes, which are then air dried. The amount of salt added is that which is optimum for cake formation with the species of fish used and is related to the water binding capacity of the fish (Del Valle and Gonzalez-Inigo, 1968). The technique was designed for use in developing countries and requires little processing equipment. Air drying of pressed cakes, however, requires drying times too long for an industrial process with a throughput sufficient to handle seasonal abundances of fish within a short processing period. Moreover, processing with this technique is carried out under conditions which favor oxidative deterioration—exposure to air and metal catalysts during grinding, salting, pressing and drying.

TECHNIQUE

SKINLESS FILLETS were ground in a Waring Blendor (1-gal capacity) in saturated salt solution (fish to brine ratio 1:1 weight to volume) to which sufficient excess salt (25g/100g fish) had been added to bring about the greatest fluid loss (syneresis) when the brine was diluted with the water of the fish (Table 1). Blending for two 15-sec periods with the blendor on "Hi" and a Variac setting of 40, using a plastic baffle placed just under the surface of the salt solution to prevent foaming (Dyer et al., 1950), was sufficient to grind the fish to a particle size similar to ground beef (hamburger). Between periods, the mix was stirred by hand to ensure even comminution. The mixture was allowed to stand about 15 min to ensure complete salt penetration (Table 2). The mixture was stirred occasionally during salting. Free fluid was then drained through cheesecloth. The drained, salted product was either (1) spread on trays of plastic screening and dried for 5 hr in an air-circulating oven set at 37°C or (2) passed two or three times through a laboratory model vacuum drum dryer with steam input set so that with the short on-drum dwell time, cooking did not occur (about 30 min required for small-scale batch processing).

The dried product was packaged in mylar pouches under vacuum and packed in cardboard cartons (browning of the product is catalyzed by light).

The salted product was prepared for use in fishcakes by adding water, bringing the mixture to a boil and discarding the free fluid. Fish cake mix consisted of reconstituted salt fish and rehydrated potato flakes (fish to potato 1:1) with spices and dried onion and parsley flakes as seasonings. Fish cakes were served deep-fat fried.

Fish cakes made from salted whiting stored 5 months at room temperature were rated very acceptable and equal to fishcakes made from good quality commercial salt cod.

Table 1—Relation of additional salt to fluid loss and gelling during salting of 800g of ground whiting is saturated brine for 20 min

Salt added to brine (g)	Fluid loss on draining (ml)	Amount of gel[a]
0	0	No free fluid; gelled mass
50	510	Drained fish appeared gelled
100	685	Small amount gel in drained fish
200	865	No gel
300	815	No gel

[a]Amount of gel was determined by visual examination

Table 2—Salt contents of drained salted whiting prepared with various salting times[a]

Salting time (min)	Salt content (%)
0	27.0
5	28.5
10	27.9
24	27.3

[a]Salt content was determined in accordance with section 18.010, AOAC (1965).

DISCUSSION

THE TECHNIQUE described brings together the most advantageous conditions for rapid salt-curing of fish, provided vacuum drum drying is used:

1. Grinding of the fish greatly increases surface area exposed for salting and

decreases diffusion distance, an advantage shared with the Del Valle-Nickerson technique.

2. The fish is covered with brine during grinding so that exposure to oxygen is virtually excluded and all surfaces of the fish are brought into contact with the brine immediately, features not shared by the Del Valle-Nickerson technique, in which formation of pickle or brine by action of salt on the ground fish must occur before salt is in contact with all exposed surfaces.
3. Use of additional salt to maintain saturation when the brine is diluted with the fluid of the fish provides a large salt concentration gradient until salting has taken place throughout the ground fish. Thus salting takes place in as little as 5 min (Table 2).
4. Syneresis results when the muscle protein is dehydrated in the presence of high concentrations of salt. Its occurrence indicates that the saturated salt solution diffuses so quickly that "salting-in" of the muscle protein is followed immediately by "salting-out," with no visible gel formation.
5. The small particle size of the ground fish is retained throughout processing. This greatly facilitates drying so that drying time is short. In addition, reconstitution of the dried product is accomplished so simply that the product is considered a convenience item.

In an industrial process, the use of a meat-bone separating device to obtain the ground fish efficiently is indicated. Even with immediate transfer of the separated fish to brine, some subsequent quality loss may result from exposure to air and metal catalysts during comminution. The use of antioxidants may be indicated.

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A Research Note

CONTROL OF CHLOROPHYLL AND SOLANINE SYNTHESES AND SPROUTING OF POTATO TUBERS BY HOT PARAFFIN WAX

INTRODUCTION

IN RECENT YEARS, the persistence and in some instances, intensification, of bitter taste and solanine poisoning have been associated with the technological developments in handling, transportation and merchandising of potatoes. In most supermarkets potatoes are displayed under artificial light to appeal to the customers.

Potatoes exposed to light either before or after harvest gradually turn green at the surface as chlorophyll develops (Larson, 1949). Although chlorophyll is harmless and tasteless, greening can be a serious defect in potatoes during marketing. Green potatoes are often bitter. Solanine, the poisonous glycosidal alkaloid, is responsible for the bitter taste (Hilton, 1951). Solanine develops in parenchyma cells of periderm and cortex of the tubers as chlorophyll and often light duration, intensity, and quality of both incandescent and fluorescent bulbs or tubes stimulate the development of the compounds. Sprouting is another aspect that confronts the potato industry. It not only decreases the nutritive value of the potatoes but makes them unfit for human consumption. Therefore, the control of greening, solanine formation and sprouting becomes a problem of major concern in storage and marketing. Many physical and chemical agents have been employed to control the formation of solanine, chlorophyll and sprouting. These include lowering the temperature, avoiding exposure to light, γ-irradiation, controlled atmosphere of increased CO_2 and decreased O_2, and chemicals (Larson, 1949; Schwimmer and Weston, 1958; Yamaguchi et al., 1960; Ziegler et al., 1968; Patil et al., 1971; Patil et al., 1971a, b). However no method seems satisfactory to control these defects.

In this paper, we describe a simple and effective method of inhibiting chlorophyll, solanine, sprouting and weight loss of potato tubers.

MATERIALS & METHODS

RUSSET BURBANK potatoes were used in the experiment. Nine treatments including control, waxing at 60°, 80°, 100°, 120°, 140° and 160°C, and heating in air at 160°C for 3 or 5 min were arranged in a completely randomized block design, each with three replications. Each replication consisted of eight tubers of uniform size and maturity. Paraffin wax (American Oil Co., Chicago, Ill.) was used to coat the tubers. The waxing was accomplished by a short time (½ sec) dipping the tubers in the paraffin bath at the temperatures and treatments mentioned heretofore. Tubers were exposed to the fluorescent light of 200 ft-c for 10 days at 16°C and 60% R.H. Peels (1 mm thick) were removed for determination of solanine and chlorophyll. Chlorophyll was determined by the method of AOAC (1965). The method for solanine extraction and determination was that of Gull and Isenberg (1960). For measuring sprouting and respiration, the tubers were stored in darkness at 16°C and 60% R.H. The respiration of the peel was measured by employing a Gilson Respirometer. Weight loss was determined by measuring the weight of tubers at the beginning and the end of storage. The analysis of variance was made and the means were compared according to Tukey's ω-procedure (Steel and Torrie, 1960).

RESULTS & DISCUSSION

AS SHOWN IN Figure 1, waxing potatoes for ½ sec at relatively lower temperatures (60° and 80°C) did not have significant effect on inhibition of chlorophyll and solanine formation. Waxing at relatively

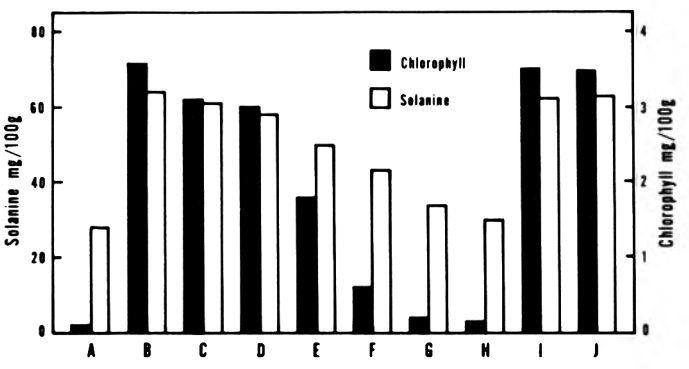

Fig. 1—Effects of waxing and heating at different temperatures on chlorophyll and solanine formation in peels of potato tubers after exposure to 200 ft-c light intensity for 10 days at 16°C and 60% R.H. (A) Original, zero-time sample; (B) Control, nonwaxed potatoes; (C) Waxing at 60°C; (D) Waxing at 80°C; (E) Waxing at 100°C; (F) Waxing at 120°C; (G) Waxing at 140°C; (H) Waxing at 160°C; (I) Heating with air at 160°C for 3 min; (J) Heating with air at 160°C for 5 min.

Table 1—Effects of waxing and heating at different temperatures on sprouting, respiration and weight loss of potato tubers after storage for 50 days at 16°C and 60% R.H.[a]

Treatment	Sprouting (mg sprout/100g)	Respiration (μl O_2/g/hr)	Wt loss (%)
Original (zero-time sample)	1.5**	104[ns]	—
Control (nonwaxed potatoes)	404.5	109	5.8
Waxing at 60°C	614.9**	98[ns]	1.8*
Waxing at 80°C	687.7**	105[ns]	1.9*
Waxing at 100°C	125.5**	110[ns]	1.3**
Waxing at 120°C	2.3**	102[ns]	1.4**
Waxing at 140°C	1.7**	112[ns]	1.1**
Waxing at 160°C	1.1**	107[ns]	0.9**
Heating at 160°C for 3 min	37.6**	92[ns]	7.7[ns]
Heating at 160°C for 5 min	20.8**	101[ns]	6.4[ns]

[a]Analysis of variance and comparison of means by Tukey's ω-procedure
*Significantly different from control at 5% level
**Significantly different from control at 1% level
[ns]Not significantly different from control at 5% level

high temperatures (100° and 120°C) significantly inhibited chlorophyll and solanine formation when compared with nonwaxed potatoes (control). Waxing at higher temperatures (140° and 160°C, especially at 160°C) almost stopped the solanine and chlorophyll formation. Heating at 160°C in air for 3 or 5 min did not inhibit chlorophyll and solanine formation. It is the combined treatment of waxing and heating which effectively inhibited solanine and chlorophyll formation. The coated wax is a barrier for gas exchange of the tubers, because waxing creates a modified atmosphere containing low O_2 and high CO_2 inside and around the tubers as a result of respiration.

It has been reported that a wax treatment (cold wax) did not reduce the greening of potato tubers (Howard et al., 1957). This is in agreement with our findings. However, only hot wax (above 120°C) inhibits chlorophyll and solanine formation.

Table 1 shows that the waxing of potato tubers at 60°C and 80°C stimulated sprouting when compared with the control; however, sprouting was inhibited when waxed at 100°C. Waxing above 120°C completely inhibited the sprouting. This may be due to the injury of the meristematic tissues of the potato buds. Table 1 also indicates that waxing significantly reduced weight loss of potato tubers during storage. This may be due to the impermeability of the coated wax to water vapor. The respiration of the peels was measured to evaluate the detrimental effect of hot waxing on the tissue. It is apparent from Table 1 that there was no significant difference in the respiratory rate of peels of nontreated and hot-wax treated potatoes when stored for 50 days at 16°C and 60% R.H. This indicates that hot waxing for ½ sec apparently has no destructive effects on the peels of the potato tubers.

Since paraffin wax does not create residue problems, like most physicochemical treatments, and coated wax can be easily removed by peeling the tubers before processing or cooking, therefore, from the point of practical application, it can be concluded that with proper combinations of hot waxing (above 120°C for ½ sec) and subsequent storage at lower temperature (5°C) should efficiently control chlorophyll, solanine and sprout formation as well as weight loss of potato tubers.

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A Research Note

A METHOD FOR CLEAN-UP OF RAW POTATO EXTRACTS FOR REDUCING SUGAR DETERMINATION

INTRODUCTION

THE ANALYSIS of raw potato extracts for reducing sugar is a critical step in many potato receiving and processing operations. A 1% maximum reducing sugar level is often imposed for processing. Potatoes for potato chip manufacture, for example, must have reducing sugar levels less than 1%. Consequently, raw material disposition is generally dependent on the analytical results of reducing sugar analyses. Speed and accuracy in the analysis of these low levels of reducing sugar are important to the processor. Both highly accurate and rapid methods have been developed. However, accurate methods are tedious and the rapid methods are of questionable accuracy because of variations in moisture levels, interference of nonsugar reducing substances (especially in unpeeled potatoes) and enzymic reactions.

Traditionally, the fried color of chips obtained under standardized frying has been used as an estimate of reducing sugar (Schwimmer et al., 1954; Lauer and Shaw, 1970). A rapid tablet test for potato juice for relating reducing sugar to chipping quality of potatoes with less than 1% reducing sugar has been developed (Wisler and Free, 1968). Samotus and Kujaioski (1970) reported the use of anthrone without clarification of the potato extract for rapid routine analysis of fructose, glucose and sucrose. The presence of sucrose in potatoes and its potential interference with reducing values can limit the use of anthrone for studies with potato extracts.

Accurate analyses have been obtained by copper reduction tests following hot ethanol extraction and a lead acetate deproteinization clean-up step (AOAC, 1970; Schwimmer et al., 1954). Recently, Oborn et al. (1971) reported analyses of dried potato products with an automatic analyzer by forming 2,4-dinitrophenolate derivatives following clarification with lead acetate and sodium phosphate/potassium oxalate. The standard deviation of the determination was reported to be 0.052%. Interference by aldehydes, ketones and amino acids is a limitation of this method. The method is not satisfactory for raw potatoes.

Ion-exchange resins have been successfully employed in other biological systems for clarification purposes (Furst, 1970), and their use in the clean-up step for potato extracts has been considered. Expense and time requirements resulting from the use of columns and regeneration of resins limit the ion-exchange procedure. Although ion-exchange appeared to be a feasible clean-up method, the greater ease and economy of charcoal as an adsorbant led to the clean-up method reported herein.

Charcoal is a standard decolorizing adsorbant in sugar processing, but its use as a clean-up tool is fairly novel. Charcoal is used for separation of mono and disaccharides (AOAC, 1970) and was the method of choice for clean-up of noncarbohydrate interfering substances in conifers (Ebell, 1969). This work reports the investigation and development of a charcoal clean-up technique for raw potato juice to be followed by any applicable colorimetric procedure for quantitative determination of reducing sugar content.

EXPERIMENTAL

Reagent preparation

Copper reagent A. 25g potassium sodium tartrate; 25g anhydrous sodium carbonate; 200g anhydrous sodium sulfate; and 20g sodium bicarbonate. Add to 800 ml distilled water and dilute volumetrically to 1:1; filter if necessary.

Copper reagent B. Dissolve 15g cupric sulfate in solution of 2 drops concentrated sulfuric acid and 85 ml distilled water; store in amber glass container.

Arsenomolybdate reagent. Dissolve 25g of ammonium molybdate in 450 ml of distilled water; add 21 ml of concentrated sulfuric acid and mix; add 3g of sodium orthoarsenate heptahydrate to 25 ml of distilled water; mix well and add to molybdate solution. Incubate at 37°C for 24 hr; store in amber glass container.

Anthrone solution. 1.5g of anthrone was dissolved in 1 liter of solvent (see reagent B). Anthrone solution was discarded if a greenish or bronze tinge appeared.

Anthrone solvent. 20% ethanol:conc H_2SO_4 (1:3 v/v).

Stock sugar solution. Vacuum dried reducing sugars were dissolved in distilled water at concentrations ranging from 15–50 mg/100 ml.

Preparation of potato samples

Potatoes were washed and diced. A 100-g sample was added to 100 ml distilled water. The potatoes were finely chopped with a cycle consisting of 30 sec chop, 10 sec mince and 10 sec grind using a household blender. The resulting slurry was filtered through a double thickness of cheesecloth, and 20 ml of the filtrate was pipetted into a 50 ml centrifuge tube. Bone charcoal (MCB # CX635) was added to clarify the filtrate. The amount of bone charcoal added ranged from 4.6–13.8g, depending on the color of the raw juice. The sample and bone charcoal were thoroughly mixed with a vortex mixer for 1 min. The tubes were allowed to stand for 10 min for initial settling and to obtain a rough estimate of the supernatant color. If necessary, additional charcoal or further agitation can be applied. The tubes were then centrifuged for 5 min at 10,000 rpm. The clear supernatant was filtered through a 0.45μ, 25 mm millipore filter into a 125 ml filtration flask. 1 ml of filtrate was collected and diluted to 50–100 ml with distilled water for anthrone colorimetric method or 10 ml for copper colorimetric method.

Colorimetry

Copper reduction method. The colorimetry was performed according to Shallenberger's modification of the Nelson procedure (Shallenberger and Mootes, 1957). Immediately before use, 25 ml of Copper reagent A is added to 1 ml of Copper reagent B to make "mixed sugar reagent." A 3-ml sample of diluted potato filtrate was pipetted into a 25 ml blood sugar tube and 1 ml of mixed sugar reagent was added. A blank was prepared using 3 ml of water and used to set the 100% transmittance reading. The blood sugar tube was capped with a glass marble and placed in a boiling water bath for 20 min. The tube was cooled in running tap water until well chilled and then 1 ml of arsenomolybdate reagent was added. The tube was shaken well and allowed to stand for 10 min with intermittent shaking. The sample was diluted to 10 ml with water and read at 500 nm. The effective range was 0.6–5.0 mg of reducing sugar.

Anthrone method (for samples where sucrose will not interfere). A 1-ml aliquot of diluted filtrate or stock solution was pipetted into a pyrex 20 cm × 0.25 cm test tube. The tubes were placed in an ice bath and 10 ml of anthrone reagent was added. Samples were mixed by swirling the tubes and immediately placed in a boiling water bath for 8 min. Samples were removed to an ice bath, chilled and then allowed to return to room temperature. Absorbance was read at 625 nm. The effective range of colorimetric analysis was .005–.100 mg reducing sugar/ml of diluted sample.

RESULTS & DISCUSSION

UNPEELED raw potatoes generally produce dark colored extracts, the interfering constituents consisting of peel components or products of enzymatic

browning reactions. The clean-up procedure described produces a clear potato extract suitable for reducing sugar analysis in a relatively short period of time (approx 30 min) from raw, ground, unpeeled potatoes. By using decolorizing charcoal followed by millipore filtration to remove the charcoal and adsorbed material, clear extracts are obtained without resorting to pretreatments to inactivate enzymes, such as heating or addition of sulfite. Sulfite prevents discoloration but may adversely affect test values. The method is less tedious than previous clean-up methods without sacrificing accuracy. The use of both copper reduction and anthrone was reported because of the desirability of the copper method in the presence of sucrose or other potentially hydrolyzable sugar fragments and disaccharides. The anthrone procedure offers greater sensitivity provided the samples do not contain sucrose or other hydrolyzable sugars.

Our experience indicated that the decolorizing step was an important factor in accurate analysis of low reducing sugar levels. Uncleaned extracts produce erroneous values. Samotus and Kujaioski (1970) reported analysis of potato tuber juice for glucose, fructose and sucrose using anthrone at a reaction temperature of 100°C. Their method was performed without clarification and good sensitivity was reported. Ebell (1969) reported that decolorizing was essential for accurate analysis of low levels of reducing sugar in conifers due to the formation of a complex of anthrone, tryptophan and carbohydrate.

In this work, no significant differences were found in the readings from samples with varying levels of charcoal, providing the amount of charcoal was sufficient to decolorize the sample. No significant losses by adsorption were observed when using known concentrations of sugar solutions.

With anthrone, the reproducibility of the method was dependent upon the precision of the individual performing the test due to the high sensitivity of anthrone reagent. Reaction conditions other than those specified herein may result in incomplete color development by glucose (Nakamura, 1968), and old reagent can produce excessive darkening of samples and reagent blank under the reaction conditions. The copper reagent, although less sensitive than anthrone, allows scanning of a wider range of reducing sugar concentrations without rediluting the samples.

The reaction conditions described produce reproducible and equivalent curves from glucose, fructose and mannose for either colorimetric method. Replicate samples of mixed tubers had an average deviation of ± .07% reducing sugar (wet weight basis).

Glucose and fructose represent the major reducing sugars in potatoes. Galactose values do not fall on the anthrone curve, but this reducing sugar is not normally a contributor to reducing sugar values of potatoes.

In performing the analysis it is important to adjust concentration of sugar to fall within the most linear transmittance range of the standard curve, 30–90% transmittance (blank = 100% transmittance). The standard curve should be checked weekly with either reagent.

The entire process can be completed in about 1 hr, assuming adequate supplies of glassware. Ten or more samples can be run as a batch in essentially the same period of time.

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A Research Note
EFFECT OF HANGING POSITION ON SOME PROPERTIES OF DRY SAUSAGE

INTRODUCTION

DRY SAUSAGE is manufactured in a wide range of varieties. This type of sausage is of two distinct types: one type being smoked and the other not smoked, sometimes known as the Italian type. In general, this type of sausage is uncooked, and its keeping qualities depend upon curing ingredients, spices and removal of moisture from the product by drying (Anonymous, 1966; MacKenzie, 1966; Anonymous, 1968).

In the processing of dry sausage, the general procedure is to suspend the sausage on sausage sticks that are in turn suspended on sausage racks in the drying room. The conditions under which the product is most favorably processed in the drying room are from 45–55°F (7.2–12.8°C) DB and 70–74% RH (MacKenzie, 1966). The moisture can only be removed from the product at the rate at which the moisture comes to the surface of the casing. Any attempt to speed the drying rate results in over drying the surface of the sausage, resulting in a condition known as case hardening. This condition precludes any further attempt to remove moisture from the interior of the sausage.

Horizontal processing of slicing stick products, e.g., bologna, is being evaluated by industry for maintaining uniform stuffed diameters over the entire length of the product (Anonymous, 1966; 1970). No information is available concerning the effect of hanging position on dry sausage products; therefore, the present study was undertaken to investigate the effect of hanging position on some properties of dry sausage.

Table 1—Effect of hanging position on percentage of shrinkage of sausage[a]

Drying time in days	Hanging position	
	Vertical %	Horizontal %
0	0.0 ± 0.0	0.0 ± 0.0
1	8.0 ± 0.1	9.7 ± 0.1
2	14.6 ± 0.1	15.9 ± 0.2
3	19.5 ± 0.1	20.3 ± 0.1
4	22.8 ± 0.2	23.4 ± 0.1
7	29.6 ± 0.2	29.9 ± 0.1
10	33.8 ± 0.2	34.1 ± 0.1
14	38.2 ± 0.2	38.5 ± 0.1
17	40.8 ± 0.3	41.1 ± 0.1
21	43.4 ± 0.2	43.7 ± 0.1
Avg	27.8 ± 0.2	28.5 ± 0.1

[a]Mean of percentage of shrinkage from six individual determinations ± standard error

EXPERIMENTAL

BONELESS PORK shoulders, containing 75–80% lean, were frozen to comply with APHIS regulations of pork to destroy trichinae. Sausages were prepared using a modified Genoa Salami formulation and processing procedure. Approximately 400–500g of the ground product was stuffed into each of 12, 52 mm D.S. fibrous casings. The sausages were then divided into two groups of six each: one group hanging in the conventional vertical position and the second group in a horizontal position. The second group was supported by using a sling made from plastic ham nets. All sausages were dried in an environmental room for 21 days at a temperature of 55°F (12.8°C) DB; and at a relative humidity of 70% with 30–40 fpm of air passing over the product. External visual observations and moisture loss determinations were made at 1, 2, 3, 4, 7, 10, 14, 17 and 21 days after preparation of product. Internal visual observations were made after the 21-day drying period.

RESULTS & DISCUSSION

Shrinkage

From the data on weights of individual sausage before and during the drying period the percentage of shrinkage and the mean percentages of shrinkage were calculated. The results of the effect of hanging position on shrinkage during the drying period are given in Table 1. Shrinkage was significantly higher ($P < 0.01$) for the samples of sausage hung in the horizontal position than for the samples hung in the conventional vertical position (Table 2). The results would indicate that this was due to the increased surface area exposed to the flow of air over the product. There was a significant interaction ($P < 0.01$) between positions and drying time, the difference between positions being greater during the earlier part of the drying cycle. Also, there was more shrinkage during the first 7 days of drying time than during the last 14 days.

Table 2—Analysis of variance of shrinkage for sausages hung in vertical and horizontal positions

Source of variation	df	MS	F
Positions (P)	1	11.08	14.3**
Drying Time (DT)	8	1757.76	2282.8**
P × DT Interaction	8	0.77	5.1**
Error	90	0.15	

**$P < 0.01$

Subjective observations

The surface of the sausage samples exposed to the direct flow of air developed a cured color faster than the surface which was not in the direct flow of air. As expected, the sausage hung in the horizontal position had a larger and redder cured color area than those samples hung in the vertical position. This was due to the increased surface area exposed to the flow of air.

In general, the samples of sausage hung in the horizontal position exhibited less incidence of hollow centers than the samples held in the vertical position. The samples with hollow centers had a poor cured color that was tannish brown. Casings were easily removed from all sausage samples regardless of hanging position.

Further studies need to be conducted on the effect of hanging position and other environmental conditions on the physical and chemical characteristics of dry sausage.

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Reference to a product name does not imply approval or recommendation of the product by the USDA to the exclusion of others that may be suitable.

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A Research Note
METMYOGLOBIN REDUCTION AND FORMATION IN BEEF DURING AEROBIC STORAGE AT 1°C

INTRODUCTION

DURING AIR STORAGE, autoxidation of myoglobin to metmyoglobin (metMb) occurs at the surface of beef. At 1°C the concentration of metMb in whole muscles remains virtually constant from 5 to at least 28 days storage. However, this concentration varies from muscle to muscle (Ledward, 1970; 1971). Stewart et al. (1965) have described a technique for measuring the metMb reducing activity (MRA) of anaerobically stored minced meat following ferricyanide oxidation. Ledward and Macfarlane (1971) found that aerobic reduction of metMb occurred in meat slices in which high initial metMb concentration had been induced by storage in atmospheres containing about 1% O_2.

This note reports on the relationship between both MRA and aerobic reducing ability (ARA) of beef muscles, and the formation of metMb during storage in air.

EXPERIMENTAL

THE SEMITENDINOSUS muscle and the highly pigmented central portion of the biceps femoris were removed from a beef carcass immediately after slaughter and their outside surfaces were flamed. After 3–6 days storage at 1 ± 1°C, 16 slices of thickness 2.0 ± 0.5 mm (Kaess, 1961) were cut from each muscle and stored in an atmosphere of 1% O_2/99% N_2 (Ledward, 1970). Two slices (15 ± 1 mm thick) were wrapped in polyethylene film (0.0015 in.) and stored in air. All storage was in the dark at 1 ± 1°C and care was taken to avoid bacterial contamination.

After 24 hr storage in 1% O_2 the metMb concentration, expressed as a percentage of the total heme pigments, was determined in a randomized sample of eight thin slices (Ledward, 1970). The metMb concentration in the other eight slices was determined after a further 24 hr in air. Each determination was the mean of 32 readings. As differences occurred in the metMb concentration of different muscles after 24 hr in 1% O_2, ARA was defined as

$$\frac{\text{Observed decrease in metMb conc}}{\text{Initial metMb conc}} \times 100$$

Only muscles having metMb concentrations of 50–70% after 24 hr in 1% O_2 were used (11 out of 16 muscles from 8 animals).

After 7 days storage the "equilibrium" metMb concentration in the thick slices was determined as the mean of 16 readings. Total heme pigment concentration ([heme]) of the muscles was determined by the method of Hornsey (1956).

After 10–13 days storage the MRA was measured as the percentage of metMb reduced 1 hr after ferricyanide addition (Stewart et al., 1965). Duplicate samples from the interior of the muscles were used. During this period no significant change in the MRA occurred.

RESULTS

THE HIGHLY SIGNIFICANT correlation (r = −0.94) between the equilibrium concentration of metMb in thick slices and ARA, determined on thin slices (2 mm) to ensure O_2 penetration throughout the samples, is shown in Figure 1.

The correlation of equilibrium concentration of metMb in the thick slices and observed decrease in metMb concentration in the thin slices is −0.88 ($P < 0.001$).

No significant correlation between the MRA levels and metMb formation was found (Fig. 2).

The mean MRA of the semitendinosus and biceps femoris muscles were not significantly different although, as observed previously (Ledward, 1971) the

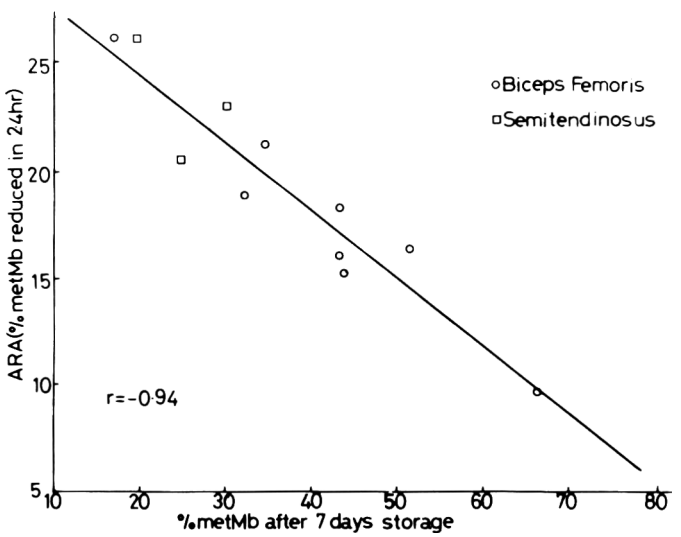

Fig. 1—Relationship between the metMb reducing activity under aerobic conditions (ARA) of post-rigor muscle slices (2.0 ± 0.5 mm thick) and the concentration of metMb on 15 ± 1 mm thick slices after 7 days storage in air. ARA is the percentage reduction in metMb over 24 hr in air after storage for 24 hr in 1% O_2/99% N_2 atmospheres. All storage was at 1 ± 1°C and the muscles were from eight carcasses.

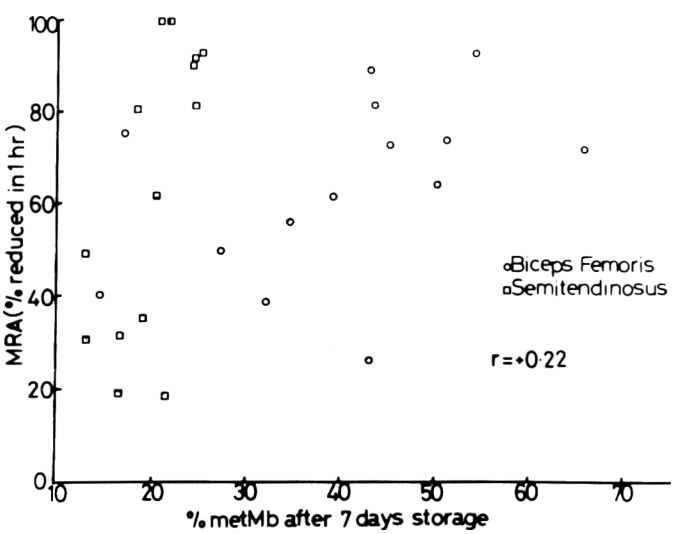

Fig. 2—Relationship between metMb reducing activity (MRA) of post-rigor muscle and the concentration of metMb on 15 ± 1 mm thick slices after 7 days storage in air at 1 ± 1°C (r = +0.22). MRA is the percentage metMb reduced, at 22 ± 1°C, during 1 hr following ferricyanide oxidation of the twice minced muscle. The muscles were from 14 carcasses including the eight used to obtain the data in Figure 1.

mean metMb concentration, [metMb], differed significantly ($P < 0.001$).

Positive correlations between [heme] and [metMb] formed were found. The linear regression was [metMb] = 1.44 + 4.5 [heme] for the biceps femoris ($P < 0.05$) where [heme] was in the range 7–12 $mg \cdot g^{-1}$ wet tissue and [metMb] = 13.9 + 1.2 [heme] for the semitendinosus ($P < 0.05$), where [heme] was in the range 2.5–9 $mg \cdot g^{-1}$. These separate regressions fitted the data better than one overall regression.

DISCUSSION

AS ARA WAS DETERMINED in the presence of O_2 it must represent the resultant of a reduction/oxidation mechanism. Thus, the high negative correlation between ARA and metMb would arise if the differences in metMb formation between muscles originated from differences in rates of oxidation and/or reduction. In solution the rate of autoxidation of bovine myoglobin in air is about 0.004 hr^{-1} at pH 5.5 and 1°C (Brown and Mebine, 1969). If this rate of oxidation occurs in fresh beef, then in samples with 50–70% metMb a further 4.5–2.8% will form by autoxidation over 24 hr. In meat of pH > 5.5 less autoxidation will occur. Thus, unless the oxidation is catalyzed in meat, differences in rates of oxidation will not explain why ARA can vary from less than 10% to over 30% (Fig. 1). If catalysis of the oxidation is responsible for the differences in ARA (and metMb formation) then the positive correlation between [heme] and [metMb] would require an increase in the ratio catalyst: [heme] with increasing [heme]. This seems to be unlikely and so some, or all of the differences in metMb formation, are probably due to differences in the effective reducing mechanism. The correlation between [heme] and [metMb] would then reflect the occurrence of decreased pigment to reductant(s) ratios.

The lack of correlation between the MRA and metMb formation agrees with the observation that increasing the MRA of meat by the addition of glutamate had no significant effect on the accumulation of metMb (Saleh and Watts, 1968). However, Hutchins et al. (1967) found meat of high MRA tended to form less metMb than meat of normal MRA during 4 days storage ($r = -0.44$ on 30 samples), while Greene (1969) reported that enzymically active samples browned more readily during storage. These results suggest that either the technique for MRA estimation does not accurately measure the activity of the enzymic reduction or that the effective aerobic reduction does not occur by this mechanism. Perhaps further insight into the effective metMb reducing system in meat could be obtained by a study of the reduction under other conditions, e.g., the anaerobic reduction of metMb in beef slices and mince following storage in 1% O_2.

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A Research Note
EVALUATION OF A METHOD TO DIFFERENTIATE BETWEEN NONFROZEN AND FROZEN-AND-THAWED MEAT

INTRODUCTION

KÖRMENDY et al. (1965) found two isoenzymes of glutamic-oxalo-acetic transaminase (GOT; aspartate amino transferase; E.C.2.61.1) in bovine and porcine skeletal muscle. They showed that one of these isoenzymes is localized in the mitochondria (GOT_M), the other in the sarcoplasm (GOT_S). The GOT activity of press juice from nonfrozen muscle is almost entirely due to the presence of GOT_S, whereas press juice of frozen-and-thawed muscle shows considerable GOT_M activity, released from damaged mitochondria (Hamm and Körmendy, 1969). Hamm and Körmendy (1965; 1966) developed a routine method to differentiate between nonfrozen and frozen-and-thawed meat, based on these findings: electrophoresis of muscle press juice, spraying with GOT reagent and viewing under UV shows only a GOT_S band with nonfrozen meat, while two bands show ($GOT_S + GOT_M$) with frozen-and-thawed meat. A modification of the method, involving quantitative determination of both GOT_S and GOT_M activity after electrophoresis, was described recently for bovine and porcine muscle (Hamm and Körmendy, 1969) and for poultry muscle (Hamm et al., 1971). However, de Fiellietaz Goethart (1968) reported the occasional presence of GOT_M in press juice of fresh porcine meat, and questioned the usefulness of the method with pork. He related his results to the wide-spread incidence of stress susceptibility in pigs, probably linked to a defective mechanism of oxidative phosphorylation and damage of the mitochondria (Sybesma et al., 1971). Hamm (1968) stated that the proportion of GOT_M and GOT_S in muscle press juice is apparently a good indicator for damage of the mitochondrial membranes. Using electron microscopy, Van den Hende (1970) recently established that the number of intact mitochondria in porcine muscle decreases considerably with body weight. This suggests that damage of mitochondria is a common defect in pig muscle, which may be reflected in the release of GOT_M into the sarcoplasm. Preliminary work, however, showed no effect of PSE conditions on the activities of total GOT, GOT_M and GOT_S in porcine muscle (Hamm, 1968).

The experiments described in this note were carried out to evaluate the effectiveness of the method proposed by Hamm and Körmendy (1965; 1966) with porcine meat.

EXPERIMENTAL

Muscle samples

Samples of skeletal muscles were obtained from animals (pigs and cattle) bought by the Research Center for Meat Production, Ghent, and designated for consumption in the University Restaurant. Before sampling, carcasses were held for 48 hr at 4°C. When frozen, samples were kept 24 hr at −17°C, wrapped in aluminum foil.

Extraction of total GOT activity

1g of muscle tissue was homogenized for 3 min in 10 ml of 0.1M phosphate buffer (pH = 7.6) using a Bühler apparatus (50,000 rpm). The supernatant, after centrifugation (90 min, 15,000G) was used. All operations described were carried out at 3°C (cold room).

Muscle press juice

Press juice was obtained by squeezing a 3-g sample of muscle tissue between the metal blades of a hand-operated press, constructed in the laboratory: 1.4–1.5 ml of juice were collected and a very thin and white film of dry material was left between the blades. After addition of 5 ml of 0.1M phosphate buffer (pH = 7.6), the juice was centrifuged for 90 min at 15,000G.

Determination of GOT activity

Determination was carried out at 25°C as described by Bergmeyer (1962) in a total volume of 3 ml containing 2.5 ml of 0.25M asparaginate in 0.1M phosphate buffer (pH = 7.6), 50 µl of 0.2M α-ketoglutarate, 50 µl of 0.012M NaOH, 50 µl of malate dehydrogenase (0.5 mg/ml; C.F. Boehringer U. Söhne, GMBH, Mannheim, Germany) and 50 µl of extract diluted as necessary. Activities are expressed in Bücher units per gram of tissue (BU/g).

Electrophoresis of GOT isoenzymes

Separation of GOT_S and GOT_M was achieved by electrophoresis at 3°C (150v, 14 hr) on cellulose acetate membranes in 0.03M phosphate buffer (ph = 7.6). Bands of GOT_M and GOT_S were visualized under UV by spraying with the mixture of reagents used for determination of GOT activity. Relative proportions of GOT_M and GOT_S were determined by cutting the membranes into 5 mm wide strips which were eluted in 2.75 ml of 0.25M asparaginate in 0.1M phosphate buffer (pH = 7.6) for 60 min at 3°C. Determination was carried out on the eluates and results expressed as percentage of total activity recovered.

RESULTS & DISCUSSION

PRELIMINARY DETERMINATIONS in nonfrozen bovine muscle showed good reproducibility although lower activities were found than reported by Hamm et al. (1969). The extracts could be stored at 3°C for 48 hr, resulting in a small decrease in activity (Table 1).

In press juice, only 20–25% of total GOT activity was recovered. This activity was due to GOT_S, as no GOT_M could be detected after electrophoresis. The low proportion of GOT_S was confirmed by electrophoresis of total extracts of nonfrozen bovine and porcine muscle (Table 2). To examine the possibility of incomplete extraction of GOT_S in the press juice, the material remaining after collection of the juice was homogenized to recover remaining activity. Electrophoresis of this homogenate revealed only the presence of GOT_M. Although contra-

Table 1–GOT activity of nonfrozen bovine M. Semispinalis (BU/g)

Total	Sarcoplasmatic	Experimental conditions
772 ± 16[a]	180 ± 3	12 detm on 1 extract
1038 ± 65	175 ± 6	5 detm on 5 extracts of 1 muscle
779	169	Fresh extract
671	167	Same extract kept 48 hr at 3°C

[a]Mean value ± Standard deviation

Table 2–GOT_M and GOT_S (% of Total) in extracts of nonfrozen bovine and porcine muscle

Species	Muscle	GOT_S	GOT_M
cattle	M. Diaphragma	19.8	80.2
pigs	M. Psoas	23.2	76.8
		25.0	75.0

Table 3–GOT_M (% of total activity) in press juice of muscle tissue

Species	Muscle	Nonfrozen	Frozen-and-thawed
cattle	M. Spinalis	–[a]	35.3
	M. Longissimus dorsi	–	32.6
		–	54.7[b]
pigs	M. Psoas	10.8	46.0
		24.0	51.8
		13.9	41.6
		16.0	49.0
		24.5	45.7
		14.0	41.0

[a]Could not be detected
[b]Sample immersed in liquid Nitrogen (–190°C)

dictory to data published by Hamm et al. (1969), who suggest a GOT_M/GOT_S ratio of near unity, these findings may be related to the lower optimal pH for GOT_S activity, reported to be 6.0 (Latner and Skillen, 1968).

Although no GOT_M could be detected in press juice of nonfrozen bovine muscle, considerable amounts of this isoenzyme were found in the press juice of nonfrozen porcine muscle, indicating damage of mitochondria (Hamm, 1968). Freezing and thawing of the muscles resulted in the appearance of GOT_M in bovine press juice and in an increase of the GOT_M concentration in porcine press juice (Table 3).

These data were obtained with six samples taken at random during a period of 3 months. Although insufficient to permit a definite conclusion, they are in line with the reported wide-spread incidence of stress susceptibility in pigs, correlated with mitochondrial damage (Van den Hende, 1970). Consequently, they indicate that the simple qualitative method proposed by Hamm and Körmendy (1965; 1966) may not be appropriate for porcine meat.

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3. To indicate more than two authors, use et al. after the name of the first author:
   Example: This was first observed by Smith et al. (1966) and later confirmed by Smith and Jones (1972).

Complete titles of works should not be cited in the text.

In the list of References. This section is headed References. The entire list MUST be typed double spaced. Each individual citation in the list should begin flush left (with no paragraph indentation). If the citation requires more than one line, indent runover lines about six characters (½ in.).

Arrangement shall be strictly alphabetical, by author(s) LAST name, and where necessary, by order of citation in the text in the case of multiple references to the same author(s) in any one year.

EXAMPLES of use in a Reference list are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is **NOT** to be included in the reference list.

(Book—example)

AOAC. 1970. "Official Methods of Analysis," 11th ed. Association of Official Agricultural Chemists, Washington, D.C.

(Bulletin, circular—example)

Adams, J.Z. and Brakestone, I.J. Growing apple tissue in a hydrotropic environment. Bull. 1478, Geneva Agric. Exp. Stn., Geneva, N.Y.

(Chapter of book—example)

Dickerson, R.W. Jr. 1968. Thermal properties of foods. In "The Freezing Preservation of Foods," Vol 2, p. 26. Avi Publishing Co., Westport, Conn.

(Journal—example)

Katz, M.H. 1972. Functional properties of carbohydrates. Food Technol. 26(3): 20.

Tsai, R., Cassens, R.G. and Briskey, E.J. 1972. The emulsifying properties of purified muscle proteins. J. Food Sci. 37: 286.

(Non-English reference—example)

Wolfsohn, J.D. 1923. "Apfelgeruch und die Chemischen Bestandteile des Apfels." Springer Verlag, Stuttgart, Germany.

(Paper accepted—example)

Delft, J.R. 1972. Apple essence recovery. Food Technol. In press.

(Paper presented—example)

Parrish, F.C. Jr., Young, R.B. and Miner, B.E. 1972. Effect of postmortem conditions on certain properties of bovine muscle. Presented at the 32nd Annual Meeting of the Institute of Food Technologists, May 22—24, at Minneapolis, Minn.

(Patent—example)

Roth, T.L. 1972. Coating of frozen foods. U.S. Patent 3,607,313.

(Secondary source—example)

Wehrmann, K.H. 1961. Apple flavor. Ph.D. thesis, Michigan State University, East Lansing, Mich. Quoted in Wehrmann, K.H. 1966. "Newer Knowledge of Apple Constitution," p. 141. Academic Press, New York.

—or—

Ogandzhanyan, A.M. 1966. Carbohydrate and nitrogenous fractions of corn ears (in Russian). Tr. Arm. Nauch.-Issled. Inst. Zhivotnovod. Vet. 7-8: 283. [In Chem. Abstr. 1968, 68(2): 967.]

(Thesis—example)

Taumer, U. 1965. Identification of volatile constituents of aged Delicious apple juice. Ph.D. thesis, Cornell University, Ithaca, N.Y.

(Unpublished data/letter—example)

Douglas, B. 1963. Unpublished data. Natl. Bur. Stand., Washington, D.C.

Douglas, B. 1963. Private communication. Natl. Bur. Stand., Washington, D.C.

- Single author precedes same author with co-authors.
- Use only initials of first and middle names and always place them after the last name.
- Do not use et al. in the reference list. List all authors by name as described above.
- Government agencies or similar organizations may be cited as authors: e.g., ACS, AAAS, IFT, USDA.

Order of listing shall be as follows: author(s); year; title (plus title of larger work, where applicable); bibliographic reference. Cite only the volume number if pages in the source are numbered consecutively; if not, give the issue in parentheses. Use a colon after the volume number (or issue). Use only the page number of the first page of the referenced item in all listings: e.g., J. Food Sci. 37: 621; Food Technol. 26(3): 27.

- In citing titles of articles or chapters, capitalize only the first word and any proper nouns. Place book titles inside quotation marks and capitalize the first letter of all significant words.
- Never underline any part of the reference listing.
- Refer to examples cited in this Style Guide.

Unpublished papers. Reference to papers presented at scientific meetings but not published should indicate the year of presentation. Similarly, references to papers accepted for publication but not yet published should show the journal name and, if known, the probable year of publication, and state "In press." If possible, avoid reference to papers that have been submitted for publication but not (yet) accepted. If reference is made to such papers they should be listed as "Submitted for publication." If the papers so cited are the author's, the author should advise the Scientific Editor of any change of status of his referenced paper during the time his new paper is being processed.

Foreign language publications. References to publications in languages other than English should be given in the original language, where this is possible, using English alphabet characters; transliterations or translations are acceptable in the case of references originally in Cyrillic alphabet. This applies to journal articles, book titles, etc.

Journal abbreviations shall conform to the *International List of Periodical Title Word Abbreviations* prepared by Standards Committee Z39, American National Standards Institute. Do not abbreviate journal titles consisting of a single word. Do not abbreviate personal names when they begin a journal title. Retain the original title order when abbreviating, but omitting articles, conjunctions and prepositions.

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Type each table on a separate sheet. Use Arabic numerals to number tables. Provide a descriptive caption, with descriptive but short column and/or side heads, giving recognized units. Keep the number of columns to a MINIMUM; do not include columns derived from others by simple arithmetic manipulation. Keep footnotes to a MINIMUM; identify them by a superior lower case letter in the body of the table and preceding the footnote below the table.

Avoid duplication. Do not present the same material in both tabular and graphic form.

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Unacceptable illustrations. These include slides, transparencies, color prints, original recorder trace chart strips, poorly executed line drawings, mimeograph copies of illustrations and the like.

BIBLIOGRAPHY

American National Standards Institute, Standards Committee Z39. 1970. International list of periodical title word abbreviations. (Available in N. and S. America from Chemical Abstracts Service, Marketing Dept., University Post Office, Columbus, OH 43210; elsewhere, ICSU-AB Secretariat, 17 rue Mirabeau, 75-Paris 16e, France.

Committee on Form and Style of the Conference of Biological Editors. 1970. "Style Manual for Biological Journals," 2nd ed. American Institute of Biological Sciences, 3900 Wisconsin Ave. NW, Washington, D.C. 20016.

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