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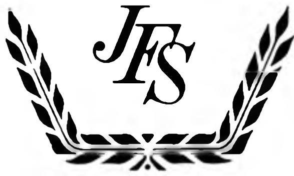

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Memo *FROM THE SCIENTIFIC EDITOR*

THREE INNOVATIONS for the *Journal of Food Science* in 1972 were the publication of • a review paper, • a symposium presented at the IFT Annual Meeting, and • two Scientific Status Summaries of the IFT Expert Panel on Food Safety and Nutrition. We plan to continue these new features of *JFS* in 1973.

As far as our regular "bill of fare" is concerned, more than 90% of the research paper manuscripts published in *JFS* in 1972 went through the publication process—from receipt by the Scientific Editor to publication—in less than 7 months.

A total of 324 manuscripts were submitted between July 1, 1970 and June 30, 1971, and 442 manuscripts were submitted between July 1, 1971 and June 30, 1972. Six issues of *JFS* were sufficient to prevent any backlog of manuscripts from occurring during 1972. However, if the number of manuscripts submitted continues to increase, an additional issue of *JFS* may be required in 1973 to maintain our present rapid processing and publication of research papers and prevent an accumulation of unpublished manuscripts.

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The quality of manuscripts, from both a scientific and literary point of view, has improved during the year. The rejection rate of manuscripts is about 25%. Many of the manuscripts are rejected because of their poor clarity and presentation. This problem could be reduced by more careful preparation and in-house review of the manuscripts before they are submitted to the Scientific Editor.

The unsung heroes of *JFS* are the members of the Board of Editors and the reviewers, who commit many hours of their busy schedules to the review of manuscripts. My thanks to both the Board of Editors and to the persons listed below who have served as reviewers for one or more manuscripts during the period of July 1, 1971 to June 30, 1972. If anyone has been omitted from the list, please accept my apologies.

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ABSTRACTS:
IN THIS ISSUE

PREPARATION OF INSTANT ORANGE JUICE BY FOAM-MAT DRYING. R.E. BERRY, C.J. WAGNER JR., O.W. BISSETT & M.K. VELDHUIS. *J. Food Sci.* 37, 803–808 (1972)–Factors of interest in commercialization of foam-mat dried instant orange juice (IOJ) were studied. These included (1) Drying conditions for commercial concentrates; (2) Concentrates with unusual properties; (3) Retention of orange oil during drying; (4) Rate of moisture removal; and (5) Materials balance. IOJ containing 1.10–1.50% moisture was prepared from commercial orange concentrates using 0.45% methylcellulose and temperatures of 160°F or less for 12 min on a "crater–type" drier. Orange concentrates with added cold-pressed peel oil resulted in retention of up to 73% of the oil in the dried product. Amount of oil before drying made little difference in initial quality of product but samples without encapsulated oil did not have satisfactory storage stability. Measure of product loss during drying indicated about 85% orange solids were recoverable as final product, but most of the losses would be reducible in commercial operation. Based on these studies a high quality foam-mat dried instant orange juice is commercially promising.

RECOVERY OF NATURAL ORANGE PIGMENTS–AN IMPROVED METHOD APPLIED TO CITRUS PROCESSING WASTES. R.E. BERRY, C.W. WILSON, III & O.W. BISSETT. *J. Food Sci.* 37, 809–811 (1972)–An improved method for purifying pigments was applied to several different peel materials. The hexane-extracted pigment was dissolved in 2-propanol, water added to make 60% 2-propanol, causing precipitation of carotenoids which were separated. Previous steps of saponification, neutralization and extensive washing were superseded. Pineapple, Temple and Valencia orange flavedo materials yielded 1009, 812 and 622 mg pigment/kg starting material, respectively. Other materials yielded 554 to 232 mg pigment/kg. In flavor studies, using paired comparison, all except pigment from Hamlin oranges resulted in acceptable products when added to FCOJ.

PRE-FREEZING PROCESSING OF GOLDEN DELICIOUS APPLE SLICES. J.D. PONTING & R. JACKSON. *J. Food Sci.* 37, 812–814 (1972)–Golden Delicious apple slices were treated by dipping, soaking or vacuum infiltration of solutions containing ascorbic acid or sulfur dioxide to protect color, and calcium and/or sugar to maintain firmness. It was found that soaking overnight in suitable solutions resulted in a better quality frozen product than dipping or vacuum infiltration. Both calcium and sugar in the soaking solution increased the firmness of frozen and thawed slices and their effects were additive. Enzymic browning of the slices could be prevented by either ascorbic acid or sulfur dioxide in the soaking solution. These compounds, especially SO_2, caused a decrease in firmness at higher concentrations, but at very low concentrations they prevented browning with little effect on texture or flavor. The highest quality slices after freezing and thawing resulted from soaking in a solution containing 20–30% sugar, 0.2–0.4% calcium and 0.2–1.0% ascorbic acid or 0.02% sulfur dioxide. Very rapid freezing further improved the crispness of the slices.

EFFECT OF CARBON DIOXIDE ATMOSPHERE ON THE COURSE OF ASTRINGENCY DISAPPEARANCE OF PERSIMMON (Diospyros kaki Linn.) FRUITS. S. GAZIT & I. ADATO. *J. Food Sci.* 37, 815–817 (1972)–The absorption curve of methanolic extracts from nonastringent and astringent persimmons was determined in the 225–375 mμ wavelength range. The height of the absorption peak at 277 mμ was found to be directly correlated with the degree of astringency as determined by tasting. Using this finding, the disappearance of astringency was followed in 'Triumph' persimmon fruits which had been treated for 0, 1, 2, 3, 6, 9, 12, 18 or 24 hr in a CO_2 atmosphere, followed by a period of shelf-life. Astringency disappeared only when fruit had been in CO_2 for at least 6 hr. A decrease in astringency was first observed 12 hr after the end of the 6-hr treatment and the fruit was free from astringency 24 hr after the start of the treatment. The rate of disappearance of astringency was the same in fruit which had been treated with CO_2 for 9 hr followed by shelf-life and those treated with CO_2 for more than 9 hr. All these fruits lost their astringency approximately 15 hr after the start of treatment.

CARBOHYDRATES, ORGANIC ACIDS AND ANTHOCYANINS OF Myrciaria jaboticaba, Berg. L.M. TREVISAN, F.O. BOBBIO & P.A. BOBBIO. *J. Food Sci.* 37, 818–819 (1972)–Glucose, fructose, sucrose, citric and oxalic acids were identified in extracts of the fruit from *Myrciaria jaboticaba*, Berg. Peonidin and peonidin 3-glucoside were the only anthocyanic pigments present in the fruit skin.

CHANGES IN QUALITY AND NUTRITIONAL COMPOSITION OF FOODS PRESERVED BY GAS EXCHANGE. T. BESSER & A. KRAMER. *J. Food Sci.* 37, 820–823 (1972)–Evacuation of intra-tissue atmosphere followed by flushing with CO and C_2H_4O was studied as a means for preserving fresh-like quality and nutritive value of mushrooms, sliced peaches and ground beef. Because of the fragile nature of these products, only partial evacuation was tolerated and oxidative browning by C_2H_4O was not avoided. By use of partial evacuation followed by CO flushing and low temperature storage in sealed containers, shelf life of mushrooms was extended to 20 days, beef patties to more than 75 days and peach slices to more than 60 days. Thiamine retention was improved in mushrooms and beef patties, and ascorbic acid in peaches. No definite pattern was found for any of the gas treatments on protein content or value, or total and reducing sugars.

RANCIDITY IN ALMONDS: SHELF LIFE STUDIES. N.E. HARRIS, D.E. WESTCOTT & A.S. HENICK. *J. Food Sci.* 37, 824–827 (1972)–Rancidity in almonds was studied by storing diced unroasted and roasted nuts (1969 and 1970 crops) for periods up to 6 months at 0°F and 100°F. Both glazed and unglazed samples were stored in hermetically-sealed cans in air packs and assayed by chemical and sensory methods. Data showed that diced unroasted almonds remained acceptable to a consumer panel for 6 months at 100°F. On the other hand diced roasted almonds became unacceptable to a technical panel after 3 months at 100°F. Confectioner's glaze was of little benefit in protecting diced unroasted or roasted almonds against sensory detectable rancidity. Moreover, it was found that the glaze provided no additional protection to almonds embedded in a sweet chocolate confection. Changes in free fatty acid, iodine value and moisture content were not useful indicators of rancidity in diced unroasted or roasted almonds during storage. However, oxygen content of the headspace gas of almonds stored in cans at 100°F, as measured by a gas chromatographic technique, agreed well with a sensory technical panel in judging quality of almonds.

SURVIVAL OF SALMONELLAE AND Escherichia coli DURING THE SPRAY DRYING OF VARIOUS FOOD PRODUCTS. D.L. MILLER, J.M. GOEPFERT & C.H. AMUNDSON. *J. Food Sci.* 37, 828–831 (1972)–The effect of spray drying on the survival of salmonellae in

certain food products was studied. Product temperature during drying and powder particle density were demonstrated to be the key factors influencing the rate of destruction of salmonellae during spray drying. A greater fraction of the contaminant flora remained viable when low dryer temperatures were employed and when more dense powders (i.e., thicker crusted particles) were prepared. Interestingly, high fat-containing products did not protect salmonellae from destruction during spray drying and even seemed to enhance the rate of destruction. Possibly, this is due to longer retention of heat in the high fat powders. It is evident that spray drying per se cannot be counted upon to supplant adequate pasteurization and post-drying sanitary procedures.

DATA ANALYSIS: INTERBLOCK AND INTRABLOCK ESTIMATES OF VARIANCE ON TASTE PANEL DATA. M.C. GACULA JR. & J.J. KUBALA. *J. Food Sci.* **37**, 832–836 (1972)–This work was conducted to assess the importance of interblock and intrablock information in the analysis of sensory panel scores. The data from three studies designed as a balanced incomplete block with repetitions was obtained as a model for the study. The result shows that the variance is reduced by interblock and intrablock recoveries. However, the absolute reduction in variance is not large enough to effect changes in the interpretation of result. An intrablock analysis of panel scores is therefore sufficient. A numerical illustration of the interblock and intrablock analyses of data is presented.

DEHYDRO-IRRADIATION PROCESS FOR WHITE POMFRET (Stromateus cinereus): SYNERGISTIC EFFECTS OF BLANCHING WITH PRESERVATIVES, PARTIAL DEHYDRATION AND LOW DOSE IRRADIATION. S.R. AGARWAL, U.S. KUMTA & A. SREENIVASAN. *J. Food Sci.* **37**, 837–840 (1972)–A process is described consisting of cooking pomfret fillets in 20% brine containing 0.1% propyl paraben for 5 min at boiling temperature, partial air-dehydration to 35–40% moisture level, packaging under anaerobic conditions and irradiation at 0.5 Mrad. This product was preserved for more than 4 months at ambient temperature when judged in terms of total bacteria and mold counts, TMAN, TVBN, TBA, carbonyl value, discoloration and organoleptic score.

CHANGES IN QUALITY OF CHANNEL CATFISH HELD ON ICE BEFORE AND AFTER PROCESSING. E.K. HEATON, J. PAGE, J.W. ANDREWS & T.S. BOGGESS JR. *J. Food Sci.* **37**, 841–844 (1972)–Sensory scores and shear press firmness measurements showed significant differences in quality associated with fasting vs. feeding and time of holding fish on ice (0–96 hr) before processing. Dulling and graying of the raw and cooked fish were apparent after 12 hr and increased with holding time. Quality reduction in nonprocessed channel catfish held off ice (75°F) for 4 and 8 hr was approximately equivalent to 24 and 48 hr on ice. Based on processing characteristics, laboratory data and observations, it was concluded that channel catfish may be held in crushed ice for 24 hr and processed into a high quality product. Dressed channel catfish retained good quality when held as "Ice-pack" for 12 days and as "Chill-pack" for 8 days.

EFFECT OF TIME AND TEMPERATURE OF SMOKING ON MICROORGANISMS ON FRANKFURTERS. M.G. HEISZLER, A.A. KRAFT, C.R. REY & R.E. RUST. *J. Food Sci.* **37**, 845–849 (1972)–The effect of different smoking schedules on microorganisms on frankfurters was studied. Frankfurters were smoked to internal temperatures of 140°F (60°C), 150°F (65.8°C), 160°F (71.1°C) and 170°F (76.8°C). Prolonged smoking up to 30 min at 150°F (65.8°C) was also investigated. In general, survival or growth of organisms during subsequent storage at 5°C was inversely related to temperature or time of smoking. Greatest rate of reduction of organisms occurred during smoking to 140°F (60°C) although further decreases were observed with heating to higher temperatures. Additional advantage of smoking to temperatures up to 170°F (76.8°C) was obtained during later refrigerated storage.

BONE DARKENING IN FROZEN CHICKEN BROILERS AND DUCKLINGS. V. HATCH & W.J. STADELMAN. *J. Food Sci.* **37**, 850–852 (1972)–Frozen broilers are characterized by an undesirable darkening of the bones. The effect of the following treatments upon darkening was determined: freezing rates, thawing rates, cooking method, refrigerated aging time, % bone ash, calcium and phosphorous in the femurs, dietary calcium level, and microwave preheating of broilers and ducklings. The freezing rate did not affect darkening. Cooking directly from the frozen condition gave less darkening than rapid or slow thawing. Aging prior to freezing for over 5 days gave increased darkening. Birds cooked rapidly with microwaves exhibited less darkening than those deep-fat fried. Increased dietary calcium decreased darkening, but not through increased bone calcification. The percentage bone ash of broiler femurs was significantly less than duckling femurs, but the percentage of calcium or phosphorous was not different between the species. No darkening occurs in ducklings. No significant correlation between bone ash or percentage calcium vs. darkening could be shown.

EFFECT OF TOCOPHEROL SUPPLEMENTATION ON THE QUALITY OF PRECOOKED AND MECHANICALLY DEBONED TURKEY MEAT. R.W. WEBB, W.W. MARION & P.L. HAYSE. *J. Food Sci.* **37**, 853–856 (1972)–Large White male and female turkeys were administered orally or subcutaneously the equivalent of 10 or 100 IU of alphatocopheryl acetate (vitamin E) per pound of ration consumed from 8 wk of age to market age. Samples representing turkey breast and thighs and mechanically deboned turkey racks were tested for storage stability. Tocopherol supplementation was effective in retarding oxidative rancidity development during cooking and frozen storage of precooked whole turkey parts, and during storage of meat from mechanically deboned turkey racks at 5°C. Weekly subcutaneous injections of tocopheryl acetate were most effective in retarding rancidity development, but the addition of 100 IU of tocopheryl acetate per pound of ration was effective enough to also warrant its recommendation. Even with these positive results, vitamin E supplementation to the live turkey does not appear to be the complete answer in stabilizing mechanically deboned turkey (MDT). Samples from turkeys receiving the tocopherol showed off-odor and off-color development after only 3 or 4 days of storage at 5°C.

AN EVALUATION OF THE ARMOUR TENDEROMETER FOR AN ESTIMATION OF BEEF TENDERNESS. R.L. HENRICKSON, J.L. MARSDEN & R.D. MORRISON. *J. Food Sci.* **37**, 857–859 (1972)–99 Angus steer carcasses with an average weight of 541 lb were evaluated for tenderness of the longissimus dorsi muscle. Breeding, feeding and environmental treatments of the animals were similar. All animals were slaughtered soon after their arrival at the plant. The carcasses were ribbed after a 24 hr chill at 0°C and tenderometer measurements were made on the longissimus dorsi of the wholesale rib. The influence of muscle temperature, repeated penetration, quantity of fat, iodine number, fiber diameter, degree of fiber kinkiness and Warner-Bratzler shear values were evaluated.

EFFECT OF CONDENSED PHOSPHATES ON pH, SWELLING AND WATER-HOLDING CAPACITY OF BEEF. G.W. SHULTS, D.R. RUSSELL & E. WIERBICKI. *J. Food Sci.* **37**, 860–864 (1972)–The effects of various levels of phosphates (0.25–1.0%) with and without addition of salt (0.5–10% NaCl) on pH, swelling and water-holding capacity (WHC) (reciprocal of meat shrinkage) were studied using ground loin and round muscles of chilled beef. The phosphates studied were sodium salts of tripolyphosphate (TPP), pyrophosphate (PP), hexametaphosphate (HMP), metaphosphate (MP) and two blends of commercial phosphates Curafos 22-4 (TPP and HMP) and Kena FP-28 (TPP, PP and sodium acid pyrophosphate). All phosphates, with the exception of MP, increase the pH and swelling of the meat. The greatest effect resulted from PP. The WHC at 60°, 65° and 70°C heating temperatures was only slightly affected when phosphates were added to the meat without salt. However, the addition of salt with the phosphates, particularly with PP and TPP, greatly increased WHC. A plateau in the increase of WHC was reached

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with the addition of 0.5% phosphates to the meat. When salt is added, alone or with the phosphates, a plateau was reached at 3–5% NaCl addition followed by a decrease in WHC with higher salt concentrations. The most effective phosphates for increasing WHC (decreasing the loss of natural juices on heating) of beef were PP, TPP and a mixture of ¾ TPP and ¼ HMP.

EFFECT OF FLUCTUATING STORAGE TEMPERATURES ON MICROORGANISMS ON BEEF SHELL FROZEN WITH LIQUID NITROGEN. C.R. REY, A.A. KRAFT & R.E. RUST. *J. Food Sci.* 37, 865–868 (1972)–Studies were made of the effect of fluctuating temperatures during holding of frozen beef loins on microbiological quality of retail cuts prepared from the loins. Cryovac-packaged loins were shell frozen by spraying with liquid nitrogen or completely frozen by conventional air blast and then held in insulated containers at varying temperatures representing transport and holding conditions. Changes in environmental temperature did not greatly influence heat absorption throughout the frozen meat. Steaks from loins frozen by either method had lower bacterial counts than did fresh steaks during the first 3 days of storage at 5°C; differences were significant. Shell freezing was successful in preserving the meat for several days.

EFFECT OF CONTROLLED GAS ATMOSPHERES AND TEMPERATURES ON QUALITY OF PACKAGED PORK. J.R. ADAMS & D.L. HUFFMAN. *J. Food Sci.* 37, 869–872 (1972)–144 boneless pork chops, 1.5 cm thick, were packaged in gas permeable film and allotted at random to four storage treatments: 2.2°C air; 2.2°C gas (mixture of ≈ 70% N_2, 25% CO_2 and 5% O_2); –2.2°C air; –2.2°C gas for 15 days. Aerobic, anaerobic and lactic acid producing bacteria were enumerated at 0, 5, 9 and 15 days post packaging. Dilutions were made following a 5 min washing in 99 ml water on a shaker. Surface area was calculated from acetate tracing, and counts were reported as number per cm^2. Color was evaluated by panel and reflectance spectrophotometry. Overall acceptability of the cooked chops was evaluated by a taste panel. Aerobic counts increased more rapidly and were higher ($P < 0.20$) at 2.2°C than at –2.2°C and increased more rapidly and were higher ($P < 0.20$) in air than in gas. At the end of storage, both gas treatments had lower aerobic counts than the air treatments. The –2.2°C gas treatment had the lowest ($P < 0.15$) aerobic count (slightly greater than the initial count). No differences were shown in anaerobic or lactic acid counts for any treatment. Color became progressively lighter ($P < 0.01$) during storage by both color panel scores and reflectance measurements. The color panel indicated that chops stored at –2.2°C were darker ($P < 0.01$) than chops stored at 2.2°C. Sensory evaluation indicated that chops decreased ($P < 0.01$) in acceptability during storage. Chops stored at –2.2°C were least acceptable ($P < 0.05$).

ANTIOXIDANT EFFECT OF PROTEIN HYDROLYZATES IN A FREEZE-DRIED MODEL SYSTEM. S.J. BISHOV & A.S. HENICK. *J. Food Sci.* 37, 873–875 (1972)–Protein hydrolyzates derived from autolyzed yeast (AYP) and from acid hydrolyzed vegetable protein (HVP) alone and in combination with phenolic antioxidants, α-tocopherol or butylated hydroxyanisole (BHA), inhibited the autoxidation of antioxidant-free corn oil in a freeze-dried model system containing a carboxy-methyl-cellulose (CMC) matrix. Inhibition was demonstrated by measuring oxygen uptake by gas chromatography of headspace samples during incubation at 65°C to the end of the induction period. Several commercial samples of both AYP and HVP were screened for antioxidant activity. Those with the greatest antioxidant activity were fractionated on a series of molecular sieves. All fractions had antioxidant activity, with those in the range below 700 and between 700 and 1500 having greater activity than those between 1500 and 5000. Antioxidant activity of hydrolyzate fractions was determined over the range of about 10 to 50% oil basis in a system composed of 1:1 corn oil and CMC. Maximal effect was observed at about 25%. The most active fraction increased the induction period by a factor of 10. Synergism was observed in the combined effects of AYP with BHA or α-tocopherol.

SENSORY EVALUATION USING COMPOSITE COMPLETE–INCOMPLETE BLOCK DESIGNS. J.A. CORNELL & F.W. KNAPP. *J. Food Sci.* 37, 876–882 (1972)–The complete block (CB) and incomplete block (IB) designs presently used for sensory evaluations are inadequate for testing and measuring the panelists × treatments (P × T) interaction (the difference in magnitude of variations in judgements). This testing is possible with a composite complete-incomplete (C-I) block design in which each CB is augmented with an IB; i.e., some of the samples in the former are replicated in the latter. Removal of the P × T interaction allows the estimation of pure error in the analysis of variance. The use of pure error only, when comparing sample effects, leads to a more efficient test than can be attained with the CB or IB designs. The efficiency increases as the ratio M.S. S × T interaction/M.S. error increases, provided the ratio remains nonsignificant.

ASCI PRODUCTION BY *Byssochlamys fulva* ON A SYNTHETIC MEDIUM. R.J. HEBERT & A.D. LARSON. *J. Food Sci.* 37, 883–885 (1972)–A liquid synthetic medium composed of Czapek's basal salts mixture, sucrose (20.0g/l), calcium chloride (5.5g/l), riboflavin (9.0 mg/l), nicotinamide (100 mg/l), and ascorbate (200 mg/l) supported on a dry weight basis asci production to the extent of 30–50% obtained with potato extract sucrose broth (PSB). Asci from the synthetic medium and PSB exhibited the same quantitative reaction to heat shock. Asci production in the synthetic medium was consistent with different spore crops when the initial pH was 4.0. Erratic asci production occurred if the initial pH was 2.0 or 5.0 or if glucose was substituted for sucrose.

CHEMISTRY OF THIAMINE DEGRADATION. Mechanisms of Thiamine Degradation in a Model System. B.K. DWIVEDI & R.G. ARNOLD. *J. Food Sci.* 37, 886–888 (1972)–Degradation products of autoclaved thiamine-S^{35} samples were separated by thin layer chromatography and quantitated by a Geiger-Mueller chromatogram scanner. At pH 6.0 or below, thiamine breaks down at the methylene "bridge" between thiazole and pyrimidine moieties, producing 4-methyl-5-(β-hydroxyethyl) thiazole and a pyrimidine derivative, probably 2-methyl-4-amino-5-hydroxymethyl pyrimidine. Above pH 6.0 small amounts of the pseudo base of thiamine and/or the thiol form of thiamine exist along with free thiamine. Hydrogen sulfide appears to be the main degradation product under these conditions. Some complexes of thiamine are also likely formed under these conditions.

GAS CHROMATOGRAPHIC ESTIMATION OF THIAMINE. B.K. DWIVEDI & R.G. ARNOLD. *J. Food Sci.* 37, 889–891 (1972)–A gas chromatographic method for thiamine estimation in food products and pharmaceutical preparations has been developed. Thiamine, if present in bound form as thiamine pyrophosphate, is extracted by refluxing the sample in 0.1N HCl, followed by enzymatic hydrolysis of the pyrophosphate group with Takadiastase or Mylase-P. Extracted thiamine is quantitatively cleaved into 4-methyl-5-(β-hydroxyethyl) thiazole and a pyrimidine moiety by incubating the thiamine solution in the presence of bisulfite at pH 6.0. 4-Methyl-5-(β-hydroxyethyl) thiazole is extracted

with chloroform/methanol solvent, converted into its TMS derivative, and quantitated by gas-liquid chromatography using a flame ionization detector.

MEAT TENDERNESS: AGE RELATED CHANGES IN BOVINE INTRAMUSCULAR COLLAGEN. M. SHIMOKOMAKI, D.F. ELSDEN & A.J. BAILEY. *J. Food Sci.* 37, 892–896 (1972)–Results of a study to investigate the changes in the aldehyde-derived crosslinks of intramuscular and tendon collagen fibres of various bovine muscles with increasing age demonstrated that the proportion of crosslinks reducible by borohydride increased from the foetal stage to reach a maximum at about 1 yr, and then gradually decreased. At maturity of the animal the reducible crosslinks had virtually disappeared. The solubility of collagen as gelatin follows a similar pattern. The results clearly indicated that these reducible crosslinks are labile intermediates in the stabilization of the collagen fibre, and the amount present at any given time is directly related to the growth rate. The proportion of labile and stable crosslinks present at a particular age provides a rational basis for the age related changes in the tenderness of meat. Comparison of intramuscular and tendon collagen revealed a consistently higher proportion of the more heat stable crosslink intermediates and a lower solubility in the former. No significant differences could be detected in the nature or extent of the crosslinking of the various active and less active muscles examined. It was not possible, therefore to convincingly correlate the nature of the crosslinks with the classification of the muscles according to meat toughness, although a tentative relationship with the proportion of the most stable Schiff base crosslink was suggested.

EFFECTS OF POST-MORTEM AGING AND STRETCHING ON THE MACROMOLECULAR PROPERTIES OF COLLAGEN. N.E. PFEIFFER, R.A. FIELD, T.R. VARNELL, W.G. KRUGGEL & I.I. KAISER. *J. Food Sci.* 37, 897–900 (1972)–Bovine longissimus muscles were stretched and/or aged 1 or 21 days. Acrylamide-gel electrophoresis of guanidine hydrochloride-soluble intramuscular collagen indicated that stretching and aging decreased the number of covalent cross-links. The correlation between α component and shear value was significant ($P < 0.01$) indicating that tenderness of meat might be increased by reducing the number of covalent cross-links in the collagen network. Heat labile collagen yield was not affected by stretching and/or aging, but differences in heat labile yield between intramuscular and epimysial samples were significant ($P < 0.01$). The data indicate that tenderness of meat is more closely associated with cross-linking of soluble collagen than with amount of heat labile collagen.

MORAXELLA-ACINETOBACTER AS CONTAMINANTS OF BEEF AND OCCURRENCE IN RADURIZED PRODUCT. N.P. TIWARI & R. B. MAXCY. *J. Food Sci.* 37, 901–903 (1972)–Food products contain gram-negative diplococci and physiologically closely related diplobacilli, but the population density has not been determined. These bacteria have been identified as belonging to various genera, but the most appropriate identification for this work was *Moraxella-Acinetobacter*. Selective media were not usable for these organisms, therefore colonies were picked by random design from Trypticase Soy Agar plates. Identification of the isolates indicated the frequency of occurrence and allowed calculation of the population density. *Moraxella-Acinetobacter* accounted for approximately 3% of the total microflora of poor quality ground beef. In high quality ground beef these organisms accounted for a greater percentage of the total population. When ground beef was stored at 5°C or 25°C *Moraxella-Acinetobacter* grew slower than other members of the microflora. Various parts of carcasses and sources of beef were found to contain *Moraxella-Acinetobacter* indicating the organisms were not limited to specific parts of the animal. *Moraxella* were more resistant than *Acinetobacter* or most other members of the microflora to radiation. The proportion of *Moraxella* was therefore increased by radurization (68 Krad) of ground beef. During storage of radurized ground beef, *Moraxella-Acinetobacter* were overgrown by other members of the microflora.

EMULSIFYING CAPACITY OF MUSCLE PROTEIN: PHASE VOLUMES AT EMULSION COLLAPSE. J.C. ACTON & R.L. SAFFLE. *J. Food Sci.* 37, 904–906 (1972)–Oil phase volumes at emulsion collapse ranged from 72.0 to 81.1% increasing as the concentration of salt-soluble muscle protein increased from 2.6 to 16.8 mg/ml. Accompanying the 13% increase in oil phase volume was a 75% decrease in emulsifying capacity and an 88% increase in emulsified volume of oil. Examination of previous reports of emulsifying capacities of meat proteins showed that the collapse point is primarily dependent on the basic method of emulsification.

CERTAIN CHEMICAL AND PHYSICAL PROPERTIES OF HAM MUSCLE PORTIONS AFTER THERMAL PROCESSING. D.G. TOPEL, F.C. PARRISH JR., R.E. RUST & D.G. WILSON. *J. Food Sci.* 37, 907–908 (1972)–Five normal-to-dark hams and five pale, soft, watery hams were boned and separated into three major muscle portions: (I) quadriceps femoris; (II) semimembranosus, gracilis and pectineus; (III) semitendinosus and biceps femoris. These fresh ham portions were subsequently processed through a conventional ham canning operation. The influence of muscle portion on percentage moisture, fat and protein was highly significant. Portion II was highest in moisture and protein and lowest in fat percentage. Conversely, portion III was highest in fat and lowest in protein and moisture. Portion I was intermediate. The muscle portions from the pale hams had a higher percentage purge (quantity of fluid released into container after heat processing) than did the portions from the normal colored hams. Highest percentage purge was found in portion II, and, lowest, in portion III. Purge from pale, watery hams was significantly higher in specific gravity than was the purge from normal ham muscles. Significant differences existed for sodium and potassium levels in the purge from the three muscle portions, but no significant difference was obtained between pale and normal colored muscles.

PORCINE AND OVINE MYOGLOBIN: ISOLATION, PURIFICATION, CHARACTERIZATION AND STABILITY. L.D. SATTERLEE & N.Y. ZACHARIAH. *J. Food Sci.* 37, 909–912 (1972)–Purified myoglobins from ovine and porcine muscle were isolated and characterized. Throughout the study both myoglobins were compared to bovine myoglobin. Ovine and bovine myoglobin were found to be very similar by their behavior during purification and with respect to their amino acid composition, isoelectric point and stability to acid denaturation. The oxymyoglobins from all these muscle sources differed in their autoxidation rates, whereas all of the myoglobins were equally stable toward heat denaturation. Porcine myoglobin definitely differs from ovine and bovine myoglobin in its behavior during purification, its amino acid composition, isoelectric point and sensitivity to acid denaturation.

EFFECT OF CERTAIN PHYSICAL AND CHEMICAL TREATMENTS ON THE MICROSTRUCTURE OF EGG YOLK. R.J. HASIAK, D.V. VADEHRA, R.C. BAKER & L. HOOD. *J. Food Sci.* 37, 913–917 (1972)–The structural changes induced in yolk by various chemical and physical treatments were studied by scanning and transmission electron microscopy. Scanning electronmicrographs showed that the surface structure of the frozen and concentrated yolk samples was much more "open" than that of normal yolk. Transmission electronmicrographs showed changes in both the low density (LDF) and high density (HDF) fractions. The background continuous phase (LDF) showed a greater disorganization while the electron dense particles (HDF) showed changes in shape and organization in both the frozen and concentrated samples. Urea produced changes similar to those caused by freezing, while NaCl tended to stabilize the LDF and break up the HDF particles. Possible alteration of the structure of water or the removal of water may result in lipoprotein destabilization and aggregation. The photomicrographs suggest that the freezing process induces aggregation of the low- and high-density yolk lipoproteins. This aggregation results in the formation of a three-dimensional structure which entraps large quantities of water, and may result in an increased yolk viscosity.

THE RELATIONSHIP OF LYSOZYME CONTENT OF EGG WHITE TO VOLUME AND STABILITY OF FOAMS. E.A. SAUTER & J.E. MONTOURE. *J. Food Sci.* 37, 918–920 (1972)–Eggs from hens previously indexed for lysozyme content of egg white were used to study stability and volume of foams. Eggs from individual hens were identified as to production date and stored at 3.3°C for up to 8 wk. Eggs were equilibrated to room temperature, weighed, broken out and Haugh Units determined. Yolks and whites were separated; whites from two eggs/hen were blended to obtain a uniform consistency. Five μliter samples used for

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each foam were subjected to electrophoresis. Fractions were quantitated by staining with Ponceau S, eluting and determining optical density at 540 mμ. Similar determinations were made on foam drainage. 60 ml samples of egg white were whipped at 336 rpm for 90 sec, foams transferred to funnels, volume determined and foams allowed to drain for 60 min. Foam volume was significantly less for high lysozyme eggs than for low lysozyme eggs (212 ml compared with 265 ml), through 4 wk of storage but not thereafter. Foam volume was negatively correlated with both lysozyme content (r = −0.884) and with drainage (r = −0.517). Electrophoretic patterns indicate that albumins represent relatively more and lysozyme less of the protein in foam drainage than in egg white.

FUNGAL DECAFFEINATION OF ROAST COFFEE INFUSIONS. S. SCHWIMMER & R.H. KURTZMAN JR. *J. Food Sci.* 37, 921–924 (1972)–Caffeine was removed from infusions of both ground and soluble commercial roast coffee by fermentation with a caffeine-utilizing strain of *Penicillium crustosum.* Fungal decaffeination was stimulated by sugars and inhibited by organic buffer anions. The rate of decaffeination was optimal at pH 4.8. The effect of initial caffeine concentration, initial volume and other variables were also examined.

THE NATURE AND CONFORMATION OF THE CAFFEINE-CHLOROGENATE COMPLEX OF COFFEE. I. HORMAN & R. VIANI. *J. Food Sci.* 37, 925–927 (1972)–Caffeine and the chlorogenate ion form a hydrophobically bound π-molecular complex in D_2O with an association constant of 16.9 kg (soln) · $mole^{-1}$ at 40°C as determined by the complexation-induced displacement of NMR absorptions. The spectra show that the complex is formed between the entire caffeine molecule and the aromatic ring of the caffeoyl moiety of the chlorogenate ion.

THERMAL DETECTION OF SPOILAGE IN CANNED FOODS. L.E. SACKS & E. MENEFEE. *J. Food Sci.* 37, 928–931 (1972)–The heat liberated during spoilage of some canned foods inoculated with common spoilage bacteria has been monitored by placing thermistors on the tops of inoculated cans and a reference can, connecting them to a Wheatstone bridge and recording temperature difference as a voltage. Temperature increases of 0.02–0.07°C were detected during spoilage. Possibilities for thermal detection of spoilage of processed foods are discussed. The method would have the advantages of a nondestructive technique, capable of detecting spoilage at the time of occurrence. Limitations of the method (viz., slow growth at low temperature, with low rates of heat evolution, and arrested growth due to exhaustion of essential nutrients, or accumulation of metabolic products) are discussed.

QUANTITATIVE ANALYSIS OF BETACYANINS IN RED TABLE BEETS (Beta vulgaris). J.H. VON ELBE, S.H. SY, I.-Y. MAING & W.H. GABELMAN. *J. Food Sci.* 37, 932–934 (1972)–A quantitative method was developed to determine stability and variability of betacyanins obtained from several breeding lines of red table beets. The method employs paper electrophoresis of aqueous beet extracts. Individual bands are quantified by determining peak areas with densitometry (550 nm). Amounts of pigment(s) are calculated by comparing peak areas of unknown samples to those of known betanin concentrations. Values within the range of 2–9 μg had a standard deviation of 0.4 μg. Recovery of pure betanin was 98% when added to a sample of raw beet extract. This method permits determination of individual betacyanins, which is not possible with other existing procedures.

INFLUENCE OF SELECTED 5'-NUCLEOTIDES ON FLAVOR THRESHOLD OF OCTANAL. D.J. SCHINNELLER, R.H. DOUGHERTY & R.H. BIGGS. *J. Food Sci.* 37, 935–937 (1972)–Octanal exhibits an orange-like flavor in water at a concentration of approximately 10 ppb, and is found in fresh orange juice at about 60 ppb. 5'-Nucleotides, noted for their flavor-enhancing potential, are relatively abundant in orange juice. This study determined the influence of selected 5'-nucleotides on the flavor threshold of octanal. Ten screened and trained panelists evaluated combinations of five concentrations of octanal (0.1, 0.3, 1.0, 3.0 and 10 ppb) in six nucleotide (ATP, ADP, AMP, GTD, GDP and GMP) solutions, plus a control with octanal only. The nucleotides were used at 10 ppm, the approximate concentration of each in fresh juice. The flavor threshold in ppb obtained for octanal in distilled water (control) was 1.38. Of the 5'-nucleotidestested, the thresholds for octanal in ADP and GMP were significantly lower, 0.85 and 0.86, respectively. ADP and GMP are postulated as intensifying orange flavor. GMP has been reported to possess flavor-intensifying properties while ADP was reported to lack this activity.

CHARACTERIZATION OF LIPIDS FROM SEEDS OF THE ROSACEA FAMILY. T. GUTFINGER, S. ROMANO & A. LETAN. *J. Food Sci.* 37, 938–940 (1972)–Composition of fatty acids, sterols and tocopherols in lipid extracts from kernels of apricot, peach and almond were determined by thin-layer and gas-liquid chromatography. All three oils were composed mainly of oleic and linoleic acids and were also similar in the composition of sterols (β-sitosterol was the main component) and in squalene content. α-tocopherol was the principal component in the extracts from almond and peach kernels, while γ-tocopherol was the major tocopherol in the apricot oil. A small amount of δ-tocopherol was detected only in apricot oil. Similarities in the oils' composition make possible substitution of the relatively expensive almond oil with apricot or peach oils.

ABSORPTION OF AQUEOUS BISULFITE BY APRICOTS. A.E. STAFFORD, H.R. BOLIN & B.E. MACKEY. *J. Food Sci.* 37, 941–943 (1972)–The sulfur dioxide content of fresh apricots dipped in bisulfite solutions was measured and the rate of penetration of the bisulfite determined with the catechol test. These studies showed a linear relationship between dip solution concentration and sulfur dioxide content of the treated apricots. The rate of bisulfite absorption by apricots was significantly increased by lowering the pH of the dip solution along with deeper penetration of the bisulfite into the fruit during dipping. The pit and cut surfaces of the apricots absorb the bisulfite rapidly during the first few seconds of immersion, but the rate of absorption diminishes rapidly thereafter.

FREE AMINO ACIDS IN RAW AND PROCESSED TOMATO JUICES BY ION EXCHANGE CHROMATOGRAPHY WITH A LITHIUM CITRATE COLUMN FOR SEPARATION OF GLUTAMINE AND ASPARAGINE FROM THREONINE AND SERINE. F.H. STADTMAN.

J. Food Sci. 37, 944–951 (1972)–Automatic ion-exchange chromatography has been used to determine the free amino acids of tomato juice before and after processing. A procedure is described for the ion-exchange separation of glutamine and asparagine from threonine and serine using a resin column equilibrated and eluted with lithium citrate buffer. In raw tomato juice 31 compounds giving positive reactions with ninhydrin were separated. These include ammonia and 20 identified amino acids. The results of this study have been compared with those reported in eight other publications. Discrepancies in the various results have been discussed in detail.

EFFECTS OF SUB-ATMOSPHERIC PRESSURE STORAGE ON RIPENING OF TOMATO FRUITS. M.T. WU, S.J. JADHAV & D.K. SALUNKHE. *J. Food Sci.* 37, 952–956 (1972)–Sub-atmospheric pressure storage inhibited the ripening of 'green-wrap' tomatoes and thus extended the storage life. Inhibition was proportional to the reduction in pressure. Physiological changes associated with ripening (e.g., losses of chlorophyll, starch, the formation of lycopene, β-carotene, flavor, and sugar) were delayed. Tomatoes could be stored at 102 mm Hg for 100 days and then ripened at 646 mm Hg in 7 days.

EFFECTS OF FUNGICIDES IN COMBINATION WITH HOT WATER AND WAX ON THE SHELF LIFE OF TOMATO FRUIT. J.A. DOMENICO, A.R. RAHMAN & D.E. WESTCOTT. *J. Food Sci.* 37, 957–960 (1972)–Ten fungicides were tested by each of three dip treatments: in water at room temperature, in hot water and in fruit wax. Storage was for 8 wk at 50–55°F. Each treatment was evaluated for effectiveness in reducing infection spread, controlling causal organisms and increasing yield. It was found that captan, OPP, thiram and dithane M45 showed promise in extending tomato shelf life.

FATTY ACID CONTENT OF FRANCHISE CHICKEN DINNERS. W.P. DONOVAN & H. APPLEDORF. *J. Food Sci.* 37, 961–962 (1972)–Fatty acid composition of franchise chicken dinners was determined by gas-liquid chromatography. Five dinners were analyzed from each franchise. Five fatty acids accounted for 98% of the total fatty acids present in extracted fat. Mean values and ranges for relative percent fatty acid content were: palmitic acid 20% (17–23%), palmitoleic acid 2% (1–3%), stearic acid 7% (5–10%), oleic acid 47% (44–54%) and linoleic acid 23% (19–28%). Linoleic acid contributed an average of 10% of the total caloric content of the dinners. The average ratio of unsaturated to saturated fatty acids was 2.5 to 1.

THE EFFECT OF FLAVOR ENHANCERS ON DIRECT HEADSPACE GAS-LIQUID CHROMATOGRAPHY PROFILES OF BEEF BROTH. J.A. MAGA & K. LORENZ. *J. Food Sci.* 37, 963–964 (1972)–This study was designed to measure by direct headspace gas-liquid chromatography (GLC) the possible odor interactions of beef broth and several common flavor modifiers [monosodium glutatmate (I), equal mixtures of disodium 5′-inosinate and disodium 5′-guanylate (II) and equal mixtures of I and II]. Control broth was prepared from soup bones and water. Upon cooling, 0.05% of I, II and equal proportions of I and II were added, stirred and the samples refrigerated for 24 hr. The samples were equilibrated to room temperature and 2 ml of broth heated at 60°C for 15 min in a 5 ml stoppered serum vial. 2 ml of vapor were withdrawn and injected into a GLC unit and the peak areas measured with an automatic integrator. In all cases enhancers increased peak areas. The total increase for I was 1.66 times that of the control; II increased 2.30 times; and equal mixtures of I and II increased total peak area 2.35 times.

WATER BINDING OF SOME PURIFIED OILSEED PROTEINS. R.D. HAGENMAIER. *J. Food Sci.* 37, 965–966 (1972)–The amount of bound water at 84% relative humidity, for a group of purified oilseed proteins, decreased in the order: cottonseed isolate I, coconut, soybean, peanut, sunflower, cottonseed isolate II. A group of animal proteins, selected for comparison, adsorbed more water than the oilseed proteins. Results suggest that the large amount of amide nitrogen in oilseed proteins is responsible for their low water binding.

IMPLICATION OF Bacillus subtilis IN THE SYNTHESIS OF TETRAMETHYLPYRAZINE DURING FERMENTATION OF COCOA BEANS. D.L. ZAK, K. OSTOVAR & P.G. KEENEY. *J. Food Sci.* 37, 967–968 (1972)–A strain of *Bacillus subtilis* isolated from fermenting cocoa beans yielded 6×10^{-7} μg of tetramethylpyrazine (TMP) per viable cell when cultured on synthetic medium. No TMP was produced when uninoculated media was incubated. During fermentation of cocoa beans TMP concentration increased linearly with time and paralleled the growth of *B. subtilis*. Addition of labeled carbon-14 glucose showed labeled TMP only when cell growth occurred. Quantitation of TMP in unroasted cocoa beans may find application as an index of fermentation and flavor quality.

EFFECTS OF LIGHT AND TEMPERATURE ON THE FORMATION OF SOLANINE IN POTATO SLICES. D.K. SALUNKHE, M.T. WU & S.J. JADHAV. *J. Food Sci.* 37, 969–970 (1972)–Formation of solanine in peeled potato slices was stimulated when stored at 15° and 25°C in the dark or light (200 ft-c). The slices held under light developed nearly three to four times more solanine than those in the dark. Significantly higher concentrations of solanine were formed in the late (after 24 hr) than in the early stage of storage period. Hence, it can be concluded that when potatoes are sliced for chips or French fries, they should be processed immediately before the glycoalkaloid–solanine–is synthesized in higher concentrations.

EFFECT OF VARIOUS SUGARS AND THEIR DERIVATIVES UPON THE GERMINATION OF Bacillus SPORES IN THE PRESENCE OF NISIN. K.G. GUPTA, R. SIDHU & N.K. YADAV. *J. Food Sci.* 37, 971–972 (1972)–Germination of the spore of *Bacillus cereus*, *Bacillus megaterium* and *Bacillus stearothermophilus* to selected carbohydrates and their derivatives was studied both in the presençe and absence of nisin. Ribose, mannose, sorbose, arabinose, glucose, mannitol, fructose, arbutin, mucic acid, glucuronic acid, raffinose, sucrose, lactose, melibiose, inulin and glycogen were all more or less effective stimulants for the germination of spores of all the three *Bacillus* species. But in the presence of 100 μg/ml of nisin, the maximum germination of spores of each of the species was produced by the following carbohydrates: *B. cereus*, glucose and inulin; *B. megaterium* sorbose and inulin; *B. stearothermophilus*, arabinose and sucrose. The remaining sugars were less effective or had no effect.

BOTULISM. A Scientific Status Summary. IFT EXPERT PANEL ON FOOD SAFETY & NUTRITION. *J. Food Sci.* 37 985–988 (1972)–A brief review of the incidence of botulism is followed by a description of the microorganism and the illness, its causes and distribution in nature, and methods for determining the cause of suspected outbreaks. Most botulism outbreaks are attributed to home-preserved foods; commercial processing methods sufficiently control the problem. General procedures to keep foods safe from botulism are reviewed concluding that proper food safety can be achieved by following proper procedures exactly, use of good sanitation, and most importantly, good common sense.

NITRITES, NITRATES AND NITROSAMINES IN FOODS–A DILEMMA. A Scientific Status Summary. IFT EXPERT PANEL ON FOOD SAFETY & NUTRITION. *J. Food Sci.* 37, 989–992 (1972)–Attention is currently being focused on nitrosamines, a group of chemicals shown to be carcinogenic, which can be formed in food products as a reaction between nitrites and certain amines. Presents a general review of nitrite and nitrate toxicity studies emphasizing the difficulty encountered in analyzing for nitrosamines. In answer to the question why not eliminate amines and nitrites from foods, suggests acquiring more data as quickly as possible so a sensible, considered judgment can be made.

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APPLIED SCIENCE and ENGINEERING

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PREPARATION OF INSTANT ORANGE JUICE BY FOAM-MAT DRYING

INTRODUCTION

FOAM-MAT DRYING is useful for producing dehydrated products from heat sensitive liquid-form foods or those in which high sugar content causes stickiness at temperatures used in other dehydration methods (Morgan et al., 1961). Foam-mat drying has two definite advantages. First, the use of foams greatly speeds moisture removal and permits drying at atmospheric conditions in a stream of hot air in a short time. The other advantage is that even though the product may be sticky at drying temperatures, it can be transferred to a cooling zone and crisped before it is scraped off the surface. Several commercial processes currently producing instant fruit juice products are variations of foam-mat drying (Beck, 1968).

Scalf (1967) has proposed a large scale commercial "carousel" design for perforated tray-type foam-mat driers. A perforated stainless steel belt-type foam-mat drier has been in commercial operation in Corvallis, Oregon (Foamat Foods, Corp.) for several years. Although citrus is not one of the principal products, instant orange juice has been produced there. A solid steel belt-type commercial operation is installed at Citrus World, Inc., Lake Wales, Fla., and is devoted almost exclusively to citrus juices and by-products. This unit has been in successful operation for the past 2 yr.

Bissett et al. (1963) described conditions for preparing foam-mat dried orange juice on a pilot scale fiber-glass belt-type drier of low capacity. Berry et al. (1965a) described conditions for preparing grapefruit powder on a similar drier and in 1967 (Berry et al., 1967b) described conditions for producing dried grapefruit on a "crater-type" tray foam-mat drier which appeared more practical for commercial use.

Inasmuch as the "crater–type" foam-mat drier enabled production of a low moisture product (where in-package desiccation was not required) and maintenance of flavor quality, a study was needed of conditions for producing instant orange juice (IOJ) on this type drier. Studies were needed on variations which might be encountered in commercial orange concentrates. This information would enable IOJ producers to modify process conditions for a given concentrate or to outline specifications of concentrates best suited for foam-mat drying. Because prescribed amounts of orange oil are needed for flavor fortification, information was also needed on retention of added oil after drying and the effect of peel oil in the concentrate. Although commercial encapsulated oils are available to add to the dried product for flavor reinforcement, their necessity might be circumvented if cold-pressed peel oil in orange concentrates were retained after drying. This would require study of the effect on flavor when added in this manner, however.

Further, because of increasing interest in commercial possibilities studies were needed on the rate of moisture removal and product losses. Such information could be helpful in pointing up specific developments needed to improve efficiency.

Experiments were planned to study these questions as related to instant orange juice production.

EXPERIMENTAL

Fruit

Seventy field boxes (90 lb fruit/box) of Valencia oranges obtained locally with juice of about 13° Brix and about 14:1 Brix/acid ratio were randomized and subdivided to prepare experimental concentrates as described below.

Experimental concentrates

Juice was extracted using three different type extractors. Three units of fruit (≈ 900 lb per unit) were extracted using a rotating pressure-reamer type extractor (Brown juice extractor) produced by Automatic Machinery Corp., Winter Haven, Fla.; three units were juiced on a serrated cup pressure-piston type extractor (FMC type in-line fruit extractors), FMC Corp., Lakeland, Fla., and one unit of fruit was prepared on a rotary press juice extractor (Goulds rotary juice extractor). Although this machine is no longer used in the United States, similar machines are used in other countries. (The yield of juice is somewhat lower than with the other machines and the peel oil content is higher.) Juice was produced using relatively low, medium and high extraction pressures. The juice from the FMC extractor after being partially finished during extraction, was finished by pressing it through a stainless steel screen with .020-in. diam holes on a screw-type juice finisher (Chisholm-Ryder Co., Niagara Falls, N.Y.). The juice from the AMC extractor since extraction did not include partial finishing, was finished using a screen with .015-in. holes on a screw finisher as above, followed by a paddle-type super finisher operated at 1580 rpm (F.N. Langsenkamp Co., Indianapolis, Ind.). The juice from the rotary press was finished using a screw finisher with screen of .020-in. holes. In all cases, juices from higher extraction pressures contained more pulp, and were given higher finishing pressures. The extent of finishing treatment was determined by the relative dryness of the finisher pomace, measured by amount of water retained by the pomace under standard conditions. This is the "quickfiber" method described by FMC Corp. (1964).

The finished juices were concentrated on a small falling film vacuum evaporator (≈ 25 gal. concentrate capacity) at a pressure of about 15 mm Hg, ≈ 70°F to 58–60° Brix. These concentrates were stored at –5°F in 46-oz cans until required for foam-mat drying experiments.

Three other samples were prepared using commercial Valencia frozen orange concentrate, 58° Brix 16:1 Brix/acid ratio and 0.005% oil. Commercial cold-pressed Valencia orange peel oil was added with stirring, to adjust these samples to the levels of 0.106, 0.054 and 0.026% oil (ml oil per 100 ml reconstituted juice).

Foams

Preliminary evaluation of foam suitability for drying was carried out by the method of Berry et al. (1965b). Foaming agents were prepared and foams produced batch-wise using a 2-qt Hobart rotary wire beater-mixer. After formulations gave foams suitable in viscosity and "bubble size index" for foam-mat drying, sub-

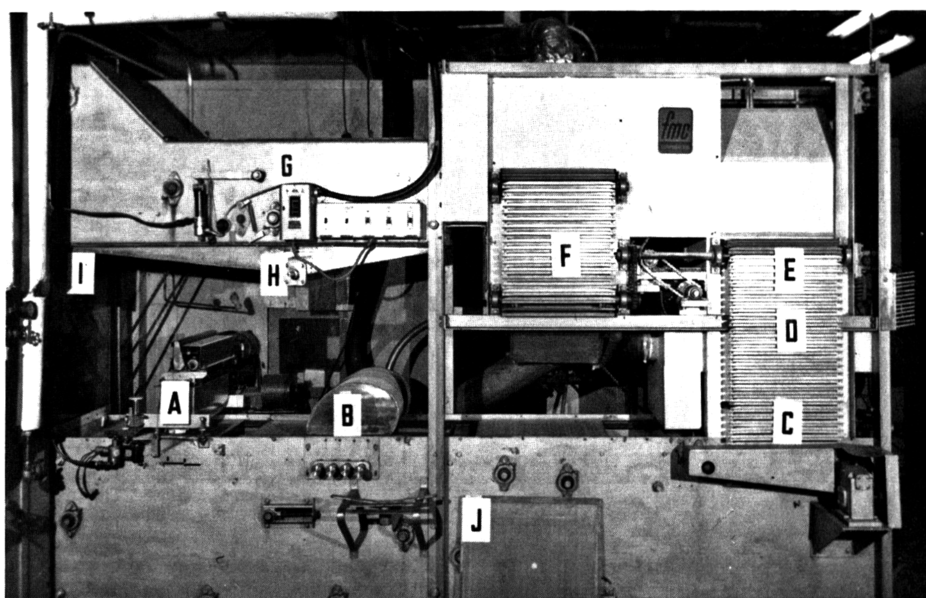

Fig. 1–"Crater-type" foam-mat drier used for production of instant orange juice (IOJ): (A) Foam feeder; (B) Air nozzles and "cratering" section; (C,D,E) Drying section; (F) Cooling section; (G) Product scraping section; (H) Conveyor for product into dry room; (I) Tray return elevator; (J) Full view of a perforated tray.

sequent foams were prepared on an 8 in. Oakes continuous mixer (The E.T. Oakes Co., Long Island, N.Y.) using foaming techniques described by Berry et al. (1967a). (See Discussion section for detailed foaming procedures.) The foam was fed to the "crater" foam-mat drier.

Preparation of IOJ

IOJ was prepared through three basic steps: (1) dehydration; (2) densification; and (3) particle size classification. Dehydration was carried out on an experimental "crater" tray-type foam-mat drier. The modified equipment used in these studies is shown in Figure 1. Earlier versions were pictured by Platt et al. (1965) and Berry and Veldhuis (1968), and described in detail by Berry et al. (1967b). In this process, liquid foods such as frozen orange juice concentrate are mixed intimately with air or N_2 and a foam stabilizer solution, causing formation of a thick, stable foam with about the consistency of marshmallow cream. This foam is fed in a thin sheet (approx. 1/8-in. thick) onto perforated stainless steel trays (Fig. 1–A). These trays, loaded with foam are then transported across air nozzles, perforating the foam sheet. (Fig. 1–B, shown in detail in Fig. 2). The tray loaded with a thin sheet of perforated foam is then transported into a vertical drying stack (Fig. 1–C,D,E). The tray progresses up this stack for about 12 min, while it is surrounded by hot (250°F) air through the bottom third slightly cooler (200°F) in the center, and warm air (≈ 160°F) in the top third of the drying section. It is then transferred to a short, cooling section (Fig. 1–F) where a flow of cool, dehumidified air for about 3–4 min crisps the foam making it easier to remove by scraper blade after transfer to the next section (Fig. 1–G). After the dried product has been removed by scraper blade and transferred into a dehumidified area by screw conveyor (Fig. 1–H) for further processing, the scraped tray is carried by return elevator (Fig. 1–I) back to the initial point of entry to receive more foam and proceed through the process again. Temperatures of drying air, cooling air and product at various points in the drying process were measured by appropriate thermocouples and recorders.

The IOJ was put through a process to increase its bulk density by passing it into the nip of two hardened steel rolls 9-in. diam × 11 5/8-in. long. A feeder was arranged to guide the powder into the center 2 3/4-in. width and 3,500 lb of pressure was applied to each bearing so that a total of 7,000 lb was applied to the 2 3/4-in. length of the nip being used for densification. The hard, dense flakes of dried orange solids removed from these rolls by a spring-loaded scraper blade were ground to pass through 35 mesh and be retained on 60-mesh screens (U.S. Standard Sieve Size).

Using the experimental concentrates described above, IOJ was prepared by foam-mat drying. The conditions required and the characteristics of the products were compared. The experimental concentrates with varying amounts of peel oil were foam-mat dried and oil content of the IOJ was compared to initial oil values.

A commercial Valencia orange concentrate, 58° Brix 16:1 Brix/acid ratio and 0.005% oil was used in the preparation of IOJ for the development of standard drying conditions. After standard drying conditions had been developed for this product, rate of moisture loss during drying was determined as follows: the vertical drying stack of the foam-mat drier contains 24 trays which progress up the drying stack over a period of about 12 min, i.e., a tray enters this stack at the bottom and another leaves at the top about every 30 sec. With heat on, as soon as the drying stack was filled and steady state had been established as determined by measurement of temperatures of air entering and leaving the drying sections, the lower section of the drying stack was opened and tray number 2 was removed and rapidly taken to a dehumidified area where samples were removed for moisture determination. The drying section was again closed up, allowed to fill with trays and reach equilibrium. After steady state was again established, the lower section was opened and tray No. 4 was removed and samples taken as before. This was continued progressively up the stack with alternate trays. Moisture determinations were carried out on samples removed from these trays.

In another experiment measurements were made to determine losses taking place during drying. Foam lost during start-up, and that around the air perforator lost due to splatter, was measured. Product on trays when the system was not at steady state, during start-up or

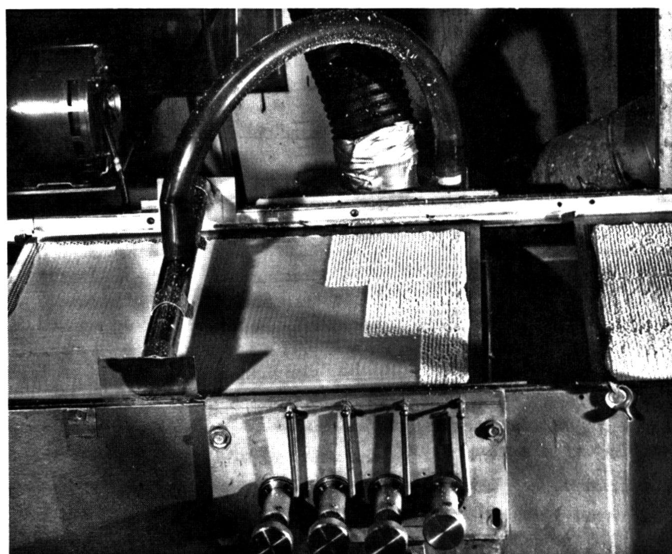

Fig. 2–Detailed view of "cratering" technique. Air blowing upward through perforations in the steel tray perforates the foam layer leaving an extremely porous foam layer.

Table 1—Characteristics of orange concentrates, foams and IOJ

Juice			Concentrate		Foam			IOJ
Extractor type	Relative extraction pressure	Finish** Quick fiber	Viscosity (cps)	Oil content % x 10^{-3}	Agents required Methocel %	Other	%	Moisture %
Cup reamer	a[a]	144	500	3	0.49	Kelzan	1.06	1.3
Cup reamer	b	117	975	4	0.49	Kelzan	0.16	1.8
Cup reamer	c	164	1300	5	0.48	none		1.8
Serrated cup-piston	a[a]	166	170	1	0.53	CMC	1.19	1.4
Serrated cup-piston	b	109	220	5	0.49	Kelzan	0.64	2.0
Serrated cup-piston	c	161	820	3	0.75	Kelzan	1.05	2.0
Rotary press		180	200	16	1.53	Kelzan	1.19	2.3
Commercial control			1500	5	0.45	none		1.1

[a]Rel. extraction press: a = very light; b = light; c = medium; pressures are not directly comparable between extractor types.
[b]Higher numbers represent lighter finishing treatments, i.e., "wetter" finisher pomace.

shut-down, was measured. Powder adhering to trays on the second, third and fourth cycles through the drier and material accumulating as dust on top, bottom and sides of the scraping area were also measured. These measurements were determined as percentages of original input solids.

Analytical methods

Relative finishing treatment of juices was determined from relative dryness of the finisher pomace (i.e., pulp expelled from the finisher) using the "quick fiber" method (FMC Corp., 1964). This test was carried out by adding 200g water to 200g finisher pomace, shaking under standard conditions and measuring the water which remained unabsorbed by the pomace. The relationship between these test results and finishing treatments are as follows: quick fiber less than 130 = very tight finish (i.e., most of the water soluble components had been squeezed from the pulp); 150–180 = moderate finish; and > 210 = very loose finish.

Oil in concentrates and in juice reconstituted from IOJ samples was determined by the Bromate titration method as described by Scott and Veldhuis (1966). Moisture in powder was determined by a modification of the Karl Fisher method described by McComb and Wright (1954) in which formamide was the extracting agent.

Viscosities of concentrates and foams were measured on a Brookfield Synchrolectric viscosimeter and converted to centipoise (cps) readings.

Flavor evaluations were carried out between samples stored at 85°F and controls (stored at −5°F) using the triangular procedure of Boggs and Hanson (1949).

RESULTS & DISCUSSION

THE FIRST STEP of the foam-mat drying process, foam formation, was reported earlier (Berry et al., 1967b) to have been developed to a point where satisfactory foams could be produced with no more than 0.5% of a single foam stabilizer (methylcellulose) for the production of grapefruit crystals. This was also found applicable to the production of instant orange juice as will be noted in Table 1 under "commercial control" where a satisfactory foam was formed using 0.45% of methylcellulose with a typical commercial frozen orange concentrate. Careful foam adjustment procedures which had been described for grapefruit were also found necessary for orange. Amounts of foaming agents, nitrogen gas (or air) and 50° Brix orange concentrate had to be regulated carefully so as to maintain a satisfactory back-pressure to enable suitable foam formation. The details of this procedure were outlined by Berry et al. (1967b). Foam start-up usually involved the following steps: The foam stabilizer (methylcellulose solution) was started at the full rate, the gas and concentrate at about half the desired rate and pumped into the Oakes mixer with mixing head rotor at desired rate. When back pressure reached about 5–6 psig, gas and concentrate were slowly increased in small discrete changes so that, in about three increases, they would reach the full desired rates. If back pressure began to drop, gas was increased and concentrate decreased slightly until it was regained, and if it began to rise too rapidly, this could usually be compensated by decreasing gas flow slightly. Too high back pressure for more than a few minutes was usually followed by an escape of large bubbles of gas and foam breakdown. Results were usually best when a back pressure of around 13 psig was maintained. The foaming agent was a 4.3% solution of Methocel 10 cps (Dow Chemical Co., Midland, Mich.) in water. After careful adjustment had been made and a satisfactory foam was produced, the rates of the three foam components were as follows: 50° Brix concentrate at 32°F, 246g/min; 4.3% Methocel at 65°F, 13g/min; nitrogen or air 850 cc/min. After equilibrium was established in almost all cases the system would remain stable over long periods and only very slight adjustments were required during the drying run. Temperature of the foam at the outlet of the mixing head from the Oakes 8-in mixer was about 46°F and the density of the typical foam was about 0.30g/cc, average bubble diameter about 25μ. Loading rate of foam onto trays for drying was maintained at about 100g/tray (± 5%).

Although most developmental work was carried out using nitrogen gas for foaming, air may be used without any significant changes in product quality or storage stability. Experiments carried out in the pilot plant, comparing IOJ samples which were identical in every respect, except in one air was used and in the other nitrogen was used for foaming, resulted in no detectable difference between the samples by triangular taste tests. When samples of each, stored at 85°F were compared to controls which had been kept at −5°F, both N_2 and air foamed samples developed earliest detectable changes at the end of 8 wk. At that time the 85°F stored samples from nitrogen and air foaming were still indistinguishable from each other by triangular taste tests as well. Thus, air could be used for foaming if desired, and would be less costly.

Generally, although the experimental orange concentrates varied considerably in viscosity and oil content as indicated in Table 1, the principle difference between them in suitability for foam-mat drying was related to the stabilizer requirements to achieve a foam satisfactory for drying.

The samples were prepared to provide juices covering the range of values which might conceivably be encountered in commercial operation. Drying suitability did not appear to be affected by finishing treatment. From these experimental concentrates, using standard drying conditions developed for the commercial control, IOJ was produced with moisture contents from 1.3–2.3%.

As Table 1 indicates, the foam-mat drying process was found accommodating to orange concentrates which varied over a wide range of viscosity, from 170–1300 cps as prepared on the reamer and piston-type extractors, but this required using appropriate foaming agent and stabilizer combinations. Foam stabilizers are usually required for foam-mat drying in order to maintain the foam structure during the drying process. Without them the hot air usually causes the foam to collapse before all moisture has been removed. Generally, the lower the viscosity, the greater amounts of foaming agents required, but these had to be used without changing the solids content significantly. This often required change in agents as greater viscosity was needed, and sometimes required combinations of agents. The greatest change in foaming formulation was required with the lowest viscosity sample, where 1.19% carboxymethylcellulose (CMC) plus 0.53% low viscosity methylcellulose and 7.36% glycerine were required. The latter was required as a mutual solubilizing agent to enable incorporation of the high viscosity CMC. Such a low viscosity in orange concentrate would not normally be encountered in commercial products. The sample with highest viscosity, (1300 cps) required no foaming agents other than 0.48% methylcellulose. This sample was similar in foaming characteristics to typical commercial FCOJ from which a number of samples of IOJ have been prepared by foam-mat drying in the pilot plant in the past. Its similarity to a typical commercial concentrate can be observed by comparing the values in Table 1. Past experience had shown most problems were encountered when the orange concentrate was too low in viscosity so this study was designed specifically to determine foaming needs for low viscosity FCOJ, and experimental concentrates were not prepared to attempt to duplicate commercial concentrates. Other samples of orange concentrate varying from 220–975 cps in viscosity required the addition of an algin derivative (Kelzan–The Kelco Co., Los Angeles, Calif.) along with methylcellulose for proper foam consistency and stability. For most commercial concentrates, however, the algin derivative would not be required. In both types of extractors viscosity increased with extraction pressure. Although the agents used here were successful, other types or combinations probably would suffice.

The concentrate from the rotary press extractor was especially high in oil content while its finishing treatment was relatively light and its viscosity was fairly low. This high oil level and low viscosity required the addition of 1.19% algin derivative with 1.53% methylcellulose to prepare a suitable foam for drying. This product had one of the higher moisture contents as well, and this may be due to a combination effect of the high orange oil and relatively low viscosity.

When these samples were compared for relative storage durability by triangular taste evaluation, for significant detectable differences between samples stored at –5°F and those stored at 85°F, there was no difference in stability between the IOJ samples made from different types of experimental concentrates. All samples developed minimal detectable changes within 4–6 wk. However, when judged as satisfactory/unsatisfactory, all remained acceptable for 3–9 months at 85°F. Both initial consumer acceptability and storage stability of IOJ are greatly influenced by oil level. In tests carried out at Statistical Reporting Services, Washington, D.C., using untrained tasters, oil levels as high as 0.035–0.040% were found desirable. When oil levels in IOJ which has previously been prepared at 0.020%, were increased to 0.035% storage stability at 85°F before detectable change, increased from about 6 wk to about 4 months, and samples remained acceptable up to about 6 months (Berry and Veldhuis, 1968). This is in marked contrast to findings with frozen concentrated orange juice where more extensive studies with untrained consumers indicated oil levels of 0.020–0.030% were preferable and levels as high as 0.040% were relatively unacceptable (Marketing Research Report #946, 1972). The oil added was "locked-in" Permastable obtained from Sunkist Growers, Ontario, Calif.

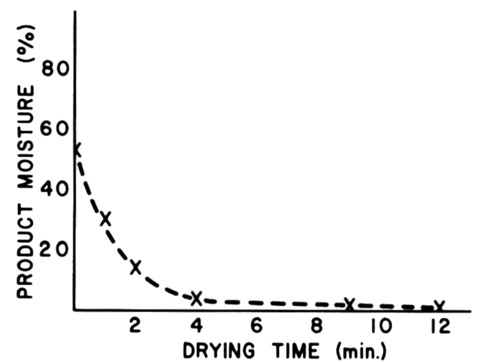

Fig. 3–Moisture content of foam-mat dried instant orange juice (tray-type "Crater" drier), after different drying times.

Oil recovery in IOJ

In a study of oil recovery in IOJ, a considerable portion of oil added to the

Table 2–Retention of orange oil during drying

Oil level (% × 10^{-3}) concentrate	powder	Retention %	IOJ moisture %
26	16	62	1.9
54	30	56	1.7
106	78	73	1.6

Table 3–Materials balance in foam-mat drying of orange juice

	Solids in (lb)	Solids out (lb)	Recovery (%)
Solids in			
146.25 lb 50° Brix conc	73.13		
8.59 lb 4.3% Methocel soln.	.37		
To be accounted for	73.50		
Solids out			
Final product recovered		52	
Dust from walls & bottom scraper section and from densifier		10.5	
Total recovered		62.5	85.3
Losses[a]			
Foam in Oakes mixer, start-up		3.8	5.2
Splatter around blower		0.6	0.9
Foam on trays for start-up & shut-down		5.5	7.4
Residue on trays when removed for cleaning		1.1	1.2
		11.0	100.0

[a]All losses are probably reducible (see text); Total drying time 5 hr 19 min

original concentrate was found to be recovered in the resultant IOJ, and this increased with higher original oil level, based on measures of d-limonene. These results are shown in Table 2. A typical commercial concentrate with viscosity about 1500 cps and oil content about 0.005% was used for these experiments. Cold-pressed orange peel oil was added to adjust batches of this concentrate to three 0.026, 0.054 and 0.106%. Recovery increased with higher initial oil levels and amounted to about 56, 62 and 73%. When these samples were placed in storage at 85°F and compared to controls which contained "locked-in" orange oil, the experimental samples were found to develop terpeney off-flavors within 2–4 wk. Thus, although it was possible to prepare suitable foams and IOJ from concentrates containing relatively high amounts of oil, it may not be advisable from a flavor stability standpoint. Of course, the oil recovery data refers to measurements of d-limonene and recovery of other oil components may not necessarily follow.

Rate of water removal during drying

Study of rate of water removal from orange juice during foam-mat drying indicated most of the moisture is removed during the very early drying stages. As shown in Figure 3, the original moisture level of about 52% was reduced to about 31% after 1 min and to about 15% after 2 min. Thus, well over half the moisture was removed during the first 2 min and about 90% had been removed by about 5 min drying time. The reduced rate toward the latter half of the drying cycle is partly due to the fact that lesser amounts of heat must be applied during these stages to prevent flavor damage. As the moisture content of the orange solids becomes less, the sensitivity of the remaining material to flavor damage by overheating becomes greater as does its binding capacity for the remaining moisture. Thus, almost half the drying time is required to remove the final 1–3% of moisture to obtain a commercially satisfactory product. This might be reduced somewhat, but most attempts to increase throughput rate or shorten drying time by increasing heat during the latter stages have resulted in unsatisfactory heat damage to flavor.

As indicated by Berry et al. (1967b), the relative humidity (R.H.) in the final drying section was so high it was not possible to remove the final residual moisture when ambient air was 90°F or above and ambient R.H. was 70% or more. The equilibrium R.H. of foam-mat dried IOJ is about 13% (Wagner, 1961). If ambient air were 90°F and 70% R.H., the R.H. would still be 17% when the air was heated to 140°F. Thus, during warmer, wetter months when these conditions might be expected, a refrigeration-type dehumidifier was required for treating the air before heating it to enter the final third of the drying section. This assured the air in the final drying section would be no greater than about 7% R.H. at the usual drying temperatures. The installation of the dehumidifier in the air stream to the last drying section made it possible to readily reduce moisture content of the final product to 1.5% or below and eliminate the need for an in-package desiccant. Drying to this low level in a continuous process and elimination of the in-package desiccant represents a marked economic advantage for the foam-mat process.

Material balance study

In order to point out places where efficiency might be improved, a material balance study was carried out over 5 hr, 19 min drying time. The results are indicated in Table 3. When about 150 lb of 50° Brix concentrate were foam-mat dried, the resultant total solids input amounted to about 73.5 lb. Final product recovered from first-time processing of this material amounted to 52 lb. This was supplemented with accumulated IOJ dust and "fines" from walls and bottom of the drier scraper-section and from the densifying operation, all of which could be converted into final product. This netted an additional 10.5 lb or a total recoverable final product of 62.5 lb (85.3% recovery).

When the various stages of the process were studied for losses, sources and methods of recovery, the following were encountered:

Foam in Oakes mixer at start-up. When the Oakes mixer was begun, some orange concentrate was used before concentrate, foam-stabilizer and gas were in equilibrium and satisfactory back pressure was built-up to assure continuation of stable foam production. This start-up foam was not of sufficient density or stability for drying and was usually discarded. This amounted to 3.8 lb of solids or 5.2% of the total solids input.

Splatter around foam crater blower. In the vicinity of the air nozzles, perforating the foam layer on each tray, some foam splattered onto surrounding surfaces and was lost. This blower area was usually surrounded by a wire basket which retained such splattered foam. Studies indicated about 0.6 lb solids or 0.9% were lost in this manner.

Foam on trays for start-up and shut-down. At the beginning and end of a drying run, the first and last trays of product were dried when the system was not in equilibrium and therefore the initial and final trays were over-heated. This product was unusable and amounted to 5.5 lb solids (7.4%).

Tray residue. Trays were recycled several times but eventually, after three to four rounds, had to be replaced. When trays were removed, they had slight residues of dried orange solids on them and scraping and weighing of these residues indicated total solids lost in this manner amounted to about 1.1 lb or 1.2%.

In a commercial operation, most of these losses would be highly reducible. For example, foam prepared in start-up of one operation could be incorporated into the foam mix of the next operation or blended back into the concentrate feed tank. Splatter around blower cannot be totally prevented but can be held to an absolute minimum by careful observation and adjustment of blower nozzles by the foam-mat drier operator. This should easily be held to about 0.5% or less. Foam on trays for start-up and shut-down might be difficult to recover since most of this has been overheated and of poor quality. It might be possible, however, to raise the heat of the drying section slowly and progressively as the first trays are coming in so that only very few trays are lost in this way and reduce heat gradually on shut-down. Residue on trays can be held to a minimum by recycling trays as many times as possible. Weights of residue on trays indicated 1.9g solids/tray after one cycle, 3.6g after two cycles, and 4.8g after three cycles (average of 10 trays). Each tray had been loaded to about 100g of solids to begin with. A more efficient tray scraping system or a built in tray-cleaning and drying system with some means of recovering the residual powder left on the tray (e.g., by a vacuum, brush system) might increase this recovery. Most of this loss was from IOJ which became imbedded in the indentions around the holes in the tray. Losses from foam start-up and foam on trays at beginning and end would be nearly constant for any given production run and would therefore tend to become less percentage-wise as more extended runs were carried out. The studies were carried out on a drying run which lasted 5 hr and 19 min. A commercial run could last 12–24 hr or longer which would in itself cut these particular losses down to around 1% or less. Experienced commercial runs on two different types of driers indicate an expected loss of 5% or less is reasonable. These results coupled with flavor quality and stability of the product indicate this process has a high degree of commercial feasibility.

When produced under the recommended conditions, the product from both orange and grapefruit (Berry et al., 1967b) usually had around 1.25% moisture or less. This is recommended to be ground to particle sizes to pass through 35 mesh and on 60 mesh (U.S. sieve size) for optimum solubility, and should be densified by the method of Berry et al. (1967a) to a bulk density of about

0.6g/cc. IOJ prepared in this manner may be stored at 85°F for 6–9 months before becoming unacceptable and at 70°F or lower for more than a year (Berry et al., 1966; Berry and Veldhuis, 1968). Flavor quality can also be upgraded with increased acceptability through use of encapsulated peel oil and concentrated citrus essences. IOJ with these characteristics has been used successfully on all space flights for moon exploration since Apollo 12.

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RECOVERY OF NATURAL ORANGE PIGMENTS: AN IMPROVED METHOD APPLIED TO CITRUS PROCESSING WASTES

INTRODUCTION

DUE TO increased interest in natural orange pigments as potential sources of color for food products, methods for obtaining such pigments have recently been under development. Most methods used to date have several inherent disadvantages, however, chief among which is the need for large volumes of water. There is a need for improved methods, minimizing water usage and simplifying the process.

Previous methods were too complex for easy commercial scale-up. Fisher et al. (1966) reported a relatively complicated laboratory procedure and Kew and Berry (1970) reported a more simplified hexane extraction method, applicable to Kg batches of peel. Wilson et al. (1971) reported on pilot scale adaptation of a somewhat simplified version of Kew's hexane extraction method using three forms of Valencia citrus peel. Ting and Hendrickson (1968, 1969) reported a method using original acetone extraction followed by a solvent/solvent transfer to hexane.

A method for pigment extraction should be applicable to most forms of commercially available peel materials. From different types of citrus juice processing operations, three forms of citrus peel are discharged which should serve as good sources of natural orange color. These are: (1) whole orange peel expelled from juice extractors; (2) orange flavedo (the outer colored layer of orange peel); and (3) "frits." Whole peel consists of flavedo, albedo and adhering membrane. Flavedo is obtained from whole peel by a special slicing device separating the color-containing outer layer (flavedo) from the albedo and membrane. "Frits" are rather coarse peel particles, mostly flavedo, expelled from a screw finisher used to obtain peel oil as a byproduct from an FMC orange juice extraction and finishing operation. These forms of citrus peel have been described in more detail by Wilson et al. (1971).

A study was made to develop an improved carotenoid purification method which would be simpler, require less water usage and still result in efficient pigment yield. The developed improved method was then tested for its applicability to citrus peel from different orange cultivars, different forms of peel and different degrees of maturity.

EXPERIMENTAL

Materials

(1) Peel "frits" (described above), (2) FMC extractor peel (FMC Corp., Lakeland, Fla.), (3) Brown extractor peel (Automatic Machine Co., Winter Haven, Fla.), and (4) Flavedo (outer layer of orange peel obtained from an AMC peel shaver, Automatic Machine Co.) were obtained from Hamlin, Temple, Pineapple and Valencia oranges, and Dancy tangerines. From each of the five cultivars listed, three 60-lb batches were extracted and the extracts pooled for purification and concentration. All treatments were duplicated.

Methods

Pilot plant extractions followed the method reported by Wilson et al. (1971). Peel "frits" were small enough that grinding was unnecessary. All other samples were ground (Hobart meat grinder Model 4256c, The Hobart Mfg. Co., Troy, Ohio) prior to extraction. Three 60-lb batches of peel were mixed 1:1 (w/w) with hexane, stirred 10 min and the hexane extract decanted. The extracted peel was passed through a juice finisher (Chisholm Ryder Co. Inc., Niagara Falls, N.Y.) (0.030 screen) to remove residual hexane. An emulsion was expelled through the screen at this step and formed a separate layer. The hexane layer was separated and combined with the previous hexane extract. From three 60-lb batches of starting material, the hexane extracts were combined and concentrated in vacuo at 50°C and 28 in. Hg (Wilson et al., 1971). The peel residue, emulsion layer, hexane extract and recovered hexane were weighed and recorded. Peel oil was removed from the crude pigment mixture by distillation over water at 50°C at reduced pressure of 10 mm Hg as previously described by Wilson et al. (1971).

Carotenoid purification

The carotenoids in the crude mixture were purified by precipitation from a solution of 2-propanol and water. The crude fraction was dissolved in a minimum of 2-propanol (ca. 30 ml 2-propanol per g of crude pigment mixture) and the resultant solution was diluted with water to a final concentration of 60% 2-propanol and 40% water. In some cases, large quantities of flavonoids interfered at this stage and when these were present (e.g., tangerine peel) the 2-propanol solution was allowed to stand for about 1 hr (to precipitate flavonoids) and filtered before dilution (Tatum and Berry, 1972). After dilution the precipitated carotenoids were removed from solution and dried under vacuum at 40°C for about 1 hr.

Thin-layer chromatography (TLC) of color concentrates

Each pigment concentrate was spotted on 2 cm × 8 cm, Silica Gel G plates (Analtech, Inc., Wilmington, Del.), developed in hexane-acetone solvent (75: 25 v/v), air dried in a fume hood and examined by long and short wavelength UV light.

Treatment of emulsion

The emulsion layers that formed during the finishing operation were treated with acetone as

Table 1—Yields of pigment obtained by 2-propanol precipitation[a]

Cultivar	Yield of color (mg/kg starting material)		
	Hexane extract	Acetone extract of emulsion	Total
Tangerine "frits"	414	69	483
Hamlin ground whole peel			
11–70	200	95	295
1–71	224	8	232
2–71	308	10	318
Temple "frits"	672	140	812
Pineapple			
Flavedo	954	55	1009
Ground whole peel	342	24	366
"Frits"	326	98	424
Valencia			
Flavedo	606	16	622
Ground whole peel	550	4	554
"Frits"	268	99	367

[a]All results are averages of duplicate determinations.

described by Wilson et al. (1971) to recover the residual color and precipitate pectinous materials and phospholipids.

Color evaluation

Visual comparison was made with USDA standard color tubes (see methods in "U.S. Standards for Grades of Frozen Concentrated Orange Juice," USDA, 1968) and instrumental measurement with a Color Eye Reflectance Spectrophotometer Model D-1 (Instrument Development Labs., Kollmorgan Corp., Attleboro, Mass.). Poorly colored frozen concentrated orange juice (FCOJ) prepared from early season Hamlin oranges was used as the vehicle for visual comparison. Based on previous findings that 1/6000 dilution of pigment concentrates resulted in substantial color improvements (Wilson et al., 1971), samples were prepared by mixing 0.087g pigment with 100g FCOJ then diluting to 12.5° Brix with about 420g water. For comparison a control was prepared in the same way, without the pigment. These samples were compared to the USDA standard color tubes (USDA, 1968).

Instrumental determinations were made by the procedure reported by Berry et al. (1971). Samples of concentrated pigment (0.12g) were weighed into 10 ml volumetric flasks and diluted to 10 ml with hexane, stoppered and mixed. A 4.25 cm diameter Whatman No. 1 filter paper disc was soaked in the color solution for 10 min, air dried for 2 min, placed in the instrument and read after 1 min. In the instrument, the disc was backed by two layers of Whatman filter paper and a white Vitrolite plate. A white Vitrolite plate was inserted in the other side of the instrument as a reference.

Taste panel evaluation

Samples of pigments were evaluated for flavor effect by paired comparison. 48 judgements were obtained for each evaluation. Samples were prepared for testing by thoroughly mixing 0.25g of pigment, 0.041g of cold pressed Valencia orange oil and 367.5g of 50° Brix FCOJ. This mixture blended and dispersed easily with stirring, and was then diluted to 12.5° Brix with water. Control samples for comparison were prepared by adding 0.20g cold-pressed Valencia orange oil (this amount made them equivalent to experimental samples, accounting for oil in pigment concentrates) to 367.5g 50° Brix FCOJ and diluting to 12.5° Brix.

RESULTS & DISCUSSION

PRINCIPAL IMPROVEMENT was in separating and purifying the crude carotenoid fraction by solution and dilution-precipitation with 2-propanol/water, replacing the previously used steps of saponification, neutralization and extensive solvent-water washing as reported by Wilson et al. (1971). This new method was a modification of that reported by Higby (1962, 1963) and Bernath and Swisher (1969), and had been applied by Tatum (1970) on a small scale to remove carotenoids from an analysis mixture. The Higby and Bernath methods extracted carotenoids from the 2-propanol solution with hexane, but the modified procedure simplified removal by pricipitating carotenoids directly from 2-propanol by water dilution and separating them by filtration. This had the additional advantage that impurities such as waxes and flavonoids precipitated separately before the carotenoids, and could easily be removed while further dilution precipitated the carotenoids. No problems were encountered in filtering off the impurities before carotenoid precipitation.

Yields of pigment obtained by the precipitation method varied with the amount of solvent, which in turn depended on amount of peel oil contained in the crude pigment fraction. It was found helpful to remove as much peel oil as possible during the preceding distillation step (without unduly risking product heat damage). The less solvent required to affect precipitation, the more efficient the recovery was found to be. For example when about 15–20 ml of 2-propanol/g of crude pigment was used a loss of about 1–2% carotenoids was found. However, when 30 ml or more of 2-propanol/g of crude pigment was used, losses were 5–10% or more. These are not total losses however, because the remaining solutions could be added back to subsequent batches and recycled, but this would reduce efficiency.

Yields of carotenoid pigments obtained by the precipitation method varied with different citrus cultivars, form used as starting material, and season, as shown in Table 1. Among citrus cultivars, the highly colored Temple orange had one of the highest pigment yields (812 mg/kg "frits") but this is primarily a fresh market commodity and only limited amounts are processed. Tangerine "frits" also showed a good yield, but this too is a limited commodity. Among the most commonly processed cultivars, Valencia showed the highest average pigment yields followed by Pineapple and the relatively low colored Hamlin. With regard to form of peel used as pigment source, flavedo resulted in highest yields with ground whole peel next and "frits" lowest. The difficulty in handling "frits" because of emulsion formation probably accounts for their lower pigment yield. Because early season oranges (Hamlin) are relatively low colored at earliest processing maturity but increase in color as they become more mature, extractions were made on this cultivar at increasing stages of maturity to see if yield would improve. Although some improvment was noted the most mature samples tested were still well below yields of other cultivars. Thus, a barely mature sample, (Nov. 1970), a mature sample (Jan. 1971) and a very mature sample (Feb. 1971) yielded about 295, 232 and 318 mg pigment per kg ground whole peel, respectively. Regardless of peel form, the two most widely processed cultivars, Pineapple and Valencia oranges resulted in good yields of pigment. Of all samples tested, the highest yields (800 to 600 mg/kg) were from Pineapple flavedo followed by Temple "frits" and Valencia flavedo, respectively, as compared to around 400 for other cultivars and forms.

Several times during the 1970–71 citrus season, winter freezes were experienced and some samples were processed

Table 2–Material balance for pilot scale hexane extraction of orange peel pigment

Cultivar	Solids recovery (% wet weight)			Emulsion found % by wt	Solvent recovery (% by wt)	
	Peel residue	Pectin from peel	Total recovered peel solids		Hexane	Acetone
Tangerine "frits"	71	10	81	36	80	57
Hamlin ground whole peel						
11–70	75	25	100	33	82	79
1–71	96	3	99	10	84	48
2–71	94	3	98	8	81	72
Temple "frits"	67	10	77	40	84	53
Pineapple						
Flavedo	81	9	90	25	89	78
Ground whole peel	92	6	98	11	78	75
"Frits"	70	30	100	36	73	85
Valencia						
Flavedo	95	5	99	19	74	85
Ground whole peel	96	1	98	3	71	75
"Frits"	73	10	93	36	71	80
					Avg 79	Avg 71

Table 3–Relative qualitative color differences by tristimulus values

Cultivar	X[a]	Y[a]	Z[a]
Tangerine "frits"	97	82	25
Hamlin ground peel	101	91	36
Temple "frits"	100	90	37
Pineapple			
Flavedo	101	92	36
Ground whole peel	102	94	43
"Frits"	101	91	35
Valencia			
Flavedo	100	88	25
Ground whole peel	103	99	66
"Frits"	102	97	59

[a]X = redness; Y = greeness; Z = blueness

earlier than normal. This was especially true of Pineapple oranges and Valencias and pigment yield might have been higher had the peel from sound mature fruit been available throughout the processing season. However, these studies served to indicate even freeze-damaged fruit could be successfully processed by this method.

Carotenoid concentrates were examined by TLC for changes in composition and gross impurities. On visual inspection of TLC plates, all extracts appeared identical except tangerine. Some pigments were absent from tangerine extracts. Only traces of noncarotenoid impurities were noted in the pigment concentrates, including probable small amounts of steroids and lipid.

Recoveries were also considered from the standpoint of different cultivars, form of peel used as starting material and maturity. Material balances for hexane extraction of different forms and types of peel indicated nearly total recovery of peel solids and around 80% recovery of solvents for most experiments (see Table 2). Pectin from peel was combined with wet peel residue for determining solids recovery. With regard to cultivars, there was relatively little difference in total recovered peel solids which generally ranged around 100% except for tangerine and Temple "frits." Pectin data in Table 2 indicate amounts of this additional byproduct which might be available. Both hexane and acetone recoveries were around 75–85% except acetone recovery from processing of tangerine and Temple "frits" and January Hamlins was considerably lower. This was probably due to larger amounts of emulsion formed, especially with the "frits." With regard to type of peel used as starting material, recoveries also did not vary much except tangerine and Temple "frits" were most difficult materials to handle. When considered from the standpoint of maturity there was little difference in total peel solids accounted for from Hamlins whether processed in November, January or February. They all yielded more than 80% recovery of hexane as well. However, the acetone recovery from processing of the January sample was considerably reduced. Thus, peel in any of the three forms from the two most widely processed cultivars, Pineapple and Valencia oranges, would provide good sources for orange peel pigment resulting in relatively high yields and reasonable recoveries of solvents. Due to lower yields, the peel from Hamlins even when they are extremely mature would not be a very favorable pigment source. Due to limited availability, problems in emulsion formation and difficulties in solvent recovery, the two limited specialty cultivars, Temple oranges and tangerine also would not be favorable sources of pigment, in spite of fairly good pigment yields.

Solvent recoveries sustained during these studies are less than optimum since these extractions and other processing operations were conducted, for the most part, in the open to avoid unsafe accumulations of solvent vapors in the pilot plant. Many solvent losses could be reduced substantially by using enclosed equipment specifically designed for such operations and by recovering additional solvent left in peel residues and emulsions. Yield of pigment could also be increased by additional material recovered from separate treatment of the formed emulsions. The results of acetone treatments to recover pigment from these emulsions are shown in Table 1. Additional yield ranged from about 25 mg/kg material for Pineapple ground whole peel to about 140 mg/kg material for Temple "frits."

Flavor studies using these pigment fractions with cold-pressed orange oil added to juice at 1/6000 dilution indicated that with one exception, a favorable product was obtained. The exception was pigment from Hamlin peel, where the resultant product was significantly different from the control sample at the 0.01% level of confidence and was judged by the panel to be unsatisfactory in flavor. Significant differences from the control were not obtained for the remaining samples.

Thus, ground whole peel, chopped flavedo or "frits" may serve as good sources of natural orange pigments. Other materials such as "frits" containing considerable water and causing more than usual emulsion formation, can still be used as pigment sources providing appropriate processing steps are taken. The 2-propanol precipitation method is an effective, efficient means of purifying the extracted pigment fraction, minimizes water usage, and could be the basis of an efficient, larger scale process. This new method is applicable to most common orange and tangerine cultivars, to the three most available peel forms, and to fruit of different maturities. Although Hamlin oranges are not a promising source of orange pigment, Pineapple and Valencia oranges resulted in good pigment yields and efficient recoveries.

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PRE-FREEZING PROCESSING OF GOLDEN DELICIOUS APPLE SLICES

INTRODUCTION

FREEZING AND THAWING disrupts the cellular structure of most plant tissues, including apple slices. Thus frozen and thawed apple slices are softer than the fresh and lack crispness. The Golden Delicious variety is relatively soft in the fresh state, and when frozen commercially under present practices, it often yields an undesirably soft thawed product, at least with western apples. However, since it is a major processing variety in Washington and other western states, it would be desirable to have a pre-freezing process for this variety which would yield a thawed product closely resembling the fresh in texture, flavor and color. This report describes our efforts toward developing such a process.

Calcium treatment of apples is the logical and historical method for increasing firmness. It has been used for this purpose by Powers and Esselen (1946) for McIntosh apples to be frozen or canned, and by Archer (1962) and Wiley and Lee (1970) for canned apples. Our own recent work on refrigerated apple slices (Ponting et al., 1971; 1972) showed that a calcium dip was effective in preserving texture over an extended storage period, as well as having a synergistic effect with ascorbic acid or sulfur dioxide in preventing browning. Powers and Esselen (1946) also found such an effect with frozen McIntosh apples. Therefore, we used these compounds in our pre-freezing treatments. Sugars were also used in the treating solution for their effect in lowering the freezing point of the apple tissue, with the hope of preventing some breakage of cells by maintaining them in only a partly frozen state. Wiley and Lee (1970) found that sugar increased the effectiveness of calcium in firming canned apples.

MATERIALS & METHODS

GOLDEN DELICIOUS apples (3 in. diam) which had been stored in a controlled atmosphere for 3–6 months were used in these studies. They were peeled, cored and sliced radially into twelfths. Sample lots of 200 or 250g were immediately placed in twice their weight of treating solution. Treatment consisted of short dips of 3–5 min or longer soaking for up to 24 hr. In some experiments the solution was vacuum-infiltrated into the apple slices. Treating solutions contained ascorbic acid (0.05–2%), sulfur dioxide (0.01–0.05%), calcium (0.1–0.5% as $CaCl_2$) and sugars (10–40%) either alone or in various combinations. After treatment the samples were drained and then frozen in polyester bags, usually at −10°F in still air.

Samples were removed from frozen storage after 1 or more days, thawed in the bag for 6 hr at room temperature and observed for color, flavor and texture. Additional tests were made as follows.

Drained weight

Bags were weighed before and after opening and draining for 1 min, and the percentage recovery of drained apples was calculated.

Shear strength

Two-slice samples were tested in triplicate in a L.E.E.-Kramer shear press, with a 200-lb ring and a calibration setting of 200. Results were expressed as mean percent of full scale reading.

Other tests (as pertinent)

pH; degrees Brix (percent sugar) by refractometer on expressed juice; penetration of SO_2 by catechol test (Ponting, 1944); residual SO_2 (Ponting and Johnson, 1945).

RESULTS & DISCUSSION

RESULTS are reported in Tables 1–6 and Figures 1 and 2. Results of preliminary experiments were used to guide the

Table 1—Effect of ascorbic acid and calcium concentrations on texture (Slices soaked 17 hr)

	Ascorbic acid, %	Calcium (%)	Drained wt (%)	Shear strength[a]	Observed texture
	0	0	84	9	Mushy
Water	0.05	0.2	80	34	Soft
	0.2	0.3	82	32	Fibrous
	0	0	97	17	Mushy
	0	0.2	97	39	Soft-Firm
30% Sucrose	0	0.4	99	84	Firm
	0.05	0.2	99	58	Firm
	0.1	0.2	97	57	Firm
	0.05	0.3	97	70	Firm
	0.2	0.3	97	70	Sl. Soft
	0.1	0.4	100	102 est.	Fibrous

[a]Shear strength of fresh slices > 100 in this system

Table 2—Effect of ascorbic acid and calcium concentrations on quality (Slices soaked 17 hr in 30% sucrose plus ascorbic acid and/or calcium)

Ascorbic acid, %	Calcium (%)	Drained wt. (%)	Shear strength	Observations		
				Texture	Color	Flavor
0.05	0.3	99	34	Firm-Good	Sl. Tan	Fair
0.2	0.3	97	54	Good	Good	Good
0.5	0.3	97	41	Soft	Good	Good
2.0	0.3	97	17	Soft	OK-Light Y	Good
1.0	0.4	98	48	OK-Very Firm	Good	Good

Table 3—Effect of time of treatment on quality (Sliced apples soaked in 30% sucrose, 0.05% ascorbic acid, 0.2% calcium)

Time	Drained wt. (%)	Shear strength	Observations		
			Texture	Color	Flavor
Control (5 sec)	93	17	Mushy	Med. Br.	Fair
3 Min	94	9	Mushy	Lite Br.	Fair
12 Min	94	16	Mushy	Lite Br.	Fair
1 Hr	96	19	Very Soft	Sl. Br.	Fair-Good
3 Hr	97	21	Soft	Lite Br.	Good
6 Hr	99	31	Soft-Firm	Lite Br.	Good
16 Hr	98	66	Firm	V. Sl. Br.	Good
24 Hr	100	61	Firm	Good	Good

Table 4–Effect of sugars on drained weight and shear strength (Slices soaked 17 hr in soln containing sugar plus 0.2% ascorbic acid and 0.3% calcium)

Kind of sugar	Conc (%)	°Brix	Drained wt. (%)	Shear strength
None	0	7.7	82	32
	15	15.4	94	47
Sucrose	20	16.0	95	47
	25	17.3	98	59
	30	19.0	97	70
	40	21.8	88	65
	15	15.9	95	66
Dextrose	20	17.3	100	55
	30	22.8	100	76

Table 5–Effects of sugar and concentration of sulfur dioxide on quality (Slices soaked in solution containing 0.5% malic acid, 0.4% calcium)

Treatment	SO_2 (%)	Drained wt (%)	Shear strength	Residual SO_2 (ppm)	Observations Texture	Color	Flavor
Water-17 hr	.02	85	33	84	Woody	Good	Poor
	.04	82	6	159	Mushy	Good	Poor
30% Sucrose-17 hr	.02	99	41	30	Firm	Good	Fair-Good
	.05	95	14	114	Firm	Good	Fair
30% Sucrose-heated to 120°F, 1 hr	.02	99	23	4	Firm-Crisp	Good	Fair

investigation, as follows: (1) Vacuum infiltration of solutions into apple slices was abandoned because it resulted in a translucent or water-logged appearance rather than the opaque appearance of fresh slices; (2) Calcium chloride was found to cause an off-flavor at concentrations of 0.5% or above; therefore, this was the upper limit used in our experiments; (3) Mixtures of ascorbic acid or sulfur dioxide and calcium in water were satisfactory for protecting color of frozen and thawed apple slices but not the texture, no matter how long they were treated. Increasing calcium concentration caused an increase in shear press readings but after a certain point the slices became woody, while the juice still leaked out easily. On the other hand, when sugar was used in the treating solution, leakage was greatly reduced and the soft tissue was firm and gel-like. Consequently, sugar was incorporated into the treating solution.

Reeve (1970) stated that the mushy texture of frozen and thawed apple slices is caused mainly by damage to the large thin-walled parenchyma cells. The smaller fiber cells are not seriously damaged by freezing and thawing. Sugars have a protective effect on parenchyma cells, thus reducing the leakage. Calcium has a hardening effect mainly on the cell walls but juice can still leak through them. The quality of firmness or crispness of soft tissue that is so different between fresh and frozen fruit (Sherman, 1972) cannot be measured instrumentally at present, but an indirect measure can be obtained from the amount of leakage or the drained weight (Wolford et al., 1967). A higher percentage of drained weight indicates less damage to the parenchyma cells and consequently a firmer texture in the soft tissue. The two measurements, drained weight and shear strength, taken together give an objective assessment of texture which approaches the organoleptic method fairly closely. The effects of various treatments on leakage or drained weight are shown in Figure 1.

The effects of ascorbic acid and calcium concentrations in water and in 30% sucrose solution are shown in Table 1. Sugar increases drained weight even without calcium, although both shear strength and observed texture are poor. With 0.2–0.4% calcium, the observed texture is good. Color is better with increasing amounts of ascorbic acid, but the minimum that can be used is variable, depending on the time the apples are to be held after thawing. In our experiments the packages of apples were thawed for 6 hr at room temperature, and a concentration of ascorbic acid of 0.05% in the treating solution was usually satisfactory with a treating time of 16–17 hr. However, this concentration of ascorbic acid is not sufficient to completely inactivate the browning enzyme polyphenol oxidase, and further holding of the apples results in gradual internal browning. Use of more ascorbic acid is not only more expensive but also causes softening, as does sulfur dioxide. Therefore, more calcium must be used with more of the enzyme inhibitors. Complete enzyme inactivation requires

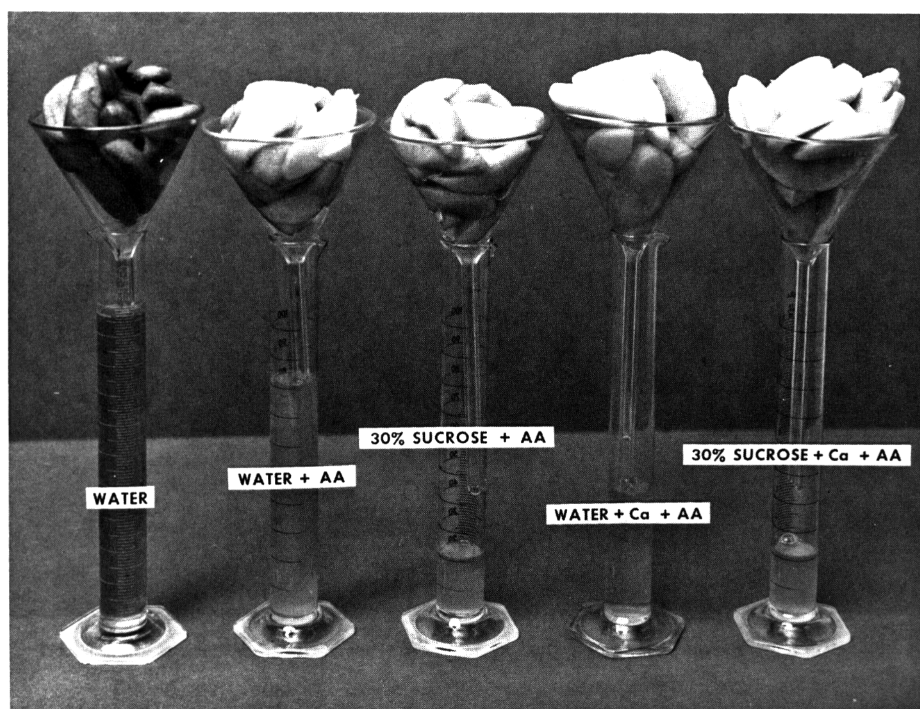

Fig. 1–Effect of ascorbic acid, calcium and sugar on leakage of frozen and thawed apple slices.

about 1% ascorbic acid, and this in turn requires about 0.4% calcium for balance (Table 2). A combination of 0.2% ascorbic acid and 0.3–0.4% calcium provides a good quality product and is probably more practical for most purposes.

The effect of treating time is shown in Table 3. The penetration rate of the chemicals used is quite slow and their effects gradually increase with treating time up to 24 hr or more. The minimum satisfactory treating time under our conditions was about 16–17 hr at room temperature. This raises the shear strength into the desirable range of 50–70. Some experiments with heated solutions containing sulfur dioxide indicated that heating would greatly increase the rate of penetration (Table 5).

Table 4 shows the effects of varying concentrations of sucrose and dextrose. Best results are obtained with concentrations of 20–30% of either of these sugars. Dextrose is slightly more effective. At a concentration of 40% there is some osmotic dehydration and the drained weight decreases. Apple slices soaked in syrups containing 20–30% sugar are increased in sugar content 2–8% from an original 13–14%.

Table 6–Effect of freezing rate on texture of thawed apple slices (Slices soaked 17 hr in 30% sucrose, 0.2% ascorbic acid, 0.3% calcium)

Freezing method	Drained wt % Recovery	Shear strength	Observed texture
Still air, –10°F	99.5	66	Slightly soft to firm
Air blast, –28°F	98.8	65	Slightly soft to firm
Dry ice snow	98.8	100	Very crisp

Sulfur dioxide treatment is different from ascorbic acid treatment in that SO_2 must penetrate clear to the center of the apple slice; otherwise there will be noticeable browning of the unpenetrated portion. The catechol test (Ponting, 1944) was used to show the degree of penetration in a soak time of 17 hr (Fig. 2). It can be seen that the pH must be held to a low value for satisfactory penetration of SO_2. With 0.5% malic acid in the treating solution (pH 2.4), 0.02% SO_2 is sufficient for complete penetration. Table 5 shows that at this concentration the residual SO_2 is only 30 ppm., which would not be detectable after cooking. At 0.05% the residual SO_2 is higher and the flavor becomes poorer. Heating the slices for an hour at about 120°F in a bath containing SO_2 with sugar and calcium offers an alternative to the long soaking treatment.

Further improvement in the texture of thawed apple slices can be obtained by faster freezing, as shown in Table 6. Freezing individual slices in granulated dry ice improved the crispness markedly and brought it close to that of raw slices. The shear strength increased above the desirable range for slow-frozen slices, but this high shear strength did not correspond to a woody or fibrous texture as with other samples.

The treatments described above offer the possibility of processing Golden Delicious apple slices with SO_2 or with only essential nutrients to produce thawed slices which can be used interchangeably with fresh. Although the processing method is slow and somewhat cumbersome, the increase in quality of product may well justify its use.

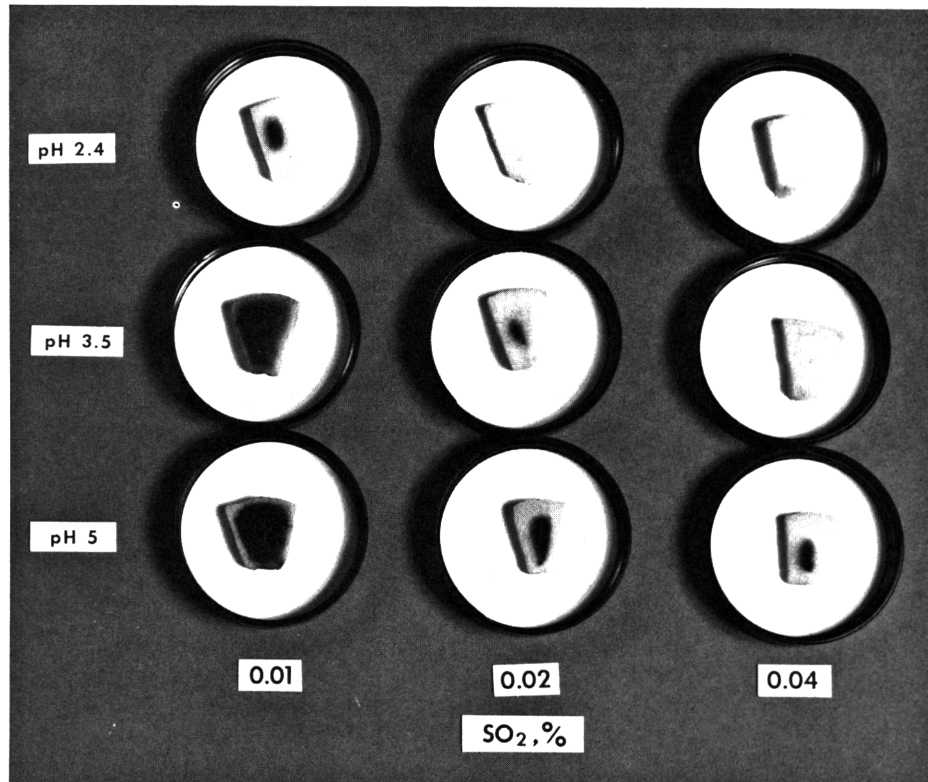

Fig. 2–Effect of pH and SO_2 concentration on penetration of SO_2; apple slices soaked 17 hr.

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EFFECT OF CARBON DIOXIDE ATMOSPHERE ON THE COURSE OF ASTRINGENCY DISAPPEARANCE OF PERSIMMON (Diospyros kaki Linn.) FRUITS

INTRODUCTION

AT THE BEGINNING of the century, Gore (1911) found that astringency could be removed from persimmons by keeping them for 3–5 days in an atmosphere with a high concentration of CO_2. Tarutani and Manabe (1957) also found that astringency is removed when persimmon fruit is kept 3–4 days in an atmosphere containing 78% CO_2.

Gazit and Levy (1970) recognized two distinct stages in the disappearance of astringency following treatment with CO_2. The first stage is an inductive one in which fruit has to be kept a certain minimum period in CO_2 atmosphere, whereas the second stage, in which the fruit actually loses its astringency, can occur also after removal from the CO_2 atmosphere. The duration of time between fruit removal from the CO_2 and complete disappearance of astringency depends on the length of time the fruit was in the CO_2 atmosphere. The longer the latter period, the shorter is the time required for complete disappearance of astringency up to a point where the fruit comes out of the CO_2 in a nonastringent condition. The present study is a continuation of the above research, and follows stages in the disappearance of astringency both during the CO_2 treatment and after it.

EXPERIMENTAL

Fruit sampling and extraction of tannins

Persimmon (*Diospyros kaki* Linn. cvs. Triumph, Hiratanenashi, Monpei, Saijo and Hyakuma) fruits were cut transversely. A piece of filter paper soaked in 5% $FeCl_3$ was placed on the cut surface of the proximal half, so that an impression of the position and quality of tannins present was obtained (Gazit and Levy, 1963). A 2-g sample was taken from the other half of the fruit from a region which appeared most astringent according to the tannin "print." The samples were homogenized and extracted for 1 min with 30 cc absolute methanol in a Virtis 45 homogenizer. After centrifugation, the supernatant was made up to a volume of 50 ml with methanol.

Tannin analysis

The tannins found in persimmons, are condensed tannins (Bate-Smith and Swain, 1953; Ito, 1962) which have a specific absorbance in the UV spectrum range. The methanolic extract of persimmon fruit was made up to a concentration of 2 mg fresh weight per 1 ml, and the absorption curve between 225 and 375 mμ, was determined with a Unicam SP.800 spectrophotometer. Fruit (cv. Triumph) was also tasted and graded according to the following grades: very astringent, intermediate in astringency, slightly astringent and nonastringent.

Adsorption by insoluble PVP (Polyvinylpyrrolidone)

Polyclar AT (obtained from General Aniline & Film Corp., Dyestuff & Chemicals Div., 435 Hudson St., New York, N.Y.) was purified of substances which absorb light in the UV range of the spectrum, with absolute methanol and acetone. An equal volume of water was added to astringent persimmon-methanolic extracts. The methanol was evaporated from the solution in a rotavapor. 10 ml of the aqueous residue was brought to pH 3.5 by the addition of acetic acid (Andersen and Sowers, 1968) and 1g Polyclar AT was added. The mixture was shaken for 30 min. After filtration the absorption curve of the solution at a concentration of 2 mg fresh weight per 1 ml, was determined in the UV range.

Disappearance of astringency

Persimmons (*Diospyros kaki* Linn) cv. 'Triumph' were picked when fully colored. Groups of 50 fruits were placed in air-tight plastic containers. CO_2 was introduced into the containers in sufficient quantity to ensure complete replacement of the air. Temperature during the treatment and after was kept at 21°C. Fruit

Table 1—Comparison between the absorption peaks of methanolic extracts from persimmon fruits (cv. Triumph), and the degree of astringency as determined organoleptically

Degree of astringency by taste	Absorbance at 277 mμ Mean	Range	No. of fruits tested
Very astringent	0.97	0.70–1.16	10
Moderately astringent	0.56	0.48–0.70	6
Slightly astringent	0.27	0.12–0.44	12
Non-astringent	0.05	0.00–0.12	8

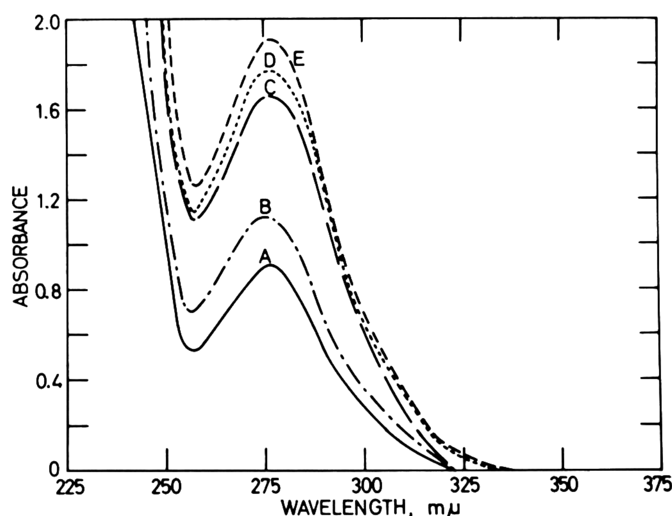

Fig. 1—UV absorption curves of methanolic extracts from different cultivars of persimmon fruit: (A) Hyakuma; (B) Saijo; (C) Monpei; (D) Hiratanenashi; (E) Triumph.

Table 2–Absorption at 277 mμ of methanolic extracts from untreated astringent persimmon fruit, fruit treated for removal of astringency and naturally nonastringent fruit

Cultivar	Treatment[a]	Fruit condition at analysis	Absorbance at 277 mμ
Triumph	25 hr in H_2O + 24 hr shelf-life	Firm	0.12
Triumph	14 hr in 100% CO_2	Firm	0.15
Triumph	20 hr in acetaldehyde fumes	Firm	0.14
Triumph	–	Firm	0.90
Hyakuma	–	Firm grey flesh[b]	0.02
Hyakuma	–	Firm normal flesh	0.60
Saijo	–	Soft[c]	0.04
Saijo	–	Firm	0.60
Fuju	–	Firm[d]	0.16

[a]Astringency-removing treatments
[b]Grey nonastringent tissue surrounding seeds in an astringent fruit
[c]Fruit loses astringency naturally on softening
[d]Naturally nonastringent cultivar

remained in the CO_2 atmosphere for 0, 1, 2, 3, 6, 9, 12, 18 or 24 hr. The intensity of the astringency was determined by the absorbance of the methanolic extract at 277 mμ. Results were analyzed statistically according to completely randomized F and Q tests.

RESULTS

Tannin analysis

The absorption curve of the methanolic extract from astringent fruit between 225 mμ and 375 mμ was determined. All the cultivars tested–Triumph, Hiratanenashi, Monpei, Saijo and Hyakuma–produced only one absorption peak, at 277 mμ (Fig. 1).

A correlation was sought between the degree of astringency by taste and the absorbance at 277 mμ of the methanolic extract from the same fruit. The correlation obtained was clear-cut and direct (Table 1).

Methanolic extracts of nonastringent fruit had a very low absorbance at 277 mμ in comparison with those of astringent fruits (Table 2).

The results presented in Figure 2 indicate that PVP adsorbs and precipitates approximately 73% of the substances causing light absorbance at 277 mμ. This corroborates the conclusion that this absorbance is mainly due to tannin-like substances (Loomis and Battaile, 1966).

Course of astringency disappearance

The method described above for tannin analysis lent itself well to following the disappearance of astringency in fruits. As seen in Figure 3, the treated fruits may be divided into three groups: those kept 0–3 hr in CO_2; those kept 6 hr; and those kept 9 or more hr in CO_2 (Fig. 3).

During the whole period of shelf-life tested, no significant decrease in astringency was found in fruits treated in CO_2 for 0–3 hr. The group treated for 6 hr was astringent immediately after removal from the CO_2, but after 18 hr of shelf-life there was a significant decrease in astringency which reached that of fruit which was kept 24 hr in CO_2. Fruits which were kept 9 or more hr were significantly less astringent than nontreated fruits on removal from the treatment.

DISCUSSION

A QUANTITATIVE METHOD of measuring astringency is essential for a study of astringency disappearance. Ito (1962) reported that the main constituent causing astringency in persimmons is a leucoanthocyanidin which has an absorption peak at 277 mμ. In this study a good direct correlation was found between the height of the absorption peak at 277 mμ and the degree of astringency as graded by taste (Table 1). Furthermore, treatments that reduced astringency also caused an equivalent drop in absorbance of methanolic extracts at the same peak (Table 2). The tannin-like characteristics of the substances responsible for this specific light absorbance are further indicated by their great affinity to PVP (Fig. 2). These findings enable us to follow the course of astringency disappearance by determination of the change in absorbance at 277 mμ.

The effectiveness of CO_2 as an astringency removal agent is well known (Gore, 1911; Tarutani and Manabe, 1957). In the present study the two-stage process of astringency removal (Gazit and Levy, 1970) has been studied in detail.

It was found that full induction occurred after 9 hr in CO_2 atmosphere and

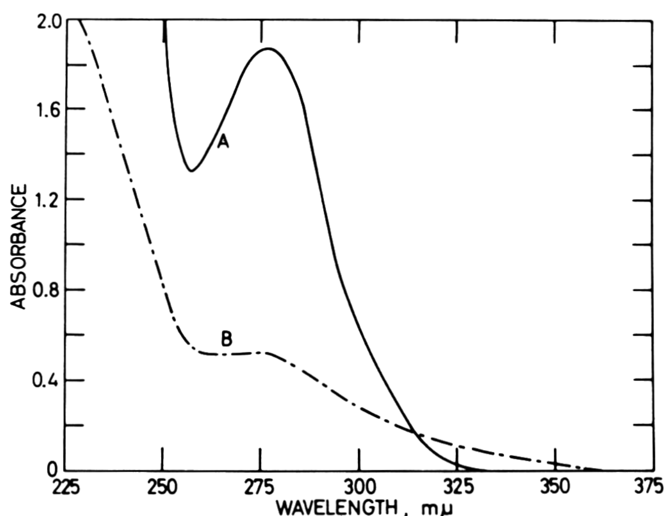

Fig. 2–UV absorption curves of methanolic extract from astringent persimmons: (A) Before and (B) after the addition of PVP.

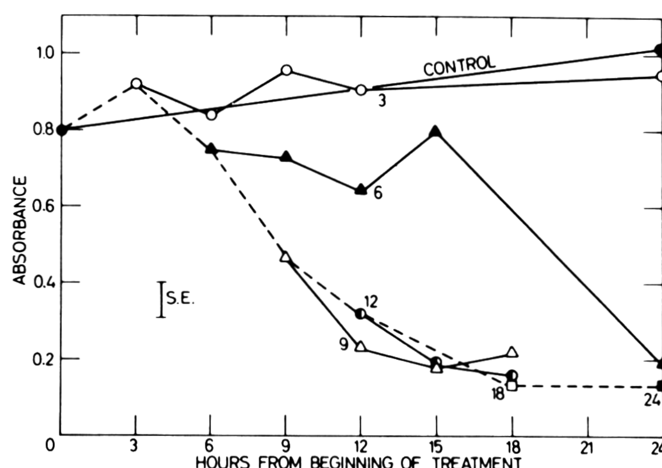

Fig. 3–The effect of CO_2 treatment (3, 6, 9, 12, 18 and 24 hr in CO_2 atmosphere) on the absorption at 277 mμ of methanolic extracts from 'Triumph' persimmon fruit. (– – –) Immediately after removal from CO_2; (—) During shelf-life.

thereafter astringency disappearance rate was independent of CO_2 presence. Induction was not complete in fruit held for 6 hr in CO_2 and as a result astringency disappeared at a slower rate while the shorter treatments, 3 hr and less, were not effective (Fig. 3). These results may be explained by the assumption that in the inductive stage CO_2 induces a process in which a certain astringency-removing substance is synthetized quantitatively. Once present, the substance functions without the continued presence of CO_2. The effectiveness of this process depends on the length of time the fruit has been kept in CO_2. The 3-hr period was apparently too short a time for completion of the process or for production of a large enough quantity of the substance. The lag period found in the 6-hr CO_2 treatment (Fig. 3) was probably due to the time required for the astringency-removing substance to build up to a certain level before its activity commenced. Prolonging the period of CO_2 treatment increased the quantity of substance formed and consequently led to a faster removal of the astringency. When the astringency-removing factor reaches a certain maximum, removal of astringency no longer depends on further synthesis of the substance, and therefore prolonging the CO_2 treatment beyond 9 hr in this particular case did not change the rate of loss of astringency.

It may be assumed that the process mentioned above is connected with anaerobic respiration since the products of alcoholic fermentation, e.g. acetaldehyde and ethanol, remove the astringency of persimmons very efficiently (Gore, 1911; Tarutani and Manabe, 1957). Furthermore, the fact that a CO_2 atmosphere is far more effective than nitrogen (Tarutani and Manabe, 1957), indicates that CO_2, apart from producing anaerobic conditions, has an additional specific function in the process described. Further work is required to elucidate this function.

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CARBOHYDRATES, ORGANIC ACIDS AND ANTHOCYANINS OF *Myrciaria jaboticaba*, Berg

INTRODUCTION

MANY TROPICAL fruits from the family Myrtaceae (e.g., *Myrciaria jaboticaba*, Berg), can be used for making preserves, or their juices are employed in the preparation of pectin jellies. Although botanical studies of jaboticaba have been the subject of two recent papers (Ferri, 1971; Moura, 1971), its chemical composition has not been investigated except for a general analysis of the fruit (Almeida, 1966).

Using ripe fruits from *M. jaboticaba*, Berg, we identified the soluble carbohydrates, organic acids and anthocyanins present in the fruit pulp and skin.

EXPERIMENTAL

Extraction of sugars and organic acids

100g of fruits free from seeds were extracted at 30°C with four 200 ml vol of water. A fifth extraction gave a negative test for reducing sugars (Fehling).

The combined extracts were decolorized with 50g of Dowex 50W-X_4, 100–200 mesh, H^+. The pH of the colorless product was brought to pH 7.5 and the volume reduced to ca. 200 ml at reduced pressure.

The same procedure was applied to two additional 100g batches of fruit and each extract worked up separately for the identification of carbohydrates and organic acids.

Identification of soluble sugar

40 μl of the extract were submitted to electrophoresis (Hashimoto et al., 1952) in paper strips using 0.03M borate buffer with 350v for 2 hr after which three zones were visualized by spraying each dried strip with anisaldehyde H_2SO_4 reagent. Elution of each zone from several strips with hot water produced enough material for paper chromatographic identification of the three sugars using n-PrOH/EtOAc/H_2O (6:1:3, v/v) as solvent (Roberts, 1957).

Identification of organic acids

The concentrated aqueous extract was acidified to pH 3.0 and spotted on silica-gel G plates, using formic, acetic, propionic, caproic, caprilic, citric, tartaric, malic, oxalic and galacturonic acids as standards. Plates were developed with MeOH/NH_4OH 2.5% (95:5, v/v) (Lynes, 1964) or water saturated n-butanol/formic acid (95:5, v/v).

Extraction of anthocyanins

50g of skins from *M. jaboticaba* were macerated with three 100 ml portions of MeOH containing 0.1% HCl for 12 hr under N_2. The combined extracts were washed with Et_2O and then passed through a column of Dowex 50W-X_4, 100–200 mesh, H^+ form. After washing out the sugars from the column the pigments were eluted with solutions of HCl in MeOH of concentrations increasing from 0.1–1%. The acid solution from the column was concentrated at 25°C under reduced pressure while protected from light.

Separation and purification of anthocyanins

The solution containing the anthocyanins was applied on a Whatman No. 1 paper sheet (18 × 48 cm) and the chromatogram developed for 16 hr in acetic acid/water/conc HCl (15:3:2, v/v) (AWH). The four zones (A, B, C, D from the starting line) that separated were eluted from 100 chromatograms with 0.1% HCl in MeOH. Zones A and C contained a smaller amount of pigments judging from the intensity of their color on the paper. Each eluate was concentrated under vacuum at 25°C and stored under N_2 at 15°C.

The purity of each fraction was checked by descending paper chromatography of a pigment extract which had not been treated with cation exchange resin, with n-BuOH/acetic acid/water (4:1:5, v/v upper layer) (BAW) and with 1% aqueous HCl.

R_f of pigments B and D

The purified pigments B and D were chromatographed (Harborne, 1958a) on Whatman No. 1 paper in a descending direction with BAW for 18 hr, 1% HCl for 4 hr and n-butanol/2N HCl (1:1, v/v, upper layer) (BuHCl) for 24 hr. A sample of malvidin 3,5-diglucoside was used as reference. The dried chromatograms were examined under UV light, and color changes observed when treated with a 5% $AlCl_3$ solution in 98% EtOH.

Absorption spectra

Spectra of the purified pigments were recorded using methanol solutions of the pigments at pH 5.0 (HCl) (Harborne, 1958b). Shifts in absorption peaks caused by addition of $AlCl_3$ solution in ethanol were measured.

Identification of anthocyanin components

Acid hydrolysis. 3 ml of the concentrated purified eluates from zones B and D were cooled to room temperature, mixed with Dowex 50W-X_4 and Dowex 1-X_1 and filtered.

Identification of sugar moiety. The colorless filtrate was used for the identification of carbohydrates by paper chromatography. Samples of glucose, arabinose, rhamnose, galactose and xylose were used as references. The chromatograms were developed with BAW and n-butanol/pyridine/water (1:3:1, v/v) (Bu-Py-W) for 14 hr.

Identification of the aglycone. The anthocyanidins were eluted from the resin with 0.1% HCl in MeOH. The eluate was concentrated at 25°C under reduced pressure and used for the determination of the R_f values of the anthocyanidins by paper chromatography with two solvent systems: acetic acid/conc HCl/water (30:3:10, v/v) (Forestal) for 13 hr and formic acid/conc HCl/water (5:2:3, v/v) (formic) for 6 hr.

RESULTS & DISCUSSION

MINOR MODIFICATIONS of the conditions described by several authors (e.g., Hashimoto et al., 1952) for separation of carbohydrates by electrophoresis gave a faster and sharper separation of glucose and fructose than paper chromatography or TLC, making possible the use of higher levels of sugars in the mixtures.

Comparison of the electrophoretic patterns of the jaboticaba extracts with mixtures of pure glucose, fructose and sucrose identified these three sugars in jaboticaba. The identification was confirmed by paper chromatography of the eluates from each zone from the electrophoresis.

Only citric and oxalic acids were found in the water extracts of jaboticaba. Using TLC with solvents commonly employed for paper chromatography of organic acids (Buch, 1952), a good resolution of mixtures was achieved. Special

Table 1—R_f values and spectral data of anthocyanin pigments in jaboticaba

Pigment	Solvent system R_f BAW	BuHCl	1% HCl	Color Visible	UV	λ max (nm)	O.D. 440 / O.D. max (%)
B	0.69	0.68	0.03	pink	dull	530	28
D	0.40	0.27	0.07	lt. pink	dull	520	30
Peonidin[a]	0.70	–	0.06			532	–
Peonidin[a] 3–G	0.41	0.28	0.09			523	–

[a]Reported by Harborne (1958a, b)

Table 2–Products of acid hydrolysis of jaboticaba anthocyanins

Pigment	Anthocyanidins			Carbohydrates
	Forestal	Formic	BAW (R_f)	Bu-Py-W (R_f)
B	0.63	0.30	–	–
D	0.62	0.30	0.16	0.99
Glucose	–	–	0.16	1.00
Arabinose	–	–	0.20	1.19
Rhamnose	–	–	0.36	1.62
Galactose	–	–	0.14	0.88
Xylose	–	–	0.30	1.29

care was exercised to avoid losses of ascorbic and low molecular weight acids but none could be detected by chromatography of the extracts.

Using Dowex 50W-X_4 resin for the purification of pigments as suggested by Chen and Luh (1967) improved the chromatographic separations of the anthocyanins. However, the adsorption of the anthocyanins in Dowex 50W-X_4 and elution with diluted HCl was probably responsible for the formation of zones A and C not present in the original extract of jaboticaba. A similar result was reported by Hetmansky and Nybom (1969).

Table 1 presents the R_f determined in three solvent systems and spectral data for the purified pigments B and D. The variation of the R_f in alcoholic solvents and in aqueous solvent is indicative of an anthocyanin and of anthocyanidin (Seikel, 1961).

Data presented in Table 1 and other results from the experimental section indicated the presence of a free OH group on carbon 5 in the aglycones in both pigments, and the absence of chelating vicinal OH groups. The absorption maxima for B and D, the lack of any shift in the λ max upon addition of $AlCl_3$, and the values for the ratio of O.D. at 440 nm and at λ max indicated that fraction B was peonidin and fraction D, peonidin 3-glucoside. Acid hydrolysis of fraction B (which produced no sugar) confirmed that it was an anthocyanidin. Acid hydrolysis of fraction D followed by R_f measurements of the aglycon and identification of glucose (Table 2) as the sugar moiety proved that fraction D was peonidin 3-glucoside.

CONCLUSION

JABOTICABA, the fruit of *M. jaboticaba*, Berg, contains glucose and fructose together with smaller amounts of sucrose. The only organic acids found in the ripe fruit are citric and oxalic.

The skin contains an anthocyanin, peonidin 3-glucoside and the corresponding anthocyanidin.

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CHANGES IN QUALITY AND NUTRITIONAL COMPOSITION OF FOODS PRESERVED BY GAS EXCHANGE

INTRODUCTION

PREVIOUS STUDIES in our laboratories (Kramer et al., 1968) demonstrated that peeled, sliced or diced potatoes and apples could be preserved in a fresh-like condition by means of gas exchange. In order to approach total stabilization of these products, it was necessary first to completely evacuate oxygen not only from the atmosphere surrounding the products, but from within the product as well. The evacuated gas was then replaced with carbon monoxide, an enzymocidal agent, followed by ethylene oxide, a bactericidal agent. When the order of application of the gases was reversed, that is, when ethylene oxide was applied in advance of carbon dioxide, texture and flavor were preserved equally well; however, enzymatic browning was sharply accelerated so that the peeled and comminuted apples became dark brown and the potatoes pitch black within several minutes.

Kaffezakis et al. (1969) explained these results by demonstrating that ethylene oxide was an effective bactericidal agent, but also an oxidizing agent. It was therefore necessary to first inactivate the oxidative enzymes present in the potato and apple tissues. Then, after the intratissue oxygen was removed by evacuation, and the phenolases inactivated by carbon monoxide, ethylene oxide could be applied to complete the destruction of microflora without causing browning. This last gas exchange was necessary since the carbon monoxide was not effective against anaerobic microorganisms.

The work reported here was to determine whether a similar procedure would be effective in preserving fresh-like quality of more fragile fruits and vegetables as well as foods of animal origin. Another objective of this study was to determine the effect of such gas exchange treatment on retention of nutrients.

MATERIALS & METHODS

PEACHES (V. Redskin) were picked at the green-ripe stage and held 1 day at 3°C before processing. The fruits were then rapidly peeled and sliced in ambient atmosphere and temperature, and placed into bell jars in which the gas exchange treatments were performed (Fig. 1). Because of their fragile nature, the preliminary evacuation procedure had to be limited to 5 min at 27 in. Subsequent flushing with carbon monoxide (pure) was for a 15-min period followed by similar flushing with ethylene oxide. Following a final flushing of residual gases with nitrogen, the jars were removed from the apparatus and stored at 3°C. After 2, 7, 21, 31 and 62 days, duplicate jars were tested for headspace gases, color, texture, refractive index, ascorbic acid, alcohol insoluble solids, total sugars and reducing sugars.

White type button mushrooms (V. MGA) were similarly processed within 2 hr of harvest. For these exceptionally fragile vegetables, the vacuum treatment was reduced to 15 in. Two storage temperatures were used: −2°C and +3°C. Nutritional analyses included thiamine, total protein and relative protein value (RPV) (Stott et al., 1963).

The beef was taken from one carcass and processed 2 days after slaughter. Meat from the shoulder was ground three times and formed into 50g patties. Three patties were placed in each jar. Vacuum was limited to 15 in., and no ethylene oxide was used. Instead, carbon dioxide was included. Both storage temperatures of −2°C and +3°C were used, and determinations were made after 1, 3, 9, 31 and 75 days. Nutritional analyses consisted of thiamine, protein and RPV. After 75 days storage the patties were evaluated by a taste panel consisting of seven panelists. The patties were fried in vegetable oil until well done and presented to the panel for paired comparison with the standard being freshly ground chuck.

With the exception of the one taste panel

Fig. 1—Apparatus used for gas exchange.

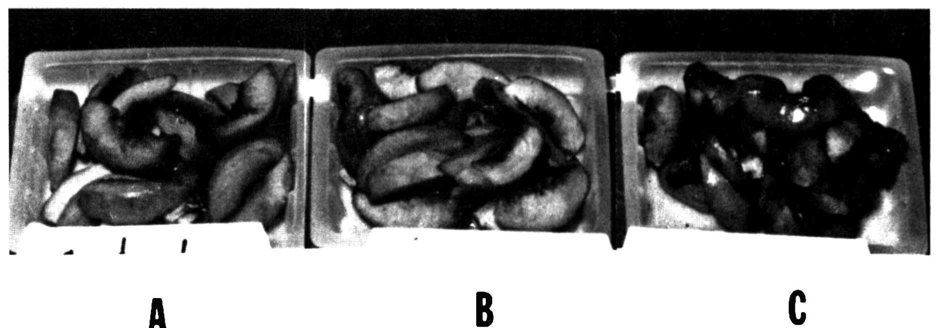

Fig. 2—Appearance of sliced peaches held at 3°C for 21 days in sealed jars: A—control; B—evacuated and flushed with CO; C—evacuated and flushed with CO and C_2H_4O.

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for flavor of the beef, panel evaluations were limited largely to visual inspection for appearance and sniffing for odor. These were supplemented by objective methods for measuring color attributes by the Hunter Color difference meter, texture by the use of the shear-press, and headspace gases by the use of a Fisher-Hamilton GLC partitioner. Since the objective tests added little to the sensory-panel data, the objective quality data were not included in the following results.

The visual-sniffing inspection was performed by the same seven-member panel immediately upon removal of each sample from storage, just prior to the performance of the objective quality tests and the nutritional analyses. Each panelist scored for overall quality on the basis of a 5-point scale: (1) Excellent, not different from freshly harvested control; (2) Good, but slightly poorer than freshly harvested; (3) Acceptable, but obviously poorer than freshly harvested; (4) Marketable for cooking or further processing purposes, but not acceptable as a fresh product; (5) Unmarketable for any purpose. Thus shelf life was calculated (or interpolated) as the time at which the appearance and/or odor of the sample reached the value of 3, i.e., acceptable as a prepared fresh product.

RESULTS

Peaches

As demonstrated in Figure 2, sliced peaches which were evacuated, and treated with carbon monoxide, then flushed with nitrogen and stored at 3°C for 21 days remained indistinguishable in quality from freshly sliced peaches. It should be noted that the control consisted of sliced peaches stored in sealed jars so that they were in fact in "controlled atmosphere" storage. Thus the shortest shelf life of 2 days was for untreated sliced peaches stored in an open jar, whereas the "control", stored similarly but in sealed jars, remained acceptable for 30 days. Slices treated with carbon monoxide and ethylene oxide retained a shelf life of 13 days, while those treated with carbon monoxide only remained acceptable, although significantly poorer in quality than fresh peach slices at the end of the 62-day storage period.

Of all the nutrients analyzed, only ascorbic acid content was influenced by the gas treatments. As demonstrated in Figure 3, ascorbic acid content was reduced to approximately half of its original level after 7 days in storage for all treatments. The carbon monoxide-ethylene oxide treated slices, however, continued to lose ascorbic acid for the first 3 wk of storage, at which time the ascorbic acid content leveled off to little above zero. The sealed control and the carbon monoxide treated slices, on the other hand, leveled off after the first 7 days of storage, and ascorbic acid was maintained at approximately the same level (4 mg %) until the end of the storage period.

It was therefore concluded that both from the standpoint of sensory and nutritional quality, ethylene oxide treatment for sliced peaches is not advisable. This may have been due to the fact that a more complete evacuation closer to 30 in. was not feasible because of the fragile nature of the peach flesh. The surprisingly good performance of the sealed control was obviously due to the rapid reduction in oxygen content resulting from respiratory activity of the slices within the sealed jars. The superior performance of the carbon monoxide treatment may have been due in part to the rapid evacuation of oxygen as well as the rapid inactivation of the browning enzymes by the gas.

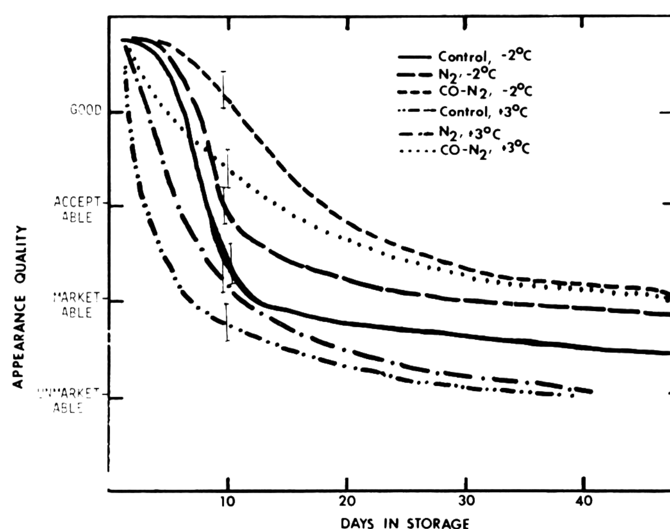

Fig. 4–Appearance of mushrooms stored in sealed jars after flushing with various gases.

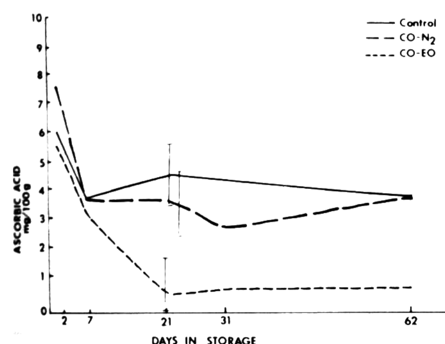

Fig. 3–Retention of ascorbic acid in stored peach slices flushed with various gases.

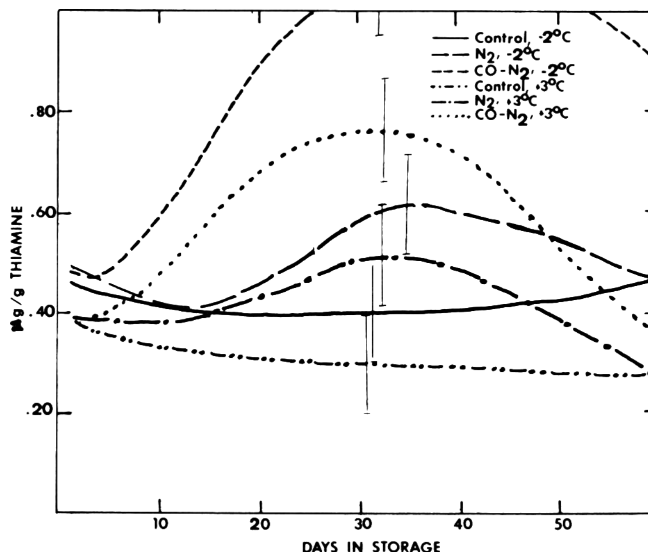

Fig. 5–Changes in thiamine of mushrooms stored in sealed jars after flushing with various gases.

Mushrooms

Since oxygen evacuation from mushrooms and beef patties was even less thorough than for peach slices, ethylene oxide treatment for these products was not even attempted. Results for mushroom shelf life are shown in Figure 4. As might have been expected, poorest shelf life (3 days) was for the sealed control held at 3°C. When evacuated and stored in nitrogen, shelf life was extended to 6 days, and when treated with carbon monoxide, to 15 days. When stored at −2°C, all treatments performed somewhat better (9, 12 and 20 days respectively).

Gas treatments had a profound effect on changes in thiamine content of mushrooms in storage (Fig. 5). The sealed controls at both storage temperatures showed little change in thiamine content throughout the storage period. When stored in nitrogen, thiamine content appeared to increase by about 25% after 1 month's storage then gradually decreased to original levels during the second month's storage. A similar tendency was noted for the carbon monoxide-treated mushrooms, but to a much more substantial degree with the increase at the end of the first month's storage being practically 100%. For all treatments at all storage periods, thiamine content was higher at the lower storage temperature. Total protein content showed no significant trends; nor did the relative protein value. As shown in Figure 6, the sealed control and nitrogen stored mushrooms showed a sharp rise in relative protein value from approximately 75% (of casein) to about 90% after about 2 wk storage. The protein value then declined to approximately the original value after 1 month's storage at which time it again increased perceptibly. In contrast to these variations in relative protein value during storage, the CO-treated mushrooms maintained a relatively constant protein value throughout the storage period.

It may therefore be concluded that carbon monoxide does not materially affect nutrient retention in mushrooms, but does substantially improve shelf life from 2–3 days to 20 days when stored at, or slightly below 0°C.

Beef

Undoubtedly the most spectacular beneficial effect of carbon monoxide treatment was with the beef patties. The beneficial effect of flushing with carbon monoxide on retention of redness in beef is well known (El Badawi et al., 1964). Figure 7 illustrates a less known phenomenon, which is that the retention of redness by flushing with carbon monoxide is more effective at a higher temperature of +3°C than at −2°C. The beneficial effect of CO treatment on eating quality of prepared patties is demonstrated in Figure 8. Even after a 2-½ month's storage period all CO treated samples were still acceptable, although significantly poorer than the fresh ground equivalent, while the sealed controls were no longer acceptable.

From the appearance standpoint (Fig. 9) the best shelf life is obtained with carbon monoxide treated patties at the

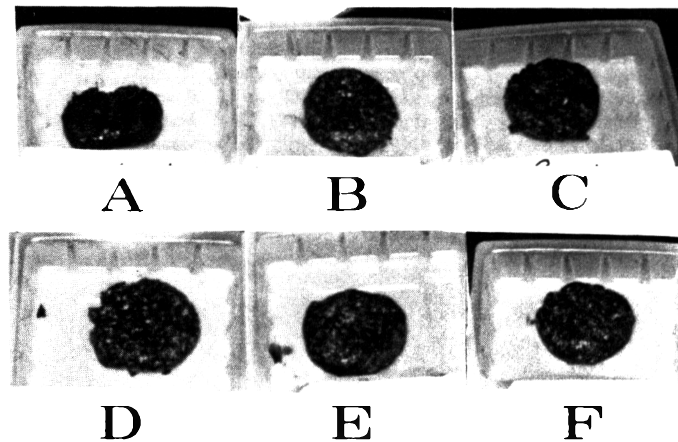

Fig. 7–Appearance of fresh beef patties treated with various gases after storage in sealed jars for 31 days: A–air control, 3°C; B–CO, 3°C; C–nitrogen control, 3°C; D–air control, −2°C; E–CO, −2°C; F–nitrogen control, −2°C.

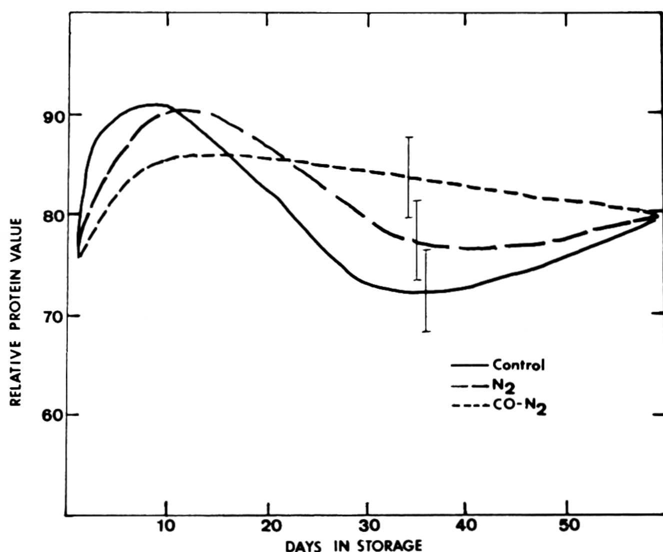

Fig. 6–Changes in relative protein value of mushrooms stored in sealed jars after flushing with various gases.

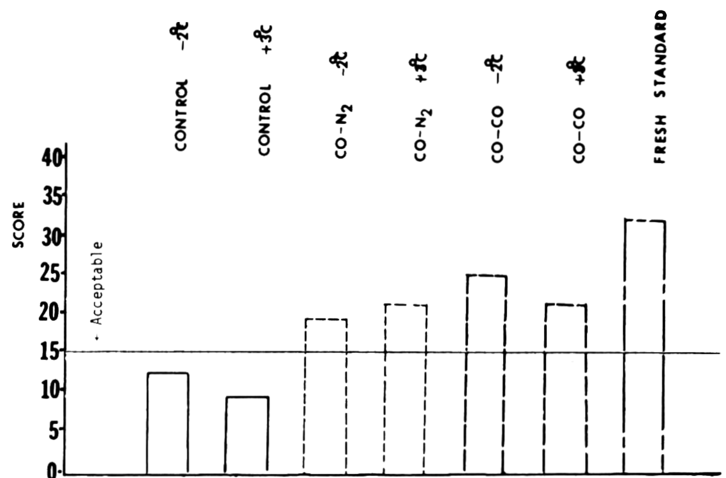

Fig. 8–Taste-panel evaluation of cooked beef patties treated with various gases after storage in sealed jars for 75 days.

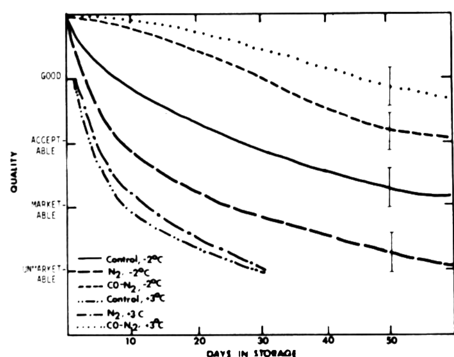

Fig. 9–Shelf life (appearance) of fresh beef patties treated with various gases.

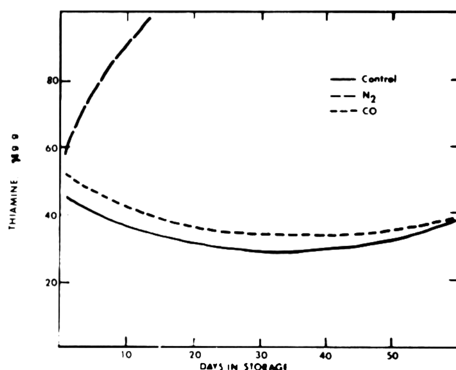

Fig. 10–Changes in thiamine of fresh beef patties stored in sealed jars after flushing with various gases.

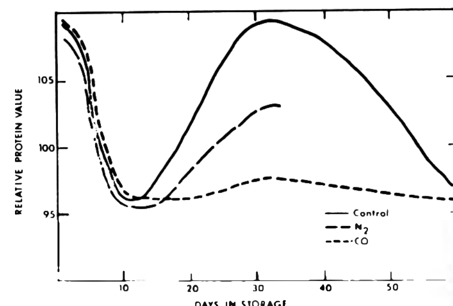

Fig. 11–Changes in relative protein value of fresh beef patties stored in sealed jars after flushing with various gases.

higher +3°C storage temperature where good appearance is retained for approximately 7 wk and acceptable appearance is retained for well over 2 months. At the lower storage temperature of −2°C, supposedly optimal storage temperature for beef, shelf life in terms of appearance is significantly shorter for the CO treated patties, being in the good category for only 1 month and acceptable for only 2 months. When the patties were not treated with carbon monoxide, the lower temperature was superior. Thus, the sealed controls at the lower storage temperatures retained acceptable appearance for 4 wk, but for less than 1 wk at the higher temperature. There was less temperature effect for the nitrogen flushed samples where the shelf life at the low temperature was 2 wk, and 1 wk at the higher temperature (Fig. 9).

Thiamine content (Fig. 10) for the sealed control and carbon monoxide treatments remained fairly stable throughout the storage period. The nitrogen flushed patties, on the other hand, showed a sharp increase in thiamine content (well over 100%) during the first month's storage. During the second month's storage, thiamine content tended to drop, but was not reduced to the original levels even after 2-½ month's storage at both high and low temperatures. Relative protein values for beef patties showed spectacular changes during the storage period. All treatments showed about 15% decline during the first 2 wk of storage. The carbon monoxide treatment then leveled off at approximately 95% RPV for the balance of the storage period. The nitrogen flushed treatment then increased to over 100% (compared to casein) after 1 month's storage. The sealed control showed a similar trend peaking at the original protein value after 30 day's storage, then dropping back again to little more than 95% at the end of 2 month's storage.

It may therefore be concluded that ground beef benefits tremendously from carbon monoxide flushing where acceptable sensory quality is maintained for well over 2 months at relatively high storage temperatures as compared to not more than 1 wk for controls. From the nutritional standpoint about all that can be stated is that most interesting and peculiar changes occur in stored ground beef which are stabilized by flushign with carbon monoxide. At the least, further studies should be conducted to determine why both thiamine and protein value appear to increase during the first 2 months of cold storage.

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RANCIDITY IN ALMONDS: SHELF LIFE STUDIES

INTRODUCTION

THE DETERMINATION of quality of shelled almonds is based primarily on the U.S. Standards for Grades of Shelled Almonds (1960). Paragraph 51.2122 states, "Rancidity means that the kernel is noticeably rancid to the taste." Unfortunately, according to Mattei (1969), little is known about quality of oils in oleaginous nuts. Moreover, Mattei commented that Schaal Oven and A.O.M. tests conducted at a temperature exceeding 110°F. were not reliable. He observed that accelerated high temperature tests produce a different type of change in quality than that found in almonds stored at lower temperatures. Therefore, he suggested quality deterioration in almonds be measured by storing them below 110°, and analyzing the "cold pressed" oil contained therein for free fatty acids and iodine value. No such studies have been reported. Almonds generally contain more than 50% fat as oil (Watt and Merrill, 1963). When autoxidation of the oil occurs in fresh almonds, the free fatty acid content will increase above 0.5% (as oleic), and the iodine value will decrease in magnitude because of oxidation at the double bond site in the unsaturated oil.

The specific objectives were:

(a) To study the aforementioned chemical factors in diced unroasted and roasted almonds.
(b) To use a simplified gas chromatographic method for measuring oxygen absorption. It was compared with sensory tests to determine if this test related to changes in quality of diced roasted almonds.
(c) To determine the effect of a confectioner's glaze containing mono- and diglycerides as a coating on diced unroasted and roasted almonds per se.
(d) To compare glazed and unglazed diced roasted almonds when added to enriched sweet chocolate disks for use in military rations.

EXPERIMENTAL

Diced unroasted almonds

Six 600g quantities of diced unroasted almonds from a single source were pan coated (1.2% by wt) with a confectioner's glaze of the following composition (Gutterson, 1969):

Ingredient	% by Wt
Ethanol	63.66
Zein (a corn protein)	24.00
Mono- and diglycerides	12.00
BHA (antioxidant)	0.17
BHT (antioxidant)	0.17
	100.00

Each lot was air dried and packed in hermetically sealed 401 × 411 cans. Three cans were stored at 0°F and three at 100°F.

Six 600g quantities of almonds from the same batch were left unglazed, packed and stored as above.

After 0, 3, or 6 months one can each of glazed and unglazed treatments from each storage period were opened. 100g aliquots of the contents of each can were analyzed for free fatty acid and mean iodine value (see "Chemical tests"). 20g aliquots of the almonds were served to each member of a 30-member consumer taste panel as a 4 of 4 complete block design. Samples were rated using the hedonic scale Peryam and Pilgrim (1957).

Analysis of variance of data was calculated using a split plot design, with storage time as the between variable, and nut sample as the within variable.

Table 1—Hedonic rating[a] of diced, unroasted almonds (glazed or unglazed) stored for 0, 3, or 6 months at 0° or 100° F

Storage time (months)	Stored at 0°F		Stored at 100°F		Main withdrawal effect (Mean)
	Nonglazed	Glazed	Nonglazed	Glazed	
Initial (0-Time)	6.5	6.5	6.4	7.0	6.6
3	6.9	6.9	7.1	6.5	6.9
6	7.0[b]	6.2	6.7	6.5	6.6
Main treatment effect (mean)	6.8	6.5	6.7	6.6	

[a]Hedonic rating ranges from "dislike extremely" (1) to "like extremely" (9) (Peryam and Pilgrim, 1957).
[b]Product rated significantly better than 0° F coated sample. L.S.D. (0.05) = 0.7.

Diced roasted almonds

15g of diced roasted almonds (glazed and unglazed from the same batch as used in the disks) were packed in quadruplicate in separate hermetically-sealed 300 × 200 cans. The samples were packed in air and stored at 0° and 100°F for 0, 1, 2, 3, 5 and 6 months. Then the cans were withdrawn at the end of each storage period, brought to room temperature, and the atmospheres in the cans analyzed for oxygen content by the gas chromatographic method of Bishov and Henick (1966).

Another batch of almonds from the same lot as above were treated in the same manner as mentioned in "Diced unroasted almonds," except that the samples were withdrawn at 0, 1, 2, 3, 5 and 6 months and evaluated by a technical panel of 12 judges. Samples were obtained from the California Almond Growers' Exchange from the 1969 and 1970 crops. The technique for sensory measurement is described by Pilgrim and Peryam (1958). Normative judgement was made for flavor on a 1–9 point scale, with 1 rated as extremely poor and 9 excellent. Analysis of variance was made on sensory data collected at each withdrawal period, comparing either the samples stored at 0°F or 100°F. Duncan's (1955) multiple range test was used to find differences between the means of samples. For checking correlation of oxygen content as noted previously with sensory panel ratings, "x" was used as oxygen content and "y" as panel score. 100g aliquots of the contents were analyzed for free fatty acid and mean iodine value and moisture as noted in "Chemical tests."

Diced roasted almonds in enriched sweet chocolate disks

Two lots of the chocolate disks containing 20% by weight of diced roasted almonds were produced following requirements of MIL-C-10928D (1969) by a commercial chocolate company:

Lot 1: Chocolate disk containing unglazed almonds
Lot 2: Chocolate disk containing glazed almonds

In Lot 2, the almonds were pan coated with a confectioner's glaze prior to their addition to the chocolate disks. The prepared candies of each lot (50 1-oz disks individually wrapped in aluminum foil packed in a standard paperboard container) were stored for 0, 3 and 6 months at 100°F. When the samples were withdrawn they were served in random fashion as ¼ oz pieces to a 10-member taste panel. A triangle test was used to determine differences between lots as described by Amerine et al. (1959). The number of correct decisions in triangle tests necessary for significance was determined according to Merck (1963).

Chemical tests

Percent free fatty acid content (as oleic) and mean iodine values were determined on the oil expressed from samples of diced unroasted al-

monds at the same withdrawal periods noted under "Diced roasted almonds." The oil was expressed from the almonds by placing samples in a plunger die assembly and subjecting it to pressure via a hydraulic press. The oil was collected and stored in a glass beaker under nitrogen until determination of free fatty acids by assay method of AOCS Ca 5a-40 (1947). The Wijs iodine value was determined according to AOCS Cd 1-25 (1956). Moisture of almonds was determined using AOAC vacuum oven procedure (1970).

RESULTS & DISCUSSION

NONE OF THE diced unroasted almonds, except for those frozen and unglazed were judged to be significantly different in consumer acceptance as the result of varying coating, temperature, or withdrawal time (Table 1). The glazed product frozen and stored at 0°F after 6 months was found to be poorer in acceptability than its unglazed counterpart. Hedonic ratings of the almonds ranged from 6.2 (like slightly) to 7.0 (like moderately) depending on treatment and withdrawal time.

Results of triangle test run at each withdrawal period of 0, 3 and 6 months at 100°F on enriched sweet chocolate disks containing either glazed or unglazed almonds indicated no significant differences at any storage period by panel members. Even after 6 months storage at 100°F all of the chocolate disks containing 20% by weight of diced roasted almonds were judged to be of good quality by a hedonic test (data not presented). Known factors responsible for protecting almonds in a chocolate confection of this nature are the hydrophobic nature of fats (cocoa butter and added fat contained in the chocolate) surrounding the almond pieces, plus naturally occurring antioxidants in cocoa butter, and added antioxidants (such as BHA and BHT) specified for military candy. In this regard Kempf (1964) noted that cocoa butter contains unidentified natural antioxidants which inhibit rancidity development in chocolate for 2–5 yr. As early as (1954) Stuckey reported that phenolic antioxidants inhibit rancidity development of almonds in candies.

Unglazed or glazed diced roasted almonds of 1969 and 1970 crops were stored at 100°F in hermetically-sealed cans without chocolate and withdrawn for oxygen analysis after 0, 1, 2, 3, 5 and 6 months at both 0° and 100°F (Table 2). Data show that for the 1969 crop there were significant differences between glazed and unglazed samples indicating that the unglazed samples absorbed more oxygen. Nevertheless, despite a higher oxygen uptake of the unglazed samples of almonds, they were similar in taste to the glazed samples stored at 100°F according to the sensory panel (Table 5). For example, they could detect off flavor in glazed almonds even when small changes in headspace oxygen occurred. To support this finding comparing oxygen content and panel score, it was found that the correlation coefficient value for the 1969 crop of unglazed almonds stored at 100°F was 0.63. Its glazed counterpart correlated better with a value of $r = 0.83$. In 1970 however, using the same parameters as above, the unglazed product had a value of $r = 0.90$ and its glazed counterpart a 0.96. Little or no association was found between oxygen content of almonds stored at 0°F and sensory panel results which indicates temperature dependence.

For the 1969 crop, oxygen absorption was most rapid in the period of 1 to 5 months when almonds were exposed to a temperature of 100°F. For the 1970 crops, there were no significant differences between glazed or unglazed samples for the treatments stored at either 0°F or 100°F.

Diced unroasted almonds (glazed and unglazed) were assayed for free fatty acid changes and mean iodine value (Table 3). During the 6-month period at 0°F the free fatty acid content of oil from unglazed almonds increased 177%. This value was approximately double the level for glazed almonds. After 6 months storage at 100°F for unglazed almonds, the free fatty acid content increased 268%, or almost two times that of samples stored at 0°F. A decrease in iodine value indicates oxidation. However, the iodine value showed a greater drop for the glazed sample. The reason for this inconsistency cannot be explained.

Table 2–Oxygen absorption of coated and uncoated diced, roasted almonds from 1969 and 1970 crops stored in hermetically-sealed cans

	Headspace Oxygen (%)[a]						
	Storage Time (months)						
Treatment	0	1	2	3	5	6	
	1969 Crop						
Storage at 0°F							
Unglazed	20.6	20.4	20.0	20.0	20.0	19.9	**
Glazed	20.8	20.7	20.5	20.5	20.5	20.6	
Storage at 100°F							
Unglazed	20.6	18.0	15.3	10.2	1.4	1.3	*
Glazed	20.8	19.5	18.3	16.2	12.4	12.1	
	1970 Crop						
Storage at 0°F							
Unglazed	20.9	20.9	20.9	20.9	20.9	20.9	NS
Glazed	20.9	20.9	20.9	20.9	20.9	20.9	
Storage at 100°F							
Unglazed	20.9	20.6	20.3	19.9	19.6	19.3	NS
Glazed	20.9	20.7	20.3	19.9	19.4	19.0	

[a]Average of four samples
*Significant at 5% level
**Significant at 1% level
NS = Not significant

Table 3–Effects of glazing and storage temperature upon free fatty acid (% oleic) and mean iodine values on diced, unroasted almonds

	FFA (%)			Iodine value[a]		
Treatment	0 Months[b]	6 Months	% Inc	0 Months	6 Months	% Dec
Storage at 0°F						
Unglazed	0.22	0.61	177	96.8	92.8	4.1
Glazed	0.22	0.43	95	91.6	89.9	1.9
Storage at 100°F						
Unglazed	0.22	0.81	268	92.2	90.5	1.8
Glazed	0.22	0.23	5	96.8	92.0	5.0

[a]Mean of duplicates
[b]A single determination (0.22) was made of oil of almond from original single source.

Table 4—Effects of glazing, storage temperature and crop upon moisture, free fatty acid (% Oleic) and mean iodine values on diced roasted almonds stored in hermetically-sealed cans

Treatment	Moisture (%)[a]							FFA (%)[a]							Iodine Value[a]						
	Months							Months							Months						
	0	1	2	3	5	6	Signif.	0	1	2	3	5	6	Signif.	0	1	2	3	5	6	Signif.
1969 Crop																					
Storage at 0°F																					
Unglazed	1.0	.77	.75	.85	.92	.76	NS	.25	.31	.30	.30	.30	.30	NS	102.8	102.7	102.1	102.3	100.6	99.4	NS
Glazed	.92	.91	.84	.95	1.1	.88		.25	.30	.30	.30	.30	.40		102.8	103.3	102.1	102.6	101.9	100.4	
Storage at 100°F																					
Unglazed	1.0	.84	.74	.77	.91	.69	NS	.25	.35	.30	.30	.30	.35	NS	102.8	102.2	102.1	101.2	100.6	99.7	NS
Glazed	.92	.94	.97	.90	.98	.93		.25	.30	.30	.30	.30	.40		102.8	102.9	102.3	101.5	102.2	99.7	
1970 Crop																					
Storage at 0°F																					
Unglazed	1.0	1.3	1.0	1.1	1.1	1.0	**	.40	.40	.40	.30	.30	.30	NS	104.8	103.5	102.8	103.8	104.4	102.7	**
Glazed	1.4	1.5	1.4	1.6	1.7	1.4		.40	.30	.40	.30	.35	.30		104.6	102.7	102.4	103.2	102.7	101.9	
Storage at 100°F																					
Unglazed	1.0	1.3	1.0	1.0	1.2	.97	**	.40	.40	.30	.30	.35	.30	NS	104.8	102.7	103.7	103.8	103.0	101.9	NS
Glazed	1.4	1.3	1.3	1.4	1.5	1.2		.40	.30	.30	.40	.40	.40		104.6	102.1	102.8	103.1	103.9	101.5	

[a]Mean of duplicates **Significant at 1% level NS = Not significant

Effects of glazing, storage temperature (0° and 100°F) and crop year upon moisture, free fatty acid content and mean iodine values on diced roasted almonds stored in hermetically-sealed cans were compared (Table 4). Data indicated that for the 1969 crop there were no significant differences for moisture, free fatty acid content or iodine value when almonds were stored at either 0°F or 100°F. Changes in free fatty acid content between glazed and unglazed samples of the 1970 almond crop stored at either temperature were not significantly different. For the 1970 crop, however, the glaze treatment reduced moisture loss of almonds stored at both 0°F and 100°F.

Gross changes in the free fatty acid content were greater for the diced unroasted product in 1969 than the roasted products of the same year. Mean iodine values for the 1970 crop stored at 0°F differed significantly between glazed and unglazed almonds. This difference, however, was not evident in 1970 almonds stored at 100°F or in the 1969 crop stored at 0°F or 100°F. There was an overall decrease in the iodine values in almond samples for both years stored at either 0°F or 100°F.

Table 5—Sensory technical panel rating[a] for flavor of diced roasted almonds 1969 and 1970 crops

Storage time (months)	1969 Crop		1970 Crop	
	Frozen (0°F) storage			
	Unglazed	Glazed	Unglazed	Glazed
0	6.8	6.0	6.2	6.3
1	7.0*	5.0	6.2	5.9
2	6.0	4.2*	5.1	4.6
3	6.8*	4.8	5.3	5.1
5	7.1*	5.8	6.1	5.9
6	6.1	5.3	5.4	5.0
	Unfrozen (100°F) Storage			
	Unglazed	Glazed	Unglazed	Glazed
0	6.2	6.3	6.2	6.3
1	5.8	5.0	6.0	5.6
2	6.7	5.8	5.9	5.4
3	4.9	4.1	4.6	4.6
5	All samples considered unacceptable			
6	Test discontinued			

[a]Mean of 12 scores
[b]Line under mean scores indicates no significant difference
*Significant difference at 5% level

Panel ratings for flavor of diced roasted almonds of the 1969 and 1970 crops (glazed and unglazed) stored for 0, 1, 2, 3, 5 and 6 months at either 0°F or 100°F were determined at each withdrawal period (Table 5). Data indicated that there was no difference between the control and glazed treatment of the 1969 and 1970 crops stored at 0°F at the initial or the 6 month withdrawal period. However, the 1969 unglazed almonds were rated significantly better than all of the other treatments at 1, 3 and 5 months. A reason for this could be that the glazed almonds had a detectable residual alcoholic taste and a bitter note. The bitter note was apparently formed in roasting and was accentuated by the glaze treatment. After 3 to 5 months at 0°F, the 1969 crop appeared to rate higher than after 2 months storage. This difference apparently was due to variation in panel scoring, but was offset by comparing scores of each session.

Samples stored at 100°F comparing glazed with unglazed nuts of the 1969 and 1970 crops showed no significant difference as to flavor at any withdrawal period (Table 5). All of the samples got

progressively worse in flavor and at the 2–3 month period, they rated less than fair, above poor. They became so unacceptable after 5 months that testing was discontinued.

It is interesting that the unglazed almond crop harvested in 1969 got the best technical panel ratings for flavor throughout the study with one exception. At the 1 month withdrawal at 100°F the 1970 control crop was rated slightly better in quality. It remained at an acceptable level of below good, above fair, even after 3 months in air pack at 100°F. Even though the 1969 crop was a year old when first stored, it was thought to be more stable to autoxidation (off flavor) than the 1970 crop.

In summary, the gas chromatographic method of Bishov and Henick (1966) proved to be a valuable chemical technique in most cases for testing for rancidity development in glazed or unglazed diced almonds. Nevertheless, the "tried and true" taste test for flavor using a trained technical panel by the method of Pilgrim and Peryam (1958), was more reliable in judging quality loss in almonds.

A glaze coating prevented oxygen absorption in the 1969 crop of almonds but was less effective on the 1970 crop. Also, it was found that glaze provides no additional protection to diced roasted almonds embedded in a chocolate confection.

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SURVIVAL OF SALMONELLAE AND Escherichia coli DURING THE SPRAY DRYING OF VARIOUS FOOD PRODUCTS

INTRODUCTION

IN 1965, outbreaks of salmonellosis that were traced to contaminated nonfat dry milk reiterated the fact that dried foods could serve as vehicles for salmonellae. Since then, several other dried foods (e.g., eggs, whole milk, yeast, buttermilk, etc.) have been found to contain salmonellae. Most of these foods have been dried by either the roller or spray process.

Investigations conducted in various food drying plants have shown that contamination of product can and does occur in numerous ways. It also appears that many processing plants have single and unique means by which the offending organisms gain entry into the product but seldom are these means the same in any two plants. Because of this, recommendations to the industry aimed at reducing or eliminating salmonellae are usually general in nature, i.e., adopt good sanitation programs, maintain facilities in good repair, and reduce or eliminate residual moisture in and around equipment.

There remain many unanswered questions regarding the survival of salmonellae during food processing operations. One of the key questions concerns survival during the spray drying process. At present, there are few data in the literature describing the survival rates of salmonellae during the spray drying of foods. Recently, the survival of salmonellae during the drying of skim milk concentrates was described by LiCari and Potter (1970a,b). They found that survival of salmonellae decreased as the spray drying temperature was increased. A variation in the percentage survival of the four strains tested at a given temperature was also noted. This study was undertaken to extend the observations of LiCari and Potter and to provide data on the survival of salmonellae during the drying of other food products.

MATERIALS & METHODS

Bacterial cultures

All cultures used in this study, i.e., *Salmonella typhimurium*, *S. alachua*, *S. newbrunswick*, *S. senftenberg* 775W, *S. montevideo*, *S. cubana*, *S. anatum*, *S. manhattan*, *S. newport*, *S. newington*, *Escherichia coli* 044, *E. coli* 070, *E. coli* 085, *E. coli* 0104, *E. coli* 0127 were obtained from the culture collection of the Food Research Institute.

Maintenance of stock and working cultures

Stock cultures were stored at room temperature on nutrient agar slants in screw-capped tubes. Transfers were made at intervals of approximately 6 months. Working cultures were transferred every 24 hr into Trypticase Soy Broth (TSB) and incubated without agitation at 35–37°C.

Sources of food materials

Skim milk concentrates were prepared by reconstituting powder from a single lot of antibiotic-free nonfat dry milk. The skim milk was obtained from the University of Wisconsin dairy plant and dried in the University of Wisconsin Spray Dryer (Amundson, 1967). It had a moisture content of 2.0% and contained less than 1% butterfat. Whole milk powder and sweet whey powder were obtained from Land o'Lakes, Inc., Minneapolis, Minn. The whole egg, egg white and egg yolk were pasteurized products obtained from Mazo Egg Products, Inc., Middleton, Wis. The dry yeast used in this study came from a single lot of inactive *Torula* yeast prepared by Amber Labs., Juneau, Wis. Yeast concentrates were prepared by suspending dry yeast in sterile distilled water to obtain a final concentration of 25% (w/w) yeast solids.

All products were found to be free of *Salmonella* and *E. coli* prior to use in this study.

Inoculation of concentrates prior to drying

For the inoculation of the milk products, the test organisms were grown to the stationary phase in 10 ml of concentrated skim milk (20% or 40% total solids). Immediately before spray drying, this material was added to a given quantity of fresh concentrate to achieve an initial *Salmonella* level of approximately 1×10^7 organisms per g of milk solids. The egg products, yeast and whey concentrates were inoculated in a similar manner except that the inoculum was grown in TSB rather than in the food concentrate. To ensure homogenous distribution of the cells in the high fat products, the inoculum was homogenized into the products with a hand type Lodgeman Mill Homogenizer.

Spray dryer and drying procedure

Concentrates were dried in a Nerco-Niro portable spray drying unit. Inlet air temperatures were 165 ± 2°C and 225 ± 2°C and outlet air temperatures were 67 ± 2°C and 93 ± 2°C. Air flow through the dryer was 35 Cfm at STP. The atomizer was controlled at 50,000 rpm. The powder was collected in a cyclone collector. The drying air was heated by a direct gas heater. The dryer was operated in such a way as to produce two different powder moisture levels, i.e., a conventional powder moisture level of 3.0–3.7% and a high moisture level of 6.0%.

Enumeration of salmonellae and E. coli

Salmonellae were enumerated by both the plate count and Most Probable Number (MPN) procedures. The level of salmonellae present in the food concentrates prior to drying was deter-

Table 1—Effect of drying temperature and final moisture content on the destruction of salmonellae and E. coli during the spray drying of concentrated skim milk

Organism	Total solids in conc (% w/w)	Powder moisture (% w/w)	Number of viable cells[a] in Conc milk	Number of viable cells[a] in Dry powder	Log reduction of viable cells
S. typhimurium	40	3.0 ± 0.2	1.7×10^7	9.3×10^3	3.3
		6.0 ± 0.2	7.0×10^7	4.3×10^5	2.2
S. alachua	40	3.0 ± 0.2	9.6×10^6	9.3×10^2	4.0
		6.0 ± 0.2	1.5×10^7	1.1×10^6	1.1
E. coli 0104	40	3.0 ± 0.2	1.0×10^7	3.3×10^5	2.5
		6.0 ± 0.2	1.0×10^7	2.1×10^3	3.8
S. typhimurium	20	3.7 ± 0.2	2.3×10^7	2.4×10^1	6.0
		6.0 ± 0.2	3.0×10^7	3.9×10^2	4.9
S. alachua	20	3.7 ± 0.2	2.5×10^7	4.6×10^2	4.7
		6.0 ± 0.2	1.9×10^7	2.4×10^4	2.9
E. coli 0104	20	3.4 ± 0.2	1.3×10^7	3.0×10^2	4.7
		6.0 ± 0.2	1.7×10^6	1.2×10^3	3.2

[a]Counts expressed on a per gram milk solids basis

mined by surface plating 0.1 ml aliquots of the appropriate peptone-water dilutions onto MacConkey agar. These plates were examined for typical *Salmonella* colonies after incubation at 35–37°C for 24 hr. Typical colonies were confirmed by slide agglutination procedures employing 0 antisera. Levels of *Salmonella* are expressed on a dry basis, i.e., number of cells per gram of moisture-free food solids.

All spray dried powders were held at room temperature and examined microbiologically within 2 hr after being dried. An MPN procedure was used to determine the number of salmonellae present in the dried product. This was accomplished by aseptically removing 11g of the powder that had been collected in a sterile sample bottle. For the analysis of the milk powders, 11g of sample was reconstituted in 99 ml of sterile buffered water according to the method described in the *Standard Methods for Examination of Dairy Products* (APHA, 1967). In the work done on the egg products, 11g of the powder was reconstituted in 99 ml of physiological saline solution (0.85% NaCl). The yeast powder was reconstituted in peptone water. After reconstitution, three 1 ml aliquots of suitable peptone-water dilutions were placed into individual lactose broth preenrichments. These were then incubated at 35–37°C for 18 hr. One ml of each preenrichment tube was then transferred to 9 ml of selenite-cystine broth. The enrichment broths were incubated 18–24 hr at 35–37°C. A loopful of each enrichment was streaked onto MacConkey agar and the plates were incubated at 35–37°C for 24 hr. The three tube MPN determinations were based on the appearance of typical salmonellae colonies on the selective agar following confirmation by serological procedures, i.e., 0 agglutination tests.

The number of *E. coli* in the concentrates prior to drying was ascertained by plating aliquots of suitable peptone water dilutions on violet red bile agar. Plates were examined after 18–24 hr at 35–37°C. *E. coli* in the spray dried product were enumerated by the method described in *Standard Methods for the Examination of Dairy Products* (APHA, 1967).

Moisture determinations

All moisture determinations were made using a Cenco Moisture Balance that had been standardized by the toulene distillation method (ADMI, 1948).

RESULTS & DISCUSSION

TWO INLET-OUTLET air temperature combinations were employed throughout this study. The first, i.e., 225 ± 2°C inlet and 93 ± 2°C outlet, resulted in a nonfat dry milk powder containing 3.0 ± 0.2% moisture when a 40% solids containing concentrate was dried and 3.4–3.7 ± 0.2% moisture when a 20% solids containing concentrate was dried. The second combination, i.e., 165 ± 2°C inlet and 67 ± 2°C outlet air temperature, resulted in powder containing 6.0 ± 0.2% moisture regardless of whether 20 or 40% solids containing concentrates were fed to the dryer.

Two strains of salmonellae and a single strain of *E. coli* were grown as described and inoculated into the concentrates for spray drying. The results of a single representative trial are shown in Table 1. It is quite obvious that greater destruction of the test organisms occurred when conditions were adjusted to produce the low moisture product. This occurred regardless of the total solids level of the concentrate fed to the dryer.

The greater destruction of the test organisms might be due to (1) greater thermal shock upon introduction into the hotter inlet air, and/or (2) higher product temperature during the final stages of drying to the lower moisture level. Previous studies (Bradford and Briggs, 1963) have shown that milk proteins may be dried at an inlet air temperature of 315°C and still escape denaturation if they are cooled rapidly. In our experimental dryer approximately 90% of the moisture removal occurred within the first 10% of the vertical drop in the dryer, and the remaining moisture was removed in the final 90% of the vertical drop. Since the product being dried approaches the temperature of the outlet air and since the outlet air temperature of the low moisture products was 93 ± 2°C it is evident this product was subjected to the higher temperature. The dryer residence time was the same for both products.

It is also apparent that particle density is a significant factor in determining the degree of survival by these enteric organisms. LiCari and Potter (1970a) suggested that the width of the powder particle wall may be an important factor influencing the survival of salmonellae. The effect of particle crust diameter was studied by comparing the degree of microbial destruction in powders dried from 20 and 40% total solids concentrates of skim milk. Since the powder particles produced by drying a 20% solids-containing concentrate have thinner crusts (Hall and Hedrick, 1966) and a greater degree of cell destruction was obtained in the powders produced from these concentrates, a correlation is apparent (Cf. Table 1). It can be seen that at comparable moisture levels (i.e., 6%), a greater degree of destruction was observed in the process of drying the 20% solids concentrate. Moreover, even though the moisture content of the powder produced at the lower

Table 2–The survival of Salmonella and E. coli during the spray drying[a] of concentrated skim milk (40% total milk solids)

Organisms	Number of viable cells/g in Concentrate	Number of viable cells/g in Dry powder	Log reduction in viable cells per g of milk solids
S. alachua	9.6×10^6	9.3×10^2	4.0
S. anatum	2.9×10^7	9.3×10^2	4.4
S. cubana	1.8×10^7	7.5×10^2	4.4
S. manhattan	1.8×10^7	4.6×10^3	3.6
S. montevideo	9.5×10^6	2.4×10^3	3.9
S. newbrunswick	2.0×10^7	2.4×10^4	2.9
S. newington	2.3×10^7	4.3×10^3	3.7
S. newport	2.7×10^7	4.3×10^2	4.8
S. senftenberg 775W	1.0×10^7	4.3×10^2	4.4
S. typhimurium	1.7×10^7	9.3×10^3	3.3
E. coli 044	8.5×10^6	2.4×10^2	4.6
E. coli 0104	1.0×10^7	2.1×10^3	3.8
E. coli 085	3.5×10^7	1.2×10^3	4.4
E. coli 0127	1.5×10^7	1.6×10^4	2.9
E. coli 070	2.2×10^7	3.6×10^3	3.8

[a]Results shown are those obtained in a single representative trial.

Table 3–The survival of S. typhimurium during the spray drying of various milk products[a]

Substrate	Number of viable salmonellae/g in Concentrate	Number of viable salmonellae/g in Powder	Log reduction in viable cells (per g dry food solids)
Skim milk	1.7×10^7	9.3×10^3	3.3
Whole milk	1.8×10^7	4.3×10^2	4.6
Whey	1.6×10^7	4.6×10^3	3.5

[a]Results shown are those obtained in a single representative trial.

drying temperature was greater in the materials made from the 20% solids concentrate than that made from the 40% solids concentrate, the survival of the test organisms was much less in the less dense product. It would seem that a combination of (1) a higher relative humidity in the air stream surrounding the thinner crusted particle (thereby resulting in a moister heat) and (2) less resistance to the penetration of the moist heat in the particle crust may be responsible for the greater observed cell destruction in the less dense particles.

The relationship between particle size and destruction of salmonellae remains unclear. In this study and in that reported by LiCari and Potter (1970a) the powder particles produced were significantly smaller than those normally produced in industrial practice and should be considered as 'fines.' Moreover, the very small range of particle sizes studied by LiCari and Potter do not permit an accurate determination of the influence of particle size on microbial destruction. The relationship (or lack of same) remains questionable at this time.

Previous studies on the heat resistance of salmonellae in moist environments have indicated a significant influence of growth temperature on the thermal resistance (Dega et al., 1972; Ng et al., 1969). The influence of growth temperature on the resistance to destruction by salmonellae during spray drying was examined by comparing the survival of *S. typhimurium* cells grown at 25°C, 37°C and 44°C to drying in a 40% solids concentrate at 225 ± 2°C inlet air temperature. The log reductions in viable salmonellae were 3.5, 3.3 and 3.0 at 25°C, 37°C and 44°C, respectively. This would indicate that there is perhaps some effect of incubation temperature on the heat resistance during spray drying but that it is not nearly as pronounced as in the case of moist environments.

In most cases, there has proven to be a considerable difference in the resistance of various strains of salmonellae to adverse environmental conditions. Ten strains of salmonellae and five strains of *E. coli* were tested for their ability to survive spray drying. The cells were added to concentrated skim milk (40% total solids) prior to drying at 225 ± 2°C inlet air temperature. The results are shown in Table 2. These data are in agreement with those of LiCari and Potter (1970a) who demonstrated a variation among strains of salmonellae with respect to their ability to survive spray drying. Our results do not indicate any significant difference in the resistance of salmonellae and *E. coli*. What is significant is the degree of variation of resistance among the strains. It would not be correct to consider this a serotype or species variability since the taxonomical status of these organisms is dependent on their antigen content which, most likely, has little effect on thermal resistance. More appropriately, this is a strain variation and it would not be at all surprising to find the same degree of variation in resistance among numerous strains of the same species or serotype.

It was of interest to compare the influence of product composition on the ability of salmonellae to survive spray drying. A single strain of *Salmonella*, *S. typhimurium* was added to 40% solids-concentrates of skim milk, whole milk and whey. The concentrates were then spray dried at an inlet air temperature of 225 ± 2°C to ensure as nearly as possible uniformity in moisture content, particle size and particle crust thickness. The results are shown in Table 3. Surprisingly, the high fat-containing product, i.e., whole milk, was least protective and the skim milk and whey were comparable in protective effect. This observation would indicate that fat is not protecting the cells from heat during spray drying. We cannot explain the seemingly sensitizing effect that fat has other than to suggest that the longer retention of heat in the fat may cause the destruction of more salmonellae.

Next, the survival rate of *S. typhimurium* during the drying of various egg materials was studied. The inlet air temperature was 225 ± 2°C and the total solids levels in the concentrates were 12.2, 26 and 40% for egg white, whole egg and egg yolk, respectively. As shown in Table 4, the greatest destruction occurred in the egg yolk. It is difficult to compare these data with those obtained from the trials with the milk products because of the variation in solids level in the concentrates. However, it can be pointed out that whole egg and whole milk were about equally protective when dried from 40% solids concentrates. It is possible that the greater kill in egg white could be attributed to the thin particle crust resulting from the low solids level in the concentrate. Moreover, it is also possible that the high fat content and low moisture level (resulting from higher product temperature in the latter stages of drying) are the reasons for the great degree of cell death occurring during the drying of egg yolk.

Drying concentrated yeast (25% solids) resulted in approximately the same degree of *Salmonella* death as was characteristic of that observed when skim milk (20% solids) was dried under similar conditions (Cf. Table 4).

The results of this study and the previous work of LiCari and Potter (1970a) indicate that several factors are important in deciding to what extent enteric microorganisms survive the spray-drying process. First, product temperature during the drying process is quite significant. It would appear that in spite of the evaporative cooling effect, high product temperature and microbial cell destruction are closely related. Second, particle density or particle crust diameter will influence the cell destruction rate. Thinner particle crusts will result in greater killing of the cells. Strain variation with regard to sensitivity to spray drying is considerable. The reasons for the varying sensitivities are not understood. High fat content in the product being dried appears to enhance the destruction of the enteric bacteria. Quite possibly, this is due to a longer retention of thermal energy in the lipid material.

As expected, the factors influencing cell death during spray drying are complex and interrelated. It is obvious that no single parameter is governing the behavior of salmonellae or *E. coli* during the drying process. Because of this complexity, it may never be possible to set a processing schedule for all food products which guarantees the elimination of salmonellae during the drying process and yet yields an economically, physically and organoleptically suitable final product. Finally, it is evident that the food processor cannot and should not depend upon the drying process to take the place of adequate pasteurization prior to drying and care must be exercised to avoid contamination during and after drying. Indeed, survival of the spray drying step yet remains an unknown quantity in the overall problem of *Salmonella* contamination in dried foods.

Table 4–The survival of S. typhimurium during the spray drying[a] of various egg products and inactive yeast

Substrate	Solids level in conc (%)	Final product moisture (% w/w)	No. of viable salmonellae/g in Concentrate	No. of viable salmonellae/g in Powder	Log reduction of viable cells (per g dry food solids)
Whole egg	40	3.0 ± 0.2	1.3×10^7	2.4×10^3	4.4
Egg white	12.2	7.0 ± 0.2	5.7×10^7	1.1×10^2	5.7
Egg yolk	26	2.0 ± 0.2	1.2×10^7	7.5×10^0	6.2
Torula yeast	25	3.4 ± 0.2	1.8×10^8	2.4×10^3	4.9

[a]Results shown are those obtained in a single representative trial.

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DATA ANALYSIS: INTERBLOCK AND INTRABLOCK ESTIMATES OF VARIANCE ON TASTE PANEL DATA

INTRODUCTION

THE NEED FOR better control of variability in the comparison of several treatments is necessary in many experimental investigations. For instance, we often require a specified number of litters per genetic group or a specified number of males and females of the same parents in one experimental run. Due to the difficulty in obtaining these types of experimental conditions, we often arrange treatments in incomplete blocks where each block does not contain all the treatments in the experiment. In some experiments, we use human beings as the experimental unit. When the experiment consists of several treatments and the response measurement is based on subjective scoring, human fatigue enters into the judging process resulting in biased scores. An incomplete blocking allowing, perhaps, two or three treatments per panel would lessen judging fatigue. A solution to this problem is provided by incomplete block designs.

The use and validity of incomplete block designs in sensory testing of foods is documented in the literature (Galinat and Everett, 1949; Hanson et al., 1951; Marquardt et al., 1963). There is however, a unique aspect of this class of design that requires attention in data analysis; that is, the relevance of the recovery of interblock and intrablock information. As far as the authors are concerned, there is no published inquiry into the importance of interblock and intrablock analyses of taste panel scores.

The recovery of interblock and intrablock information in an incomplete block analysis of variance is dependent upon the following relationships (Yates, 1940; Federer, 1955; Cochran and Cox, 1957): (1) if the degrees of freedom (DF) associated with block sum of squares (E_b) is less than 12 and E_b is larger than intrablock error (E_e) it is recommended that interblock information be ignored and treatment means adjusted only for intrablock information; and (2) if E_b is greater than E_e and the DF associated with E_b greater than 12–14, it is recommended that both interblock and intrablock information be utilized. Since the introduction of incomplete block designs by Yates (1936) about 35 years ago, interblock recovery was a computational problem. At present, where computers are readily available, we have the opportunity of studying the advantage of recovering both intrablock and interblock information and evaluating their contributions to increasing design efficiency in sensory testing.

This work was conducted to assess the importance of interblock and intrablock analyses of sensory panel scores.

EXPERIMENTAL

BALANCED INCOMPLETE block design (BIBD) is frequently used in sensory testing of foods at the Food Research Lab. of Armour Food Co. Specifically, we have been utilizing Type III BIBD of Cochran and Cox (1957) which involves repetition of the basic design. Three of the experiments available were selected as models for an in-depth study of interblock and intrablock analyses.

Experiment I

This study concerns the comparison of five treatment groups of frankfurters with varying levels of fat content. The response variables were appearance, flavor, texture and overall preference. An 8-point scale, with 1 = dislike extremely, and 8 = like extremely, was used. The design parameters for a basic design (Table 1) following the conventional notation are:

Number of treatments, t	5
Number of blocks, b	10
Number of replications, r	6

Table 1—Basic design for Experiment I[a]

Panelists (blocks)	Treatments 1	2	3	4	5
1	x	x	x		
2	x	x			x
3	x			x	x
4		x	x	x	
5			x	x	x
6	x	x		x	
7	x		x	x	
8	x		x		x
9		x	x		x
10		x		x	x

[a]Each x represents a sample.

Table 2—Block eliminating treatment (E_b) and intrablock mean squares (E_e)

Experiment	Period	Variable	E_b	E_e
I		Tenderness	3.5899	1.2174
		Juiciness	2.2492	.6806
		Flavor	1.2339	.6008
II		Appearance	1.3351	.7589
		Flavor	2.1821	.6346
		Texture	2.8885	.7160
		Preference	2.2523	.8741
III[a]	0	Off-Flavor	1.0769	.5833
	1		1.1622	.7097
	2		2.1244	.3444
	3		2.0683	.6431
	4		2.5058	.8167
	5		3.2196	.4028
	6		1.9676	.5542
	7		3.2712	.8208
	8		4.1106	.5278
	0	Preference	1.6843	.8819
	1		1.5311	.5653
	2		1.8833	.4667
	3		1.5712	.8153
	4		2.7404	1.1042
	5		3.5362	.5319
	6		2.5231	.7042
	7		3.7000	.8250
	8		5.4369	.8861

[a]Intrablock mean square E_e was found homogeneous by Bartlett's test.

Number of experimental units per block, k 3
Number of times each pair of treatments occur in the same block, λ 3

The total number of observations is denoted by N, obtained from r(t) or b(k). With repetitions of the basic design the parameters of this experiment are:

$$t = 5 \quad p(r) = 36 \quad k = 3 \quad p(b) = 60 \quad p(\lambda) = 18$$

where p is the number of repetitions. The value of k is the number of samples each panelist evaluates at one sitting.

Experiment II

In this experiment, four groups of steaks were evaluated on the basis of tenderness, juiciness and flavor on an 8-point scale. The design parameters were as follows:

$$t = 4 \quad p(r) = 72 \quad k = 2 \quad p(b) = 144 \quad p(\lambda) = 24$$

Experiment III

The stability of five experimental groups of sausage over a period of 8 wk was studied by using a BIBD with design parameters:

$$t = 5 \quad p(r) = 16 \quad k = 2 \quad p(b) = 40 \quad p(\lambda) = 4$$

The response variables were degree of off-flavor and of preference.

Statistical procedure

An observed panel score can be described by a linear model,

$$x_{ijk} = \hat{\mu} + t_i + b_j + r_k + e_{ijk}$$
$$i = 1,2,\ldots,t \text{ treatments}$$
$$j = 1,2,\ldots,b \text{ blocks (panelists)}$$
$$k = 1,2,\ldots,p \text{ repetitions}$$

where x_{ijk} is an observation of the ith treatment in the jth incomplete block and in the kth repetition, $\hat{\mu}$ is the general mean, t_i is the effect of the ith treatment, b_j is the effect of the jth block, r_k is the effect due to the kth repetition, and e_{ijk} is a random error effect assumed to be normally and independently distributed, $N(0, \sigma^2)$.

The allotment of the total DF is shown in Appendix A. In this model, the expected value (E) of the blocks is $E(b_j^2) = \sigma_b^2$, which defines an interblock variance. The block effect is assumed to be random, otherwise the extraction of the interblock information is not valid. The $E(e_{ijk}^2) = \sigma_e^2$, which defines an intrablock variance, is always assumed to be a random component.

There are two estimates of treatment difference in BIBD. The first is derived from an intrablock comparison which utilizes the intrablock error and in which treatment means are adjusted for block effects. The second estimate is derived from block differences and utilizes interblock and intrablock variances in adjustment of means and in significance testing. In the present work, we have estimated the interblock and intrablock variances following the procedure outlined by Finney (1960). We would like to state that the writings of Yates (1936; 1939; 1940), Kempthorne (1952), and Federer (1955) basically formed the core of the present work.

RESULTS & DISCUSSION

Condition of balance

Unlike the more common experimental designs, e.g., completely random and randomized block (RCBD), where there is no restriction as to the number of replications, BIBD possesses a design restricting relationship that must be satisfied. This relationship means that for a given t and k, the required r and b is fixed by the resulting basic design instead of being fixed by the research investigator. This is a point that investigators should realize in the use of this class of design so that experimental materials and other instrumentation would be prepared accordingly. Once the basic design is constructed, we can discuss the number of p repetitions of the basic design.

According to Yates (1940), the relations shown below should hold in BIBD:

$$tr = kb$$
$$\lambda(t-1) = r(k-1)$$
$$\lambda = \frac{r(k-1)}{t-1}$$

Table 3—Variance component estimates and percent gain by inclusion of interblock information

Experiment	Product	Period	Variable	Interblock $\hat{\sigma}_1^2$	Intrablock $\hat{\sigma}_2^2$	Combine $\hat{\sigma}_3^2$	Gain by inclusion of interblock estimate, %
I	Frankfurters		Appearance	.2297	.0253	.0228	9.92
			Flavor	.3367	.0212	.0199	5.91
			Texture	.5119	.0239	.0228	4.45
			Preference	.3615	.0291	.0270	7.46
II	Steaks		Tenderness	.1222	.0253	.0209	17.16
			Flavor	.0548	.0123	.0100	18.27
			Juiciness	.0935	.0173	.0146	15.58
III[a]	Sausage	0	Off-Flavor	.1911	.0583	.0447	23.39
		1		.1730	.0710	.0503	29.10
		2		.3413	.0344	.0313	9.17
		3		.3581	.0643	.0545	15.22
		4		.3973	.0817	.0677	17.05
		5		.4655	.0403	.0371	7.96
		6		.3589	.0554	.0480	13.38
		7		.5271	.0821	.0710	13.47
		8		.6233	.0528	.0487	7.81
		0	Preference	.2894	.0882	.0676	23.36
		1		.2611	.0565	.0465	17.80
		2		.2883	.0467	.0402	13.93
		3		.2446	.0815	.0611	25.00
		4		.4405	.1104	.0871	19.78
		5		.6212	.0532	.0478	7.70
		6		.4051	.0704	.0600	14.81
		7		.6420	.0825	.0731	11.39
		8		.9013	.0886	.0807	8.95

[a] $\hat{\sigma}_2^2$ was found homogeneous by Bartlett's test.

The condition of balance in BIBD will be fulfilled if each pair of treatments (t) occurs together in the same block an equal number (λ) of times. This relationship can be verified using the basic design in Table 1. The value of λ in this design is 12/4 = 3. Therefore, any pair of treatments should appear with equal frequency three times in the design. Thus, treatments 1 and 2 appear in three blocks (blocks 1,2 and 6) and treatments 1 and 3, in blocks 1, 7 and 8. The other pairs may be verified similarly. Another requirement in BIBD is that the numbers t, k, r, b and λ must be integers.

Total panel effect

In this study, the block effect represents variation due to panelists, and we term it as total panel effect. It is represented by "block eliminating treatment" sum of squares (E_b) in the analysis of variance table (Appendix A). The estimates of total panel effect E_b and intrablock error E_e are given in Table 2. In all cases, E_b was greater than E_e, suggesting that additional information about treatment differences can be obtained from interblock analysis, and also that the recovery of interblock information would appear to be of value. When $E_b < E_e$, it is assumed that the total panel effect variance σ_b^2 is zero, and therefore only intrablock adjustment is made on treatment means.

With two exceptions (1.2174 and 1.1042), the intrablock error mean square exhibited a small variation and showed independence from E_b. The fairly constant value of E_e validates the use of most statistical methods in significance testing of sensory scores.

The panel effect showed an increasing trend with time. This trend is attributed to differences in threshold among individual panelists. As treatment difference increases, some panelists failed to detect this difference at some point in time because of their individuality. The effects of central tendency and incomplete repeatability of score are included in panel effect. All of these effects constitute a time-trend effect, and indeed support the use of controls and reference points in sensory evaluation of this type in order to stabilize panel scores.

Interblock and intrablock analyses

The size of E_b in relation to E_e and the number of DF associated with E_b, suggest interblock and intrablock recovery. The estimation procedure for interblock and intrablock variances appears in Appendix A. The components of variance associated with interblock variance (σ_1^2), intrablock variance (σ_2^2) and combined variance (σ_3^2) are shown in Table 3.

The estimated intrablock variance component σ_2^2 appeared to vary with the product tested. The σ_2^2 was higher for sausage than for frankfurters or steaks. This result is a relevant guide in the specification of sample size. We often used the same number of panelists regardless of the product because we assumed that the variance is homogeneous. The intrablock variance for experiment III did not differ significantly using Bartlett's test. This finding substantiates the correctness of assuming homogeneous variance from one time to another as being important in the testing of hypothesis.

The combined components of variance, σ_3^2, were smaller than σ_2^2, indicating the value of interblock recovery. The gain,

$$(1 - \frac{\hat{\sigma}_3^2}{\hat{\sigma}_2^2})100$$

in information by the inclusion of interblock estimate ranges from 4.5–29.1%. To evaluate the practical implication of this result, we have selected three values of percent gain representing low, intermediate and high (Table 4). In each grouping, actual and adjusted means, F ratios, and standard errors were calculated. The result shows that the absolute change in means, F ratios, and standard errors by interblock and intrablock recoveries is negligible. Therefore, only the analyses of sensory data by intrablock recovery may be performed without loss of accuracy in the interpretation of result. An example of BIBD analysis recovering interblock and intrablock information is given in Appendix B.

Table 4–Effect of intrablock and interblock recoveries on the estimates of mean, standard error and F ratio[a]

Treatment no.	Actual $\bar{x}$	Intrablock $\bar{y}$	Weighted by interblock and intrablock $\bar{m}$
	Low (Gain=4.5%)		
1	6.25	6.17	6.19
2	6.28	6.42	6.38
3	5.86	5.81	5.82
4	5.78	5.82	5.81
5	4.97	4.93	4.94
Std Error	0.29	0.15	0.15
F statistic		13.37	14.75
	Intermediate (Gain=17.8%)		
1	5.94	5.94	5.94
2	6.01	5.89	5.97
3	6.00	6.14	6.07
4	6.13	6.19	6.16
5	5.81	5.79	5.80
Std Error	0.25	0.24	0.22
F statistic		0.51	1.85
	High (Gain=29.1%)		
1	5.06	5.25	5.15
2	5.44	5.50	5.45
3	5.50	5.40	5.48
4	5.31	5.35	5.32
5	5.38	5.25	5.35
Std Error	0.22	0.27	0.22
F statistic		0.16	1.30

[a] $F < 1.0$ is not theoretically possible. This occurs in practice due to sampling errors.

APPENDIX

A. The structure of the analysis of variance

Source of variance	Degrees of freedom
Total	N–1
Treatment ignoring block	t–1
Within treatment:	t(r–1)
Block eliminating treatment	b–1
Intrablock	N–t–b+1
Treatment eliminating block	t–1
Block ignoring treatment:	b–1
Repetition	p–1
Block within repetition (treatment component)	t–1
Remainder	(p–1)+(t–1)
Intrablock	N–t–b+1

In the above, the values for r and b have already been multiplied by p, the number of repetitions. The total panel effect E_b has two components as follows:

	DF	Expected mean squares
Total panel effect	b–1	$\sigma_e^2 + \frac{(bk - t)}{b-1} \sigma_b^2$
Treatment component	t–1	$\sigma_e^2 + Ek\sigma_b^2$
Remainder	b–t	$\sigma_e^2 + k\sigma_b^2$

The E in the expected mean squares is the ratio $t\lambda/rk$, an efficiency factor defined as the fraction of the total information contained in the intrablock comparisons, when interblock and intrablock comparisons are of equal accuracy (Yates, 1940). This ratio is the lower limit of the efficiency of BIBD relative to RCBD with values ranging from 0–1.0.

Thus, if $\sigma_e^2 < E\sigma^2$, BIBD is more precise than randomized complete block design (σ^2 is the error variance of RCBD). From the above table, total panel effect is composed of a treatment component and a remainder.

Shown below is the latter half of the analysis of variance structure used for the estimation of the interblock and intrablock variances.

	DF	Expected mean square
Block ignoring treatment	b − 1	
Repetition	p − 1	
Treatment component	t − 1	
Remainder	b − p − t + 1	$\sigma_e^2 + k\sigma_b^2$
Intrablock	N − t − b + 1	σ_e^2

The intrablock error σ_e^2 is a direct estimate of the intrablock variance and is the residual variance of observations in the same incomplete block. The remainder, purely a measure of interblock variation, has a mean square which estimates $\sigma_e^2 + k\sigma_b^2$, and is the residual variance of observations in different incomplete blocks.

The value of

$$\hat{\sigma}_1^2 = \frac{k(k\sigma_b^2 + \sigma_e^2)}{r - \lambda}$$

was obtained as an estimate of the interblock component of variance of the treatment effect, and

$$\hat{\sigma}_2^2 = \frac{k\sigma_e^2}{t\lambda}$$

as an estimate of the intrablock variance component. These quantities are tabulated in the text (Table 3). The combined variance $\hat{\sigma}_3^2$ in this table is the reciprocal of the reciprocals of interblock and intrablock variances obtained by

$$\hat{\sigma}_3^2 = \frac{(\hat{\sigma}_1^2)(\hat{\sigma}_2^2)}{\hat{\sigma}_1^2 + \sigma_2^2}.$$

B. Numerical illustration of BIBD analysis with interblock and intrablock recoveries

Design parameters: t = 4 k = 2 r = 9 b = 18 λ = 3

Session	Panelist	Treatment T_1	T_2	T_3	T_4	Panel sum $B_{.jk}$	Session sum $R_{..k}$
1	1	4	4			8	
	2			7	5	12	
	3	7		6		13	
	4		4		6	10	
	5	4			5	9	
	6		1	5		6	58
2	7	6	6			12	
	8			3	4	7	
	9	6		3		9	
	10		4		5	9	
	11	3			5	8	
	12		4	4		8	53
3	13	5	6			11	
	14			7	6	13	
	15	3		4		7	
	16		2		4	6	
	17	4			5	9	
	18		2	2		4	50
Treatment total $T_{i..}$		42	33	41	45	$X... = 161$	$\hat{\mu} = 4.47$
$kT_{i..}$		84	66	82	90		
B_i		86	74	79	83	$B. = 322$	$\bar{B} = 80.50$
$Q_i = kT_{i..} - B_i$		−2	−8	3	7	Check: $\Sigma Q_i = 0$	
$W_i = (t-k)T_{i..} - (t-1)B_i + (k-1)X...$		−13	5	6	2	$\Sigma W_i = 0$	

	Treatment T_1	T_2	T_3	T_4
Actual mean $\bar{x}_i = T_{i..}/r$	4.67	3.67	4.56	5.00
Adj. mean (intrablock) $\bar{y}_i = \hat{\mu} + (Q_i/t\lambda)$	4.31	3.81	4.72	5.06
Adj. mean (interblock) $\bar{z}_i = \hat{\mu} + [(B_i - \bar{B})/(r - \lambda)]$	5.39	3.39	4.22	4.89
Adj. mean (intrablock and interblock) $\bar{m}_i = (T_{i..} + \mu W_i)/r$	4.47	3.74	4.65	5.03

Calculation of adjusted treatment means

(1) $T_{i..}$ = total of the observations for the ith treatment from r units containing the ith treatment.

(2) $kT_{i..}$ = treatment sum multiplied by the number of experimental units per block.

(3) B_i = sum of panel totals containing the ith treatment. For example, for treatment 1,

$$B_1 = x_{111} + x_{131} + x_{151} \ldots + x_{1133} + x_{1153} + x_{1173}$$
$$= 8 + 13 + 9 + \ldots + 11 + 7 + 9$$
$$= 86$$

(4) Q_i = a measure of treatment effects free from panel effects (block). For example,

$$Q_1 = 84 - 86$$
$$= -2$$

(5) Weighting factor μ is computed from,

$$\mu = \frac{(b-p)(E_b - E_e)}{t(k-1)(b-p)E_b + (t-k)(b-t-p+1)E_e}$$

where E_b = (Block ignoring treatment SS − repetition SS)/(b − p)

$\mu = .1367$

(6) Examples of calculation of adjusted treatment means. Considering treatment 1, the three estimates are:

$\bar{y}_1 = 4.47 + (-2/12)$ = 4.31, with intrablock recovery

$\bar{z}_1 = 4.47 + [(86 - 80.50)/(9-3)]$ = 5.39, with interblock recovery

$\bar{m}_1 = [42 + .1367(-13)]/9$ = 4.47, with both recoveries

Calculation of sums of squares (SS)

(7) Correction factor $CF = X...^2/N = (161)^2/36 = 720.0278$

(8) Total SS $= \Sigma x_{ijk}^2 - CF = (4^2 + 7^2 + \ldots + 4^2 + 5^2) - CF$
$= 78.9724$

(9) Treatment ignoring block SS $= (\Sigma T_{i..}^2/r) - CF$
$= [(42^2 + 33^2 + 41^2 + 45^2)/9] - CF$
$= 8.7500$

(10) Within treatment SS = (8) − (9) = 78.9724 − 8.7500
= 70.2224

(11) Block ignoring treatment SS $= (\Sigma B_{.jk}^2/k) - CF$
$= [(8^2 + 12^2 + \ldots + 9^2 + 4^2)/2] - CF$
$= 54.4724$

(12) Treatment eliminating block SS $= \Sigma Q_i^2/kt\lambda$
$= [(-2)^2 + (-8)^2 + (3)^2 + (7)^2]/24$
$= 5.2500$

(13) Block eliminating treatment SS = (11) − (9) + (12)
= 54.4724 − 8.7500 + 5.2500
= 50.9724

(14) Intrablock SS = (8) − (9) − (13)
= 78.9724 − 8.7500 − 50.9724
= 19.2500

(15) Repetition SS $= (\Sigma R_{..k}^2/r't) - CF$, $r' = r/p$
$= [(58^2 + 53^2 + 50^2)/12] - CF$
$= 2.7224$

(16) Block (treatment component) SS $= [\Sigma B_i^2 - \{(\Sigma B_i)^2/t\}]/k(r-\lambda)$
$= [(86^2 + 74^2 + 79^2 + 83^2) - \{(322)^2/4\}]/12$
$= 6.7500$

(17) Remainder SS = (11) – (15) – (16)

= 54.4724 – 2.7224 – 6.7500

= 45.000

The analysis of variance table

Source of variance	DF		Sum squares		Mean squares
Total	35		78.97		
Treatment ignoring block	3		8.75		
Within treatment	32		70.22		
Block eliminating treatment		17		50.97	
Intrablock		15		19.25	1.2833
Treatment eliminating block	3		5.25		1.7500
Block ignoring treatment	17		54.47		
Repetition		2		2.72	
Block (treatment component)		3		6.75	
Remainder		12		45.00	
Intrablock	15		19.25		

Calculation of F statistics

(18) F ratio using intrablock information is obtained from,

$$F_{(3,15)} = 1.7500/1.2833 = 1.36$$

(19) Treatment total adjusted by intrablock and interblock information:

$$m_i = T_{i..} + \mu W_i = r(\bar{m}_i)$$

(20) Mean square for treatment utilizing intrablock and interblock information:

$$SS_T = (\Sigma m_i^2)/r) - CF$$

$$= [(40.32^2 + 33.65^2 + 41.78^2 + 45.26^2)/9] - 720.0278$$

$$= 7.8898$$

The mean square is $SS_T/t-1 = 7.8898/3 = 2.6299$

(21) Effective error variance = $E_e[1 + (t-k)\mu]$

$= 1.2833 [1 + 2(.1367)]$

$= 1.6342$

(22) F ratio using intrablock and interblock information is obtained from,

$$F_{(3,15)} = (20)/(21) = 2.6299/1.6342$$

$$= 1.61$$

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DEHYDRO-IRRADIATION PROCESS FOR WHITE POMFRET (Stromateus cinereus): SYNERGISTIC EFFECTS OF BLANCHING WITH PRESERVATIVES, PARTIAL DEHYDRATION AND LOW DOSE IRRADIATION

INTRODUCTION

BENEFICIAL EFFECTS of synergistic action of combination treatments integrated in the dehydro-irradiation process, viz. dipping shrimp in 0.5% sorbic acid, cooking in 10% brine, partial air dehydration to 40% moisture and irradiation at 0.25 Mrad, have been demonstrated from this laboratory (Gore et al., 1970; Kumta and Sreenivasan, 1967, 1970). This resulted not only in extension of shelf life of shrimp up to 4 months at ambient temperature but also in improved organoleptic attributes of the product over the traditionally sun-dried product.

Recently, Agarwal et al. (1972) showed the applicability of dehydro-irradiation to chicken and lamb meats for ambient temperature storage for over 2 months. Dehydro-irradiated, radurized and radappertized meats of chicken as well as of lamb gave identical organoleptic preferences.

White pomfret (*Stromateus cinereus*) is a medium fat containing (5–8%) fish and is susceptible to oxidative changes, leading to rancidity and yellow discoloration (Kamasastri et al., 1967). In India this fish is mainly consumed fresh and in seasons of glut it is partially preserved by freezing. This work was undertaken with a view to extending the shelf life of white pomfret at ambient temperature by dehydro-irradiation.

EXPERIMENTAL

Preparation of samples

Good quality fresh white pomfret were always used for study. Before use, the fish were dressed, filleted and washed. Fillets were cooked in water or 20% brine at boiling temperature for 5 min. Under these conditions, internal temperature of the fillets reached 85°C for a minimum period of 1 min which was enough for complete enzyme inactivation. Propyl paraben (0.1%) or p-hydroxybenzoic acid (0.1%) when desired was added directly into the cooking medium. Sorbic acid dip (0.5%, 30 min, pH 6.8) was used on the fillets when required, prior to cooking treatment.

Cooked fillets were dehydrated to final moisture content of 35–40% unless mentioned otherwise in the text and packaged under nitrogen in 4-ply laminated pouches of paper/low density polyethylene/aluminium foil/low density polyethylene. The packaged samples were divided into two lots: one was irradiated at 0.5 Mrad with 80,000 curies ^{60}Co package irradiator (IR-11) at ambient temperature (Bongirwar, 1969). The dose rate was 0.24 Mrad/hr with an overdose ratio of 1.3. Samples of the other lot served as controls.

For each of the above mentioned treatments, a minimum of 10 kg of fish were used for study.

Organoleptic evaluation

Semidried pomfret (35–40% moisture) samples obtained from 5 kg of fresh fish were reconstituted in water at room temperature for 3 hr and fried before serving to a four to five member panel. The samples were assessed on a 10-point scale (Miyauchi et al., 1964).

Chemical analysis

Sodium chloride content of the fish was estimated by the AOAC (1970) method. The VanSumere et al. (1961) method was used for propyl paraben estimation. The method described by Farber and Ferro (1956) was used for total volatile basic nitrogen (TVBN) estimation and Dyer's method (1945) was used for trimethylamine nitrogen (TMAN) determination. Carbonyl and 2-thiobarbituric acid values (TBA) of the samples were analyzed as per methods of Hanick et al. (1954) and Turner et al. (1954), respectively.

Microbial analysis

Total bacterial count (TBC) of the samples was determined by conventional pour plate technique (Corlett et al., 1965) and mold count was determined as per the Difco Manual (1966).

Discoloration

In view of the typical nature of discoloration of white pomfret in the form of uneven yellow patches (Kamasastri et al., 1967; Venkatraman et al., 1969), color of the samples was assessed by a new rapid quantitative method. This method was based on extractability of the pigment in 10% sodium hydroxide.

Semidried pomfret samples were disintegrated into small pieces varying from 1/2–1 cm in diam. 5g of the sample was soaked overnight in 25 ml of 10% sodium hydroxide. Following this, 100 ml of distilled water was added and it was filtered through filter paper. The pigment did not show any specific peak in the entire visible region. Percent transmittance of extracted pigment was taken at three arbitrary wavelengths from the straight line region of the absorption spectrum. Color of the samples was expressed as discoloration index, which was computed by $(A - B)/C \times 100$, where A, B and C are the percentage transmittance at 420, 400 and 350 mμ, respectively. Validity of this method was confirmed by gradual increase of the discoloration index of semidried pomfret samples, when arranged visually in the increasing order of discoloration.

Percent reconstitution

Semidried pomfret samples were dipped in water for 3 hr at ambient temperature, which was the optimum time for reconstitution. After removing loosely adhering water with filter paper, final moisture content of the samples was determined (AOAC, 1970). Percent reconstitution was calculated on the assumption of 100% reconstitution of freshly cooked pomfret (72% moisture).

Table 1–Shelf life of pomfret with different moisture contents[a]

Moisture content of fish	Shelf life at 25–35°C days	Spoilage characteristics
Fresh 72	1	Typical putrid odors and total bacterial count (TBC) 10^{10}/g
60	1	Typical putrid odors and TBC 10^9/g
50	1	Typical putrid odors, ammonical odors and TBC 10^9/g
40	2–3	Light green mold, ammonical odors and TBC 10^8/g
30	3–5	Heavy green mold, ammonical odors and TBC 10^7/g
20	15–20	Heavy-green-white mold, ammonical odors and TBC 10^7/g

[a]Fresh pomfret fillets were cooked and dehydrated to different moisture levels and packaged in polyethylene pouches.

RESULTS

Moisture content and shelf stability

Table 1 shows the effect of moisture content of pomfret on the shelf stability and spoilage pattern. Fresh as well as dehydrated pomfret (50% moisture) spoiled after 1 day with putrid odors. As moisture content was decreased below 30%, microbial spoilage was retarded due to decrease in water activity of the samples. Pomfret dehydrated to 20% moisture level, spoiled after 15 days mainly due to heavy mold growth and ammonical odors.

Acceptability

Both preference score and percentage rehydration were significantly influenced by extent of dehydration of pomfret (Table 2). Maximum limit of partial dehydration of pomfret with respect to sensory attributes was 30% moisture level. Further dehydration of the fish resulted in an unacceptable product mainly due to excessive yellow discoloration and toughening of the texture. Thus, semidried pomfret with 35–40% moisture was acceptable organoleptically on reconstitution but was highly susceptible to onset of microbial attack.

Tolerance dose

Optimum dose of irradiation of semidried pomfret (35–40% moisture) was studied (Fig. 1). Organoleptic scores of the product did not change appreciably at 0.5 Mrad. However, a further increase of dose resulted in a drastic fall in the score due to yellow discoloration and irradiation induced off flavors. Frying treatment was found to be superior to cooking treatment in augmenting the organoleptic score of the pomfret at all the radiation doses. At a high dose of 2.0 Mrad, traces of 'cod-liver-oil-like' irradiation odor developed which disappeared on cooking and frying treatments. This revealed the volatile nature of the irradiation odor and flavor-forming components. A dose of 0.5 Mrad was selected as an optimum irradiation dose for the semidried pomfret.

Selection of combination treatments

Since optimum dose of 0.5 Mrad gave only limited shelf life to semidried pomfret (35–40% moisture) and higher doses induced off odors and flavors, use of food additives such as sodium chloride and other chemical preservatives in conjunction with irradiation was sought. Effects of individual and combined treatments on shelf stability of semidried pomfret, stored at ambient temperature (20–34°C) are summarized in Figure 2. Shelf stability of the product was mainly based on organoleptic score. However, chemical indices viz. TMAN, TVBN, TBA and carbonyl values and total bacteria and mold counts were also determined as objective parameters.

The control, semidried pomfret spoiled after 1 day and irradiation extended shelf life to 12 days. Propyl paraben treatment of the fillets alone was not useful since onset of molds occurred after 3 days. However, combination of propyl paraben and irradiation treatment extended the shelf life of semidried pomfret to 16 days. Sodium chloride treated samples preserved for only 4 days as against 45 days when treated in combination with irradiation. The combined treatment of sodium chloride and propyl paraben delayed the onset of molds on

Table 2–Rehydration characteristics and organoleptic score of pomfret with different moisture contents[a]

Moisture content of fish	Dehydration time hr	% moisture content after rehydration	% Rehydration	Preference score[b]
72	(No dehydration)	–	–	8.2
60	3.0	64.8	90	7.5
50	5.25	57.6	80	7.2
40	7.0	54.0	75	7.0
30	10.5	48.9	68	6.8
20	14.0	36.0	50	3.0

[a]Fresh pomfret fillets were cooked, dehydrated to different moisture levels and packaged in polyethylene pouches.
[b]Rehydrated samples after steaming for 5 min were evaluated by five technological panel members on a 9-point hedonic scale (Peryam and Pilgrim, 1957).

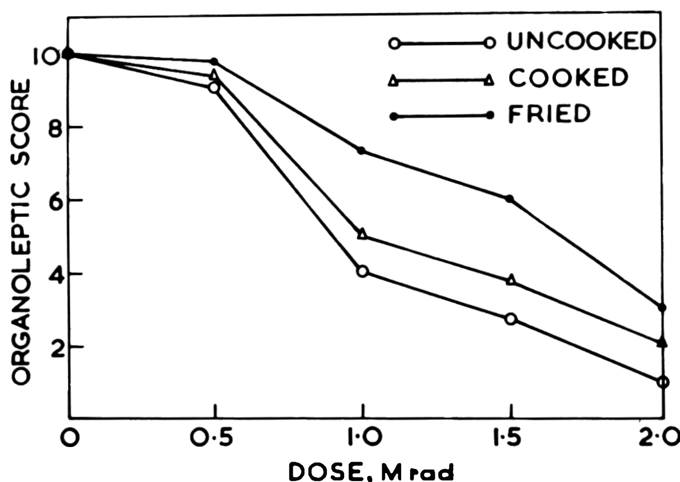

Fig. 1–Influence of radiation dose on organoleptic score of semidried pomfret. The samples were served to the panel members after reconstitution.

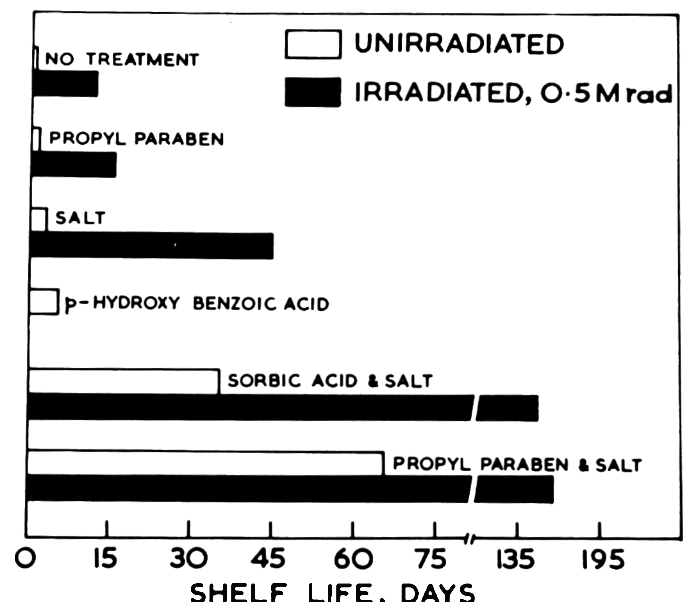

Fig. 2–Extension in shelf life of semidried pomfret with combination treatments.

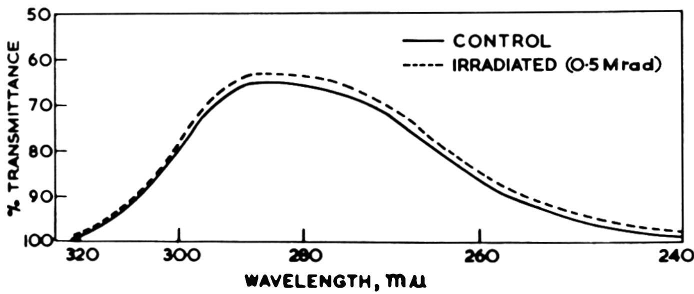

Fig. 3—UV absorption spectrum of propyl paraben. A 0.2% alcoholic solution of the preservative was irradiated at 0.5 Mrad.

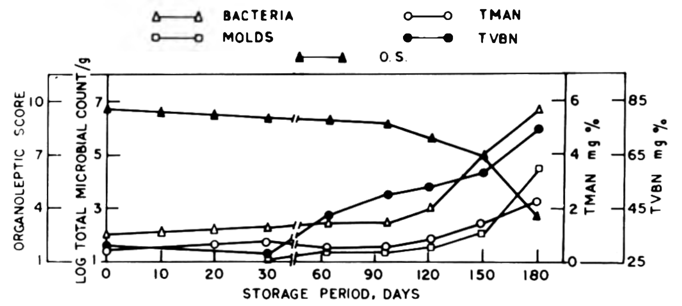

Fig. 5—Quality indices of dehydro-irradiated pomfret.

the samples by 65 days which was further delayed by 150 days in conjunction with irradiation. Sorbic acid treatment in combination with sodium chloride and irradiation also gave prolonged shelf stability of the samples for 150 days, but this treatment was not desirable since it induced yellow discoloration of the samples which further increased during storage.

Radiation stability of propyl paraben was tested in vitro by using 0.2% solution of the preservative in alcohol. Figure 3 shows identical spectra of irradiated (0.5 Mrad) and unirradiated samples of propyl paraben, thus indicating resistance of the preservative to any radiation induced degradation. Irradiation of propyl paraben samples up to 15 Mrad did not show any degradation products as evidenced by UV, IR and TLC techniques (unpublished). Thus, it can be anticipated that propyl paraben used in the product may be stable to radiation damage since no change was detected in alcohol solution.

Evaluation of the process

The appropriate combination treatment suitable for dehydro-irradiation of pomfret was sodium chloride, propyl paraben and irradiation (Fig. 4) which prolonged shelf life for more than 4 months at ambient temperature. After 5 months of storage, the product reached the marginal organoleptic acceptance level of 5 with high bacterial and mold counts and TVBN level (Fig. 5). Here, it may be noted that TMAN level did not serve as a good quality index for the product since the value was only 2 mg % even on the expiration of shelf life of the product. TBA as well as carbonyl values of the product showed increases in the beginning followed by rapid declines, while discoloration increased slowly during storage (Fig. 6). However, discoloration of the product could be completely suppressed under vacuum packaging.

Initially the product had high reconstitution of the order of 80%, but this gradually declined to 65% on storage for 5 months.

DISCUSSION

INDIVIDUAL treatments of sodium chloride, propyl paraben and irradiation were not effective in shelf stabilization of semidried pomfret having 35–40% moisture (Fig. 2). However, the product could be stabilized for prolonged ambient temperature storage when treated with all three treatments. Usefulness of combination treatments of heat (Savagaon et al., 1972) and food additives (Lee et al., 1965; Awad et al., 1965) in conjunction with radiation for extention of shelf life of flesh foods have been widely reported. However, actual mechanisms of such actions are not well understood. Ingram and Kitchell (1967) have reviewed synergistic action of sodium chloride with benzoic acid and have reported that concentration of sodium chloride required is dependent upon the type of surviving microflora of fish.

Fish stored at ambient temperature is prone to be a health hazard due to outgrowth of *Clostridium botulinum* unless process parameters ensure complete elimination of this pathogen or do not permit its proliferation. Dehydro-irradiated pomfret contains 3.3% sodium chloride, 66 ppm of propyl paraben and has low water activity. It may be noted here that concentration of sodium chloride in water present in the dehydro-irradiated pomfret (35–40%) would be in the range of 8–9%. These conditions could be considered sufficient to suppress the outgrowth of *C. botulinum*. Further, the needed cooking and other culinary heat treatments on the dehydro-irradiated pomfret after reconstitution would also control *C. botulinum* hazards (Hobbs et al., 1969). However, in order to ensure

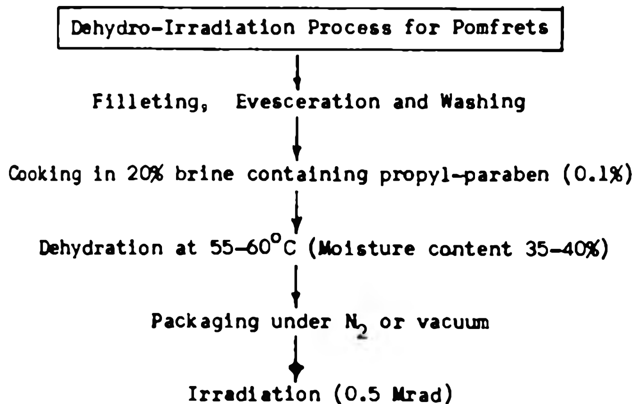

Fig. 4—Flow sheet of the dehydro-irradition process for pomfret.

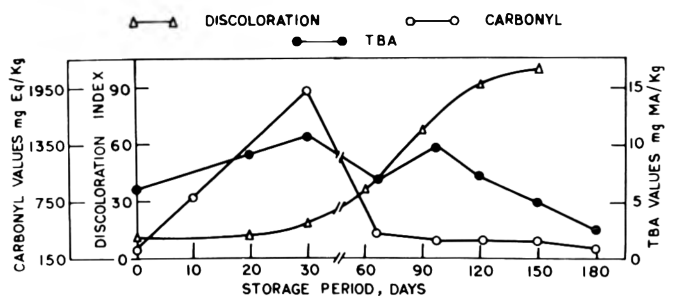

Fig. 6—Discoloration index, carbonyl and TBA values of dehydro-irradiated pomfret.

safety of the product prior to its distribution, it may be essential to determine survival kinetics of different strains of *C. botulinum* spores subjected to combination treatments.

Decline of both TBA and carbonyl values of dehydro-irradiated pomfret during storage, suggests possible participation of TBA reactive substances and carbonyl compounds in browning reactions. This pattern of TBA values during storage of fish, corroborates findings of other investigators (Kwon et al., 1965; Crawford et al., 1967). Suppression of TMAN level of the product during storage may perhaps be due to the elimination of microorganisms responsible for its production (Licciardello et al., 1967; Spinelli et al., 1967; Alur et al., 1971).

Advantages of a dehydro-irradiation process for pomfret include among others: reduction in 50–60% weight due to partial dehydration (35–40% moisture); prolonged shelf stability at ambient temperature; and better texture, color and organoleptic properties than the traditional sun-drying process.

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CHANGES IN QUALITY OF CHANNEL CATFISH HELD ON ICE BEFORE AND AFTER PROCESSING

INTRODUCTION

IN RECENT YEARS, there has been a rapid increase in the production of farm-grown fish in the southeastern states. Most of the interest has been in the culture of channel catfish, production of which now exceeds 50 million lb per year. Much progress has been achieved in the production methods, nutrition, disease and parasite control, and harvesting techniques. However, little attention has been given to the evaluation and improvement of catfish quality.

Results of a storage stability study of channel catfish conducted in this laboratory by Boggess et al. (1971) have shown that there are substantial variations in the quality of channel catfish from different sources, as well as from different processing and handling practices. These variations also influence the storage life of the frozen product.

In the early stages of commercial channel catfish processing, the fish were harvested from the ponds, hauled live to the processing plant, and then placed in holding canals until processed. This practice is being replaced by one in which the fish are hauled dead and on ice to the processing plant and held temporarily until dressed and marketed. Although much research has been reported on the icing of ocean species, only limited results are available on the icing of channel catfish. In a study on live versus dead hauling (USDI, 1970), it was concluded that dead catfish to be skinned either by hand or machine should not be held on ice for more than 3–4 days. They were difficult to skin, particularly around the neck and belly flaps. After 5 days, processing was virtually impossible either by hand or machine. Organoleptic scores were considered acceptable for those fish held up to 9 days on ice. While Boggess et al. (1971) have shown that the frozen product is relatively stable at 0°F, there has been a desire on the part of the processors and the consumers to have the fish marketed in a fresh rather than frozen condition.

Reay and Shewan (1949) in a review article on salt water fishes described quality changes in the raw and cooked fish occurring between catching and spoilage They reported the odor of fish externally and in the flesh was characteristically marine or seaweedy and in fatty species was pleasantly oily. In general, the succession of odor was fresh, "sickly sweet," stale, ammonical and finally putrid.

Fresh cooked fish have delicate, pleasant specific odors and flavors, and firm texture. As spoilage proceeds, the odors and flavors become flat, fishy, or stale and finally acrid, sour or putrid. Ungutted fish often emit odors of decomposition long before any spoilage of the flesh has taken place.

When most species of fish are held for long periods of time on ice, the flesh loses its original translucent sheen and becomes dull, grayish-yellow, with reddish-brown discoloration along the backbone and spreading back from the kidney toward the tail. Fresh fish are soft and flabby, but when rigor has set in the flesh becomes firm and elastic, and does not readily yield juices under pressure. In spoilage, the flesh became soft and watery.

The current investigation was conducted to determine the rates of change in quality of channel catfish when held on and off ice for varying periods.

MATERIALS & METHODS

CHANNEL CATFISH were grown in tank culture (Andrews et al., 1971), to about 500g live weight, under controlled conditions at Skidaway Institute of Oceanography, Savannah, Ga. After experimental treatments, the fish were frozen and transferred to the Food Science Dept. at Griffin, Ga. where quality evaluations were made.

Three separate experiments were conducted viz., (I) fasting vs. feeding prior to icing and processing; (II) holding on ice and off ice for various time intervals prior to processing; and (III) storing dressed fish as "Ice-pack" vs. "Chill-pack."

In Experiment I, a 2 × 6 factorial design was utilized. Sixty fish were fasted for 24 hr, and 60 consumed approximately 1% of their biomass of a standard high density culture catfish food (Andrews et al., 1971) before icing. Ten fish from each group were processed initially, and a similar number after 6, 12, 24, 48 and 96 hr holding time in crushed ice.

Experiment II utilized a 2 × 3 × 3 factorial that included 90 fasted fish and 90 fed fish. Fasting and feeding was the same as described in Experiment I. Fish from both of these groups were held in crushed ice for 0, 12, or 48 hr, then removed and held at room temperature (75°F) for 0, 4, or 8 hr before dressing. Ten fish from each group were used for each of the holding treatment combinations.

In Experiment III, a 2 × 6 factorial design was utilized. Sixty dressed fish were packed in crushed ice (Ice-pack) and 60 were sealed in plastic bags (Chill-pack). These were held respectively in crushed ice, and at 33°F in a refrigerator. Ten fish were removed from each treatment after 0, 2, 4, 8, 12 and 16 days then frozen and held at 0°F until all samples were accumulated.

Quality evaluations

The evaluations included percent weight loss during thawing and cooking; subjective score panel ratings for appearance, color, aroma, flavor and texture; and shear press firmness of the cooked fish.

The fish were evaluated in groups of six each and replicated four times. The four fish for the replications were selected at random from each treatment variable and arranged to allow direct comparisons with every other treatment variable within the same experiment.

Thawing

Upon removal from the freezer, each fish was weighed in the frozen condition, then allowed to thaw in a refrigerator for 24 hr. After thawing, they were removed from the plastic bags, wiped dry with a clean cloth and reweighed. The difference in frozen and thawed weight was designated as drip loss during thawing.

Cooking

Each fish was placed on heavy aluminum foil, "drug store" wrapped to minimize loss of vapors, then baked in an oven at 450°F for 35 min. After cooking, the foil was carefully unfolded and the fish rated for aroma while still hot. The liquid released from each fish during cooking was weighed and reported as drip loss in cooking.

Sensory ratings

A panel of six judges, all experienced in differentiating parameters of fish quality, rated each fish on a nine-point hedonic scale from excellent to extremely poor. A numerical rating and descriptive comments were given for appearance, color, aroma, flavor and texture. One half of each fish was evaluated by the panel, and the other half subjected to shear press firmness measurement.

Shear press firmness

Duplicate 25-g samples were taken from the side of each fish beginning at the dorsal fin and

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extending backward toward the tail. The silver-like skin and all bones were removed, to assure that only muscle was included in the samples. A Food Technology Corp. Recording Shear Press Model TP-1 was used. The instrument was equipped with a 300 lb ring; the down stroke of the shear blade was 30 sec; and the recorder was set at range 50. Data were reported as kg force per g sample. Data were subjected to analyses of variance by the methods of Snedecor and Cochran (1967) and significance was expressed at the 95% confidence level.

RESULTS & DISCUSSION

SUBSTANTIAL differences in quality, especially appearance and flavor, were evident among individual fish within the same treatment and increased with holding time on ice. Data and observations showed that under the conditions of this experiment nonprocessed channel catfish changed more in quality during the first 48 hr than between 48 and 96 hr on ice.

Observations during processing

Difficulty in skinning the fish increased with holding time in crushed ice prior to processing. Fish held for 48 hr were extremely difficult to skin, while the skin of those held for 96 hr had to be removed in small pieces. Fish held in crushed ice for 12 hr or longer before processing developed a pinkish-colored flesh indicative of poor bleeding. This condition increased with holding time through 48 hr, but showed no further increase at 96 hr.

Observations after thawing and cooking

Although the thawed fish had good appearance, changes in color became greater with increased holding time on ice before processing. The principal differences were increased dulling and darkening of the flesh, which was detectable after 12 hr, and increased in intensity at 24, 48 and 96 hr.

When cooked, dulling and graying of the flesh increased through 48 hr holding time on ice. Brown discoloration occurred externally along the side of fish just beneath the "silver skin" and internally along the backbone. Also there was dark "hair-sized" veining scattered throughout the flesh. These defects were apparent after 12 hr and increased with holding time on ice before processing.

Panel sensory evaluations

Channel catfish cooked in this manner had a characteristic mild fish flavor with traces of "musty or muddy" flavors which are usually undetected in breaded and deep fat-fried fish. The aroma and flavor changes associated with holding time on ice were first apparent as the loss of natural freshness followed by a strong fishy flavor and the development of "off flavor." This is in agreement with results on ocean fish (Reay and Shewan, 1949). As these changes in aroma and flavor occurred, a greater number of different terms were used by the panel to define them. Terms most often used included "harsh, flat, off, old, stale, strong, bitter, amines and spoiled." "Kerosene or motor oil" off flavor was noted in some individual fish, but it appeared to be unrelated to treatment.

Experiment I (Fed vs fasted × holding time). When sensory scores for the fasted and fed groups were analyzed separately by analyses of variance, only the appearance of the fasted fish and the flavor of fed fish showed significant decreases with time on ice before processing. These data appear in Table 1. Scores for aroma, color and texture showed a general decline with increased holding time on ice. The lack of statistical significant differences among treatments was due in part to the large variation among fish. Although differences in texture scores among the treatments were non-significant, comments by the panel indicated slightly different textural characteristics between the fasted and fed fish. The texture of fasted fish was described as slightly "soft and dry" which is typical for channel catfish cooked by the method used in these studies. That of the fed fish was normal at 0 and 6 hr but was slightly "tough and watery" after 12, 24, 48 and 96 hr holding time on ice before processing. Comments by the panel indicated a preference for the texture of fasted fish. These textural differences were probably associated with different post-mortem muscle changes in the fasted and fed fish.

A combined summary of the panel score evaluations for the fasted and fed groups is presented in Table 2. Significant decreases in ratings for appearance, color, aroma and flavor occurred, and sooner than previously reported (USDI, 1970).

Orthogonal comparisons (Snedecor and Cochran, 1967) of sensory scores indicate quality breaks associated with holding time on ice. Scores on fasted fish showed definite decreases in appearance, color and flavor between 6 and 12 hr, and 24 and 48 hr. Scores for the fed fish showed a rapid decrease in appearance and color between 0 and 12 hr. The greatest decrease in aroma and flavor

Table 1–Appearance and flavor scores of cooked channel catfish held in crushed ice for 0, 6, 12, 24, 48 and 96 hr before processing

Holding time on ice (hr)	Fed fish		Fasted fish	
	Appearance score	Flavor score[a]	Appearance score[a]	Flavor score
0	6.95	5.90ab	6.92a	6.47
6	6.57	6.32a	6.77ab	6.32
12	6.12	6.32a	6.37ab	5.87
24	6.27	5.85ab	5.92bc	6.37
48	6.47	5.55b	5.35c	5.60
96	5.85	5.85ab	5.95bc	6.05

[a]Values having same letters are not significantly different (P < 0.05).

Table 2–Quality scores of channel catfish held on ice before processing for 0, 6, 12, 24, 48, 96 hr

Holding time on ice (hr)	Combined, fasted fish and fed fish sensory ratings[a]				
	Appearance	Color	Aroma	Flavor	Texture
0	6.94a	7.00a	6.19a	6.19a	7.09
6	→6.67ab	→6.85ab	6.04ab	6.32a	7.06
12	→6.25b	→6.22c	→6.04ab	6.10a	6.91
24	6.10b	6.35bc	→5.96ab	→6.11a	→6.64
48	5.91b	6.22c	→5.23c	→5.58b	6.80
96	5.90b	6.26bc	5.50bc	5.95ab	6.74

[a]Values having same letter are not significantly different (P < 0.05).
→Indicates quality breaks, based on orthogonal comparisons.

Table 3–Mean firmness values of cooked muscle of channel catfish held on ice before processing

Time on ice (hr)	Shear press firmness[a] kg force/g muscle	
	Fasted fish	Fed fish
0	.867ab	.954abc
6	.935abc	1.143b
12	.877ab	1.282e
24	.810a	1.073cd
48	.844a	1.045bcd
96	.971abcd	1.116cde
Average	.884	1.102

[a]Values having same letters are not significantly different (P < 0.05).

Table 4–Drip loss during thawing and cooking of channel catfish held on ice before processing

Holding time on ice (hr)	Drip in thawing[a] Fasted fish (%)	Drip in thawing[a] Fed fish (%)	Drip in cooking[a] Fasted fish (%)	Drip in cooking[a] Fed fish (%)
0	3.42bcd	2.62ab	1.85a	4.96c
6	3.18abcd	3.08abcd	2.69ab	3.26abc
12	3.09abcd	2.37a	3.55abc	4.29bc
24	3.05abcd	2.80abc	3.22abc	4.11bc
48	3.88de	3.78cde	4.33bc	4.23bc
96	3.46bcd	4.55de	5.04c	3.13abc

[a]Values having same letter are not significantly different ($P < 0.05$).

scores occurred between 24 and 48 hr in the fasted and fed groups. A decrease in texture scores was shown between 12 and 24 hr.

Shear press data in Table 3 show that fish in the fed group were less tender. Drip loss data are presented in Table 4. In the fasted group, drip during cooking increased significantly with time of holding fish on ice.

These data show that under recommended handling practices, channel catfish may be held on ice for up to 24 hr before processing and still yield a highly satisfactory finished product.

Experiment II (Fed vs fasted × on vs off ice × storage time). Score panel evaluation data are presented in Table 5. Since the ratings for fish from the fasted and fed groups were nonsignificant, the data for the two groups were combined. A comparison of means of time on ice showed significantly lower ratings for appearance, color, aroma, flavor and texture at 48 hr holding time. Means for time off ice showed significant decreases in flavor at 8 hr and in texture at 4 hr, with no significant changes in appearance, color and aroma. Although interactions of time on × time off ice were nonsignificant, certain trends were indicated. Appearance, color and texture ratings suggested more quality loss during 8 hr off ice, than for 48 hr on ice, whereas aroma and flavor decreased less during 8 hr off ice. In those fish held on ice for 12 hr and off ice for 4 and 8 hr, appearance, flavor and texture ratings decreased slightly while color and aroma remained essentially unchanged. In fish held on ice for 48 hr, flavor and texture ratings decreased while ratings for the other factors remained unchanged. The accumulative adverse effects of time on and time off ice was not evident in flavor ratings but was indicated to some extent in the texture ratings.

Comments by the sensory panel indicated that the principal quality changes associated with holding time off ice (75°F) were increased toughening of the product and increased flat, fishy and "off" aroma and flavor.

The shear press firmness values (Table 6) were influenced by the holding times on and off ice and by the feeding treatments. The fed fish were significantly firmer than the fasted. Fish held on ice for 0, 12 and 48 hr and dressed immediately had similar firmness in the cooked product. Fish held off ice for 4 and 8 hr without prior icing resulted in a substantial firming of the product. A subsequent tenderizing effect occurred when the fish were first held on ice and then held off ice for 4 and 8 hr. As in Experiment I, the shear press values showed a marked fluctuation with the various icing practices, and may be linked with the rigor development patterns. The feeding treatments prior to slaughter had a statistically significant influence on the average shear press values which were 9.86 and 12.17 for the fasted and fed fish respectively.

The combined drip loss from thawing and cooking (Table 7) increased with the fish that were removed from the ice and

Table 5–Score panel ratings for cooked channel catfish held on ice for 0, 12 and 48 hr and off ice for 0, 4 and 8 hr before dressing

Holding time on ice (hr)	Holding time off ice (hr)	Appearance	Color	Aroma	Flavor	Texture
		Combined, fasted fish and fed fish — Sensory ratings[a]				
0	0	7.07	6.98	6.42	6.42	7.22
	4	6.51	6.67	6.26	6.21	6.68
	8	6.25	6.38	6.12	6.08	6.56
12	0	6.73	6.57	6.17	6.25	6.84
	4	6.68	6.58	6.00	5.93	6.73
	8	6.57	6.66	6.14	6.89	6.64
48	0	6.34	6.41	5.24	5.78	6.78
	4	6.18	6.19	5.67	5.73	6.43
	8	6.39	6.38	5.63	5.53	6.39
		Time on ice (mean values)				
0		6.61a	6.68a	6.27a	6.24a	6.82a
12		6.66a	6.60a	6.11a	6.02ab	6.74a
48		6.29b	6.33b	5.58b	5.68b	6.54b
		Time off ice (mean values)				
	0	6.71	6.65	6.01	6.15a	6.95a
	4	6.46	6.48	5.98	5.96ab	6.62b
	8	6.37	6.48	5.97	5.84b	6.53b

[a]Values having same letter are not significantly different ($P < 0.05$).

Table 6–Shear press firmness values of cooked muscle of channel catfish held on ice for 0, 12 and 48 hr and off ice for 0, 4 and 8 hr before dressing

Time on ice (hr)	Time off ice (hr)	Shear press firmness[a] kg force/g muscle — Fasted fish	Fed fish
0	0	.804de	.955bcd
	4	1.201bc	1.752a
	8	1.615a	1.716a
12	0	.877d	1.282b
	4	1.015bcde	1.022bcde
	8	.923de	1.068bcd
48	0	.844de	1.045bcde
	4	.845de	1.077bcd
	8	.781e	1.011bcde
		Time on ice (mean values)	
0		1.208a	1.474a
12		.938ab	1.124b
48		.823b	1.044b
		Time off ice (mean values)	
	0	.842	1.094
	4	1.020	1.284
	8	1.106	1.265

[a]Values having same letter are not significantly different ($P < 0.05$).

Table 7–Drip loss during thawing and cooking of channel catfish held on and off ice before processing

Time on ice (hr)	Time off ice (hr)	Drip loss: Fasted fish (%)	Drip loss: Fed fish (%)
0	0	2.62	3.79
	4	4.13	4.52
	8	4.43	4.51
12	0	3.48	3.33
	4	3.93	3.12
	8	4.07	3.56
48	0	3.29	4.01
	4	3.89	4.14
	8	4.02	4.84
		Time on ice (mean value)	
0		3.73	4.27
12		3.83	3.34
48		3.73	4.33
		Time off ice (mean value)	
	0	3.13	3.71
	4	3.98	3.93
	8	4.17	4.30

Table 8–Sensory rating of dressed channel catfish held in crushed ice and as chill pack at 33° F

Storage time (days)	Appearance	Color	Aroma	Flavor	Texture
		Ice pack-dressed fish[a]			
0	5.87	5.40	5.47ab	5.92a	6.85
2	6.27	6.62	5.45ab	5.77a	6.87
4	6.45	6.45	6.00a	5.90a	6.62
8	6.20	6.62	5.70ab	5.42ab	6.65
12	6.00	6.27	5.00bc	4.70b	6.82
16	6.08	6.55	4.60c	4.13b	6.10
		Chill pack-dressed fish[a]			
0	6.00a	6.32a	5.65a	5.75a	6.60a
2	5.97a	6.17a	5.30a	5.60a	6.57a
4	6.00a	6.27a	5.40a	5.67a	6.45a
8	5.67a	5.92a	6.00a	5.93a	6.67a
12	2.83b	2.93b	1.38b	1.75b	2.55b
16	1.00c	1.00c	1.00c	1.00c	1.00c

[a] Values having same letter are not significantly different ($P < 0.05$).

Table 9–Drip loss and shear press firmness of dressed channel catfish stored in crushed ice and at 33° F without ice

Storage time (days)	Drip loss (%)[a]: Ice pack	Drip loss (%)[a]: No ice (32° F)	Shear press firmness[a] (kg/g): Ice pack	Shear press firmness[a] (kg/g): No ice (32° F)
0	6.6a	8.4	1.207a	.993ab
2	12.7bc	6.7	.785bcd	.932bc
4	10.2ab	7.5	.894bc	.899bc
8	14.1c	5.9	.767cd	.853bc
12	11.4bc	7.7	.789bcd	.743cd
16	11.8bc	spoiled	.636d	spoiled

[a] Values having the same letters are not significantly different ($P < 0.05$).

held at room temperature for 4 and 8 hr. The effect was more pronounced in the fish that had no ice treatment and in the fasted fish.

Experiment III (Ice vs chill pack × storage time). Freezing and holding samples at 0°F before evaluation had no apparent effect on the sensory differences. Drake et al. (1971) had reported that in a triangular difference test, score panelists were unable to differentiate between fresh catfish and catfish frozen and stored for 1 yr.

Sensory ratings (Table 8) showed that dressed catfish could be held in crushed ice for as long as 12 days and retain acceptable quality. Those held in plastic bags at 33°F deteriorated more quickly but retained acceptable quality as long as 8 days. Fish held under refrigeration but without ice for 12 and 16 days developed a slimy appearance and objectionable odor and flavor. The texture ratings, though not statistically significant in variation, reflected an undesirable softening in the refrigerated samples held for 12 days. These samples were described by the judges as "soft and cotton-like." The chill pack fish developed a pink flesh color, which turned gray and dull when cooked.

The drip loss percentage (Table 9) for fish held in crushed ice reached a maximum with 8 days of holding. The drip loss was substantially lower and more stable in fish held without ice at 33°F.

The shear press firmness values (Table 9) for the fish in crushed ice decreased markedly during the first 2 days, levelled out, then decreased again between 12 and 16 days. The decrease in firmness values was less evident in the refrigerated samples held without ice.

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EFFECT OF TIME AND TEMPERATURE OF SMOKING ON MICROORGANISMS ON FRANKFURTERS

INTRODUCTION

THE LARGE AMOUNT of frankfurters produced yearly in the United States, as much as 1.37 billion lb in 1970 (National Live Stock and Meat Board, 1971), emphasizes the importance of this item as a meat food in the American food industry.

Many papers have been published on frankfurters and related products. These have dealt with manufacturing (Tauber, 1958), color (Tauber and Simon, 1963 and Fox et al., 1967), and texture (Simon et al., 1965). Other work has been concerned with bacteriological aspects (Ayres, 1951; Drake et al., 1958, 1959; Hall and Angelotti, 1965; Hess, 1928; Jensen and Hess, 1941; and Solberg and Elkind, 1970). Draudt (1963) reviewed the effects of the smoking process on meats.

Because of the inhibitory or bactericidal effects of curing agents, smoking and heating, the flora of frankfurters is rather select. The bacteria present on frankfurters are predominantly Gram-positive types (Drake et al., 1958). Ayres (1951) reported *Micrococcus, Sarcina, Lactobacillus, Microbacterium* and *Bacillus* as the most common organisms found in frankfurters. Hall and Angelotti (1965) isolated *Clostridium perfringens* from 19% of market samples of frankfurters and other processed meats requiring mild cooking. Yeasts are frequently encountered in packaged frankfurters. In addition to *Debaryomyces*, some other genera found in cured meats are: *Torulopsis, Candida* and *Trichosporon* (Drake et al., 1959).

During recent years, manufacturers have tended to shorten the time of curing, smoking and heating. The reasons are mainly economical and possibly because of changing "tastes" of consumers. However, little consideration has been given to the effect of reduced process time and temperature on the microbial population of processed meats.

This study was designed to evaluate the effect of smoking time and temperature on microorganisms on frankfurters after processing, and to determine differences in survival or growth of the microflora during later storage.

MATERIALS & METHODS

FRANKFURTERS for this study were specially prepared in the Meats Lab. at Iowa State University. The formula used in preparing the frankfurters was as follows: 3.6g sodium nitrite, 3.0 lb sodium chloride, 7.1g sodium nitrate, 24.6g erythorbate-neocebitate, 20.0 lb ice, 0.5 lb frank and wiener seasoning, 40.0 lb pork trimmings, 60.0 lb beef trimmings.

The frankfurters were prepared by using a continuous mechanical process, which included grinding, emulsifying and stuffing.

Treatment of samples

After stuffing, the frankfurters were divided in two main groups, designated A and B. Group A was smoked and heated to different internal temperatures: 140°F (60°C), 150°F (65.8°C), 160°F (71.1°C) and 170°F (76.8°C). Group B was smoked and heated to a single internal temperature of 150°F (65.8°C), but the time of heating was varied (0, 10, 20 and 30 min). The

Table 1—Experimental smoking schedules

	Internal temperature	Time of holding (min)
Experimental frankfurters	140°F (60°C)	0
	150°F (65.6°C)	0
		10
		20
		30
	160°F (71.1°C)	0
	170°F (76.7°C)	0

Table 2—Microbiological methods used for bacterial counts

Determination	Medium	Incubation time	Incubation temp	Plating methods
Total aerobes	Trypticase soy agar (BBL)[a] Total count agar (Difco)[b]	3 days	30°C	Pour plate
Molds and yeasts	Potato dextrose agar (Difco) Malt agar (Difco)	4 days	R. T.	Pour plate
C. perfringens	SPS medium[c]	24 hr	37°C	Pouch method[d]

[a]BBL Division of Bio Quest, Cockeysville, Md.
[b]Difco Laboratories, Detroit, Mich.
[c]Angelotti et al., 1962
[d]Bladel and Greenberg, 1965

Table 3—Microbiological methods used for detecting coagulase positive staphylococci

Medium	Incubation time	Incubation temp	Method
Enrichment broth of Wilson et al.[a]	2 days	37°C	Enrichment
Staphylococcus[b] medium no. 110 (Difco) with egg yolk	2 days	37°C	Isolation
Brain heart infusion broth (Difco)	24 hr	37°C	Confirmation enrichment before coagulase test
Bacto coagulase plasma	6 hr	37°C	

[a]Wilson et al., 1959
[b]Herman and Morelli, 1960

smoking cycle used is presented in Table 1.

After smoking, the frankfurters were cooled in ice water, drained dry, and vacuum packaged in sets of six frankfurters per package. The plastic bags used were "medium size" Visten PF, polyvinyl chloride (Union Carbide Corp., Chicago, Ill.). The frankfurters were stored in a display case at 5° ± 2°C.

Sampling procedures

Samplings were made in duplicate of fresh meat, ground meat plus ingredients, the meat emulsion and frankfurters immediately after smoking and after storage. Day 0 represented the day the frankfurters were first stored in the display case.

The procedure of sampling was as follows: Duplicate meat samples weighing 30g were blended in an Osterizer (John Oster & Co., Milwaukee, Wisc.) for 1 min at low speed and 1 min at high speed with 270 ml of sterile distilled water. Serial dilutions were made from the homogenate to obtain bacterial counts.

Microbiological methods

Counts were determined for the following microorganisms or groups of microorganisms: total aerobes, molds, yeasts and *C. perfringens*. Methods, media and incubation temperatures appear in Tables 2 and 3. Those microorganisms that produced H_2S in SPS agar (Angelotti et al., 1962) but that were either motile or did not reduce nitrate, were considered *Clostridium* spp other than *C. perfringens*. Isolates that were nonmotile and reduced nitrate were considered to be *C. perfringens*.

RESULTS & DISCUSSION

FIGURE 1 shows the counts of various microorganisms obtained at different stages of processing until frankfurters were formed. The addition of spices, water and curing ingredients did not greatly increase the numbers of aerobic bacteria, molds and yeasts per gram in the meat. Passing the meat through the emulsifier and stuffer increased the counts to a greater extent than did addition of ingredients, but only to a small degree. Apparently, the equipment and associated handling operations were a greater source of contamination than were the ingredients. Incidence of *C. perfringens* was 7.1% (1/14) and coagulase-positive staphylococci was 100% (14/14) for all samples after preparation of the emulsion and mixing with curing agents and spices. Fabian et al. (1939), Mossel (1955) and Ayres (1965) indicated that spices, water, salt and equipment can be contaminated by thousands of bacteria and bacterial spores. In the present work, the meat had high loads of organisms before addition of ingredients, and the numbers of aerobic bacteria, yeasts and molds did not increase greatly after curing agents and spices were mixed in with the meat emulsion.

As shown in Figure 2, smoking caused a reduction in numbers of microorganisms in frankfurters, at all temperatures tested. Similar findings were reported by

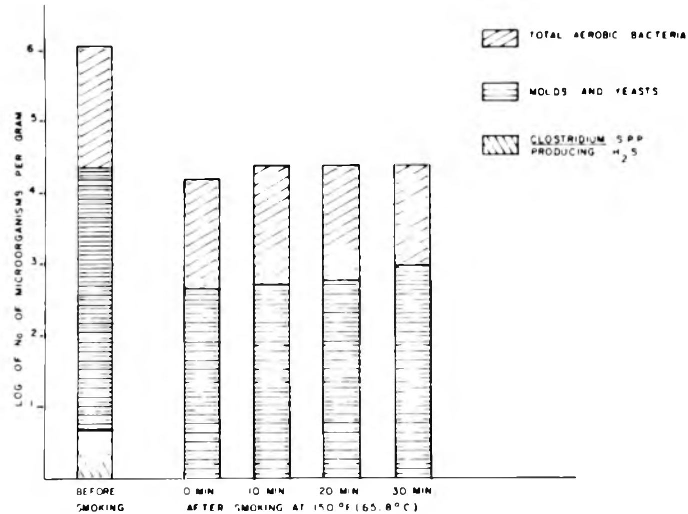

Fig. 2–Reduction in numbers of microorganisms in frankfurters after smoking at different temperatures.

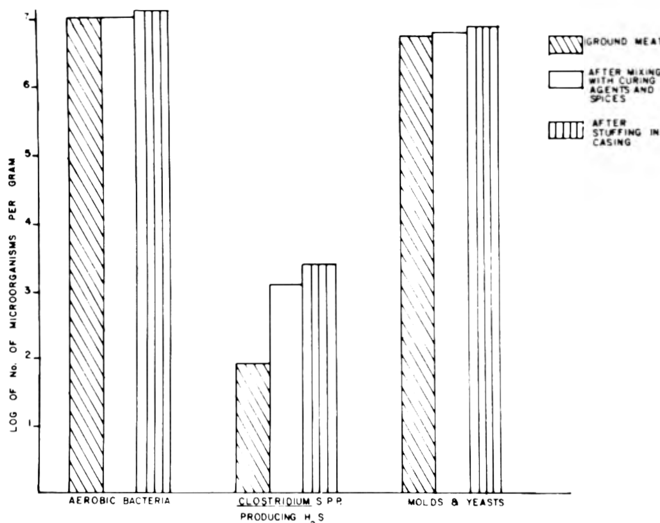

Fig. 1–Changes in bacterial counts of the meat during preparation of frankfurters prior to smoking.

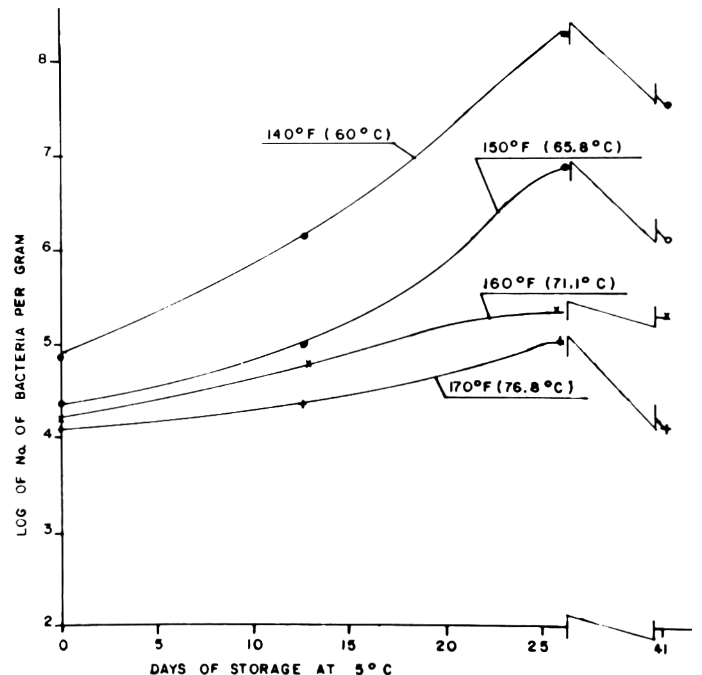

Fig. 3–Effect of final temperature of smoking on growth of total aerobic bacteria on frankfurters stored at 5°C (41°F).

Gibbons et al. (1954). The greatest increment in reduction occurred during the period in the smokehouse when the frankfurters were heated to an internal temperature of 140°F (60°C), although further reduction occurred on continued heating to 150°F (65.8°C), 160°F (71.1°C) and 170°F (76.8°C). Increasing the temperature from 140°F (60°C) to 170°F (76.8°C) was only slightly effective in reducing aerobic bacteria. Of 120 experimental frankfurters tested, only six were positive for *C. perfringens* and two samples yielded coagulase-positive staphylococci. No marked advantage was obtained in reduction of microorganisms immediately after smoking to high internal frankfurter temperatures as compared with relatively low smoking temperatures. Gough and Alford (1965) reported survival of *C. perfringens* after curing and mild smoking. However, during subsequent storage of the frankfurters at 5°C, it was observed that there was an inverse relationship between internal temperature of smoking and the capability of bacteria to multiply during storage. The higher the temperature of smoking, the lower the counts during storage (Fig. 3, 4 and 5). Possibly, the antimicrobial properties of curing agents and smoke constituents exerted an increasing influence on the injured microorganisms as the smoking

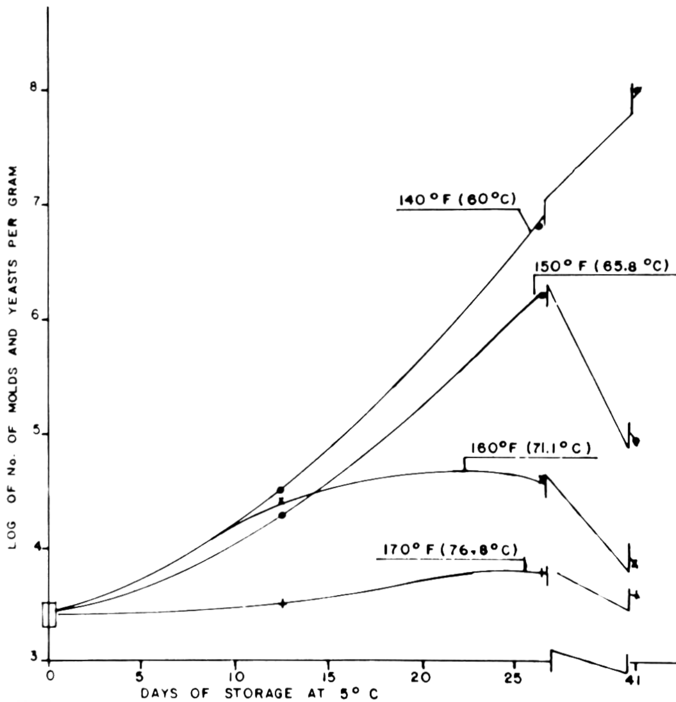

Fig. 4–Effect of temperatures of smoking on numbers of molds and yeasts growing on frankfurters stored at 5°C (41°F).

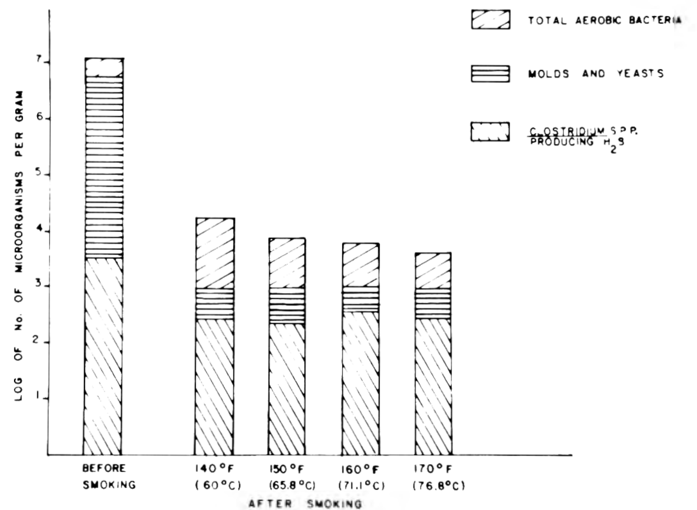

Fig. 6–Effect of time of smoking frankfurters at 150°F (65.8°C) on the survival of microorganisms.

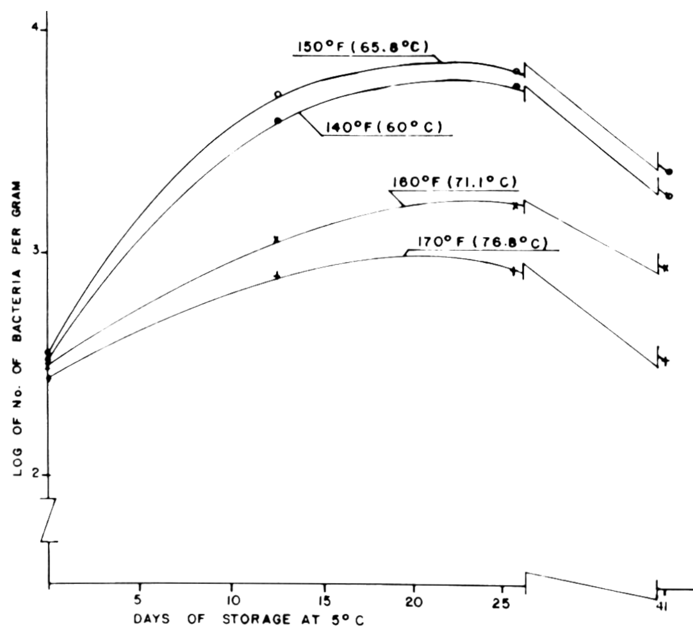

Fig. 5–Recovery of Clostridium spp. producing H_2S from frankfurters smoked at different temperatures and stored at 5°C (41°F).

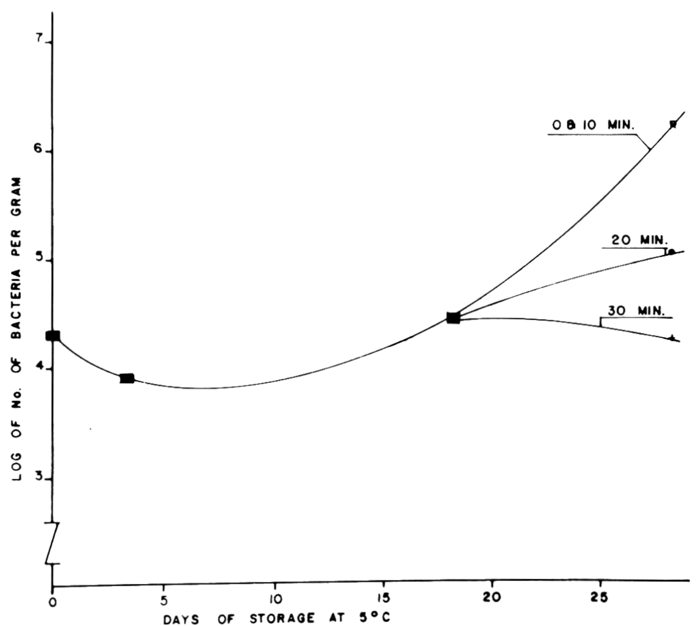

Fig. 7–Effect of time of holding during smoking at 150°F (65.8°C) on growth of total aerobic bacteria on frankfurters stored at 5°C.

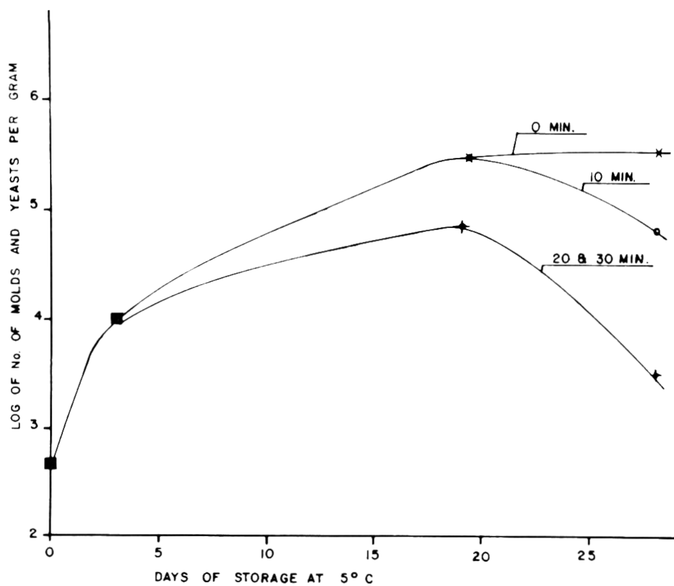

Fig. 8–Effect of time of holding during smoking at 150°F (65.8°C) on growth of molds and yeasts on frankfurters stored at 5°C.

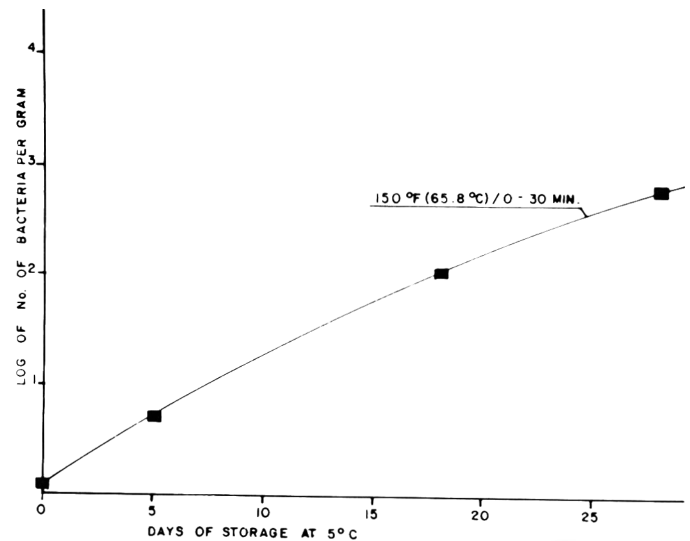

Fig. 9–Effect of time of holding during smoking at 150°F (65.8°C) on growth of Clostridium spp. on frankfurters stored at 5°C.

process was prolonged in attaining higher internal frankfurter temperatures. Residual bacteriostatic effects of smoking were discussed by Handford and Gibbs (1964). Possible changes produced in the flora and their influence on numbers of organisms will be discussed in another paper.

In view of these findings and since temperatures near 150°F (65.8°C) are used in commercial operations, further work was directed at the practical application of 150°F (65.8°C) as a minimum smoking temperature. Increasing the time of smoking at this relatively low smoking temperature did not prove to be as effective as increasing the temperature of smoking in causing immediate reduction in numbers of microorganisms in frankfurters. Figure 6 compares the effect of smoking to an internal temperature of 150°F (65.8°C) with immediate removal of frankfurters and prolonged smoking up to 30 min at the same temperature. Numbers of organisms immediately after smoking were quite similar regardless of time the frankfurters were in the smokehouse at 150°F (65.8°C). Also, of 106 samples tested, only four were positive for *C. perfringens* and one for coagulase-positive staphylococci. An unusually high proportion of yeasts and molds survived smoking. Additional work would be desirable on heat resistance of such organisms. Later determinations indicated no undue contamination during subsequent cooling and packaging. Yeasts and molds were recovered during storage of frankfurters at 5°C (Fig. 4 and 8), similar to observations of other workers (Ogilvy and Ayres, 1953; Drake et al., 1958, 1959). Isolates obtained from plates used for counts of aerobic organisms are currently being studied in detail.

Effect of time of smoking at 150°F (65.8°C) on the growth of various microorganisms during later storage at 5°C is shown in Figures 7 through 9. These results indicate that unless the holding time at 150°F (65.8°C) is longer than 10 min, time has little influence on the ability of bacteria to multiply during later prolonged low temperature storage.

The results of this study suggest that: (1) There is an inverse relationship between time and temperature of smoking and bacterial counts from frankfurters immediately after smoking and during later storage; (2) The major reduction in bacterial numbers occurs in the interval during which the internal temperature of the frankfurters reaches 140°F (60°C); (3) Incidence of coagulase-positive staphylococci is quite low after smoking (approximately 1–1.5%); (4) Prolonged smoking of frankfurters at 150°F (65.8°C) effects reductions in the bacterial counts compared with smoking only long enough for the internal temperature to reach 150°F (65.8°C) when frankfurters are subsequently stored at refrigeration temperature. Apparently the inhibitory agents associated with smoking exert an increased residual effect when smoking time is prolonged, since immediate differences were not observed for frankfurters directly after removal from the smokehouse.

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BONE DARKENING IN FROZEN CHICKEN BROILERS AND DUCKLINGS

INTRODUCTION

COOKED FROZEN BROILERS can be differentiated from cooked fresh broilers by the presence of bone discoloration. Koonz (1946) and Brant and Stewart (1950) have shown this darkening to be due to methemoglobin formation from the abundant hemoglobin in the marrow of young chickens. Freezing and thawing hemolyzes erythrocytes releasing hemoglobin, thus enabling it to pass out of the inner bone.

Many investigations to date have dealt with processing methods and their relation to bone darkening. Spencer et al. (1961) reported that time in frozen storage at 0°F (–18°C) did not affect severity of bone darkening. Streeter and Spencer (1969) reported that four different freezing methods and two different methods of catching chicken broilers did not significantly affect the degree of bone darkening. It was reported by Essary (1959) that exposure of chicken legs and wings to microwave energy for 2–4 min reduced or even eliminated bone darkening in subsequent freezing. Woodroof and Shelor (1948) reported blanching in hot oil to a joint temperature of 88°C eliminated bone darkening during subsequent freezing.

The effect of calcium and phosphorous supplementation of the diet was studied by Kotula et al. (1963). They found that neither sex nor phosphorous level affected degree of discoloration. Males showed a greater variation in response to dietary calcium levels than females. The total effect of varying calcium levels from 0.5–0.8% was small. The calcium-phosphorous ratio exerted a consistent influence on bone discoloration in both frozen and nonfrozen broilers. A higher Ca:P ratio resulted in an increased discoloration of bones.

The purpose of this research was to study several different processes that might influence degree of bone darkening in frozen broilers. An attempt was made to relate the degree of darkening to some physical characteristics of the bone such as % calcium or % bone ash.

EXPERIMENTAL

Source of birds

8-wk old chicken broilers were purchased from a local grower. Processing was in University laboratories using commercially accepted procedures. Ducks were purchased as frozen birds from a local processor. All ducks were less than 8 wk of age at the time of processing.

For birds used in studying the effects of added calcium in the diet, day-old commercial broiler strain chicks were used. 25 chicks were grown on commercial broiler starter and finisher as controls and 25 were fed the same starter with 0.85% calcium gluconate added. The finisher feed of these birds from 35 days of age to 52 days of age had 1.06% calcium gluconate added.

The broilers used in the high pressure-short time freezing were commercially processed and purchased on the Boston market as whole carcasses. Opposite halves of birds served as treated and control groups.

Bone evaluation

Both treatment and control groups were evaluated by a panel of 4–7 members depending on availability. Panelists were instructed to compare an entire treatment group to the control group. The control group consisted of cooked, unfrozen broilers. Fresh, nonfrozen broilers are relatively uniform with respect to bone color. Scoring of bone color was on a 1 to 7 basis with 1 being equivalent to the control, lightest in color. A total of seven different experiments were conducted to compare effects of the various treatments on bone discoloration. From 20–50 birds were used in each study.

Freezing methods

The four freezing methods evaluated were as follows:

1. Freezing in a home-type freezer at –18°C;
2. Freezing by submersion in liquid Freon 12;
3. Freezing by submersion in liquid nitrogen;
4. Freezing by placing the sample in a chamber which was then filled with a 1:1 mixture of water and glycerine. The contents of the chamber were then subjected to a pressure of 39,000 psig ± 2,000 psig. After being placed under pressure the chamber and its contents were cooled to –29°C. After the sample temperature had reached –29°C, the pressure then released in approximately 3 sec allowing the sample to freeze simultaneously.

Aging methods

Birds were chilled in ice water and then placed in polyethylene bags and held in a refrigerated room for from 1 to 6 days at 3°C before being frozen.

Thawing methods

Thawing was by one of the following means:

1. Thawing at room temperature;
2. Thawing for 7 min in an RCA microwave oven;
3. Thawing as a part of the cooking operation.

Cooking methods

All samples were cooked in cottonseed oil at 177°C or in a 2450 megaHz microwave oven for 20–25 min, with equal numbers being randomly assigned each cooking method.

Method for bone ash

The femurs were dried in a vacuum oven for 24 hr at 103°C and 25 in. Hg vacuum. The dried bones were then extracted with anhydrous ethyl ether in a Soxhlet apparatus for 24 hr. The extracted bones were then redried for 24 hr as above and placed into weighed crucibles. After weighing, the bones and crucibles were placed in a cold muffle furnace and heated to 425°C for 6 hr. The temperature was then increased to 870°C for 1 hr. After cooling in a desiccator, the ash and crucibles were reweighed.

Method for Ca determination

Femurs to be analyzed for calcium were first ashed by the method previously described. Ash samples of approximately 20 mg were dissolved in 2 ml of 2N HCl and brought to 100 ml with distilled water. Standard calcium solutions of 0 thru 10 ppm were prepared and 5 ml of 5% lanthanum chloride was added to samples and standards. Both standards and samples were analyzed with a Perkin-Elmer (model 303) atomic absorption spectrophotometer. Details on the procedure are reported in Perkin-Elmer (1966).

Table 1—Mean color scores of bones from chicken broilers subjected to several freezing and thawing treatments after different times of refrigerated storage

Treatment	Mean color score of tibia and femur[a]
Freezing method	
Still air at –18°C	3.3a
Liquid R12 at –30°C	3.8a
Liquid N_2 immersion	3.2a
Superchilled to –29°C then 3 sec release	3.5a
Thawing method	
Microwave	5.1a
Air	4.6a
Thawed during cooking	3.1b
Aging time: (days)	
0	3.4a
1	3.6a
2	4.1a
3	3.2a
4	4.1a
5	4.0a
6	6.2b

[a]Lightest color (nonfrozen controls) scored 1 to darkest of 7. Any treatment means not followed by same letter are significantly different ($P < 0.05$).

Method for P determination

Ash samples weighing approximately 90 mg were dissolved in 5 ml of 6N HCl and diluted to 100 ml with distilled water. A further dilution was made by adding 5 ml of the ash solution to 20 ml of distilled water. Standards containing 0, 0.02 mg/ml . . . thru 0.10 mg/ml of phosphorous were prepared. 1 ml of acid molybdate was added to 1 ml of each standard and sample. After 30 min, 1 ml of Elon's reagent was added to both standard and sample solutions and the volume was brought up to 20 ml with distilled water. Standards and samples were read on a Beckman D.U. spectrophotometer at a wavelength of 600 mμ. This is a modification of the method of Fiske and Subbarow (1925).

Table 2–Treatment means for percentage of calcium and phosphorous in ash and bone ash of femurs of control and calcium gluconate diet fed chickens and ducklings

	Chickens		
Mineral	Control (%)	Experimental[a] (%)	Ducklings (%)
Calcium	36.98a	37.83a	38.72a
Phosphorus	15.55a	15.68a	16.58a
Bone ash	43.57a	36.98a	53.68b

[a]Experimental ration had 0.85% calcium gluconate added from 0–5 wk of age and 1.06% calcium gluconate added from 5–8 wk of age. Any treatment means along lines not followed by same letter are significantly different ($P < 0.05$).

RESULTS & DISCUSSION

EXPERIMENTS comparing the four different freezing methods were repeated three times. Average color scores are listed in Table 1. In all replications the results show that there was no statistically significant difference in darkening between methods of freezing ($P < 0.05$). As results were negative the details of each experimental design are not included here but are included in the thesis of Hatch (1972). These results are in agreement with Brant and Stewart (1950) and Streeter and Spencer (1969). Ellis and Woodroof (1959) who studied freezing with dry ice do not agree with the findings.

In experiments comparing methods of thawing, the samples that were cooked without any prior thawing were significantly ($P < 0.05$) lighter in color than those samples that were thawed before cooking. The average color scores are given in Table 1. The microwave and slowly thawed samples did not exhibit a significant difference in darkening at the 5% level. These findings were in concurrence with Brant and Stewart (1950), Ellis and Woodroof (1959) and Koonz and Ramsbottom (1947) with regards to their findings on the effects of no thaw versus slow thawing and its effect on bone darkening.

Aging periods ranging from no aging, i.e., freezing immediately after slaughter, to holding in refrigeration for 6 days after slaughter were studied. Data are listed in Table 1. 6 days was thought to be a practical maximum period for refrigeration since microbial spoilage at 3°C storage occurs in approximately 9 days for fresh poultry (Shannon and Stadelman, 1957). There was no significant difference between birds frozen immediately after slaughter and those aged for up to 5 days before freezing. Holding birds for 6 days at refrigerated temperatures resulted in a significant increase in severity of bone darkening. These results are not in agreement with those of Brant and Stewart (1950). The procedures used for aging in this study are different from those used by Brant and Stewart (1950) in that birds were aged in polyethylene bags in a refrigerated room rather than in ice water where some leaching could have taken place.

In a series of experiments conducted to compare microwave and deep-fat cooking, bones of broilers cooked with microwave energy were significantly lighter than those cooked in deep fat. The observations made during this research indicate that much of the bone discoloration observed in frozen broilers takes place during thawing. If thawing time and time between thawing and heat denaturing the blood cells in the bone marrow can be minimized, discoloration can be reduced.

Bone ash, Ca and P levels in duckling femurs

Femurs from 8-wk old frozen ducklings were observed to be practically free from any bone darkening. A comparison of a sample of the aforementioned duck femurs and femurs from 8-wk old broilers that exhibited definite darkening was made with regard to percent bone ash, percent calcium and percent phosphorous. The duck femurs had significantly ($P < 0.05$) higher percent bone ash than broiler femurs (see Table 2).

The calcium content and the phosphorous content of the ash of the duck femurs was 38.72% and 16.58% while that for the control broiler femurs was 37.83% and 15.68%, respectively. These differences were not significant ($P < 0.05$) (see Table 2).

Correlation of percent bone ash and bone darkening

By ranking four femurs in order of increasing darkness (1 being the lightest and 4 the darkest) and then determining the percent bone ash in the six replications of groups of four a test was made for a correlation between percent bone ash and degree of darkness. An analysis of variance for the 24 samples and a Duncan's new multiple range test showed no significant difference in percent bone ash for the mean values of bones rated 1 through 4. With no significant difference between the ash levels of group means, the correlation value calculated was not significant. The bones ranked 1 as opposed to those marked 4 were significantly lighter but not significantly different in ash content. Ellis and Woodroof (1959) reported a correlation between darkening and bone ash in pullet tibias but the magnitude and the sign of the correlation were not reported.

In a group of 65 cooked femurs that were ranked according to degree of darkness (1 being the lightest) every fifth bone starting with 1 was analyzed for bone ash. A regression and correlation between darkening and percent ash of 0.35 was found. These data and data relative to calcium and phosphorous content of the ash of broilers and ducklings suggest that total percentage ash may be a significant determinant of bone discoloration.

Correlation of percent calcium with bone darkening

From 120 femurs that were ranked according to darkness (1 being the lightest), four groups of three bones each were evaluated for calcium with the results as shown in Table 2. An analysis of variance and a Duncan's new multiple range test of the group means at the 5% level showed no significant difference in calcium content between the groups. No significant correlation between the amount of darkening of a group and the percent calcium was indicated.

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EFFECT OF TOCOPHEROL SUPPLEMENTATION ON THE QUALITY OF PRECOOKED AND MECHANICALLY DEBONED TURKEY MEAT

INTRODUCTION

THE DEVELOPMENT of off-flavors in turkey, especially in further processed products, is a problem of increasing magnitude. This increased problem is caused primarily by a trend toward a greater production of precooked and heat-and-serve products. Off-flavor development in turkey products is due primarily to oxidative rancidity of lipids.

Mecchi et al. (1956a) suggested that the degree of unsaturation of fatty acids and the amount of natural antioxidant in the tissue govern the resistance of natural fats to rancidification. Mecchi et al. (1953) stated that the greater tocopherol content is the primary, if not the only, factor causing chicken fat to be more stable than turkey fat; they reported the fatty acid composition of turkey and chicken to be similar. Mecchi et al. (1956b) placed chickens and turkeys on a ration containing a natural level of tocopherol and found that the efficiency of tocopherol deposition in the carcass fat was much greater for chickens than for turkeys. Dicks and Matterson (1961) fed different amounts (0–320 mg/lb of ration) of tocopherols to chicks and found a direct relationship between the amount of tocopherol fed in the ration and that found in the liver.

Pickett et al. (1968) used subcutaneous implantations of vitamin E at 150, 300, 600 and 1200 IU in turkeys at 15 wk of age. 6 wk later, these birds were processed and placed in frozen storage for 2 months. Less change in TBA numbers was found in all groups that received vitamin E implantations than in birds that received no supplemental vitamin E.

Watts (1962) found that heating lean meats sufficiently to denature the protein was a factor that increased the rate of oxidation in beef and pork, and Acosta (1964) found that the rate of oxidation of total lipid varied between cooked and uncooked turkey.

Webb et al. (1971) studied the effects of feeding dl-alpha-tocopheryl acetate, Ethoxyquin and butylated hydroxy anisole on rancidity development in precooked, frozen broiler parts. Feeding broilers 5 or 10 IU of vitamin E per pound of ration for 36 days preslaughter, or 100 IU for 12 days preslaughter resulted in TBA numbers below those of controls. These researchers concluded that it is highly probable that diets and programs can be developed that will make the inclusion of synthetic or natural antioxidants economically feasible for stability improvement of precooked, frozen products.

Maxon (1971) noted that the increased use of mechanical deboning equipment led to an even greater problem of instability of meat. Because of increased surface area, bone marrow and other factors, the mechanically deboned turkey product is highly susceptible to lipid oxidation.

This study was conducted to test the effect of tocopherol supplementation of turkeys on the stability of precooked frozen turkey and of mechanically deboned turkey.

EXPERIMENTAL

LARGE WHITE MALE and female turkeys were raised to 8 wk on standard turkey starter and grower rations. Twenty turkeys of each sex were then placed in each of nine floor pens to which experimental treatments were assigned randomly (Table 1).

At 20 wk of age for females and 24 wk of age for males, five birds each were removed from duplicate pens on each treatment and 10 birds were removed from the "control" pen. The birds were slaughtered and held in slush ice for 18 hr before sampling. Raw breast and thigh samples were taken and immediately frozen at −25°C for 0 time (raw) TBA determinations (Tarladgis et al., 1960); times and temperatures for sample handling were standardized for this procedure.

Whole turkeys were roasted in groups of five in a rotary hearth oven at 162°C to an internal breast temperature of 82°C. Breast and thighs (parts) were removed from the carcasses immediately. Some samples were used for TBA analysis (0 time cooked); others were frozen (−25°C) and stored for 4 months for sensory testing and TBA analysis.

The precooked frozen samples were thawed overnight in a refrigerator (5°C) and then heated at 162°C to an internal temperature of 55°C for presentation to a trained taste panel. Samples also were taken at this time and frozen in liquid nitrogen for TBA analysis. Each panelist was presented five thigh and five breast samples at each session and was asked to evaluate them for off-odor and off-flavor on an ascending scale of 1–8, with 1 indicating imperceptible change and 8 indicating extremely pronounced change. The samples were evaluated for flavor on an ascending scale of 1–8, with 1 indicating extremely undesirable flavor and 8 indicating extremely desirable flavor.

At 26 wk of age for males and 27 wk of age for females, five turkeys were selected from the control group and 10 from treatments 2 and 4 for further testing. At the time of slaughter, the males had been off the vitamin E treatment and on the standard ration for 4 days. The females had been on the standard ration for 11 days. These birds were commercially processed and hand deboned. The remaining rib cage, sterum and neck were ground in a food chopper and mechanically deboned with a Beehive Deboner (model AV 1269MR). Samples of the mechanically deboned turkey (MDT) were collected, crust-frozen in a blast freezer, packed in ice and transferred to the Poultry Science Laboratories. Samples weighing about 40g were packed in Whirl-Pak bags and stored at 5°C or at −25°C. Triplicate TBA determinations were done daily for 7 days on samples stored at 5°C and at 0, 7, 21, 35, 49 and 63 days for samples stored at −25°C.

RESULTS & DISCUSSION

TBA analysis

Overall TBA numbers (mg malonaldehyde/1000g tissue) were significantly ($P < 0.01$) affected by treatment, storage period, meat type (breast or thigh), treatment × storage period interaction, treatment × meat type interaction and storage × meat type interaction. All tocopherol treatments except for the 10 IU oral resulted in lower TBA numbers than

Table 1—Treatment arrangement for experimental turkeys

Treatment	Description
1	Corn-soy ration (Control)
2	Control + 10 IU of vitamin E per pound of ration
3	Control + injected E equivalent to that of treatment 2[a]
4	Control + 100 IU of vitamin E per pound of ration
5	Control + injected E equivalent to that of treatment 4[a]

[a]Alpha-tocopheryl acetate in Tween 80 was administered weekly by subcutaneous injection on the back of the neck.

Table 2–Overall mean TBA numbers due to treatment

	Treatment (IU of vitamin E)			
	Oral		Injected	
Control	100 IU	10 IU	10 IU	100 IU
0.96[a]	0.93[a]	0.49[b]	0.56[c]	0.26[d]

[a]Values not followed by a common letter are different at the P < .01 level of significance.

Table 3–Mean flavor, off-flavor and off-odor responses due to treatment

			Treatment (IU of vitamin E)			
			Oral		Injected	
Parameter	Sex	Control	10 IU	100 IU	10 IU	100 IU
Flavor	F	4.79[a]	5.01[ab]	5.50[c]	5.16[bc]	5.21[bc]
	M	4.90[a]	4.89[a]	4.90[a]	5.22[a]	4.88[a]
Off-flavor	F	2.89[a]	2.66[ab]	2.13[ab]	2.41[bc]	2.04[c]
	M	2.26[a]	2.06[a]	2.11[a]	1.67[b]	2.26[a]
Off-odor	F	2.00[a]	2.05[a]	1.69[b]	1.74[b]	1.76[b]
	M	1.71[a]	1.50[a]	1.43[a]	1.40[a]	1.46[a]

[a]Values not followed by a common letter are different at the P < .01 level of significance.

those for control samples (Table 2). The lack of difference in the one instance suggests that the 10 IU oral treatment was too low to substantially increase the tocopherol content of the tissue. Webb et al. (1971) found that 5 or 10 IU of vitamin E administered orally to broilers 36 days preslaughter significantly protected precooked broiler parts during frozen storage.

Figures 1 and 2 illustrate the effect of tocopherol supplementation on the increase in TBA numbers of breast and thigh, respectively, during cooking and after 4 months of frozen storage of precooked turkey. The significant ($P < 0.01$) treatment × storage interaction and these graphs show that the increase in TBA numbers due to cooking and due to 4 months of frozen storage is much greater for the control and at the low levels of supplementation than at the higher levels of supplementation. Several researchers, Lineweaver et al. (1952), Zipser and Watts (1961) and Acosta (1964), have noted that cooking increases the rate at which meat lipids oxidize.

Breast tissue (Fig. 1), with an overall mean TBA number of 0.52 (calculated from all data used to plot Fig. 1), was less rancid than thigh (Fig. 2), with a mean value of 0.76 (calculated from all data used to plot Fig. 2). This difference, significant at the $P < 0.01$ level, was due in part to the higher lipid content and greater amount of heme pigments in thigh tissues. These results agree with those of Marion and Forsythe (1964) who found that red muscle shows a much more rapid rate of oxidation than does white muscle.

Flavor

Panelists found significant ($P < 0.01$) differences in samples from male turkeys due to days (days refer to the six tasting sessions, with one replicate group presented at each session) and meat type, but not to treatment (Table 3). Breast samples from male turkeys, with a mean flavor value of 5.18, had more desirable flavor than thigh samples, with a mean flavor score of 4.73. This agrees with TBA numbers which indicated that breast meat from males had smaller amounts of oxidation-breakdown products than did thigh meat. Panelists also found a significant ($P < 0.05$) treatment effect when evaluating samples from females for flavor (Table 3). They preferred the meat from turkeys supplemented with 100 IU oral, 100 IU injected or 10 IU injected. A difference was detected between the two levels of oral supplementation of vitamin E, but not between the two levels injected.

Off-odor

Panelists found significant differences ($P < 0.01$) in off-odor due to days and meat type. The difference between days is difficult to understand because each day's samples were replicates of the samples presented on the other 5 days of testing. This "day" effect was not noted in subsequent TBA analyses on the samples. For meat type, the panel results agree with TBA numbers. Thigh meat from male turkeys, with a mean off-odor score of 1.71, had a more pronounced off-odor than did breast samples with a score of 1.29. Female thigh samples, with a mean off-odor score of 1.98, had more off-odor development than did breast samples with a mean off-odor score of

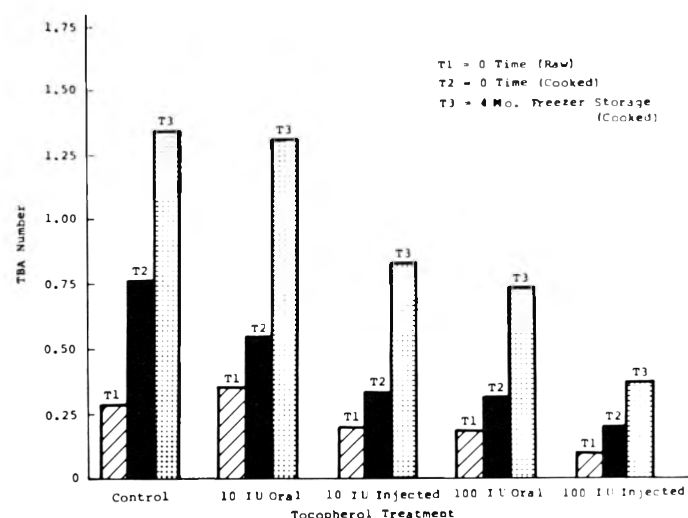

Fig. 1–Effect of treatment and storage period on TBA numbers of turkey breast.

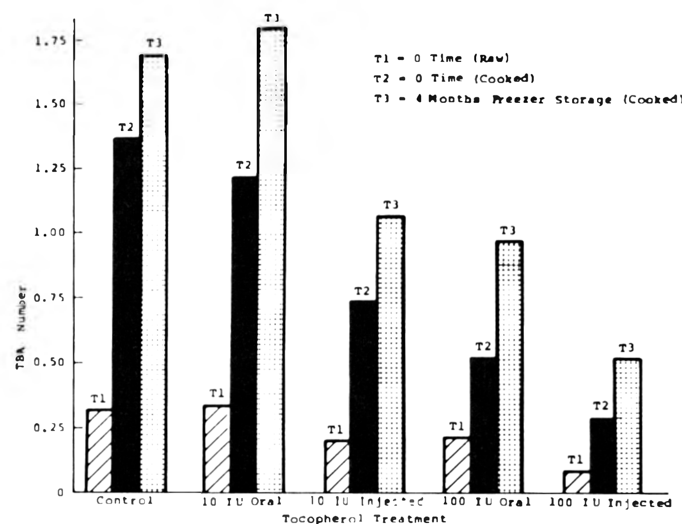

Fig. 2–Effect of treatment and storage period on TBA numbers of turkey thigh.

Table 4—Mean TBA numbers for mechanically deboned turkey

Storage temperature	Treatment (IU of vitamin E) Control	10 IU Oral	100 IU Oral
5°C	2.49[a]	1.02[b]	0.67[b]
−25°C	0.47[a]	0.53[a]	0.22[b]

[a]Values not followed by a common letter are different at the P < .01 level of significance.

1.72. These results agree with TBA numbers and are significant at the $P < 0.01$ level. Panelists also found a significant ($P < 0.01$) treatment effect on off-odor development in samples from females, but not in those from males (Table 3). Control and 10 IU oral treatment samples had greater off-odor development than did samples from 100 IU oral, 100 IU injected or 10 IU injected treatments. This suggests that tocopherol treatment at the higher levels has some stabilizing effect on off-odor development in female turkey tissues. These results agree with TBA numbers.

Off-flavor

Significant ($P < 0.01$) differences in off-flavor were found due to meat type, treatment and days. Breast samples from males, with mean off-flavor scores of 1.78 were preferred over thigh samples, with mean off-flavor scores of 2.36. The samples from male turkeys receiving the 10 IU of injected vitamin E had lower off-flavor scores than did the other treatments (Table 3).

Off-flavor in samples from female turkeys was significantly ($P < 0.01$) influenced by treatment and meat type. Breast samples, with an average off-flavor score of 2.28, were preferred over thigh samples with average scores of 2.57. The panel responses to off-flavor of female samples due to treatment show the same general trend as did the TBA numbers of these samples. The control samples had the greatest degree of off-flavor development, and the group receiving 100 IU of injected vitamin E had the least amount of off-flavor development.

No significant correlations were found between TBA values and taste panel responses for precooked frozen samples, although there were several instances in which the two methods of evaluation followed the same general trend. The lack of correlation is likely due to factors influencing the responses of the panelists. The training of the panel, for example, may have been insufficient to acquaint the members with the desired qualities of turkey. Panel members, in a few instances, seemed to prefer turkey meat that had some degree of rancidity development because it had more flavor than did fresh turkey. A longer period of frozen storage would probably have enhanced treatment differences. In future storage tests on precooked turkey meat for the detection of oxidative rancidity development, the samples should be held in frozen storage for 6 months or more.

Mechanically deboned turkey

Significant ($P < 0.01$ and $P < 0.05$) sex, treatment, days of storage, sex × treatment interaction and treatment × days of storage interaction effects were found for the mechanically deboned turkey (MDT). Table 4 shows the influence of vitamin E treatment on mean TBA numbers of MDT held at 5°C or −25°C. Figures 3 and 4 show the increases in TBA numbers with increasing storage time of samples from male turkeys held at 5°C and −25°C, respectively. Figures 5 and 6 show corresponding data on samples from female turkeys. In addition,

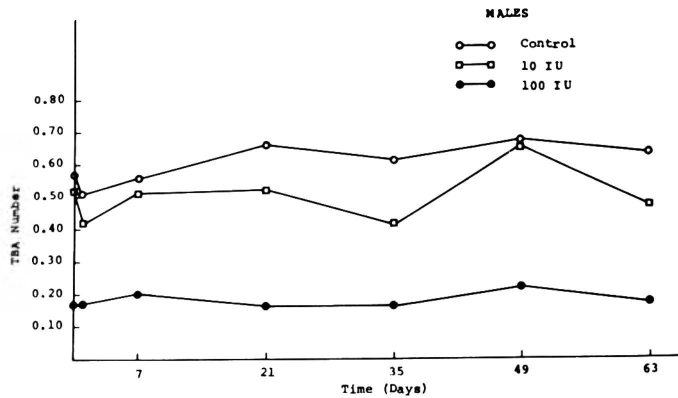

Fig. 4—Effect of −25°C storage on TBA numbers of mechanically deboned turkey from males.

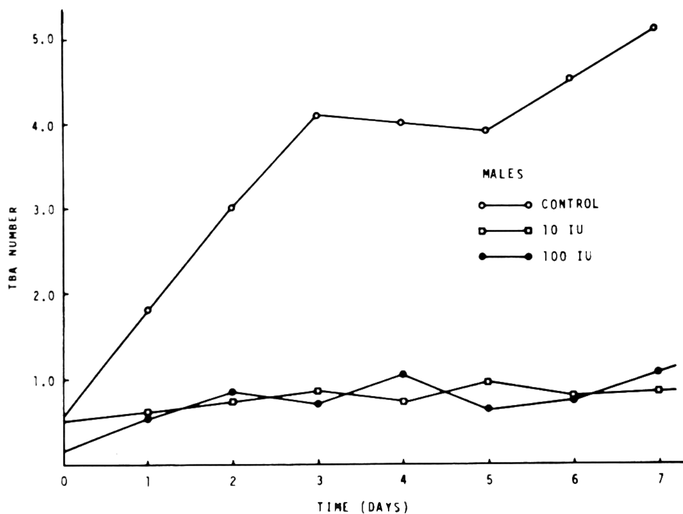

Fig. 3—Effect of 5°C storage on TBA numbers of mechanically deboned turkey from males.

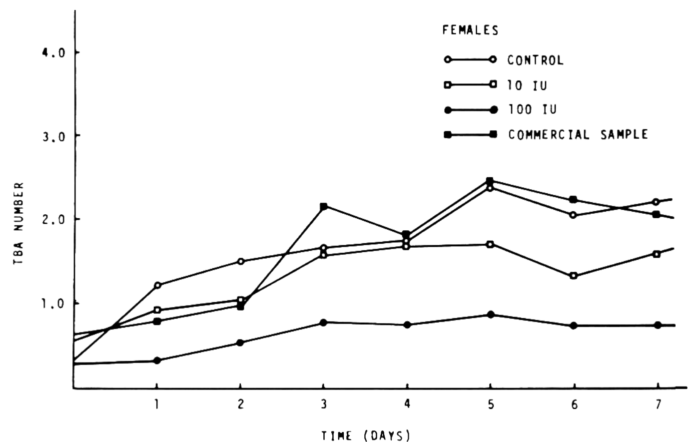

Fig. 5—Effect of 5°C storage on TBA numbers of mechancically deboned turkey from females.

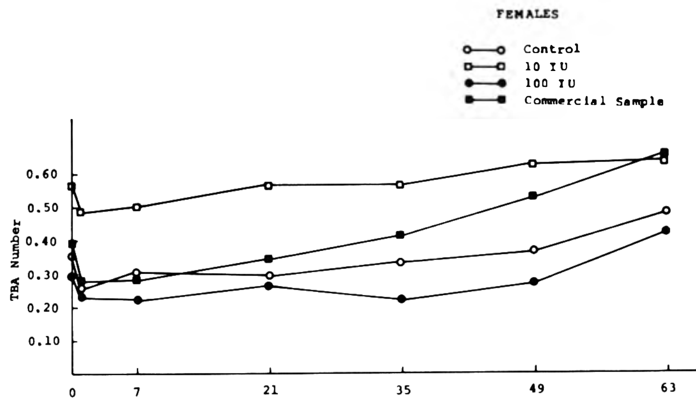

Fig. 6–Effect of –25°C storage on TBA numbers of mechanically deboned turkey from females.

Figures 5 and 6 show the change in a sample ("commercial") taken from the plant's own supply of MDT.

The data in the figures confirm the relative susceptibility of MDT to oxidative change at refrigerator temperatures (Maxon, 1971), but they also illustrate the added stability achieved with antioxidant treatment to the growing turkey. As expected, freezer storage (–25°C) minimized the rate of oxidative change, as determined by TBA number, compared with refrigerator storage. Other apparent changes in MDT, such as color or functional properties were not measured, but we would expect such properties to be unaffected by antioxidant treatment.

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AN EVALUATION OF THE ARMOUR TENDEROMETER FOR AN ESTIMATION OF BEEF TENDERNESS

INTRODUCTION

TENDERNESS is one of the most favored attributes of meat. Most of the common breeds have been selected so that fed cattle generally provide tender meat. In spite of this, the need and desire to monitor meat tenderness, in the live animal, and the carcass, has increased.

Attempts to relate objective tenderness measures of raw with cooked meat have failed to show a significant correlation (Warner, 1928; Black et al., 1931; Mjoseth, 1962; Carpenter et al., 1965). Measured tenderness values for raw meat by the wedge tenderometer, denture tenderometer and grinder tenderometer were reported by Carpenter et al. (1965). They found the correlation with a taste panel to be small.

Various types of penetrometers have been used to measure texture, firmness, and tenderness. Pilkington (1964) used several shaped penetrometer points to measure firmness and tenderness, but found no direct relationship between these two quality factors. Bourne (1966) reported that the puncture force for different punches on the same food will be a function of both area and perimeter of the punch used. A problem of great concern when using a penetrometer on raw meat has been the failure to obtain a good relationship with tenderness of cooked meat. Hinnergardt and Tuomy (1970) reported on a study which evaluated the use of a single penetration test of raw meat to predict the tenderness of cooked meat. They reported that maximum force value gave the best result.

The purpose of this research was to evaluate the Armour "tenderometer" as an instrument suitable for the measurement of raw meat tenderness.

MATERIALS & METHODS

THE BATTERY OPERATED tenderometer consisted of a probe assembly and a strain gauge. The probe assembly included 10 stainless steel needles, each 3 in. long, mounted on a manifold attached to a strain gauge, which is connected by cable to a peak force indicator. The depth of needle penetration is determined by a guard bar that regulates the penetration to exactly 2 in. The instrument was designed to be used on the raw longissimus dorsi muscle at the area of the 12th and 13th thoracic vertebrae while the carcass hangs from a cooler rail. Readings were taken on muscle chilled to a minimum of 39°F (4°C) but not more than 32°F (0°C), Hansen (1971). Consequently, a measurement was not taken until 24 hr after slaughter.

99 Angus steer carcasses weighing an average of 541 lb were evaluated for tenderness of the L. dorsi muscle. Breeding, feeding and environmental treatments of the animals were similar so as to reduce these potential tenderness variables. All animals were trucked 140 mi from the station feed-lot to the abattoir. Feed and water were provided during the 24 hr rest period prior to slaughter. 50 head were slaughtered 24 hr after arrival at the plant and 49 head slaughtered after 48 hr.

The carcasses were ribbed 24 hr after slaughter, between the 12th and 13th thoracic vertebrae, and a federal grader appraised each carcass for the amount of marbling and grade. Immediately after grading, tenderometer readings were made at the plant on the L. dorsi muscle. The internal muscle temperature was recorded using a thermometer inserted into the L. dorsi muscle. One-half of each carcass was divided into wholesale cuts (26 hr postmortem) and the rib returned to the Station research laboratory for further study and analysis. Upon arrival at the laboratory (30 hr postmortem), a second tenderometer measurement of each L. dorsi muscle, its temperature and area tracing were each recorded. When the temperature of the L. dorsi muscle had equalized to 36°F (48 hr postmortem), a third tenderometer measurement of the L. dorsi muscle was made. Care was taken in the placement of the needles to avoid previously penetrated areas of the muscle. The fourth measurement was made on the new L. dorsi muscle surface after a 2-in. thick slice had been removed (36°F, 48 hr postmortem).

A 2-in. thick slice of the penetrated L. dorsi muscle was removed from each wholesale rib, cooked in oil to an internal temperature of 150°F (65.5°C) chilled to 40°F (4.5°C), cored (2.54 cm) and evaluated using the Warner-Bratzler shear instrument. Three 2.54 cm cores were removed from each steak and each core sheared three times (Kastner and Henrickson, 1969). An adjacent 1-in. slice was removed from the L. dorsi muscle to provide tissue for histological observations and ether extract measurements.

Three 1.2 cm diam cores were removed from the dorsal, central and ventral areas of the 1-in. thick muscle sample and placed in 10% formalin. The remainder of the muscle was made into a composite paste and analyzed for its fat content and iodine number (AOAC, 1965).

The formalin-fixed tissue provided fibers to be evaluated for their degree of rigor-mortis. To harvest the fibers, a single fascicula was placed

Table 1—Mean, standard deviation and range for carcass and tissue variables of tested animals

	Mean	Std dev	Range
Carcass wt lb	541.0	57.6	406–666
L. dorsi area sq in.	10.2	1.1	8.1–12.9
Fiber diam μ	64.2	6.4	50.2–81.2
Carcass grade score[a]	10.4	1.2	8.0–13.0
Marbling score[b]	5.2	1.1	3.0–8.0
Ether extract %	5.8	1.9	2.3–11.3

[a]Carcass score ranged from 8 (average good) to 14 (top prime).
[b]Marbling score ranged from 3 (traces) to 9 (abundant).

Table 2—Analysis of variance

Source	DF	MS	F
Animal	98	15.506	5.79**
Tenderometer	3	263.364	98.39**
Error	294	2.677	
Total	395		

**P < 0.001

Table 3—Mean shear force, degree rigor fibers and tenderometer values for bovine L. dorsi muscle

		Mean	Std dev	Range
Shear force	lb	18.29	4.3	9.9–33.3
Degree rigor	%	36.37	15.3	14.0–83.4
Tenderometer				
A[a]	lb	17.10	2.6	12.0–23.4
B[b]	lb	14.72	2.5	9.0–20.0
C[c]	lb	14.96	2.3	9.7–20.5
D[d]	lb	13.12	2.2	8.4–18.4

[a]A—Muscle temperature 37.3°F, 24 hr postmortem, in plant measurement
[b]B—Muscle 41°F, 30 hr postmortem, in lab measurement
[c]C—Muscle 36°F, 48 hr postmortem, in lab measurement
[d]D—A 2-in. slice was removed and penetration was made into the new muscle surface, 36°F, 48 hr postmortem.

Table 4–Influence of fat level upon the tenderometer and shear forces

E. extract	%	2–3.9	4–5.9	6–7.9	8–9.9
Tenderometer					
A[a]	lb	16.10 ± 2.6	16.90 ± 2.8	17.33 ± 2.1	18.82 ± 2.9
B[b]	lb	14.55 ± 2.3	14.38 ± 2.6	14.75 ± 2.5	15.75 ± 2.7
C[c]	lb	14.25 ± 2.2	14.66 ± 2.8	15.11 ± 1.5	16.65 ± 1.6
D[d]	lb	12.51 ± 2.2	12.09 ± 2.1	13.99 ± 1.8	15.03 ± 1.9
Shear force	lb	20.47 ± 4.7	18.48 ± 4.3	17.49 ± 3.4	15.77 ± 4.5

[a]A–In-plant tenderometer measurement
[b]B–Tenderometer measurement 30 hr postmortem
[c]C–Tenderometer measurement 48 hr postmortem
[d]D–Muscle penetrated 48 hr postmortem using new surface

Table 5–Iodine values of fat from the bovine L. dorsi muscle

Fat[a]	Range	Iodine value
3.4%	3.1–3.8%	44.5%
9.2%	8.2–11.3%	43.3%

[a]Twelve muscle samples were used at each fat level; each measure was made in duplicate.

in a mechanical mixer and stirred at a slow speed. When the fibers were dislodged, they were placed in a petri dish to be evaluated for degree of rigor and diameter measurement at 100× magnification. Percentage rigor, or the amount of kinkiness characteristic of the fiber in rigor, was determined at the same time as fiber diameters were measured. For each fiber diameter measured a value was subjectively assigned to the condition of that fiber, this in turn was expressed on a percentage basis (Reddy and Henrickson, 1967; Gillis and Henrickson, 1969).

RESULTS & DISCUSSION

ANIMALS used in the investigation were generally homogeneous in so far as production factors were concerned. Carcass weight ranged from 406–666 lb with a mean of 541 lb (Table 1). The carcasses were generally muscular as reflected by the mean L. dorsi area of 10.2 ± 1.1 sq in., with a range of 8.1–12.9 sq in. The fat content of the L. dorsi muscle, as evidenced by the ether extract value, was 5.8 ± 1.9% (range 2.3–11.3%). The designated average federal grade was choice with the numerical score being 10.4 ± 1.2.

A highly significant difference ($P < 0.001$) due to animal variation and tenderometer reading was noted (Table 2), thus indicating that a muscle difference was being registered by the tenderometer.

The multi-point penetrometer was easy to use in the cooler while working a rail of carcasses. A measurement was rapidly made without damage to the carcass or disruption of the daily normal cooler operation. The in-plant tenderometer reading, (Table 3, A) made on the rib L. dorsi muscle, was higher than any of the three measurements made on the wholesale rib after it was returned to the laboratory (Table 3, B, C and D). The penetration force variance may be attributed to the difference in product temperature, angle of penetration, level of fat, muscle size, fiber diameter and/or the degree of fiber rigor. Since the same muscle area was penetrated a second time, tissue destruction too may have affected the reading. However, repeatability measurements were made using 20 separate rib L. dorsi muscle sections to verify this potential variable by inserting the needles from both sides of the steak. The penetration force obtained by inserting the tenderometer needles into the muscle from opposite sides of a 3-in. thick section of the L. dorsi muscle gave similar mean readings (15.3 ± 2.3 and 14.3 ± 2.1 lb). While this would not be the same as inserting the needles into the same surface, it does reflect good unit repeatability. When the muscle and cooler temperatures

Table 6–Influence of L. dorsi area upon the tenderometer and shear forces

L. dorsi areas	sq in.	8–8.9	9–9.9	10–10.9	11–11.9	12–12.9
Tenderometer						
A[a]	lb	19.33 ± 2.3	17.48 ± 2.7	17.04 ± 2.4	16.10 ± 2.4	13.40 ± 2.4
B[b]	lb	16.20 ± 2.4	15.13 ± 2.4	14.63 ± 2.6	13.75 ± 2.3	12.13 ± 2.4
C[c]	lb	16.05 ± 2.2	15.27 ± 2.1	15.22 ± 2.6	13.87 ± 1.9	13.00 ± 2.0
D[d]	lb	14.41 ± 2.0	13.60 ± 2.2	13.04 ± 1.9	12.03 ± 2.4	11.03 ± 1.3
Shear force	lb	20.01 + 3.1	18.25 + 4.5	18.13 + 4.5	17.63 + 4.0	20.96 + 6.8

[a]A–In-plant measurement
[b]B–Measurement 30 hr postmortem
[c]C–Measurement 48 hr postmortem
[d]D–Muscle penetrated 48 hr postmortem using a new surface

Table 7–Influence of fiber diameter upon the tenderometer and shear forces

Fiber diam	μ	50–59	60–69	
Tenderometer				
A[a]	lb	17.64 ± 2.7	16.91 ± 2.7	16.90 ± 2.5
B[b]	lb	15.40 ± 2.5	14.62 ± 2.6	14.12 ± 2.4
C[c]	lb	15.66 ± 2.0	14.61 ± 2.3	15.07 ± 2.5
D[d]	lb	13.14 ± 2.5	13.00 ± 2.3	13.18 ± 1.9
Shear force	lb	18.08 ± 3.7	18.70 ± 4.1	17.72 ± 5.5

[a]A–In-plant measurement
[b]B–Measurement 30 hr postmortem
[c]C–Measurement 48 hr postmortem
[d]D–Muscle penetrated 48 hr postmortem using a new surface

Table 8–Influence of degree rigor upon the tenderometer and shear forces

Degree rigor	%	10–29	30–49	50–69	70–89
Tenderometer					
A[a]	lb	17.33 ± 2.7	16.80 ± 2.4	17.86 ± 3.5	15.40 ± 2.3
B[b]	lb	14.69 ± 2.3	14.55 ± 2.7	15.62 ± 2.3	13.28 ± 1.9
C[c]	lb	14.49 ± 2.3	15.22 ± 2.4	15.72 ± 1.5	13.60 ± 3.0
D[d]	lb	12.92 ± 2.3	13.10 ± 2.3	13.78 ± 1.9	12.38 ± 0.3
Shear force	lb	17.55 + 3.7	18.50 + 4.5	18.55 + 5.5	22.49 + 1.9

[a]A–In-plant measurement
[b]B–Measurement 30 hr postmortem
[c]C–Measurement 48 hr postmortem
[d]D–Muscle penetrated 48 hr postmortem using a new surface

Table 9—Correlations among factors which tend to influence the tenderometer[a]

		Marbling	E. extract	L. dorsi area	Fiber diam	Deg. rigor	Tenderometer			
							A	B	C	D
Marbling		1.00								
Ether extract		0.32								
L. dorsi area		−0.21	−0.21							
Fiber diam		−0.04	0.01	0.08						
Degree rigor		0.14	0.04	−0.28	−0.03					
Tenderometer	A	0.23	0.05	−0.38	−0.12	−0.07				
	B	0.24	0.12	−0.34	−0.23	−0.02				
	C	0.37	0.12	−0.33	−0.06	0.04	0.56	0.58		
	D	0.44	0.12	−0.37	−0.06	0.05	0.57	0.43	0.48	
Shear force		−0.29	−0.30	−0.08	0.34	0.22	−0.15	−0.14	0.02	−0.01

[a]Animals used (99) were 13 months of age and of uniform breeding.

(36°F) were permitted to equilibrate (48 hr), tenderometer readings were not different from those recorded at 30 hr postmortem (Table 3). The in-plant tenderometer force readings were always greater than at any other time (Table 3). This may have been influenced by the angle of penetration.

The quantity of fat within the L. dorsi muscle was found to influence the tenderometer force reading. When the marbling level (ether extract) was between 2–4%, the force registered by the tenderometer was approximately 2.5 lb less than when the fat content ranged from 8–10% (Table 4). While a larger quantity of fat in the raw muscle increased the penetration force, it decreased the Warner-Bratzler shear force of the cooked meat by 4.7 lb. A partial correlation between the tenderometer and shear force, holding ether extract constant, was −0.07 ($P < 0.05$), thus, indicating an inverse relationship between the tenderometer and the shear force.

Even though the fatty acid composition of the intra-muscular fat may have an influence upon the tenderometer force, fat hardness as reflected by the iodine number was not found to vary with fat quantity (Table 5). Muscle with 3.4% fat possessed an iodine number of 44.5, while muscle with an average of 9.2% fat was 43.3, reflecting no composition difference.

The L. dorsi muscle area ranged from 8–12.9 sq in. and exerted a profound influence upon the force required to insert the tenderometer probe assembly. The penetration force decreased as the L. dorsi muscle area increased (Table 6), thus indicating that bone and quantity of external surface fat may have influenced the penetration force. A partial correlation between the tenderometer and shear force holding L. dorsi muscle area constant was −0.19 ($P < 0.05$).

Muscle fiber size was considered a factor which would influence the penetration force. Even though the fiber diameter ranged from 50.2–81.2 microns the force registered by the tenderometer did not show a significant relationship. Fibers ranging from 50–60 microns in diameter required a greater force than fibers ranging from 70–80 microns. The magnitude of difference was only 0.73 lb (Table 7). This trend agreed with the area of the L. dorsi muscle even though the magnitude of difference was not as great.

The extent to which the muscle fibers are in rigor may have an influence on muscle fiber compactness and therefore influence the penetration force. However, when the data were analyzed specifically for this parameter, no major difference was noted (Table 8). Warner-Bratzler shear force was not statistically related even though the force increased as the degree rigor increased. The quantity of fiber rigor ranged from 14.0–83.4% and warrants further more precise study. This measurement was made on the tissue 48 hr after death, recognizing that the elapsed time period would likely be sufficient for some resolution to take place. Therefore, tenderometer readings taken 24 hr after death would likely be greater and more varied than a similar reading registered after 48 hr.

Connective tissue within the muscle would also be a factor influencing the tenderometer reading. Not only would the amount of connective tissue be an important variable, but its composition would also be influential. Connective tissue with large quantities of elastin would likely provide more resistance to penetration than tissue composed only of the protein collagen. This variable was not evaluated but will be considered as the work is continued.

Correlation coefficients indicated that the Warner-Bratzler shear force machine and the Armour tenderometer were not highly related (Table 9). The units likely measure a different element of tenderness. The tenderometer measures the force necessary to separate the individual raw muscle fibers, while the Warner-Bratzler shear measures the force required to cut the cooked fibers at right angles to their long axis. A partial correlation analysis between the tenderometer and shear force holding ether extract and L. dorsi muscle area constant was −0.43 ($P < 0.01$). The degree of fiber rigor and fiber diameter were not significantly correlated with the tenderometer force. A taste panel analysis on these same samples or other techniques of using the tenderometer may have reflected a higher relationship between tenderness and the tenderometer force.

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EFFECT OF CONDENSED PHOSPHATES ON pH, SWELLING AND WATER-HOLDING CAPACITY OF BEEF

INTRODUCTION

THE EFFECT of the addition of condensed phosphates on fresh and cured meats has been reported by many investigators. Several theories have been advanced to explain the mechanisms by which the meat is affected by phosphates, alone or in combination with the common salt, sodium chloride. Water retention, water binding, water-holding capacity, cured meat volume, pH and meat swelling were studied to demonstrate the effects of the additives.

Several investigators have found a definite effect of pH on the water retention and swelling of meat samples. Water retention in meat is at a minimum at its isoelectric point, which is pH 5.3–5.5. Grau et al. (1953), Hamm (1960) and Wierbicki et al. (1963) found that increasing the alkalinity of the meat resulted in an increase in water retention properties. Hamm and Grau (1955) reported that the sequestering effects of some phosphates resulted in an increase of water retention, particularly by sequestering calcium and zinc ions of a meat (Hamm, 1956). Wierbicki et al. (1957b) reported that the addition of calcium chloride and magnesium chloride to beef increased the water retention properties when heated at 70°C. Swift and Ellis (1956) also showed an increase in water retention could be obtained by the addition of magnesium chloride. Sherman (1961a) found that fluid retention, as affected by sodium chloride or other neutral salt solutions, depended upon the degree of ion absorption by meat proteins. The anions are absorbed preferentially and are directly related to the fluid retention. With phosphate solutions, fluid retention at 0°C shows a significant statistical correlation with pH. The ionic strength of the solution was important only insofar as it controlled the rate of absorption. The greater the ionic strength, the greater the absorption of ions. Mahon (1961) found that salt concentration and not pH adjustment is the key to obtaining maximum water retention in cured meats.

The role of phosphates on the water retention and meat swelling has been studied by several investigators. Hellendoorn (1962) found that pyrophosphate and tripolyphosphate had a marked specific activity on water retention in heated meat samples at a pH range of 6.0–6.5, with pyrophosphate being slightly superior. Other phosphates such as orthophosphate and hexametaphosphate had minor specificity of several phosphates and found that tripolyphosphate had the highest activity followed by tetrapolyphosphate, hexametaphosphate, pyrophosphate and orthophosphate, in that order.

Sherman (1961b) found an overall reduction of fluid retention in samples of ground lean pork meat mixed with solutions of alkaline phosphates or sodium chloride when the samples were heated at various temperatures. He reported that in samples with the additive concentrations not exceeding 2% and heated in a range of 25–100°C, the maximum fluid retention was at 50°C, followed by an overall decrease with increasing heating temperature.

Swift and Ellis (1956) reported the importance of maintaining low temperatures in a range of 0–5°C in using alkaline phosphate additives to obtain optimum water retention activity in raw meat samples. These results showed that 16 hr at 0°C was optimum for maximum water retention. Mahon (1961) found no effect of holding time on meat samples with higher concentrations of salts and phosphate. However, at the lower concentrations, an effect of holding time was found. He also determined a synergistic effect on water retention by the addition of salt and phosphates. An increase of water retention was found when a combination of salt and phosphates was used when compared with samples with only salt or phosphates.

Results of the previous works have demonstrated the importance of both salt and phosphates in their role of improving such meat quality characteristics as water retention, pH, and meat swelling. Although some differences in the results are noted in the literature, these differences can be explained by methodologies used in determining the water-holding or water-binding capacities of meats and the natural biological differences within the samples.

Recently the use of sodium tripolyphosphate, alone or in a combination with sodium hexametaphosphate, has been allowed by the U.S. Department of Agriculture, Consumer and Marketing Service, in cooked beef and fresh beef prepared for "further cooking" (USDA, 1970).

This study was conducted to determine the relative merits of different sodium polyphosphates with and without sodium chlorides on swelling (water-binding capacity) of raw beef and water-holding capacity of beef during heating

Table 1—Effect of various phosphates, with and without sodium chloride, on pH, swelling and shrinkage at 70°C of three different USDA Choice beef muscles: longissimus (A), biceps femoris (B) and semimembranosus (C)

Phosphate (0.5%)	1% NaCl	pH			% Swelling			% Shrink		
		A	B	C	A	B	C	A	B	C
None	–	5.50	5.70	5.50	26	26	37	25	26	28
Pyrophosphate	–	5.95	6.10	5.90	83	94	83	17	20	21
Pyrophosphate	+	6.10	6.30	6.20	140	77	100	9	8	11
TPP	–	5.85	5.75	5.70	83	43	71	18	24	23
TPP	+	5.90	6.00	5.75	88	48	89	9	9	13
Curafos 22-4	–	5.85	5.90	5.75	66	48	37	20	23	24
Curafos 22-4	+	5.80	5.90	5.80	60	60	66	9	10	16
Kena FP-28	–	5.90	5.80	5.70	77	37	54	20	22	21
Kena FP-28	+	6.00	6.00	5.75	77	54	43	10	11	15
Hexametaphosphate	–	5.60	5.60	5.50	43	31	54	22	26	28
Hexametaphosphate	+	5.60	5.75	5.50	43	43	37	13	15	24

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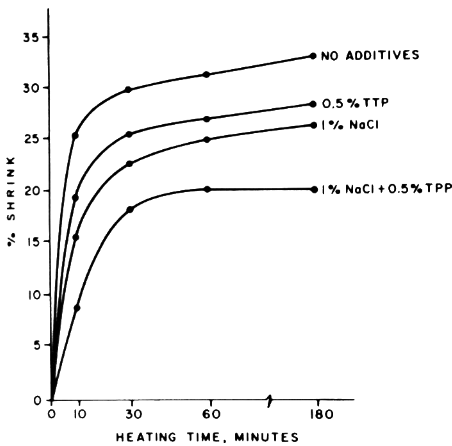

Fig. 1—Effect of heating time at 60°C on the shrinkage (water-holding capacity) of beef semimembranosus muscle.

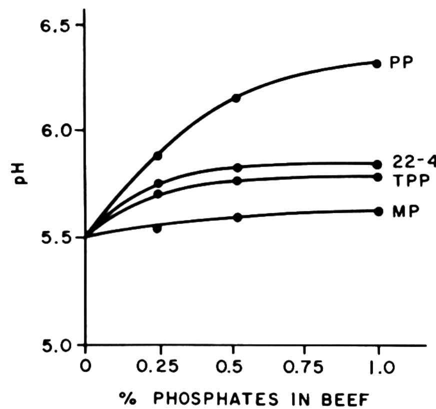

Fig. 2—Effect of condensed phosphates on pH of beef semimembranosus muscle.

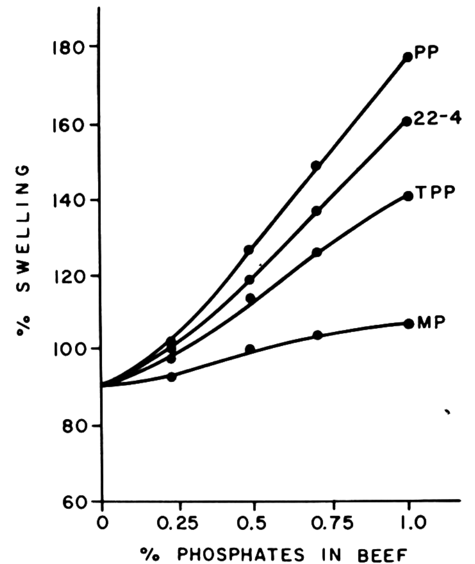

Fig. 3—Effect of condensed phosphates on the swelling of beef semimembranosus muscle.

(reciprocal of meat shrinkage), with emphasis on the water-holding capacity. The effect of the additives on pH of beef was also determined.

EXPERIMENTAL

Materials

The raw material utilized for this study was the semimembranosus, longissimus and triceps brachii muscles of USDA Commercial grade beef and in one experiment (Table 1) the semimembranosus, longissimus and biceps femoris muscles of USDA Choice grade beef. The beef was chilled (nonfrozen), 3–5 days post mortem, obtained from a local beef supplier. After removal of cover fat and visible connective tissue, the beef was ground through a 3/16 in. grinding plate and thoroughly mixed prior to each test.

The additives used were sodium chloride and the following food grade phosphates: sodium tripolyphosphate (TPP), sodium metaphosphate (MP), sodium hexametaphosphate (HMP), tetrasodium pyrophosphate (PP) and two blends of commercial phosphates, Kena FP-28 (a mixture of TPP, PP and sodium acid pyrophosphate) and Curafos 22-4 (a mixture of TPP and HMP). (All phosphates were obtained by the courtesy of Calgon Corp., Pittsburgh, Pa.) These additives were added directly to ground beef, mixed thoroughly, and held overnight in a refrigerator (at 2–4°C) prior to evaluation.

Methods

The water-holding capacity (meat shrinkage) was determined by the method of Wierbicki et al. (1957a) with the following modifications:

(a) The dimensions of the tubes were 180 mm long with a top chamber 35 mm in diameter (outside) and the bottom chamber 20 mm in diameter. The bottom section of the tube was graduated in divisions of 0.1 ml from 0–10 ml.

(b) Meat samples (with or without additives) were 20g. Each meat sample was run twice in duplicate. Each shrink datum tabulated or presented in the figures is an average of four tube readings with a standard deviation of less than ± 5% (relative).

(c) The heating times and temperatures varied, depending upon the experiment. The minimum heating time of the meat, with and without the additives, required to obtain representative shrink data was confirmed to be 30 min, as shown in a preliminary experiment on checking the methodology (Fig. 1). Unless otherwise indicated, 30 min heating time was used for the shrink determination.

(d) After heating, the samples were centrifuged at 900G (1000 rpm) for 15 min using an International Model V centrifuge and the amount of juices lost during heating and centrifugation measured. This loss of juices, or the meat shrinkage, is expressed as percent of the total weight of the samples.

The meat swelling (water-binding capacity) was determined by the method of Wierbicki et

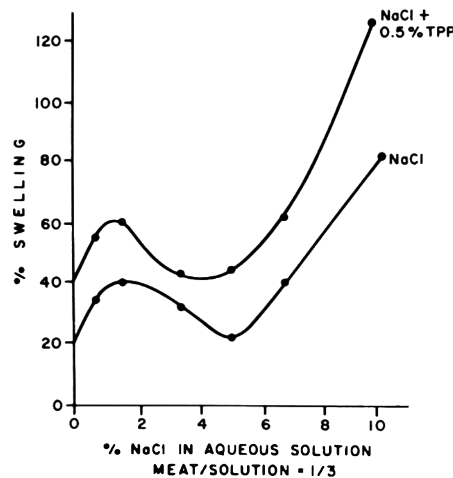

Fig. 4—Effect of sodium chloride, with and without TPP, on the swelling of beef semimembranosus muscle.

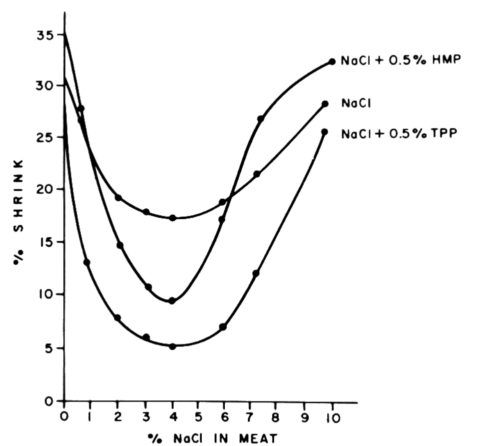

Fig. 5—Effect of sodium chloride, with and without TPP or HMP, on the shrinkage of beef semimembranosus muscle at 70°C.

al. (1962). A 50-g sample of beef, with or without additives, was blended at room temperature in a high speed blender with 150 ml of distilled water for 90 sec. 35g of the meat slurry were weighed in duplicate into 40 ml heavy glass centrifuge tubes. The samples were centrifuged at room temperature (21–25°C) for 15 min at 1000 rpm in an International Model V centrifuge. After centrifugation the volume (in ml) of the supernatant liquid was collected in a graduate. The percent swelling was determined by the following formula (Wierbicki et al., 1962) where X is the grams of absorbed H_2O per 100g meat and S is the supernatant.

$$X = 300 - (11.43 \times S) = \%\ \text{Swelling}$$

Weight of slurry 35g; weight of meat in the slurry 8.75g; weight of added water in the slurry (35–8.75)g; S = supernatant in ml. (1 ml = 1 g).

$$\%\ \text{Swelling} = [(35-8.75)-S]/8.75 \times 100$$
$$= (3-S/8.75) \times 100$$
$$= 300-(100/8.75) \times S$$
$$= 300 - (11.43 \times S).$$

All determinations were run in duplicate.

The pH of the meat samples was read directly using a Beckman pH meter. The readings were taken prior to weighing the samples for the shrink determination.

RESULTS & DISCUSSION

Effect on pH

One of the important effects of condensed phosphates on raw meats is elevation of pH from the isoelectric point of about 5.5 towards the alkaline side, thus increasing the water-holding and water-binding capacities of meats (Wierbicki et al., 1962; Sherman, 1961b; Hellendoorn, 1962; Hamm, 1960; Mahon, 1961). Figure 2 shows the effect of various phosphates on the pH of semimembranosus raw beef. Pyrophosphate was most effective in raising the pH of beef, with MP showing practically no effect. Hexametaphosphate also has a very small effect on pH (Table 1). The effect of TPP on pH is much less than that of PP. The effect of the commercial Kena FP-28 and Curafos 22-4 phosphate mixtures was comparable with that of TPP (Fig. 2 and Table 1).

Effect on meat swelling

Data shown in Figure 3 present the effect of the phosphates on the swelling of beef. The effect is greatest for PP to be followed by the commercial mixture Curafos 22-4 and TPP. The effect of MP is negligible. The effects of HMP and Kena FP-28 on the meat swelling are smaller than found for PP and TPP (Table 1). This is in agreement with the findings on HMP by others (Bendall, 1954, Hellendoorn, 1962; Yasui et al., 1964).

The relative effect of the phosphates on meat swelling is comparable to their relative effect on pH. This indicates that the elevation of the pH is one of the important effects of the phosphates in regard to the meat swelling. However, this effect of TPP (and the commercial blend Curafos containing TPP) was greater on swelling than on the meat pH. This might be due to the hydrolytic effect of muscle ATP-ase on TPP, converting part of TPP to PP, which apparently has a specific swelling effect on lean meat in addition to its (PP) pH effect and ability to split myosin B (actomyosin) (Bendall, 1954; Sherman, 1961; Yasui et al., 1964). Based on the swelling data (Fig. 3 and Table 1), it appears that PP is probably the best phosphate for meats in which the binding of the added water is of practical importance.

To determine how condensed phosphates do effect swelling of meat in the presence of different concentrations of added sodium chloride (NaCl), ground semimembranosus muscle of beef was blended with aqueous solutions of NaCl ranging in concentration from 0–10%; the ratio of meat to the solutions was 1/3. The results of this investigation are shown in Figure 4. Blending of meat with 1 and 2% NaCl solutions increased the meat swelling, followed by a decrease with 3–5% NaCl in the solution and then by a rapid increase in the swelling with increasing the NaCl concentrations in the solutions from 5% to 10%. Addition of 0.5% TPP to the NaCl solutions increased the meat swelling, but did not change the general pattern of the change in the meat swelling with the NaCl additions. The results can be interpreted as follows: initial increase in the meat swelling is due to the replacement of Ca^{++} by Na^{+} on the meat proteins; the following decrease in the meat swelling is due to the exchange of Mg^{++} and K^{+} by Na^{+}; and the rapid increase in the meat swelling with increasing the NaCl concentration in the blending solutions from 5 to 10% is strictly an ionic effect of NaCl on meat proteins. This interpretation is based on the previous work by Wierbicki et al. (1963) on the relative effects of Ca^{++}, Mg^{++}, K^{++} and Na^{+} on meat swelling.

Effect on meat shrinkage

Effect of sodium chloride. The data in Figure 5 show the effect of sodium chloride, with 0.5% TPP or 0.5% HMP and without added phosphates on the shrinkage of beef during heating at 70°C for 30 min. When no phosphates are added, the lowest shrink (the highest water-holding capacity) is achieved at 3–5% additions of NaCl. However, when used with 0.5% TPP, the great reduction in shrink is obtained already with the addition of 1% NaCl reaching the greatest reduction at 4% NaCl addition followed by a rapid increase in the meat shrinkage by increasing the NaCl additions from 5% to 10%. The combined effect on the reduction of meat shrinkage using 0.5% TPP with 1–4% NaCl is twice as great as using NaCl additions alone. The combined effect of NaCl and TPP on the water-holding capacity of meat can withstand a prolonged heating period, for example 180 min at 65°C (Fig. 1).

The effect of 0.5% HMP in combination with NaCl was not as great as that of TPP with NaCl. The relative effect, however, with the increasing additions of NaCl, followed a similar pattern as that found for TPP.

The great synergistic effect of TPP with small amounts of NaCl (0.5 to about 1.5%) suggests a possibility of obtaining precooked beef items with a low shrink and none or only a slight salty taste.

Effect of phosphates. Results on the

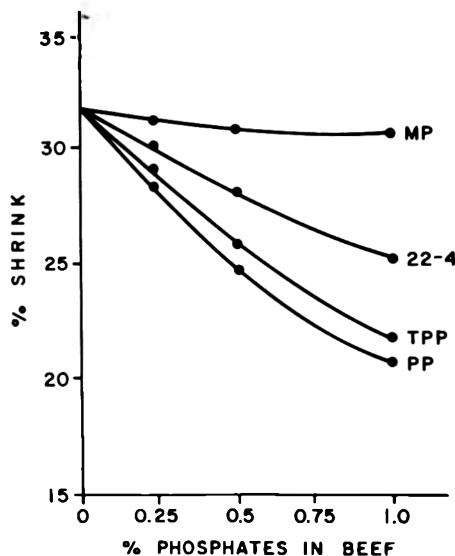

Fig. 6–Effect of various phosphates on the shrinkage of beef semimembranosus muscle at 70°C.

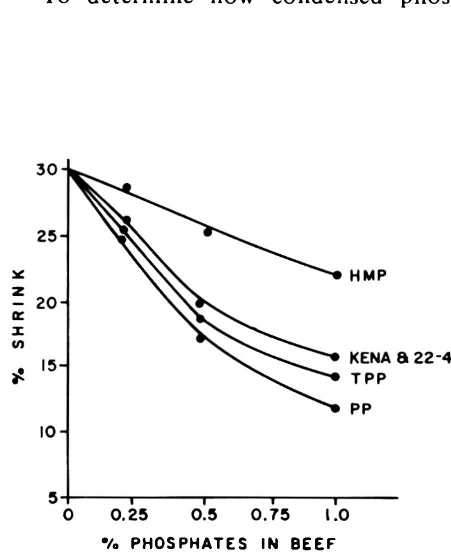

Fig. 7–Effect of HMP and Kena FP-28 in comparison with other phosphates on the shrinkage of beef semimembranosus muscle at 70°C.

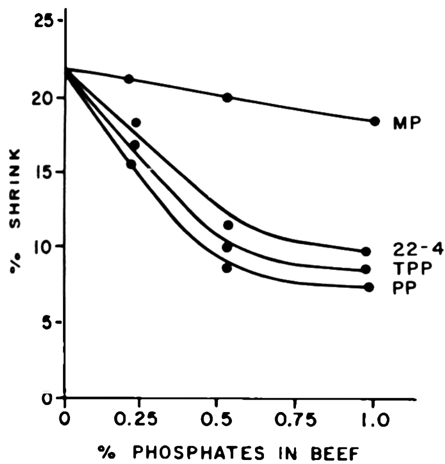

Fig. 8–Effect of various phosphates, in combination with 1.5% NaCl, on the shrinkage of beef semimembranosus muscle at 70° C.

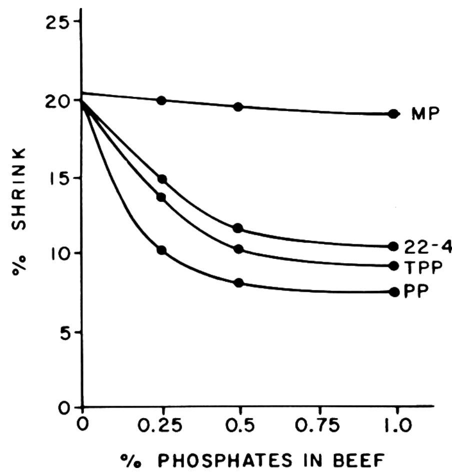

Fig. 9–Effect of various phosphates, in combination with 1.5% NaCl, on the shrinkage of beef semimembranosus muscle at 60° C.

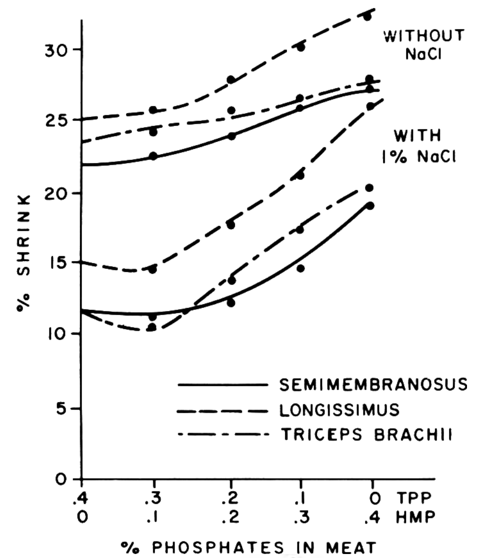

Fig. 10–Effect of various combinations of TPP and HMP, with and without salt, on the shrinkage of beef at 70° C.

effects of four polyphosphates without NaCl addition, on the beef shrinkage at 70°C are shown in Figure 6. The data indicate that PP and TPP are most effective in reducing the meat shrinkage, followed by the commercial polyphosphate mixture Curafos 22-4; however, there was practically no effect of MP.

In another experiment a comparison was made between HMP and commercial blend Kena FP-28 with the three most effective phosphates investigated previously (PP, TPP and Curafos 22-4), (Fig. 7). The results confirmed previous findings (Fig. 6) that PP has the greatest effect on shrink reduction followed by TPP and Curafos 22-4. The commercial blend Kena FP-28 had the same effect as Curafos 22-4 and was very close to the effect by TPP. However, the effect of HMP on the reduction of the meat shrinkage was very small.

Figure 8 shows the effect of different phosphates on the shrinkage of beef at 70°C in combination with 1.5% NaCl. PP, TPP and the commercial mixture Curafos 22-4 were most effective in reducing the meat shrinkage, with practically no effect by MP. However, contrary to when phosphates are used alone (Fig. 6 and 7), in a combination with 1.5% NaCl, the maximum effect of PP, TPP and Curafos 22-4 on the shrink reduction are achieved already with 0.5% additions of the polyphosphates with practically no further effect by increasing the polyphosphates additions to 1.0%. At a lower heating temperature than 70°C the maximum effect on the reduction of meat shrinkage is achieved at even lower additions of phosphates than 0.5%. Information given in Figure 9 indicates that the shrinkage of beef, with added 1.5% NaCl, is at a minimum of 0.25–0.3% additions of PP, TPP or Curafos 22-4. As shown previously (Fig. 6 and 8) MP cannot be used to control the shrinkage of beef during cooking.

Effect of phosphates on different muscles. Three muscles from the same carcass of the USDA Choice grade beef, were used in this study. They were longissimus (loin), semimembranosus (top round) and biceps femoris (bottom round) muscles. The effects of various phosphates with and without 1% NaCl, were investigated in regard to pH, swelling and the water-holding capacity (meat shrinkage at 70°C) of the meat samples. The results of this investigation are tabulated in Table 1. The data indicate that for all three muscles PP had the most pronounced effect on water-holding capacity, followed closely by TPP, Curafos 22-4 and Kena FP-28; HMP had the smallest effect. Pyrophosphate caused the greatest effect in the increase of pH and swelling in the three muscles. The two commercial blends, Kena FP-28 and Curafos 22-4 gave similar results for pH and the water-holding capacity. Hexametaphosphate was the least effective in increasing the pH and the meat swelling and reducing the meat shrinkage during cooking, thus confirming the previous results. Longissimus muscle appears to have the highest water-binding (swelling) properties and semimembranosus muscle the lowest water-holding (the highest shrink) capacity. Longissimus and biceps femoris muscles have similar water-holding properties.

Combined effect of tripolyphosphate and hexametaphosphate. The recent approval of the use of sodium tripolyphosphate and sodium hexametaphosphate by the U.S. Department of Agriculture in cooked and fresh beef (USDA, 1970) led to the testing of these two phosphates in various combinations on water-holding capacity of beef. Muscles selected for this study were the semimembranosus, longissimus and the triceps brachii of a USDA Commerical grade carcass of chilled beef. The ground meat samples, without and with 1% NaCl, were used for the shrink determination at 70°C. The data in Figure 10 show that 0.3% TPP and 0.1% HMP, when used with 1.0% NaCl, resulted in the greatest reduction in shrink for the three muscles investigated. As the amount of HMP in the phosphate mixture added to the meat increases, the shrink increases. In samples without the NaCl, the increase of shrink is also noted as concentration of HMP in the phosphate mixture is increased. In both instances, the longissimus muscle gave the greatest shrinkage (the lowest WHC) during the heating at 70°C.

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EFFECT OF FLUCTUATING STORAGE TEMPERATURES ON MICROORGANISMS ON BEEF SHELL FROZEN WITH LIQUID NITROGEN

INTRODUCTION

CHANGES in production and marketing procedures for meat in the U.S. for the past few decades have been recently described by Daly (1971). Improvements in efficiency of processing, handling and preservation of meats have come about partly because of use of temperature control and vacuum packaging. The use of liquid nitrogen for freezing with later transportation of wholesale cuts of beef may be advantageous for the continuous operations of centralized cutting and packaging of beef. Liquid nitrogen refrigerant has enabled transportation of meat over long distances with wholesomeness maintained.

Flash freezing with liquid nitrogen has been successfully used for several meat products (Kuschfeldt and Thiel, 1970; Li et al., 1969; Gray, 1967; Varner, 1966). Dixon and Robe (1966) have also reported "upgrade" arrival values of red meats by use of liquid nitrogen sprays for transportation.

Previous studies on bacteriological quality of retail cuts demonstrated that loins shell frozen with liquid nitrogen could be held at room temperature for 2 and 3 days without undue health hazard (Rey et al., 1971). Since diurnal variations in temperature are usually encountered during transport, further interest was focussed on the effect of fluctuating temperatures during holding of frozen loins.

In the present investigation, conventional air blast freezing or shell freezing with liquid nitrogen was followed by holding of the frozen primal cuts at varying temperatures. Microbiological spoilage and organisms on steaks prepared from the primal cuts were investigated.

MATERIALS & METHODS

BEEF LOINS obtained from different processing plants on the day of slaughter were divided into three wholesale cuts in the University Meats Lab. One of the wholesale cuts was divided into steaks, which served as controls. The initial quality of the fresh meat was tested by sampling immediately after arrival of the meat from the packing plant. Steaks from the fresh controls were packaged and stored under conditions similar to those in a retail store, with later analysis at intervals.

Another portion of the loins tested was shell-frozen for 6 min by a liquid nitrogen spray in a freezer tunnel. The remaining third of each loin was frozen to an internal temperature of −29°C in a cold room with forced air circulation at a temperature of −37 ± 3°C.

All the frozen loins were held in insulated boxes for 44 hr without further refrigeration.

Styrofoam boxes were used for holding the frozen loins as follows: (1) the boxes used were 14 × 14 × 14 in., with a thickness of 1¼ in., providing sufficient capacity to tightly pack the beef from each treatment, so that the box was almost completely filled; (2) empty boxes were first pre-cooled with either liquid nitrogen or air in the freezer room, in accordance with the treatment of the meat to be packed in them; (3) after sealing the liquid nitrogen and the air-frozen loins in separate boxes, the boxes were transferred through four different controlled temperature rooms in a sequence of 30°C for 8 hr, 15°C for 12 hr, 42°C for 9 hr and 25.5°C for 15 hr. This sequence of times and temperatures was chosen to represent possible environmental conditions during holding and air transport, and was considered to be a rather severe test. After holding, steaks from the frozen loins were prepared, packaged, stored and sampled under conditions identical to those of the controls. Detailed descriptions of the freezer tunnel and packaging and storage methods for the steaks were presented in our previous report (Rey et al., 1971).

Table 1—Bacteriological procedures employed

Quantitative determinations[a]	Growth media	Plating technique	Incubation	Confirmatory tests
Total aerobes	Trypticase soy agar (BBL)[b]	Pour plates	15°C, 5 days	
Fluorescent Pseudomonas	Medium B of King et al., 1954	Surface plating	15°C, 6 days	Fluorescence under UV light
Enterococci	KF Streptococcus medium (Difco)[c]	Pour plates	37°C, 2 days	
Coliforms	Violet red bile agar (Difco)[c]	Pour plates (overlayed)	37°C, 24 hr	Levine EMB agar (Difco)
Clostridium perfringens	SPS agar (Angelotti et al., 1962)	Anaerobic pouches (Bladel and Greenberg, 1965)	37°C, 24 hr	Motility and H_2S (Angelotti et al., 1962)

Qualitative determinations[d]	Enrichment	Isolation	Confirmatory tests
Salmonella	Procedure for meats (Galton et al., 1968)	BGS agar (Galton et al., 1968)	TSI and agglutination (Galton et al., 1968)
Coagulase positive Staphylococcus	Broth medium of Wilson et al., 1959	Staph 110 medium with egg yolk (Herman and Morelli, 1960)	Tube coagulase test

[a]A swab technique was used for sampling 10 cm^2 of surface.
[b]BBL Division of BioQuest, Cockeysville, Md.
[c]Difco Laboratories, Detroit, Mich.
[d]A swab technique was used for sampling 60−80 cm^2 of surface.

Bacteriological procedures employed in the analysis of the steaks are summarized in Table 1. The temperature of the frozen samples was recorded at the surface and the center during holding. An automatic recorder (Honeywell Potentiometer, Ft. Washington, Pa.) and properly calibrated copper-constantan thermocouples were used for these measurements. Analysis of variance and multiple range comparisons (Duncan, 1955) were performed on the data.

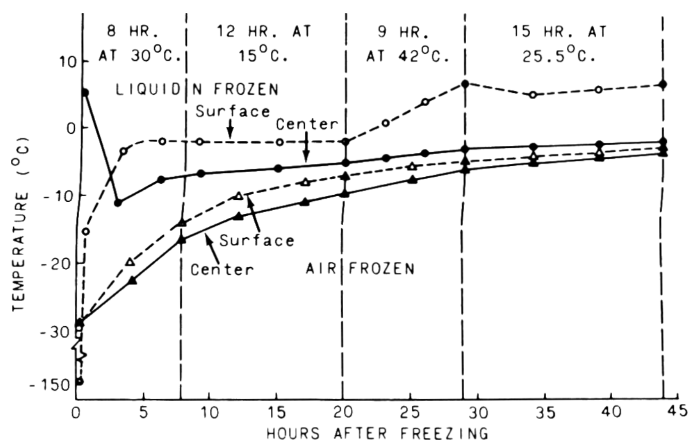

Fig. 1—Temperature changes of frozen loins during holding at fluctuating temperatures.

RESULTS & DISCUSSION

Temperature of the loins

Figure 1 shows that the loins frozen in air to −29°C were maintained in the frozen state for 44 hr during holding in insulated boxes. Only a small difference in temperature was observed between the center and the surface of these loins. Temperature fluctuations in the environment where the boxes were located during holding did not greatly influence the rate of heat absorption of the solidly frozen meat. The nature of the air blast freezing process results in complete freezing, in contrast to the shell-freezing produced by the liquid nitrogen spray.

The temperature at the center of loins shell-frozen with liquid nitrogen followed the same pattern as observed in our previous work (Rey et al., 1971). Minimum temperature was attained at about 3 hr after shell-freezing. Changes in environmental temperature did not cause fluctuations in the rate of heat absorption at the center of the loins. At the end of the holding period (44 hr), the shell-frozen loins were still solidly frozen at the center (−2°C), and the surface temperature was 6.5°C. In our previous work, the center of the loins dropped to −22.5°C, 3 hr after freezing, as compared to a minimum of −11°C in the present work. Possibly, some difference in the thickness of the ice crust at the surface may have accounted for differences in the minimum temperatures at the center, in the two phases of this investigation. Similar to previous findings, a rapid increase in temperature followed the minimum with the center reaching −2°C at the 44th hr of holding. It should also be noted that in the present work the environmental temperature of 30°C was higher than the ambient temperature of 22–28°C in the earlier experiments. This too could have influenced the difference in the minimum temperature at the center of the loins after 3 hr of holding, even in the insulated boxes. In the earlier work, the center of the loins reached a temperature of 0°C after 36 hr of holding. The loins also were completely thawed when removed from the boxes after 48 and 72 hr. In the current work, the center of the loins was still frozen after 44 hr. Differences in results as reported previously and as given here may be a result of the reduction of the ratio of air to frozen meat in the boxes. In both instances, the same type of insulating material was used for the boxes. In considering the entire temperature range in this fluctuating temperature study, it should be pointed out that the mean ambient temperature was 26.8°C, compared to 27.5°C, which was the mean of the ambient room temperature in the earlier studies. It is also of interest to note that the thickness of the boxes was less (only 1¼ in.) than that of the boxes used earlier (2½ in.). Evidently, the thickness of the box wall was not as important a determinant of temperature at the center of the meat as was the mass of shell-frozen meat itself in relation to the capacity of the box. Although the actual mass of frozen meat was approximately the same in both parts of this work, in the present study the meat almost completely filled the container since smaller boxes were used than in the earlier work. Less thermal energy from the environment within the boxes was available for absorption by the meat, and the "mass meat principle" cited by Gill (1969) created a continued refrigerating effect. The importance of filling the container to capacity, or at least nearly so, by the shell-frozen product is emphasized by these observations.

On the surface of the shell-frozen loins the temperature increased rapidly after freezing, in contrast to the center of the meat, since heat was absorbed from the environment as well as from the unfrozen center of the meat as the center became cooler. Equilibrium temperature between surface and center was −7°C, recorded 2 hr after freezing. After equilibration, the temperature of the center continued decreasing for another hour while the surface temperature increased. Once the thermal center of the meat reached its lowest reading, the rate of heat absorption on the surface greatly diminished, with practically no change in surface temperature during the remainder of the time the boxes were exposed to 30°C (5 additional hr) and then at 15°C (12 hr). When the containers were transferred to an environment at 42°C, the rate of heat absorption at the surface increased fairly rapidly, but only a slight change occurred in the rate of absorption at the center of the loins. Subsequent holding at 25.5°C caused a drop in

Table 2—Analysis of variance of bacteria recovered from steaks

		Mean squares			
Source of variation	d.f.	Total aerobes	Fluorescent Pseudomonas	Enterococci	Coliforms
A. Treatment of loins	2	7.421[a]	4.795[b]	0.702	8.522[a]
B. Storage time of steaks	4	178.246[a]	214.220[a]	29.001[a]	60.018[a]
C. Groups of loins	1	302.858[a]	838.117[a]	421.155[a]	1828.849[a]
A × B	8	1.520[b]	3.401[b]	4.501[a]	2.538[a]
A × C	2	13.653[a]	11.593[a]	36.492[a]	17.798[a]
B × C	4	11.133[a]	17.209[a]	32.966[a]	34.057[a]
A × B × C	8	2.092[a]	5.271[a]	3.807[a]	13.134[a]
Error	150	0.613	1.382	0.730	0.572

[a]Significant at 1% level
[b]Significant at 5% level

surface temperature of the meat during the first 5 hr, after which the temperature increased again, but at a very slow rate. Since the center of the meat absorbs heat from the surface at a constant rate, changes in the temperature differential between the environment and the meat surface would shift the balance between the amount of heat given up by the surface to the center and that taken up from the environment. Consequently, a fluctuation in surface temperature is produced with changes in the temperature of the surroundings, while little change occurs at the center.

Bacterial counts

The analysis of variance (Table 2) showed significant differences at the 1% level between treatments of the loins for total aerobic bacteria and coliforms recovered from the steaks, and at the 5% level for fluorescent *Pseudomonas*. Figure 2 presents numbers of total aerobes developing on the steaks stored at 5°C after being cut from the loins. Differences in bacterial numbers between treatments were not due to differences in initial counts on steaks at the beginning of storage, as may be seen from Figure 2, and as confirmed by sequential "t" tests for significance.

Total aerobic bacteria increased at a fairly rapid logarithmic rate after a very short lag phase on steaks from fresh loins (Fig. 2). In contrast, a longer lag phase was observed for organisms on steaks frozen by either liquid nitrogen or in air. The air-frozen samples, which, by the nature of the process, were maintained at the lowest temperatures during holding of the loins had a slight decrease in numbers of bacteria on the first day of storage of steaks. However, a rapid logarithmic growth phase followed, so that bacterial counts were approximately equal to those of samples from loins frozen cryogenically by the third day of storage at 5°C. Meat frozen by either of the two methods showed lower counts than did the fresh meat during the first 3 days of storage of steaks; differences were significant. This was a result of the delayed logarithmic growth observed for the frozen samples. Freezing undoubtedly caused metabolic injury to bacterial cells and some recovery time was needed for rapid growth to occur at 5°C; less injury may have resulted from cryogenic freezing that from freezing by air blast.

As with total aerobes, there was no significant difference in the initial numbers of fluorescent *Pseudomonas* on steaks from the three treatments. A longer lag phase followed by a rather rapid logarithmic growth phase was also observed for steaks from the two freezing treatments as compared with fresh meat. Figure 3 indicates that no difference was evident between treatments for initial contamination with coliforms, but the lowest coliform counts were maintained on steaks from air-frozen loins during storage of the steaks at 5°C. The relatively high susceptibility of coliforms to freezing has been well documented.

Whether differences in freezing rate between liquid nitrogen and air freezing methods, or lower temperatures attained by air freezing caused delayed growth of coliforms on air-frozen samples during storage at 5°C could not be determined from this study. Liquid nitrogen apparently had little effect on ability of coliforms to grow during later holding of meat at 5°C as compared with unfrozen beef, a finding in general agreement with our earlier results (Rey et al., 1971).

Occurrence of coagulase-positive staphylococci on steaks during simulated retail storage is presented in Figure 4. The greatest incidence corresponded to steaks from fresh loins, while the lowest was associated with air-frozen samples. The graph on the left in Figure 4 presents total percent of recoveries of coagulase positive staphylococci from steaks throughout the 7-day storage period at 5°C after the steaks were prepared from loins having different freezing treatments and from freshly cut controls. Freezing by air blast resulted in fewer samples positive for staphylococci than did shell freezing with liquid nitrogen. The graph on the right side of Figure 4 indicates that the percent recoveries of staphylococci from steaks prepared from freshly cut loins decreased progressively during storage of the meat at 5°C. For example, at the beginning of the storage period, 11 of 12 steaks (92%) from fresh loins demonstrated coagulase positive staphylococci, but after 7 days, no such organisms were recovered.

None of the samples tested yielded *Clostridium perfringens,* indicating that the temperature of the meat was sufficiently low during holding and refrigerated storage to inhibit the organism, or that sanitation practices were adequate to prevent recovery if the organism were present initially on loins before handling.

Salmonellae were recovered from 5 of

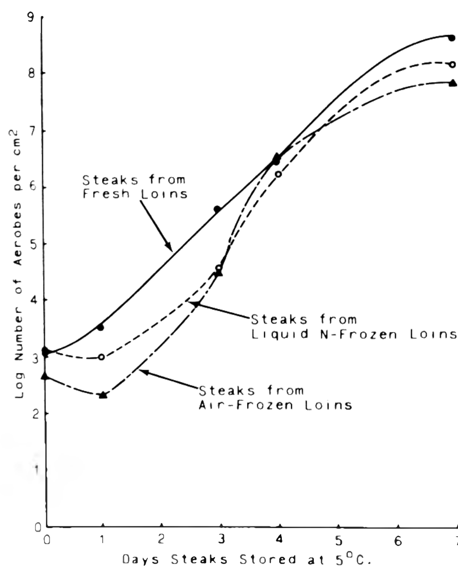

Fig. 2–Numbers of aerobic bacteria recovered from steaks prepared from fresh and frozen loins.

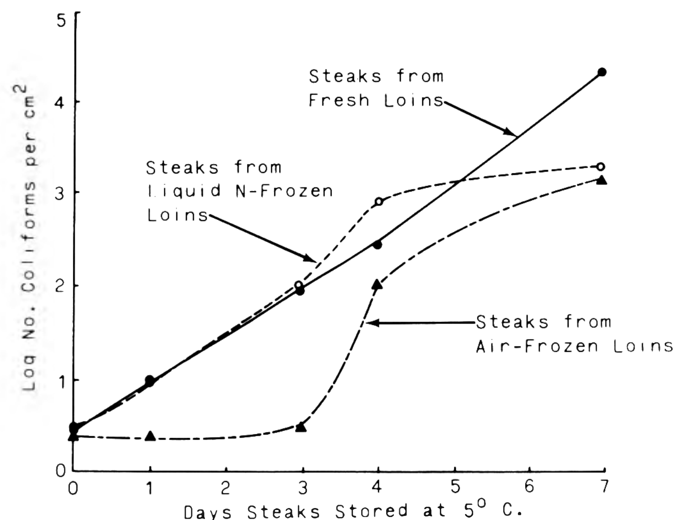

Fig. 3–Numbers of coliforms recovered from steaks from fresh and frozen loins.

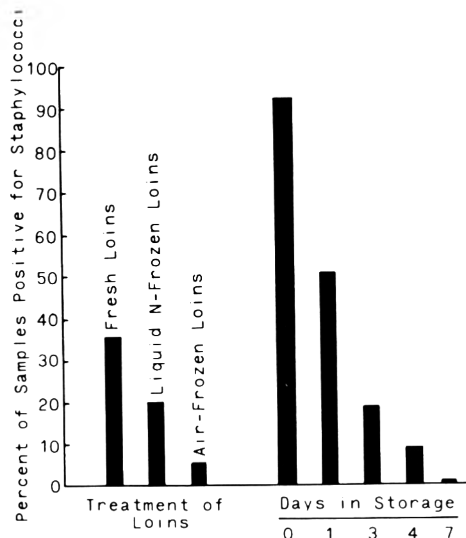

Fig. 4–Incidence of coagulase-positive staphylococci on steaks during storage at 5°C. On left: Total percent recoveries during entire 7-day storage period; on right: Percent recoveries on steaks from freshly cut loins only.

the 60 steaks analyzed from fresh loins, 10 of the 60 from liquid nitrogen-frozen loins, and 2 of the 60 steaks from loins frozen in air. However, no conclusions should be made regarding treatment effect on *Salmonellae*, since the highest recoveries were made from loins that had the greatest initial contamination with coliforms at the time steaks were prepared, regardless of treatment. The degree of contamination rather than low temperature treatment applied to the loins influenced the occurrence of the organism on the retail cuts. The meat was maintained at temperatures that are not conducive to growth of *Salmonellae*; recoveries of the organism probably were associated with degree of initial contamination.

Work done by Angelotti et al. (1961) agreed with earlier studies on growth of certain pathogens in refrigerated foods. These workers stated that no significant multiplication of *Salmonellae* or staphylococci occurred for at least 5 days in foods that they tested during storage between 4.4 and 5.6°C. In our study, comparable results were obtained for incidence of the bacteria during holding of steaks at 5°C. Low temperature storage of meat has been well established as a means of retarding development of food poisoning organisms; these results lend emphasis to this concept.

Further work is in progress in an attempt to characterize the types of organisms present and to determine if differences in flora, such as coliform types, resulted from the different freezing methods.

In summarizing this work, the following conclusions may be made: 1. The absorption of heat from the environment can be retarded for frozen meat during transport and holding in insulated boxes without refrigeration by good insulation and packing of large cuts to fill the boxes to maximum capacity; 2. From observations on the solidly frozen meat from air-blast freezing, indications are that increases in the depth of the frozen shell on liquid nitrogen treated meat would provide more efficient maintenance of the low temperature of the meat; 3. With low heat absorption maintained during transport and holding, freezing by liquid nitrogen or air blast could effect an increase in the lag phase of spoilage organisms on the meat, resulting in an advantage for preservation of subsequent retail cuts for about 3 days. This effect, however, may be neutralized during 3–4 days of continued retail storage because of later rapid growth of the surface bacteria; 4. Shell freezing with liquid nitrogen for transportation and holding of wholesale cuts of beef under conditions of marked external temperature changes such as those used here would not increase the probabilities of occurrence of food-borne disease organisms on the retail cuts.

It is emphasized that plants must operate with good sanitary practices to insure that the meat be of good bacteriological quality if the procedures described here are to be successful.

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EFFECT OF CONTROLLED GAS ATMOSPHERES AND TEMPERATURES ON QUALITY OF PACKAGED PORK

INTRODUCTION

FRESH MEAT is one major food product which is still processed and packaged at the retail level. The concept of centralized packaging has been applied in the processing and distribution of cured and cooked meats, but has only recently been applied to fresh meats. Methods of packaging fresh meats designed to enhance desirable color development also permit aerobic bacterial growth.

The most common organisms associated with spoilage of meats are of the *Pseudomonas-Achromobacter* types, which are highly aerobic and are generally surface contaminants (Ayres et al., 1950; Kirsch et al., 1952; Rogers and McCleskey, 1957; Halleck et al., 1958; Ayres, 1960; Gardner et al., 1967; Clark and Lentz, 1969; and Pierson et al., 1970). Although these are the predominant types, lactobacilli, *Micrococcus, Bacillus, Aspergillus niger, Microbacterium* and others have been reported on fresh meats (Sulzbacher and McLean, 1951; Kirsch et al., 1952; Rogers and McCleskey, 1957; Barlow and Kitchell, 1966; Stringer et al., 1969).

Oxygen concentration must be drastically reduced to affect aerobic bacterial growth (King and Nagel, 1967; Shaw and Nicol, 1969; Ledward et al., 1971; Baran et al., 1970; Clark and Lentz, 1969; Gardner et al., 1967). Carbon dioxide inhibition of aerobic bacterial growth was found to increase as temperatures were lowered (Clark and Lentz, 1969). Carbon dioxide has been found to inhibit aerobic growth while not affecting lactic acid producing bacteria (Kraft and Ayres, 1952; Ogilvy and Ayres, 1953; Gardner et al., 1967; King and Nagel, 1967; Clark and Lentz, 1969; Shaw and Nicol, 1969; Baran et al., 1970; Ledward et al., 1971). The relative proportion of lactic acid producing bacteria has been shown to increase on meats packaged anaerobically or in gas impermeable films (Jaye et al., 1962; Gardner et al., 1967; Baran et al., 1970; Pierson et al., 1970).

Color of fresh meats is of primary importance to consumers who assume that an acceptable color of lean indicates freshness. Color of meat is related to the heme pigments, particularly myoglobin (Fox, 1966). The autoxidation rate of myoglobin in vitro is a first order reaction which increases as temperature increases (George and Stratmann, 1952; Snyder and Ayres, 1961; Brown and Dolev, 1963).

The objective of this study was to investigate the effects of storage at two different temperatures in air and in another gas mixture on the microbiological, color and sensory qualities of boneless pork chops.

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MATERIALS & METHODS

Preparation and storage of samples

Pork used in this study was from pigs slaughtered and cut by conventional methods at the Auburn University Meats Lab. Three loins were chosen for a uniform color score of three for replications one and two, and a score of four for replication three using the Wisconsin (1963) standards as a guide.

During preparation of chops, loins were handled with plastic gloves, and all equipment was sanitized with a 200 ppm chlorine spray before each loin was cut. A section of each loin from the tenth thoracic vertebra to a point approximately 7.6 cm anterior to the junction of the lumbar sacral vertebrae was boned and sliced into 1.5 cm thick chops 24 hr after slaughter. Each chop was placed in a styrofoam tray, overwrapped with 0.5 mm plasticized polyvinyl chloride (PVC) film and heat sealed. Chops were randomly assigned to treatments as follows: (1) 2.2°C air (control); (2) 2.2°C gas; (3) −2.2°C air; and (4) −2.2°C gas. Chops were stored in the dark for periods of 0, 5, 9 and 15 days post packaging. Storage temperatures were maintained within 0.5°C during the storage period. A total of 144 chops were used in this study. Ledward et al., 1971, have shown that a combination of low O_2 (0.2%) and 25% CO_2 will inhibit bacteria. While it is accepted that increased O_2 tension improves color of fresh meat, Rikert et al. (1958), report no benefit from the use of O_2 tensions above that of air. Based on these reports the gas mixture used was 25% CO_2 to inhibit microbial growth, 5% O_2 for improved color development and 70% N_2 as filler.

A gas mixture was produced using a three module National simet proportional mixing valve. Packaged chops were placed in a saran bag, a vacuum of 50.8 cm Hg pulled, and the gas forced into the bag by pressure until filled. The vacuum was pulled again, and the gas was forced into the saran bag. The end of the bag was closed twice with metal clips, doubled and wrapped with a rubber band. The bags were placed at the selected temperatures for storage. No problems were encountered with bags leaking.

Microbiological analysis

At each storage time sample packages were aseptically opened, and the sample was placed in a sterile jar. The styrofoam tray was rinsed with 99 ml of sterile, buffered, distilled water which was poured into the jar containing the sample. The sample was rinsed on a Burrell wrist action shaker at a setting of 5 for 5 min. Unpublished research (Cannon, 1972) has shown that this standard procedure is adequate to remove surface organisms from fresh meat. After appropriate dilutions were made from this rinse, the chop was removed, the outline traced on acetate paper, and surface area calculated using a compensating polar planimeter to measure the surface area of the loin eye. A linear planimeter was used to determine circumference. Total surface area was calculated by adding the chop thickness times the circumference to the areas of both sides. Aerobic plate counts were made using the procedure recommended by the American Public Health Association (Sharf, 1966), and counts were expressed as numbers per cm^2 of total surface area. Plates were incubated at 32°C for 48 hr. An average of three plate counts was reported as numbers of organisms per cm^2.

Lactic acid producing bacteria were counted using McCaskey's (1971) modification of Kulp and White's (1932) method which involved mixing 0.75% calcium carbonate with tomato juice agar and counting only those colonies surrounded by a clear zone.

Anaerobic bacteria were grown on plate count agar in a Brewer anaerobic jar (Brewer and Allgeier, 1966) at 32°C for 48 hr. Since oxygen was not removed from the medium, the organisms that grew were microaerophiles, not strict anaerobes.

Color evaluation

Cool white lighting was installed to produce a color temperature of 4,000°K. and an intensity of 125 ft-c at the meat surface. A trained panel scored color using the Wisconsin (1963) scale. Objective color evaluations were made using the reflectance method of Ockerman and Cahill (1969).

Sensory evaluation

Immediately upon removal from storage overall acceptability (flavor, tenderness and juiciness) of cooked chops was rated by a six-member trained panel on a nine-point hedonic scale. Two chops from each of the four treatment groups were broiled in a preheated electric oven 5 min on the first side, turned and broiled to an internal temperature of 57°C (approximately 3 min). Chops were trimmed free of external fat, quartered, cooled to room temperature (to standardize panel evaluation) and served to the panel. Panel evaluations were made under red lights to eliminate bias because of color differences of cooked chops. Each panelist evaluated one sample from each of the four treatment groups at each session.

RESULTS & DISCUSSION

SINCE PRESENT commercial practice uses air permeable films for meat packaging and storage at a temperature of approximately 2.2°C, it is valid to consider this treatment group the control in this study.

Aerobic bacterial counts

Initial aerobic counts ranged from $7.35 \times 10^2/cm^2$ to $1.09 \times 10^2/cm^2$. Figure 1 shows the effect of temperature on the numbers of aerobic bacteria at the storage periods studied. Bacterial counts of meat stored at −2.2°C were lower ($P < 0.20$) than controls throughout storage. The lag phase was more pronounced for bacteria at −2.2°C but the numbers increased steadily from the 5-day storage period to completion. This is in agreement with reports of Jaye et al. (1962) and Gardner et al. (1967).

Figure 2 shows the difference in numbers of aerobic bacteria as affected by storage in air (control) and storage in gas. Meat stored in gas had lower ($P < 0.20$) counts for each period studied than controls. For the first 9 days, numbers of bacteria increased more rapidly on meat in air storage than in gas storage. Other investigators (King and Nagel, 1967; Shaw and Nicol, 1969; Ledward et al., 1971) have shown that low oxygen concentrations will not inhibit aerobic growth unless the concentration is less than 5%. Since the oxygen concentration in the gas mixture was 5% (to improve color), it is unlikely that low oxygen concentration was the inhibitory factor. Results of this study agree with other workers (Kraft and Ayres, 1952; Ogilvy and Ayres, 1953; King and Nagel, 1967; Clark and Lentz, 1969; Shaw and Nicol, 1969; Baran et al., 1970; Ledward et al., 1971) that carbon dioxide at the level used in this study appears to inhibit aerobic bacterial growth on pork.

The aerobic bacteriological changes occurring as a result of the interaction between temperature and gas treatments are presented in Figure 3. Meat stored in gas at −2.2°C had the lowest ($P < 0.05$) count at the final storage period.

The first changes in aerobic bacterial numbers occurred during the storage period of 5 days. Meat stored in air at −2.2°C, in gas at −2.2°C, and in gas at 2.2°C showed lower numbers than the control (air at 2.2°C). This can be attributed to the lower temperature of the gas mixture or to the interaction of these two treatments.

Neither gas treatment showed a change in percentages of aerobic bacteria. There appeared to be no decrease in bacterial numbers in the 2.2°C air storage treatment, while bacteria in the other treatments showed decreased numbers. Lag phases of bacteria in −2.2°C air and in 2.2°C gas were parallel. Aerobic bacterial numbers increased only in the control treatment at this period.

After 9 days storage the meat in the control treatment had the highest numbers of aerobic bacteria and the greatest increase over the previous storage period. The −2.2°C air and 2.2°C gas treatments also resulted in increases.

For the period 9 to 15 days, numbers of aerobic bacteria in the control treatment leveled off while numbers of bacteria in −2.2°C air and in 2.2°C gas increased most rapidly. Meat stored at −2.2°C in a gas mixture had a total count after 15 days storage that was just slightly higher than the original count.

During the entire study, meat stored at 2.2°C in air (control) showed increased numbers of aerobic bacteria and no apparent lag phase. Counts at the 9-day storage period exceeded most of the reported levels for off odor or slime (Greer, 1933; Elford, 1936; Ayres et al., 1950; Kraft and Ayres, 1952; Ayres, 1960; Stringer, 1963; Reagan et al., 1971). Only one sample at the 15-day storage period had developed off odor. None of the samples developed slime. Kirsch et al. (1952) is the only investigator reporting a higher level, 5×10^9 organisms per gram, for spoilage of beef. This study indicates a level of 10^9 organisms per cm^2 for spoilage of pork when the wash method is used to determine numbers of organisms present.

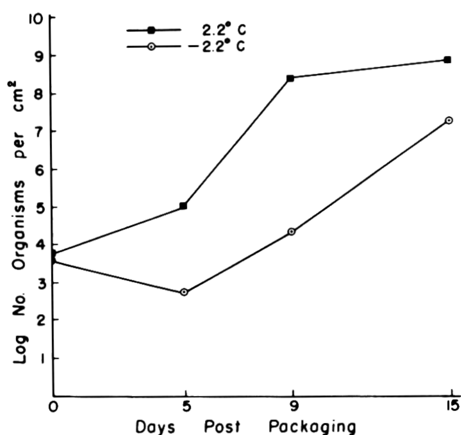

Fig. 1—Effects of temperature on aerobic bacteria.

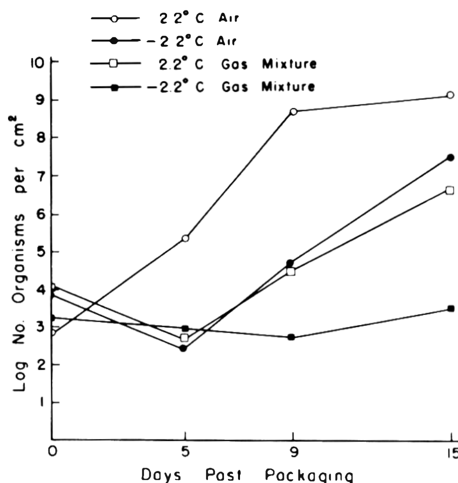

Fig. 3—Effects of temperature and storage atmosphere on aerobic bacteria.

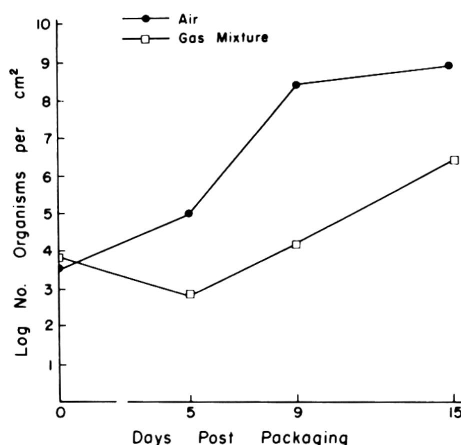

Fig. 2—Effects of storage atmosphere on aerobic bacteria.

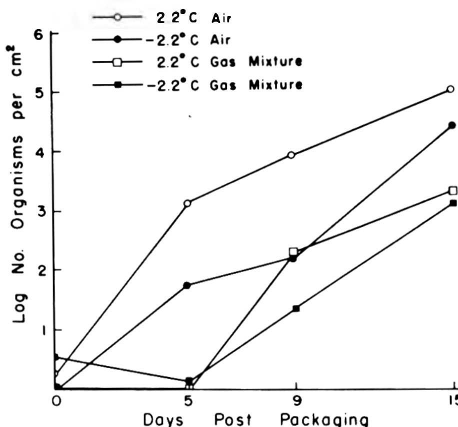

Fig. 4—Changes in anaerobic bacteria.

Anaerobic bacterial counts

Although there were no statistically significant differences in anaerobic bacterial numbers, Figure 4 graphically shows the changes that occurred during the study. The samples stored in air were higher in total anaerobic bacteria than the samples stored in gas. Storage treatment of −2.2°C gas showed increases in anaerobic bacteria at 9 days and 15 days. This effect would be expected with an increase in carbon dioxide in the gas mixture.

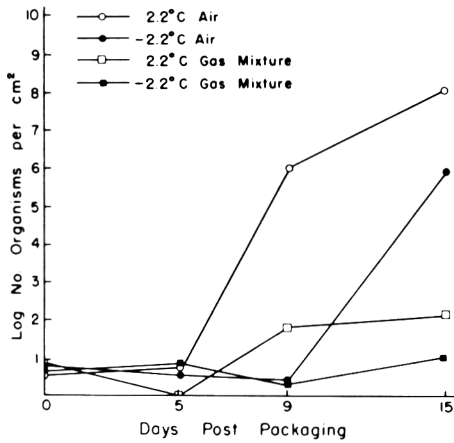

Fig. 5–Changes in lactic acid bacteria.

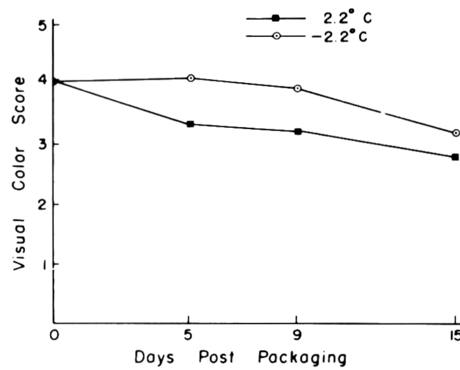

Fig. 6–Changes in color panel ratings.

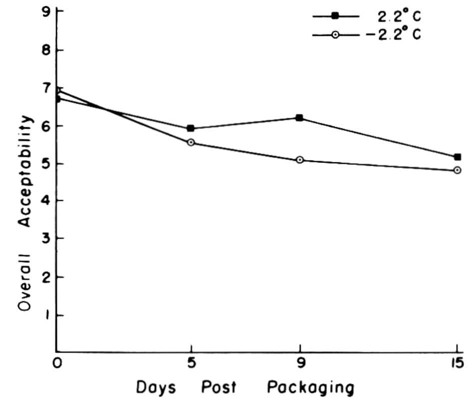

Fig. 7–Changes in sensory evaluations.

However, there was no increase in percentage of anaerobic bacteria in the gas storage at 2.2°C.

Lactic acid producing bacteria

Populations of lactic acid producers stored in air had higher counts than samples stored in the gas mixture, Figure 5. The higher numbers of lactic acid producers reported in the air storage treatments are probably a result of increased total numbers of microorganisms. Analysis of variance indicates that neither temperature nor gas storage affected relative populations of lactic acid producers.

Color evaluation

Days of storage (Table 1) and temperature treatment (Fig. 6) had a highly significant ($P < 0.01$) effect on color as rated by the panel. However, storage atmosphere did not affect color of pork chops. The average initial color score was 4.1 and decreased during the study to 2.9. The color changed, probably as a result of metmyoglobin formation, from the desirable grayish pink to a less desirable brownish or tan color as storage time increased. It is possible that dehydration also played a role in color alteration.

The −2.2°C treatments were rated darker than chops stored at the higher temperatures, otherwise changes in color during storage were similar. The data show that meat in the higher temperature storage was lighter.

It should be noted that reflectance measurements increase as the color becomes lighter, while visual color scores increase as color becomes darker. Statistical analysis of the reflectance measurements showed a difference due to the days in storage ($P < 0.01$). The difference because of the storage temperature approached significance at the five per cent level. No difference was shown as a result of the storage atmosphere.

Effects of storage time on reflectance measurements are shown in Table 1. These data agree with the color panel ratings by showing that pork color became lighter as storage time increased.

The overall correlation coefficient between the color panel score means and reflectance measurement means was −0.70 ($P < 0.05$). The range for individual panel members was from −0.45 to −0.76.

Table 1–Effect of storage on color and acceptability of boneless pork chops

Storage period (days)	Color panel score[a]	Reflectance measurements %	Taste panel score[b]
0	4.1[c]	40[c]	6.9[c]
5	3.7[c]	48[d]	7.7[c]
9	3.6[c]	46[d]	5.6[d]
15	2.9[d]	50[d]	5.0[d]

[a]Wisconsin (1963) color standard
[b]9-point hedonic scale
[c,d]Means having a different superscript are different $P < 0.05$.

Sensory evaluation

Sensory evaluation included tenderness, juiciness and flavor. Statistical analysis of sensory panel data showed that chops became less desirable during storage ($P < 0.01$).

Except for the initial evaluation, pork stored at the higher temperature was rated consistently higher than pork stored at the lower temperature (Fig. 7). A decrease in overall acceptability was noted at both storage temperatures during the study, probably because of normal deterioration of flavor as a result of storage.

No differences in sensory values were noted resulting from gas atmospheres.

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JFS BASIC SCIENCE

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ANTIOXIDANT EFFECT OF PROTEIN HYDROLYZATES IN A FREEZE-DRIED MODEL SYSTEM

INTRODUCTION

RECENT YEARS have seen the introduction of a veritable cornucopia of prepared and convenience foods for institutional feeding and for the retail consumer market. Many of these, especially the dehydrated foods, would become unacceptable very rapidly were it not for the addition of antioxidants.

Only a few antioxidant materials are approved for food use. The primary antioxidants are phenolic substances, most of these synthetic. Their use is being questioned by consumers at a rapidly increasing rate. The removal of nordihydroguaiaretic acid (NDGA) from approval (Federal Register, 1968) may fore-token other delistings. This possibility should act to spur the search for antioxidant substance from natural sources, and particularly for nonphenolic materials.

Antioxidant activity in proteinaceous materials has been reported by many workers. Bishov et al. (1960) Bishov and Henick (1961, 1962) observed stabilization by intact protein in dehydrated model systems. The effects of amino acids as antioxidants have been reported by Marcuse (1962), Bishov and Henick (1964) and Karel and Tannenbaum (1966). The latter workers found that the amino acids acted only to prolong the induction period of autoxidizing lipids, whereas phenolic antioxidants also reduced the rate of the rapid oxidation

Table 1—Gel fractionation of protein hydrolyzates

Fraction	Recovered % AYP	HVP
On G-10	85.5	37.8
On G-15	6.0	26.3
Thru G-15	8.3	39.6
Total	99.8	99.7

Table 2—Antioxidant effects of protein hydrolyzates[a]

Sample	Induction period,[b] hr 10%[c]	25%[c]
AYP-I	145	147
AYP-II	190	143
AYP-III	140	140
HVP-I	120	120
HVP-II	97	107
HVP-III	60	93
Control	24	24

[a]Freeze-dried model systems containing 1g CMC, 1g tocopherol-free corn oil and protein hydrolyzate as indicated
[b]Time to consume 50% of oxygen from air in headspace at 65.5°C
[c]Oil basis

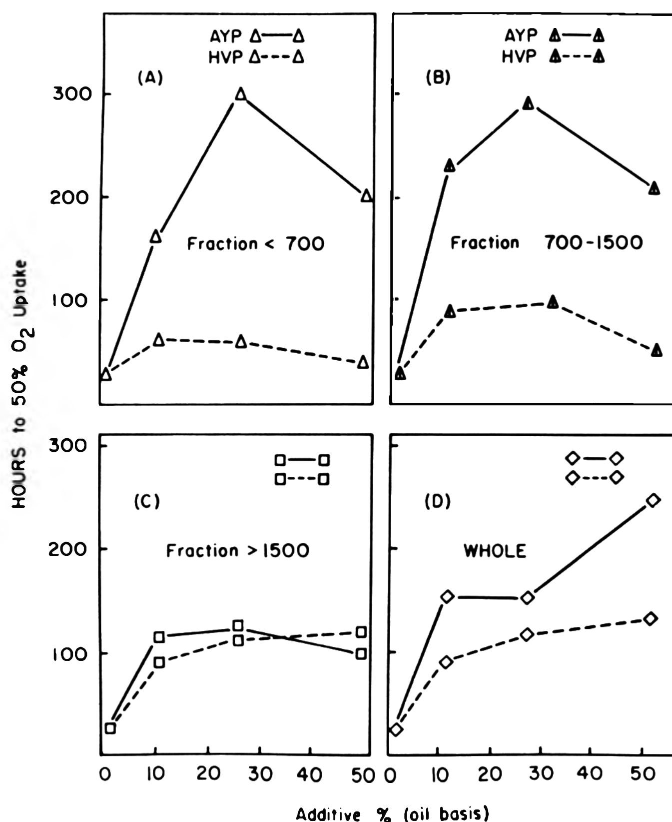

Fig. 1—Antioxidant activity of molecular sized fractions of AYP and HVP in freeze-dried emulsions.

Table 3–Antioxidant effects of nucleotides[a]

Sample	Induction period,[b] hr 10%[c]	25%[c]
AYP	145	147
Nucleotide[d]	107	138
AMP	58	68
IMP	24	47
Control	24	24

[a]Freeze-dried model system containing 1g CMC, 1g tocopherol-free corn oil and hydrolyzate fraction as indicated
[b]Time to consume 50% oxygen from air in headspace at 65.5°C
[c]Oil basis
[d]Laboratory separation from AYP

Table 4–Synergistic effects of protein hydrolyzates with phenolic antioxidants[a]

%[b]	0 I.P.[c] hr	10 I.P. hr	10 Syn%[d]	25 I.P. hr	25 Syn%	50 I.P. hr	50 Syn%
BHA			AYP%[b]				
0	39	131		140		200	
.005	59	221	38	267	47	326	37
.010	64	255	46	305	53	370	74
.020	98	307	44	447	61	498	52
			Mean = 50.2				
α-tocopherol							
0	24	78		116		132	
.02	48	156	56	198	33	200	25
.04	42	137	54	177	28	236	40
.06	55	178	58	227	39	254	40
.08	59	184	57	224	36	240	38
			Mean = 42.0				
BHA			HVP%[b]				
0	14	90		123		158	
.005	42	189	40	275	48	373	52
.010	96	253	34	333	40	440	47
.020	116	386	52	458	53	495	49
			Mean = 46.1				
α-tocopherol							
0	14	90		123		158	
.02	27	160	39	207	33	238	30
.04	33	195	47	227	40	277	38
.06	52	237	49	257	40	260	26
.08	34	162	35	204	32	242	28
			Mean = 36.4				

[a]Freeze-dried model systems containing 1g CMC, 1g tocopherol-free corn oil and antioxidant meterials as indicated
[b]Oil basis
[c]Time to consume 50% of oxygen from air in headspace at 65.5°C; average of duplicate determinations
[d]Percent of the observed effect due to synergism: Syn % = 100 $|(M-C) - (P-C) - (A-C)|/(M-C)$ where M = I.P. of combined treatment; P = I.P. of phenolic antioxidant; A = I.P. of AYP; and C = I.P. of control

phase. A most interesting, but still unexplained, resistance to antioxidation was shown in linoleate salts of lysine and arginine synthesized by Chang and Linn (1964). This phenomenon was studied at length by Koch et al. (1971). In a study of factors affecting the acceptance and stability of a soup and gravy base, Bishov et al. (1967) observed an apparent antioxidant activity in the hydrolyzed vegetable protein (HVP) component.

These findings led directly to the present work which was undertaken to determine the effectiveness of protein hydrolyzates as antioxidants and to identify, if possible, the active agents. Because of their availability, autolyzed yeast proteins (AYP) and HVP were chosen for initial investigation.

EXPERIMENTAL

Materials

Commercial AYP and commercial HVP (Vico-Asmus Products Div., A.E. Staley Manufacturing Co. and Food Ingredients Division, The Nestle Co., Inc.) were used as received and after the fractionations described in "Methods." Carboxy-methyl-cellulose (CMC) (Hercules Manufacturing Co.), tocopherol-free corn oil and butyl hydroxy anisole (BHA) (Distillation Products Industries Div., Eastman Chemical Products, Inc.), adenosine monophosphate (AMP) and inosine monophosphate (IMP) (Nutritional Biochemical Corp.) and destran gels (Sephadex G-10 and G-15) (Pharmacia Fine Chemicals A.B.) were used as received.

Methods

Fractionation of AYP and HVP by molecular size was accomplished in a batch procedure successively on Sephadex G-10 and G-15. Typically 100g of Sephadex G-10 was added, with stirring, to 100g of the hydrolyzate dissolved in 300 ml of water. After additional stirring the mixture was held overnight at 4°C and then filtered with suction. Fresh Sephadex G-10 (100g) was added to filtrate and the treatment was repeated for a total of four cycles. The final filtrate was treated in a similar manner with Sephadex G-15.

The final filtrate after treatment with both grades of Sephadex contained molecules larger than about 1500 molecular weight. The material included by the gels was eluted, from each batch separately, by successive water washings with a total volume of 1100 ml/100g of gel. Final elution was with three 100 ml portions of 95% ethanol/100g of gel. Eluates and washings from each grade of gel were combined and the volume reduced to about 250 ml on a vacuum rotary evaporator. The eluate from G-10 contained molecules smaller than about 700 molecular weight and from G-15 those between about 700 and 1500. These solids were recovered by freeze drying.

A nucleotide-rich fraction was separated from AYP by an alcohol extraction, ion-exchange procedure described by Mabrouk (1972).

Model systems for oxidation studies were prepared individually by adding (in succession) to a small blender cup, 50 ml water, the antioxidant material (AYP, HVP, their fractions, BHA, tocopherol) as required (see Tables and Figures for amounts), 1g CMC and dropwise, with intermittent mixing, 1g of tocopherol-free corn oil. The entire mixture was blended for 2 min, transferred to a 250 ml round bottom flask, shell frozen and freeze dried (Bishov et al., 1960).

Oxidation of the model system was carried out in the drying flask under an atmosphere of air at 65.5°C. The flasks were closed with rubber serum stoppers. Oxygen in the headspace was determined periodically by gas chromatography (Bishov and Henick, 1966). The end of the induction period was taken, arbitrarily, when 50% of the original oxygen had been consumed.

RESULTS & DISCUSSION

Fractionation of protein hydrolyzates

Typical recovery of fractions from the Sephadex separation of the hydrolyzates is shown in Table 1.

Antioxidant effects of protein hydrolyzates

A number of commercially-available hydrolyzates, both AYP and HVP, were screened for antioxidant effect using the freeze-dried model system. Results of a run are shown in Table 2. It can be seen that all samples show an effect and that in some the effect is concentration dependent. Sample AYP-II exhibits a bi-

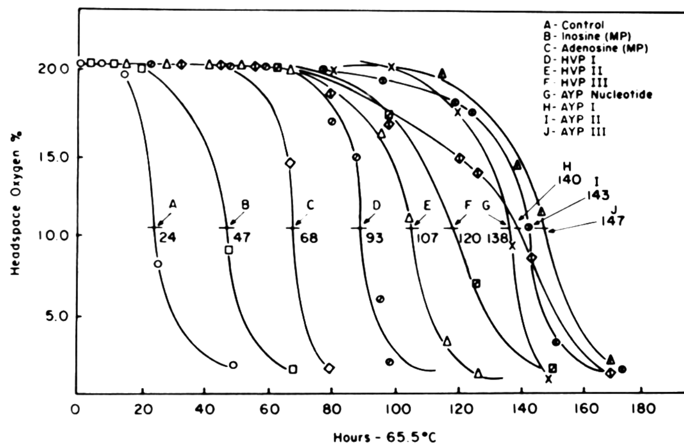

Fig. 2–Oxygen uptake by corn-oil, CMC freeze-dried emulsions with added protein hydrolyzate.

phasic behavior, decreased effect with increasing concentration, often seen in other antioxidants.

The molecular size (separated on Sephadex G-10 and G-15) fractions from one AYP and one HVP were similarly screened as seen in Figure 1. The smaller sizes, up to 1500, of AYP were more effective than the entire material.

The differences in the effectiveness of AYP and HVP are noticeable. It was thought that the difference might be due to the high salt content of HVP. Tests using salt-free HVP, however, showed that this was not the case. The milder conditions of autolysis compared to acid hydrolysis suggested that in AYP production the nucleotides might be better preserved. The effects of a nucleotide fraction isolated from AYP and of commerical AMP and IMP are seen in Table 3.

An autoxidation rate study is summarized in Figure 2. It can be seen that the sole effect of these experimental stabilizing agents is to prolong the induction period. No differences are noted, except for one sample of AYP (Curve H, Fig. 2) in the rapid rate of autoxidation after the end of the induction period.

Synergism with phenolic antioxidants

The effects of AYP and of HVP in concert with BHA and with α-tocopherol are seen in Table 4. In this system BHA is a much more effective antioxidant than is α-tocopherol. Its effect is also much more concentration dependent. These hydrolyzates are synergistic with both of these phenolic antioxidants. The extent of the combined effect due to synergism is shown in Table 4, as is the method by which it was calculated. Although the values appear to vary widely and do not follow any consistent trend, the effect is significant, reproducible and above experimental error.

This synergism may be used to good advantage in foods. Alone, 10% AYP (oil basis) appears to be the equivalent of 0.02% (oil basis) of BHA. But when combined with only 0.005% of BHA, the total effect is as great as that of 50% AYP or twice that of 0.02% BHA. Evaluation of these effects in typical foods is now underway and preliminary results appear to be promising.

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SENSORY EVALUATION USING COMPOSITE COMPLETE–INCOMPLETE BLOCK DESIGNS

INTRODUCTION

IN SENSORY EXPERIMENTS where the objective is the comparison of preferences of different samples or treatments expressed by a panel of judges, the statistical design has often been selected from the class of randomized block designs (Amerine et al., 1965). When each panelist or judge (known as the block in the design) can effectively rate each sample or treatment, the blocks are complete, i.e., each panelist or block evaluates each treatment. If on the other hand the number of treatments is larger than can be effectively rated by a panelist, then an incomplete block design is used. With both designs, a measure of the differences in preferences for the samples as well as some measure of difference among the panelists can be obtained.

When the number t of treatments is small, say $t \leqslant 5$, it may be feasible to augment the size of the block so that in addition to containing first replicates of each of the treatments, each block contains second replicates of k of the treatments $(1 \leqslant k < t)$. The construction of these blocks might be visualized as combining complete blocks of size t with balanced incomplete blocks of size k to form composite complete-incomplete blocks of size t + k. An example with t=3 and k=2 is the three block design presented in Figure 1 where A, B and C denote the three treatments.

In the construction of the composite complete-incomplete block designs, we shall require only that the incomplete block portion of the composite blocks be balanced. That is to say, if there are b incomplete blocks each of size k in which to assign t treatments $(t > k)$ at random, then each treatment will appear in (bk)/t of the incomplete blocks (as well as in all b complete blocks) and every pair of treatments will occur together in $[bk(k - 1)]/[t(t - 1)]$ incomplete blocks. Thus the numbers (bk)/t and $[bk(k - 1)]/[t(t - 1)]$ must both be integers in order for each treatment to appear r times where r = $[b(k + t)]/t$, in the b blocks each of size k+t. For example, in Figure 1, each treatment appears in $[3(2)]/3 = 2$ incomplete blocks and each pair of treatments is replicated together in $[3(2)(2 - 1)]/[3(2)] = 1$ incomplete block.

The principal advantage in using composite blocks of t + k units is that an estimate of pure experimental error from the bk replicates can be obtained separately from an estimate of block-treatment interaction. The removal of the interaction variation (however small) from the residual variation in the analysis of variance results in a more efficient testing procedure when comparing treatment effects. These separate sources of variation (interaction and error) cannot be estimated separately when only complete blocks of size t or incomplete blocks of size k are used.

Throughout we shall make reference to fixed and random effects such as fixed treatment effects and fixed or random block effects. The distinction between fixed and random effects is that with the former, a repetition of the experiment would bring the same set of effects into the new experiment and our attention is focused upon these effects and no others. With random effects on the other hand, a repetition would bring in a new set of effects but from the same population, and hence we are interested in the variability among all the effects rather than just the particular ones used in the experiment. With fixed treatment effects, we draw inferences about the specifically selected set of effects; with random effects, our inference is about the population variability.

An experiment designed to compare two treatments using blocks of size 3 was introduced by John (1962). The following year, John (1963) extended the analysis to include blocks of size t + k units where $1 \leqslant k < t$. Using formulas similar to the formulas in the analysis of Balanced Incomplete Block Designs, John presented the intrablock analysis (blocks fixed), the interblock analysis (blocks random) as well as the recovery of the interblock information by combining intra- and interblock estimates of the treatment effects. The special case where k = 1 was presented in detail.

Although brief mention on how to obtain an estimate of the block-treatment interaction was made by John (1963) in the intrablock analysis, he assumed the interaction to be nonexistent in the interblock analysis. Often in a mixed model analysis where the treatment effects are fixed but the blocks are assumed to be representative of a larger population of blocks, the fixed and random effects are not additive but rather an interaction is present. An estimate of the interaction variation, even if small in magnitude, is needed in the estimation formula for the random blocks component of variance.

Of particular interest to us is the application of composite complete-incomplete block designs arising in sensory experiments where a small number of different samples or treatments are to be compared subjectively by several panelists (blocks). To acquire panelists for this type of testing is often difficult and/or costly. Once the panelists are selected therefore, if they can effectively judge t + k samples at a sitting rather than only t samples, a savings in cost (panelists' time, monetary reward, etc.) can be made.

We now review the notation to be used throughout and discuss briefly the intrablock analysis where both the treatments and the blocks (panelists) are considered fixed effects. In a later section we shall illustrate the intrablock analysis with an example, and shall discuss the efficiency of the composite complete-incomplete block designs compared to orthogonal complete block designs. We also shall present the analysis of the mixed model where the panelists are assumed to represent a random sample from a larger population of panelists (blocks random.)

INTRABLOCK ANALYSIS

IN THE INTRABLOCK analysis, we shall be interested only in the treatments under investigation as well as removing panel-

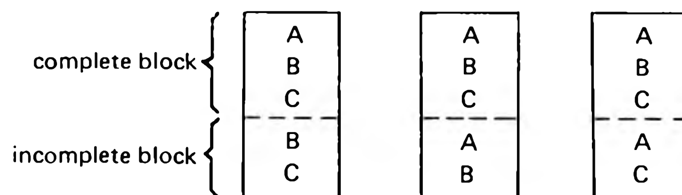

Fig. 1—A composite block design consisting of three treatments in three blocks of five units each.

Table 1a–Intrablock analysis of variance

Source	d.f.	Mean sq	Expected mean square
Treatments (Adj.)	$t-1$	$\frac{(2.13)}{t-1}$	$\sigma_e^2 + \frac{b(k+t)}{t(t-1)} \sum_i \tau_i^2$
Panelists (Adj.)	$b-1$	$\frac{(2.14)}{b-1}$	$\sigma_e^2 + \frac{(k+t)}{b-1} \sum_j p_j^2$
P x T Interaction	$(t-1)(b-1)$	$\frac{(2.12)}{(t-1)(b-1)}$	$\sigma_e^2 + \sum_i \sum_j \frac{n_{ij} I_{ij}^2}{(b-1)(t-1)}$
Error	bk	$\sum_i \sum_j \frac{d_{ij}^2}{2bk}$	σ_e^2

Table 1b–Intrablock analysis of variance (Example)

Source	d.f.	Mean sq	F
Treatments	2	25.315	30.38
Panelists (Adj)	2	2.065	2.48
P x T Interaction	4	2.405	2.89
Error	3	0.833	

ists' differences from treatment comparisons and hence both the treatments and the panelists are considered as fixed effects. The statistical model is represented by,

$$Y_{ij\ell} = \mu + \tau_i + p_j + I_{ij} + \epsilon_{ij\ell} \qquad \begin{array}{l} i = 1,2,\ldots,t \\ j = 1,2,\ldots,b \\ \ell = 1 \text{ or } 2 \end{array} \tag{2.1}$$

where $Y_{ij\ell}$ is the ℓth response to the ith treatment by the jth panelist. The other terms in model (2.1) are μ, the overall mean; τ_i, the effect of the ith treatment; p_j, the effect owing to the jth panelist; I_{ij}, the interaction parameter associated with the response to the ith treatment by the jth panelist and $\epsilon_{ij\ell}$, the random error associated with $Y_{ij\ell}$. It is assumed that the errors $\epsilon_{ij\ell}$ are randomly sampled from a Normal population with mean zero and variance σ_e^2.

Let us denote the adjusted treatment total for the ith treatment as Q_i,

$$Q_i = T_i - \sum_{j=1}^{b} \frac{n_{ij} P_j}{k+t} \tag{2.2}$$

where T_i is the total of the responses to the ith treatment, n_{ij} is the number of times the ith treatment is rated by the jth panelist and P_j is the total of the responses, over all treatments, by the jth panelist. As a check, $\sum_{j=1}^{b} Q_j = 0$. Using the restriction that the estimates $\hat{\tau}_i$ of the treatment effects sum to zero, we have for the estimate of the effect of the ith treatment,

$$\hat{\tau}_i = \frac{(t-1)(k+t)}{b[(k+t)^2 - (3k+t)]} Q_i \qquad 1 \leqslant i \leqslant t, \tag{2.3}$$

where the hat "^" denotes estimate. Furthermore, under the assumption the errors $\epsilon_{ij\ell}$ are uncorrelated with homogeneous variance σ_e^2, we have for the variance of $\hat{\tau}_i$ and the covariance $(\hat{\tau}_i, \hat{\tau}_{i'})$, $i \neq i'$,

$$\text{Var}(\hat{\tau}_i) = \frac{(k+t)(t-1)^2}{bt[(k+t)^2 - (3k+t)]} \sigma_e^2 \tag{2.4}$$

$$\text{Cov}(\hat{\tau}_i, \hat{\tau}_{i'}) = -\frac{1}{t-1} \text{Var}(\hat{\tau}_i), \quad i \neq i'. \tag{2.5}$$

An estimate of the difference between any two treatment effects, say treatment i and i′, is calculated using,

$$\hat{\tau}_i - \hat{\tau}_{i'} = \frac{(t-1)(k+t)}{b[(k+t)^2 - (3k+t)]} \left\{ Q_i - Q_{i'} \right\} \tag{2.6}$$

and the variance of the difference (2.6) is,

$$\text{Var}(\hat{\tau}_i - \hat{\tau}_{i'}) = \frac{2(t-1)(k+t)}{b[(k+t)^2 - (3k+t)]} \sigma_e^2 \tag{2.7}$$

where σ_e^2 is estimated (if unknown) using the error mean square in the analysis of variance table (Table 1a). Also, the estimate of the adjusted mean of the ith treatment is,

$$m + \hat{\tau}_i, \qquad m = \sum_i^t \sum_j^b \sum_\ell^{n_{ij}} \frac{Y_{ij\ell}}{b(k+t)} \tag{2.8}$$

where m is the overall mean of the experiment. The standard error (square root of the variance) of the adjusted mean (2.8) is

$$\sqrt{\frac{\sigma_e^2}{b} \left\{ \frac{1}{k+t} + \frac{(k+t)(t-1)^2}{t[(k+t)^2 - (3k+t)]} \right\}}. \tag{2.9}$$

Note that when comparing two treatments, one may use the treatment effects (2.6) or the treatment means $(m + \hat{\tau}_i) - (m + \hat{\tau}_{i'})$. These comparisons are identical as can be seen from their identical variances, the expression (2.7) and the quantity in (4.2).

In the intrablock analysis, the experimenter's interest lies primarily in determining if treatment effects are different. Usually the estimation of the effects of the panelists is not of interest except when these estimates are used in removing biases or differences among the panelists prior to comparing the treatments. However, the calculation of the sum of squares among panelists, adjusted for treatments, enables a check to be made on the calculation of the sum of squares among treatments, adjusted for panelists, which is of interest. The check is made by considering the identity,

$$\text{S.S. Panelists (Adj.)} + t \sum_i^t \frac{T_i^2}{b(k+t)}$$

$$= \text{S.S. Treatments (Adj.)} + \sum_j^b \frac{P_j^2}{k+t} \tag{2.10}$$

where the formulas for S.S. Panelists (Adj.) and S.S. Treatments (Adj.) are provided in (2.14) and (2.13) respectively. Also, a significant "F" ratio involving the quantities

$$\frac{\text{S.S. Panelists (Adj.)}/b-1}{\text{Mean Square Error}} \tag{2.11}$$

indicates that the panelists are different in their evaluations

and hence the removal of these differences among the panelists from the measure of experimental error has resulted in a more efficient analysis.

Table 2—Efficiency of composite block to complete block design for different numbers of treatments (t), intrablock replications (k) and ratio of MSPT/MSE (R)

No. of treatments	Values of R						
t	1	1.5	2	3	4	5	6
2							
k=1	0.889	1.333	1.778	2.667	3.556	4.444	5.333
3							
k=1	0.937	1.406	1.875	2.813	3.750	4.687	5.625
k=2	0.96	1.440	1.92	2.88	3.84	4.80	5.76
4							
k=1	0.96	1.440	1.92	2.88	3.84	4.80	
k=2	0.963	1.444	1.926	2.889	3.852	4.815	
k=3	0.979	1.469	1.959	2.939	3.918	4.90	
5							
k=1	0.972	1.458	1.944	2.917	3.889		
k=2	0.969	1.454	1.938	2.908	3.878		
k=3	0.977	1.465	1.954	2.931	3.906		
6							
k=1	0.980	1.469	1.959	2.939			
k=2	0.975	1.463	1.950	2.925			
7							
k=1	0.984	1.476	1.969	2.953			
k=2	0.979	1.469	1.959	2.938			
8							
k=1	0.988		1.975	2.963			
k=2	0.983		1.966	2.949			

Table 3—Values of d needed to attain the same efficiency where number of complete blocks = d × number of composite blocks

	R = M.S. P×T/M.S. Error						
t	1	1.5	2	3	4	5	6
2							
k=1	1.33	2.0	2.67	4	5.33	6.67	8
3							
k=1	1.25	1.88	2.5	3.75	5	6.25	7.5
k=2	1.6	2.4	3.2	4.8	6.4	8	9.6
4							
k=1	1.2	1.8	2.4	3.6	4.8	6	
k=2	1.44	2.16	2.89	4.33	5.78	7.22	
k=3	1.71	2.57	3.43	5.14	6.86	8.57	
5							
k=1	1.17	1.75	2.33	3.5	4.67		
k=2	1.36	2.04	2.71	4.07	5.43		
k=3	1.56	2.34	3.13	4.69	6.25		
6							
k=1	1.14	1.71	2.29	3.43			
k=2	1.3	1.95	2.6	3.9			
7							
k=1	1.13	1.69	2.25	3.38			
k=2	1.26	1.89	2.52	3.78			
8							
k=1	1.11	1.67	2.22	3.33			
k=2	1.23	1.85	2.46	3.69			

To obtain an estimate of the experimental error variation to be used in tests of treatment effects as well as the test in (2.11), let d_{ij} denote the range of the observations from the ith treatment by the jth panelist so that if $n_{ij} = 2$, d_{ij} is the difference between the replicates, and if $n_{ij} = 1$, then $d_{ij} = 0$. The sum of squares of the d_{ij} gives an unbiased estimate of $2bk\sigma_e^2$. The remaining portion of the residual sum of squares is associated with panelists × treatment interaction [I_{ij} in model (2.1)]. The sum of squares Interaction may be calculated either by subtraction of error sum of squares from residual sum of squares or by any of many formulas one of which is,

$$\text{S.S. Interaction} = \sum_i^t \sum_j^b \frac{Y_{ij.}^2}{n_{ij}} - \sum_{j=1}^b \frac{P_j^2}{k+t} - \text{S.S. Treatments (Adj.)} \qquad (2.12)$$

where $Y_{ij.}$ is the total of the observations on the ith treatment by the jth panelist and S.S. Treatments (Adj.) is calculated by

$$\text{S.S. Treatments (Adj.)} = \frac{(t-1)(k+t)}{b[(k+t)^2 - (3k+t)]} \sum_i^t Q_i^2 \qquad (2.13)$$

To calculate the sum of squares among panelists adjusted for treatments, we reverse equation (2.12) to get,

$$\text{S.S. Panelists (Adj.)} = \sum_i^t \sum_j^b \frac{Y_{ij}^2}{n_{ij}} - t \sum_{i=1}^t \frac{T_i^2}{b(k+t)} - \text{S.S. Interaction.} \qquad (2.14)$$

The intrablock analysis of variance is presented in Table 1a where both treatments and panelists are adjusted.

We shall now briefly illustrate the intrablock analysis with a small numerical example. In a subsequent section we discuss the efficiency of the composite block design relative to the complete block design where the treatments appear once and only once in each block.

AN EXAMPLE OF THE INTRABLOCK ANALYSIS

THREE PANELISTS (1, 2 and 3) were asked to evaluate three treatments (A, B and C). A hedonic scale which involved assigning numerical values from 1 to 9 according to the degree of preference for the treatments was used. Although only three treatments were to be compared, there was sufficient time to permit each panelist to evaluate four samples, and thus one of the treatments was replicated with each panelist. The selection of the treatment to be replicated with each panelist was done at random, resulting in treatment B being replicated with the first panelist, treatment A with the second and C with the third panelist. The data and the corresponding calculations are,

Treatments		A	B	C	P_j
Panelist	1	6	7,8	2	23
	2	5,3	7	1	16
	3	3	9	4,4	20
	T_i	17	31	11	59
	Q_i	−1.75	10.50	−8.75	
	$\hat{\tau}_i$	−0.467	2.800	−2.333	

The adjusted totals Q_i and the estimates of the treatment

effects $\hat{\tau}_i$ are calculated using (2.2) and (2.3) respectively. For Treatment A, we have

$$Q_A = 17 - \frac{[1(23) + 2(16) + 1(20)]}{4} = -1.75$$

$$\hat{\tau}_A = \frac{2(4)}{3[16-6]} Q_A = \frac{8}{30}(-1.75) = -0.467.$$

The Sum of Squares Treatments, adjusted for panelists, is computed using (2.13),

S.S. Treatments (Adj.) =

$$\frac{2(4)}{30}\left\{(-1.75)^2 + (10.5)^2 + (-8.75)^2\right\} = 50.63.$$

To calculate S.S. P × T Interaction, we refer to (2.12),

S.S. P×T Interaction =

$$\left\{6^2 + \frac{15^2}{2} + \ldots + \frac{8^2}{2}\right\} - \left\{\frac{(23)^2 + (16)^2 + (20)^2}{4}\right\} -$$

S.S. Trts (Adj.) = 356.50 − 296.25 − 50.63 = 9.62.

Panelists Sum of Squares, adjusted for treatments, from (2.14), is

$$\text{S.S. Panelists (Adj.)} = \left\{6^2 + \frac{15^2}{2} + \ldots + \frac{8^2}{2}\right\}$$

$$-3\left\{\frac{(17)^2 + (31)^2 + (11)^2}{3(4)}\right\} - 9.62 = 4.13$$

and squaring the differences between the replicates, the Error S.S. is,

$$\text{Error S.S.} = \frac{(7-8)^2 + (5-3)^2 + (4-4)^2}{2} = 2.5.$$

As a check on the calculations, the total S.S. (corrected for the mean) must equal the sum of the following sums of squares, Treatments (Adj.), Interaction, Error and Panelists (unadjusted) where the latter is computed as,

$$\text{S.S. Panelists (unadj)} = \sum_{j=1}^{b} \frac{P_j^2}{k+t} - \frac{(\Sigma P_j)^2}{b(k+t)}$$

$$= \frac{(23)^2 + (16)^2 + (20)^2}{4} - \frac{(59)^2}{12} = 6.17.$$

Numerically, we have,

$$\text{Total S.S.} = 6^2 + 7^2 + \ldots + 4^2 - \frac{(59)^2}{12} = 68.92$$

$$= 50.63 + 9.62 + 6.17 + 2.5 = 68.92$$

To test for significance, Mean Square Treatments (Adj.), Mean Square Panelists (Adj.) and Mean Square Interaction are each compared against Mean Square Error using "F" ratios. In this example, the test for treatment effects is highly significant ($P < 0.025$) as the ratio 25.315/0.833 = 30.38 is larger than the corresponding F value with 2 and 3 degrees of freedom respectively (see Table 1b).

Before proceeding, perhaps a word on the interaction variation is appropriate. Although in this small numerical example the interaction variation is not significantly larger (2.405/0.833 = 2.89) than the experimental error variation, the authors have often experienced significant interaction variation particularly when untrained or inexperienced panelists are used. Inexperienced panelists are often inconsistent in their preferences or degree of preference for a particular treatment or treatments. To illustrate with the numerical example discussed previously, suppose the evaluations had appeared as,

Treatments		A	B	C
Panelist	1	8	7,6	2
	2	5,3	7	1
	3	3	4	9,9

where now treatment A is slightly preferred by panelist 1, B by panelist 2 and treatment C by panelist 3. The lack of consistency or agreement among the panelists in their preferences for the treatments would result in a significant interaction. When confronted with a significant interaction, we might repeat the experiment with the same panelists and check on the validity of the interaction or we might select new panelists and decide to either combine the data from the two experiments or discard the opinions of the above.

We now discuss the efficiency of composite complete-incomplete block designs compared to complete block designs. We shall consider first the case where the errors $\epsilon_{ij\ell}$ are uncorrelated and then consider the case where the errors associated with the first and second responses to a particular treatment by the same panelist are correlated.

EFFICIENCY

AS WAS SHOWN in the previous section, composite complete-incomplete block designs enable the separate estimation of panelists × treatments interaction and experimental error. These sources of variation cannot be separately estimated when each treatment appears at most once ($n_{ij} = 1$) in each block with either the complete or incomplete block designs. Removal of the estimate of interaction variation from the residual variation leaving only the experimental error as an estimate of σ_e^2 results in a more efficient comparison among treatments even for small values (nonsignficant "F" values) of the ratio Mean Square Interaction/Mean Square Error. This increase in precision for comparing treatments is shown by computing the efficiency of composite designs compared to the orthogonal complete block design for different values of the ratio R = (M.S. P × T Interaction)/M.S. Error.

In an orthogonal complete block design, the variance of the estimate of the difference between two treatment means is,

$$V(\bar{\tau}_i - \bar{\tau}_{i'})_{CB} = \frac{\sigma^2_{\text{Residual}}}{r} \qquad i \neq i' \qquad (4.1)$$

where r is the number of replications of each of the t treatments and $\sigma^2_{\text{Residual}}$ is the estimate of σ^2 in the analysis of variance. In the composite design with blocks of size k+t,

$$V(\bar{\tau}_i - \bar{\tau}_{i'})_{C-I} = \frac{2(t-1)(k+t)}{b[(k+t)^2 - (3k+t)]}\sigma_e^2 \qquad i \neq i' \qquad (4.2)$$

where now σ_e^2 is the error variance (intrablock) having bk degrees of freedom. If we define the efficiency of a design as the reciprocal of the variance of the estimate of the difference between two treatment means and equalize the number of

replications of each of the treatments with the two designs, we have for the efficiency ratio,

$$\text{Efficiency C–I to CB} = \frac{V(\bar{\tau}_i - \bar{\tau}_{i'})_{CB}}{V(\bar{\tau}_i - \bar{\tau}_{i'})_{C-I}}$$

$$= \frac{t[(k+t)^2 - (3k+t)]\,\sigma^2_{Residual}}{(t-1)(k+t)^2\sigma_e^2} \qquad (4.3)$$

where the number of complete blocks of size t is $b^* = b(k+t)/t$ while the number of composite blocks of size (k+t) is b.

For different values of t and k, Table 2 presents efficiency values computed using (4.3) for conceptual values of the ratio $\sigma^2_{Residual}/\sigma_e^2$. From Table 2, we see that when the hypothesis H_o: Interaction = 0 is true resulting in R=1, (that is, R–1 is a measure of $\sum_{ij} n_{ij}I_{ij}^2/[(b-1)(t-1)\sigma_e^2]$, the composite block design is slightly less efficient than the complete block design. However, when the value of R is greater than 1, the composite block design is a more efficient design. The efficiency is an increasing function of R expressed by,

$$\text{Efficiency C–I to CB} = R \times \text{Efficiency}(H_o\text{: Int.} = 0)$$

where Efficiency(H_o: Int. = 0) denotes the efficiency under the hypothesis of no interaction. In Table 2, only nonsignificant values of the ratio R are considered since the efficiency criterion discussed is useful only in absence of a significant interaction effect. For example, when t=4, k=1 and MSPT/MSE=4 the composite block design consisting of 4 blocks is 3.84 times as efficient as the complete block design consisting of 5 blocks.

Another way of showing the superiority of composite block designs over complete block designs when some measure of interaction is present is to determine how many b* complete blocks of size t are necessary in order to obtain the same efficiency, in terms of $V(\bar{\tau}_i - \bar{\tau}_{i'})$, as b composite blocks of size k+t. Table 3 presents values of d (where b* = db) computed using the formula,

$$d = \frac{[(k+t)^2 - (3k+t)]\,R}{(t-1)(k+t)}\,.$$

As with the efficiency, the number b* of complete blocks is an increasing function of R. Again, with t=4, k=1 and MSPT/MSE = 4, approximately 19 (4.8 × 4) complete blocks are required to obtain the same efficiency as only 4 composite blocks.

To this point we have considered the errors as being uncorrelated. The question now arises, "what effect might correlated observations have on the efficiency of composite block designs?" As an example, a panelist's response to a treatment could be positively correlated with his response to the replicate of the treatment, particularly in the case of trained panelists. Furthermore, the magnitude of correlation might be different for different treatments. Although this latter problem is of interest, we shall consider at the present only the case where

$$E(\epsilon_{ij1}\epsilon_{ij2}) = \rho\sigma_e^2, \qquad 0 \leq \rho < 1 \qquad (4.6)$$

and how the degree of correlation (ρ) affects the efficiency value computed as in (4.3).

For the complete block design, the formula for the variance $V(\bar{\tau}_i - \bar{\tau}_{i'})$ shown by (4.1) does not change in form. For the composite block design with b blocks of size (k+t), rather than the form (4.2) we have,

$$V(\bar{\tau}_i - \bar{\tau}_{i'})_{C-I}$$

$$= \frac{2(t-1)\sigma_e^2}{b[(k+t)^2 - (3k+t)]}\left\{k+t + \frac{2k[(k+t)^2 - (3t+k)]\rho}{(k+t)^2 - (3k+t)}\right\}. \qquad (4.7)$$

Equalizing the number of replications of the t treatments with both designs, we have for the efficiency of the composite block design to the complete block design,

Efficiency

$$= \frac{t[(k+t)^2 - (3k+t)]^2 R}{(t-1)(k+t)\left\{(k+t)[(k+t)^2 - (3k+t)] + 2k[(k+t)^2 - (3t+k)]\rho\right\}} \qquad (4.8)$$

where again R = MSPT/MSE. Table 4 presents efficiency values for different values of R where now nonzero correlation values (ρ = .2, .5, .8) are considered.

From Table 4, we note that with a composite block design having fixed incomplete block size k, as $\rho \to 1.0$, the efficiency decreases. In fact, the larger the value k ($k \to t$), the faster the efficiency of the composite block design approaches ½ that of the complete block design as $\rho \to 1$. In other words, when $\rho > 0$.

$$\text{Efficiency}(k_i) \leq \text{Efficiency}(k_j), \text{ for } k_i \geq k_j \qquad (4.9)$$

for all values of R. This implies that if one suspects a positive correlation to be present between observations and their replicates by the same panelist, one should use k=1 for maximum efficiency.

We now discuss briefly variance component estimation in the analysis of the mixed model where the treatments are fixed but the blocks are considered to be a random sample taken from a large population of blocks. In addition to comparing the fixed treatment affects, it is again of interest to obtain an estimate of the variance among the panelists. The estimation formulas correspond exactly to the formulas arising in Henderson's (1953) method III. Henderson's methods are discussed at some length by Searle (1968; 1971).

MIXED MODEL ANALYSIS

REFERRING to the model (2.1), we now consider the terms p_j and I_{ij} as representing random variables, independently distributed, with zero means and variances σ_p^2 and σ_I^2, respectively. We also assume the I_{ij} are uncorrelated.

The analysis of variance table for the mixed model is presented in Table 5. Table 5 differs from the earlier intrablock analysis of variance table (Table 1) in the expectations of the mean squares as well as the source Reduction (Adjusted). The adjusted Reduction sum of squares consists of the reduction in total variation owing to fitting the model (2.1) ignoring both the μ and the τ_i terms, and is used in deriving the estimation equation for the block component of variance σ_p^2.

In the mixed model analysis, there are basically three questions to be answered:

(1) Is there evidence of a panelists × treatment interaction, that is, is there evidence to indicate the panelists are not consistent in their evaluations of the treatments? Do we reject the hypothesis of nonexistent interaction?

(2) In the event of a nonsignificant interaction, what is the

estimate of the magnitude of the interaction component of variance? What is the estimate of the magnitude of the among panelists component of variance?

(3) If the interaction is not significant, is the difference among the treatment effects large or small relative to the magnitude of the interaction?

Table 4—Effect of correlated observations on the efficiency of composite block design to complete block design

	R = M.S. P × T/M.S. Error						
t	1	1.5	2	3	4	5	6
3							
k = 1							
ρ = 0	0.938		1.875	2.813	3.75	4.688	5.625
.2	0.852		1.705	2.557	3.409	4.261	5.114
.5	0.75		1.5	2.25	3.0	3.75	4.5
.8	0.670		1.339	2.009	2.679	3.348	4.018
1	0.625		1.25	1.875	2.5	3.125	3.75
k = 2							
ρ = 0	0.96		1.92	2.88	3.84	4.8	5.76
.2	0.828		1.655	2.483	3.31	4.138	4.966
.5	0.686		1.371	2.057	2.743	3.429	4.114
.8	0.585		1.171	1.756	2.342	2.927	3.512
1	0.533		1.067	1.6	2.133	2.667	3.2
4							
k = 1							
ρ = 0	0.96		1.92	2.88	3.84	4.8	
.2	0.889		1.778	2.667	3.556	4.444	
.5	0.8		1.6	2.4	3.2	4	
.8	0.727		1.455	2.182	2.909	3.6	
k = 2							
ρ = 0	0.963		1.926	2.889	3.852	4.815	
.2	0.850		1.699	2.549	3.40	4.248	
.5	0.722		1.444	2.167	2.889	3.611	
.8	0.628		1.256	1.884	2.512	3.14	
k = 3							
ρ = 0	0.979		1.959	2.939	3.918	4.898	
.2	0.836		1.673	2.509	3.345	4.181	
.5	0.686		1.371	2.057	2.743	3.429	
.8	0.581		1.162	1.743	2.325	2.906	
5							
k = 1							
ρ = 0	0.972	1.458	1.944	2.917	3.889		
.2	0.911	1.367	1.823	2.734	3.646		
.5	0.833	1.25	1.667	2.5	3.333		
.8	0.767	1.151	1.535	2.303	3.070		
k = 2							
ρ = 0	0.969	1.454	1.939	2.908	3.878		
.2	0.870	1.305	1.740	2.610	3.480		
.5	0.754	1.131	1.508	2.262	3.016		
.8	0.665	0.998	1.331	1.996	2.661		
6							
k = 1							
ρ = 0	0.979	1.469	1.959	2.939			
.2	0.927	1.39	1.853	2.780			
.5	0.857	1.286	1.714	2.571			
.8	0.797	1.196	1.595	2.392			
7							
k = 1							
ρ = 0	0.984		1.969	2.953			
.2	0.938		1.875	2.813			
.5	0.875		1.75	2.625			
.8	0.820		1.641	2.461			

To test the significance of an interaction effect, the ratio (M.S. P × T Interaction)/M.S. Error is compared against an "F" value with $(b-1)(t-1)$ and bk degrees of freedom, respectively. If there is evidence of a significant interaction effect, we may still proceed with answering question (2) above. The component of variance of primary interest is the interaction component, however, as the estimate of the among panelists component of variance loses some of its usefulness in the presence of an interaction.

To estimate the variance components σ_p^2 and σ_I^2, we have from Table 5,

$$\hat{\sigma}_I^2 = \frac{1}{\psi}\,[\text{Mean Square Interaction} - \text{Mean Square Error}]$$

$$\hat{\sigma}_p^2 = \frac{t(b-1)(k+t)}{b(k+t)^2 - t(3k+t)}\,[\text{Mean Square Reduction} - \text{Mean Square Error}] - \sigma_I^2. \qquad (5.1)$$

For the simple case where $k=1$, $b=t$, the coefficients $\frac{1}{\psi}$ and $[t(b-1)(k+t)]/[b(k+t)^2 - t(3k+t)]$ in (5.1) are $[(t^2-1)(t+2)]/[t^3+3t^2-t-2]$ and $(t+2)/(t+1)$, respectively. It is of interest to note that the estimate $\hat{\sigma}_I^2$ is used in the estimation formula for σ_p^2. This latter estimation formula for σ_p^2 of course differs from John's (1963) interblock estimation formula for σ_p^2. When the nonadditivity contribution σ_I^2 is zero, however, Mean Square Reduction is Mean Square Blocks (Adjusted for Treatments) and $\hat{\sigma}_p^2$ in (5.1) reduces to John's (1963) estimate of σ_p^2,

$$\hat{\sigma}_p^2 = \frac{(b-1)(k+t)}{b(k+t)^2 - t(3k+t)}\,[\text{Mean Square Blocks (Adj)} - \text{Mean Square Error}]. \qquad (5.2)$$

The estimation formulas (5.1) were arrived at using the method of Least Squares. Unfortnately, there is no guarantee that both estimates $\hat{\sigma}_I$ and $\hat{\sigma}_p^2$ will always be greater than or equal to zero. In the event either estimate is less than zero, one may accept the negative estimate as evidence that the true value is zero or evidence that an insufficient amount of data has been collected. In the latter case, we might collect additional data and analyze the additional data either by itself or pool these data with the data that yielded the negative estimate. In either case, if the subsequent analysis also yields a negative estimate, this points strongly to the argument that the true value of the component really is zero. Four other approaches to be taken when negative estimates of variance components are obtained are discussed briefly by Searle (1971) pages 407–408.

To test the hypothesis that the differences in the treatment effects are small relative to the magnitude of the interaction effect, an approximate "F" ratio can be set up. The method is known as Satterthwaite's (1946) approximation and the approximate F ratio is constructed as,

$$F_{(\text{approx.})} = \frac{\psi[\text{M.S. Treatments (Adj.)}] - \phi[\text{M.S. Interaction}]}{(\psi-\phi)\ \text{M.S. Error}} \qquad (5.3)$$

where ψ and ϕ are given in Table 5. The value obtained in (5.3) is compared against a tabular F value with f' and bk

Table 5–Mixed model analysis of variance expressions needed to estimate random blocks and interaction components of variance

Source	d.f.	S.S.	Expected M.S.
Treatments (Adj)	$t-1$		$\sigma_e^2 + \frac{b(k+t)}{t(t-1)} \sum_i^t \tau_i^2 + \phi\sigma_I^2$
Reduction (Adj)	$t(b-1)$	$\sum_i^t \sum_j^b \frac{Y_{ij}^2}{n_{ij}} - t\sum_i^t \frac{T_i^2}{b(k+t)}$	$\sigma_e^2 + \frac{b(k+t)^2 - t(3k+t)}{t(k+t)(b-1)} \{\sigma_I^2 + \sigma_p^2\}$
Interaction	$(t-1)(b-1)$	(2.12)	$\sigma_e^2 + \psi\sigma_I^2$
Error	bk	$\sum_i^t \sum_j^b \frac{d_{ij}^2}{2}$	σ_e^2

$$\phi = \frac{(3k+t)\{(k+t)^2 + (k-t)\} - 8k(k+t)}{(k+t)[(k+t)^2 - (3k+t)]}$$

$$\psi = \frac{[b(k+t)^2 - (b+t-1)(3k+t)][(k+t)^2 - (3k+t)] - 4k(k-t)(t-1)}{(b-1)(t-1)(k+t)[(k+t)^2 - (3k+t)]}$$

degrees where f′ is computed using,

$$f' = \frac{[\text{M.S. Error} \times (F_{(approx.)})]^2}{\left\{ \left(\frac{\psi}{\psi-\phi}\right)^2 \frac{[\text{M.S. Treatments (Adj.)}]^2}{t+1} + \left(\frac{\phi}{\psi-\phi}\right)^2 \frac{[\text{M.S. Interaction}]^2}{(b-1)(t-1)+2} \right\}} \quad (5.4)$$

With large values of the ratio (5.3), we would infer that the treatment effects are in fact different and the differences are large relative to the magnitude of the interaction variation.

A much simplier approach to testing the differences in treatment effects is to assume the interaction variation is null (i.e., to assume $\sigma_I^2 = 0$) if the initial F-test for interaction variation is not significant. Then the expected mean square expressions in Table 5 will not contain σ_I^2 and the sums of squares for interaction and for error are combined to form a new error sum of squares with $bk + (b-1)(t-1)$ degrees of freedom. The mean square for Treatments (Adj.) is then compared against the new error mean square, again using an F ratio. Of course the adjustment (5.2) for estimating the random among panelists component of variance is also made in this case.

CONCLUDING REMARKS

IN THIS PAPER we have discussed some advantages in combining complete blocks of size t with incomplete blocks of size k to form composite complete-incomplete blocks of size t+k. The principal advantage to using the composite designs is that a measure of the block-treatment interaction can be obtained separate from a measure of the experimental error. By removing the interaction variation from the residual variation, leaving only the experimental error as a measure of the within treatment variation, we showed how the composite designs were more efficient than the complete block designs for comparing treatment effects in the intrablock analysis. It was also shown how an estimate of the interaction variation is used when estimating the random blocks (panelists) component of variance in the mixed model.

Throughout we have assumed that, after the assignment of the treatments to the blocks, the performance of the b(k+t) experiments is accomplished in one replication. We have not generalized the formulas in the analysis to include more than a single replication. Often however, in order that every treatment be duplicated with every panelist at one time or another, two or more replications are needed. For example, if we refer to the numerical example in the section on intrablock analysis, we might consider three days of experimentation so that each panelist is asked to duplicate each treatment. The design might look like,

		Day 1	Day 2	Day 3
Panelist	1	ABC B	ABC A	ABC C
	2	ABC A	ABC C	ABC B
	3	ABC C	ABC B	ABC A

This design is slightly more balanced than the design for a particular day since over the three days, every treatment is duplicated with every panelist.

In the analysis of the above replicated composite complete-incomplete design, the removal of day-to-day variation is necessary. In a sequel to the present paper (Cornell and Knapp, 1973) we present the analysis of the replicated composite designs. In addition, various approaches for the interpretation of panelists × treatments interactions will be discussed at more length, using real life situations.

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ASCI PRODUCTION BY Byssochlamys fulva ON A SYNTHETIC MEDIUM

INTRODUCTION

BYSSOCHLAMYS FULVA, an ascomycete of the family Gymnoascaceae, has become of increasing importance to the food industry because of the ability of the ascospores to withstand the heat-fill temperatures used in processing fruit beverages and fillings (Maunder, 1969). The first report on the heat resistance of asci of *B. fulva* was that of Olliver and Rendle (1934). Hull (1939) first reported the biphasic death curve due to moist heat for asci of *B. fulva* which was characterized by an initial slow death rate followed by rapid death on further treatment. Cultural studies on asci formation in different complex media was reported by Splittstoesser et al. (1969) and Put (1964). To date little research has been reported on the nutritional requirements for the production of heat resistant asci, and it is important that the physiological characteristics of *B. fulva* be elucidated in order to solve eventually the problem of the organism in the food industry. One significant aspect of the organism is the delineation of nutritional elements which enhance or aid heat resistant ascospore development. The present report describes a liquid synthetic medium suitable for asci formation by *B. fulva*.

EXPERIMENTAL

THE STRAIN employed was obtained by single ascospore isolation from National Canners Association strain 68–10. The culture on a medium composed of 0.5% Difco potato extract, 2% sucrose and 2% agar (PSA) or a medium composed of 0.5% Difco potato extract, 2% dextrose and 2% agar (PDA) is initially buff in color and later white flecks are observed as asci formation proceeds corresponding to the description of *B. fulva* given by Brown and Smith (1957).

Conidia were used for inoculation of media. Inocula was prepared by subculturing three times at three day intervals on PSA or PDA and the conidia from the plates of the third transfer were collected. Conidia were harvested by first flooding the surface of the plates with sterile distilled water followed by scraping the surface of the culture with a sterile bent glass rod. The material was filtered through sterile cheese cloth and washed three times by centrifugation in sterile distilled water. Inocula of 50 or 500 conidia per ml of medium was used the day of inocula preparation. In performing microscopic counts of conidia, no attempt was made to differentiate between microconidia and macroconidia.

Czapek's salts mixture with the following additives per liter: 5.5g $CaCl_2$ or 2.75g $CaCl_2$, 9.0 mg riboflavin, 100 mg nicotinamide and 200 mg ascorbate, was the basal synthetic medium. Glucose and sucrose were tested as the major carbon sources for synthetic and potato extract media. Synthetic medium, with sucrose, adjusted to three initial pH values (2.0, 4.0 and 5.0) was tested for asci formation. Other media employed were always adjusted to an initial pH of 4.0. The synthetic media was inoculated with 50 or 500 conidia per ml of medium. Only inocula of 50 conidia/ml were employed for media containing potato extract. Media was dispensed in 50 ml volumes in 250 ml flasks with cotton plugs. All pH measurements were done with a Corning model 12 pH meter. A precipitate was noted upon cooling after autoclaving of the synthetic medium, but the precipitate dissolved as fungal growth ensued.

Cultures were incubated at 34°C and mycelial mats from duplicate flasks were harvested at selected intervals for determinations of pH, dry weights and asci counts. For these determinations mats were removed with a bent glass rod and homogenized in 20 ml of distilled water in a Sorvall omnimixer at maximum speed for 7 min with the container immersed in an ice water bath. The final volume was then noted and total asci counts were done microscopically with the aid of a Spencer hemocytometer. Ten ml were removed and taken to dryness at 110°C in tared aluminum weighing pans for dry weight determinations. The particulate material in the remaining homogenate was then washed three times in distilled water and employed for heat activation studies. No attempt was made to rupture the asci, therefore heat activation results were obtained with intact asci. Suitable dilutions of asci were plated on PDA in order to cover the range of 1% to

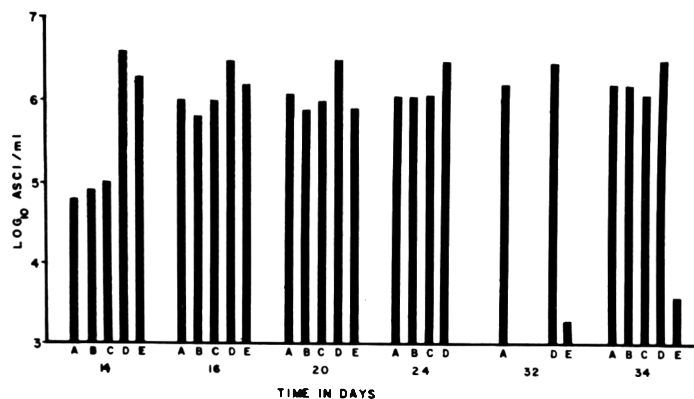

Fig. 1—Asci production in synthetic and nonsynthetic media: (A) Czapek's salts mixture with riboflavin (9.0 mg/l), nicotinamide (100 mg/l), ascorbate (200 mg/l), $CaCl_2$ (5.5g/l) and 2% sucrose. Inoculum 50 conidia/ml; (B) Same medium as in A except 2.75g $CaCl_2$/l was used. Inoculum 50 conidia/ml; (C) Same medium as in A except inoculum 500 conidia/ml; (D) Potato extract (0.5%) with 2% sucrose. Inoculum 50 conidia/ml; and (E) Potato extract (0.5%) with 2% dextrose. Inoculum 50 conidia/ml.

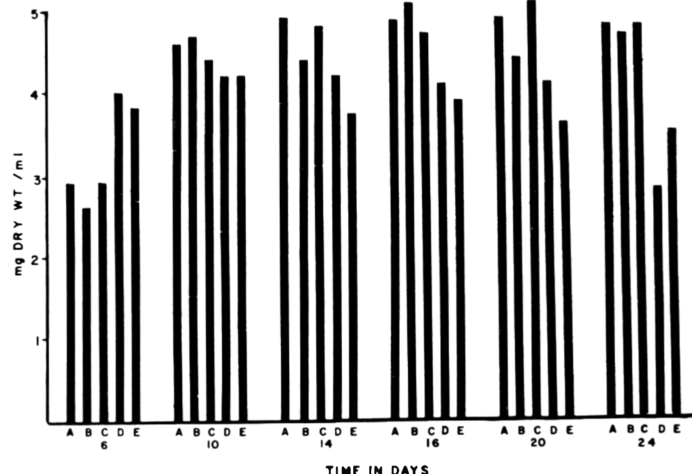

Fig. 2—Dry wt/ml in synthetic and nonsynthetic media. See legend of Figure 1 for media and inocula employed.

100% activation based on microscopic counts. For heat activation, asci were heated in 0.01M phosphate buffer (pH 7.0) for 2 hr. Microscopic asci counts performed before and after heating showed no significant breakage of asci.

RESULTS & DISCUSSION

PRELIMINARY experiments revealed that carbohydrate, riboflavin, nicotinamide, ascorbate and $CaCl_2$ were required additives to Czapek's basal salts in order to obtain good asci production. However, if any one or all of these ingredients except the sugar were omitted the cultures were not entirely lacking in asci. Heat shocking of the homogenates revealed that heat resistant bodies were present but at less than 1% of that obtained when calcium and vitamins were added.

The results recorded in Figure 1 reveal that none of the synthetic media supported asci formation to the same extent of that observed with a broth medium composed of 0.5% Difco potato extract and 2% sucrose (PSB) or a broth medium composed of 0.5% Difco potato extract and 2% dextrose (PDB). Peak asci formation was observed in 14 days with PDB and PSB whereas in the synthetic media the peak was reached in 16 to 20 days. Asci production in synthetic media on a dry weight basis ranged from 30–50% of that obtained with PSB or PDB. After asci formation peaks were reached there was a decrease in asci counts with PDB but no decrease was noted with PSB and in synthetic media containing sucrose as the main carbon source during the time of the experiment. If glucose were employed as the main carbon source in the synthetic media, asci formation was erratic. Although it has not been shown experimentally, the asci in glucose-containing media may have germinated which could account for the decrease in asci with time. Whatever accounts for decreased asci numbers with time in glucose-containing media, it is apparent that sucrose is the sugar of choice because the asci count in the sucrose-containing medium was as high as in the presence of glucose and the asci count in the presence of sucrose remained constant over an extended period of time. A decrease in spore counts with time has been reported in complex media by Splittstoesser et al. (1969). The data in Figure 1 also show no appreciable difference in asci production on synthetic media when the inoculum was increased by a factor of 10 or the calcium chloride concentration was reduced to 2.75g/l.

The results summarized in Figure 2 show that the dry weight per flask was slightly greater in the synthetic media than in the potato extract-containing media. Therefore, the synthetic media is well suited for vegetative growth. In addition, in the synthetic media there was no significant difference in dry weight increase when the inoculum was increased by a factor of 10 or the calcium chloride concentration was reduced to 2.75g/l. After 20 days, autolysis of the mycelial mats became very evident and therefore, dry weight determinations were not extended past 24 days. From the results shown in Figures 1 and 2 it may be noted that the complete synthetic medium with sucrose supports good vegetative growth and asci production.

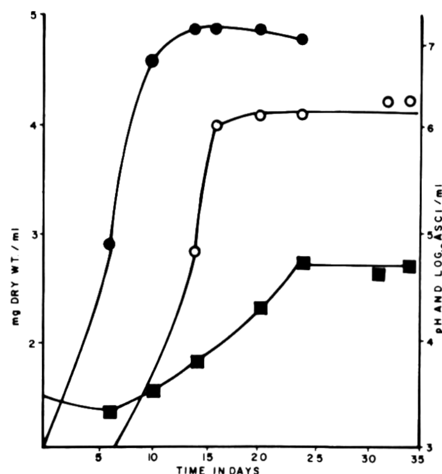

Fig. 3—Dry wt, asci production and pH in synthetic medium composed of Czapek's salts mixture, riboflavin (9.0 mg/l), nicotinamide (100 mg/l), ascorbate (200 mg/l), $CaCl_2$ (5.5g/l) and 2% sucrose. Inoculum 50 conidia/ml. ● Dry wt; ○ Asci production; ■ pH.

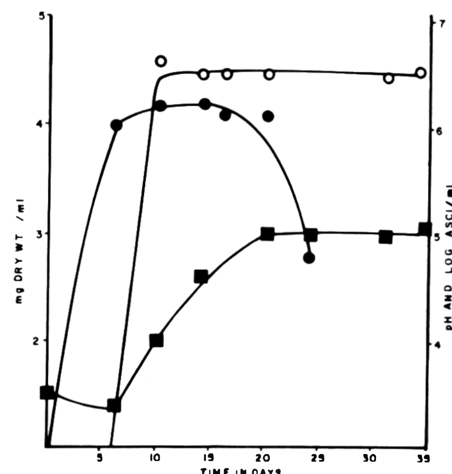

Fig. 4—Dry wt, asci production and pH in non-synthetic medium composed of potato extract (0.5%) and 2% sucrose. Inoculum 50 conidia/ml. ● Dry wt; ○ Asci production; ■ pH.

Of the different initial pH values employed in complete synthetic medium with sucrose, only an initial pH of 4.0 gave consistent results. Although the data are not reported in this paper, when an initial pH of 2.0 or 5.0 was used there was erratic asci formation. The higher ascospore counts at pH 2 as compared to pH 3 and 4 reported by Splittstoesser et al. (1969) may be due to the inherent differences between synthetic and non-synthetic media. From an initial pH of 4.0, a difference in pH values occurred during growth and asci formation between PSB and PDB media. The pH with PSB stabilized after 20 days at a pH of about 5.0 while the PDB showed a steady increase in pH values between 6.0 and 7.0. More rapid utilization of the glucose does not appear important since there is no appreciable difference in the rate of dry weight increase between PSB and PDB (Fig. 2).

The data in Figures 3 and 4 show that peak asci formation, dry weight maxima, and stabilization of the pH occurred approximately 4 days later in synthetic medium containing sucrose as compared to PSB although the same relative patterns can be observed. This suggests that some factor or factors may be present in the potato extract medium which may be responsible for triggering the earlier asci production. These factors may not be synthesized in the synthetic medium until a later time. This situation may be similar to the dispensible but promoting growth factor described by Fries (1965). The synthetic medium appears suitable for studying asci formation by *B. fulva* in terms of such growth or sporulation factors if they are involved, because such a factor(s) may alter the asci production curve in terms of total numbers and time. The effect of the $CaCl_2$ and vitamins on sporulation alone cannot be determined because the effects on vegetative growth and sporulation could not be separated experimentally. The riboflavin and nicotinamide are not essential requirements for growth and ascosporulation, but rather ancillary additives to provide for enhanced growth and ascosporulation. The reason for this deduction is that *B. fulva* will grow and produce asci on Czapek's agar, but on this medium asci production is much reduced as compared to asci formation on PDA or on our liquid synthetic medium. The function of ascorbate is probably not nutritional but rather to suppress germination of asci which are produced, thereby promoting the increased asci numbers. This role of ascorbate was proposed initially by Yates et al. (1968) in the case of ascospore germination by *B. nivea*.

The percent of the asci population which can be induced to germinate by heat shock appeared constant in the complete synthetic medium with sucrose from spore crop to spore crop and did not seem to vary over an extended period

Table 1—Viable count of asci after 2 hr at 70°C in 0.01M phosphate buffer (pH 7.0) from separate experiments

Asci from synthetic medium[a]	Age of culture (days)	Microscopic count	Viable count	Viable count × 100 / Microscopic count
	14	1.6×10^6	1.4×10^5	8.7
	16	4.0×10^5	5.4×10^4	13.5
	24	1.0×10^6	8.5×10^4	8.5
			1.1×10^5	11.0
	40	1.1×10^6	1.0×10^5	10.0
		2.5×10^6	2.9×10^5	11.6
Asci from potato dextrose broth	14	1.8×10^6	1.7×10^5	9.4
			1.6×10^5	8.8

[a]Synthetic medium composed of Czapek's salts mixture, riboflavin (9.0 mg/l), nicotinamide (100 mg/l), ascorbate (200 mg/l), $CaCl_2$ (5.5g/l) and 2% sucrose

of time (Table 1). It appears that at any given time the heat shockable population does not increase appreciably in comparison to the total population. The low percent activation we have noted for *B. fulva* has been previously reported by Splittstoesser et al. (1969).

The purpose of our research was to determine the cultural requirements of ascosporogenesis in *B. fulva* in order to delineate further the mechanism of ascospore heat resistance. The importance of ascospore maturation to heat resistance seems apparent from the report by Hull (1939) of a biphasic death curve which could mean different physiological states of ascospores or asci in terms of resistance to moist heat. This premise is further indicated by asynchronous maturation of ascospores within the ascus in *Hansenula anomala* as observed by Bandoni et al. (1966). It may be that within a heat activatable population of asci, all of the ascospores may not be affected or may be killed by the heat shock procedure.

A recent report by Denny and Brown (1969) showed that pigmentation may be correlated with different heat resistances of *B. fulva* strains. Preliminary experiments in our laboratory indicate the synthetic medium may be valuable in segregating *B. fulva* strains with different heat resistant properties. By incorporating 2% agar into the complete synthetic medium (pH 4.0) with sucrose, we have been successful in rapidly screening isolates because the differences in pigmentation reported by Denny et al. (1969) on potato dextrose agar are exaggerated and more definite on the solidified synthetic medium.

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CHEMISTRY OF THIAMINE DEGRADATION
Mechanisms of Thiamine Degradation in a Model System

INTRODUCTION

LOSS OF biological activity of thiamine resulting from thermal degradation has been studied extensively (Farrer, 1955). However, little is known about the chemical mechanisms involved. Farrer and Morrison (1949) studied thiamine destruction in buffered solutions and showed that it follows the Arrhenius equation:

$$\ln k = I - \frac{E}{RT}$$

Where E is the energy of activation, I and R are constants, k is the velocity coefficient and T is the temperature in degrees absolute.

Subsequent studies revealed that this equation can be successfully used in predicting thiamine retention in foods (Farrer, 1950, 1953; Bendix et al., 1951; Sabry and Tannous, 1961; and Agrawal et al., 1963).

Farrer (1955), in his review on the thermal destruction of thiamine in foods, commented on the very low values for E obtained with several foods. Two possible reactions were postulated, one involving cleavage of the CH_2 "bridge" leaving pyrimidine and thiazole moieties, and the other involving the breakdown of the thiazole ring with the production of hydrogen sulfide. It was not clear from this review which of the two reactions has the lower E value.

In the last two decades, numerous degradation products of thiamine from model systems have been identified (Matsukawa et al., 1951; Gaudiano et al., 1966; Arnold et al., 1969; and Dwivedi et al., 1972a,b).

The chemical properties of thiamine in relation to pH have been reviewed by Metzler (1960). In his review, Metzler postulated mechanisms of formation of compounds produced by thiamine degradation. However, the formation of compounds identified by Arnold et al. (1969) and Dwivedi et al. (1972b) cannot be explained by these mechanisms.

Dwivedi and Arnold (1971) reported quantitative data on the production of hydrogen sulfide from heat degradation of thiamine under acidic and neutral conditions. Lack of quantitative information for other degradation products has limited our understanding of the mechanisms of thermal degradation of thiamine.

The purpose of this investigation was to provide additional quantitative information on the production of major thiamine degradation products under various conditions of heating, thereby providing evidence on the mechanisms involved in thiamine breakdown.

EXPERIMENTAL

USING thiamine-S^{35} hydrochloride (Amersham/Searle Corp.), solutions (0.5 mg/ml) having specific activity of 0.05 mc/ml and buffered to pH 3.5, 5.0, 6.0, 7.0 and 8.0 with 0.1 M phosphate buffer were prepared. Thiamine-S^{35} solutions (0.5 ml each) were placed in screw-cap vials sealed with Teflon-lined caps. Duplicate samples of thiamine-S^{35} solutions were autoclaved for 15 and 30 min. One sample each from thiamine $-S^{35}$ solutions of pH 6.0 and 8.0 were autoclaved for 2 hr to study the effect of extensive heating on the nature of thermal degradation products of thiamine. Heating time was measured from the point that the solutions reached a temperature of 121°C in the autoclave. Immediately after autoclaving, tubes were cooled by refrigerating for 2 hr. Unheated thiamine samples were used as controls.

The separation of degradation products of heated thiamine-S^{35} by thin layer chromatography (TLC) was accomplished by the method of Waring et al. (1968). Heated thiamine-S^{35} samples were spotted on precoated silica gel TLC plates containing fluorescent indicator (No. 6060 Chromatogram Sheet, Eastman Kodak Co.). Since hydrogen sulfide and some highly volatile sulfur compounds are produced from heated thiamine solutions, TLC of these samples was carried out under a fume hood. Low concentrations of thiamine-S^{35} in radioactive samples did not permit direct observation under ultraviolet light. Degradation products of thiamine-S^{35} separated by TLC were located on chromatograms by comparison with chromatograms obtained from identically treated nonradioactive thiamine solutions containing 10 mg/ml thiamine. The solvent mixture for TLC analysis, consisting of acetonitrile/H_2O/formic acid:40/10/sufficient formic acid to adjust pH to 2.54, was allowed to equilibrate for 15 min in covered chromatography jars. Detection of compounds was accomplished in an ultraviolet viewing box using short wavelength (254 nm) for absorbing compounds and 366 nm for fluorescent compounds.

Dried thin layer chromatograms were cut so that 1-½ in. strips containing all the radioactive compounds were obtained. Strips were scanned with a Vanguard autoscanner, Model 880. The percentage of total radioactivity of thiamine-S^{35} contributed by individual spots was estimated by measuring the areas under each peak in the recorder tracings.

Loss of thiamine by autoclaving in each sample was calculated by comparing the areas under the radiochromatogram peak corresponding to thiamine from unheated thiamine solutions with those from heated samples.

RESULTS & DISCUSSION

THE RADIOCHROMATOGRAM resulting from autoclaved (15 min) pH 6.0 thiamine-S^{35} solution is shown in Figure 1. Only two radioactive peaks were obtained in the scan. Peaks A and B were due to thiamine-S^{35} and 4-methyl-5-(β-hydroxyethyl) thiazole-S^{35}, respectively, as determined by the R_f values and other

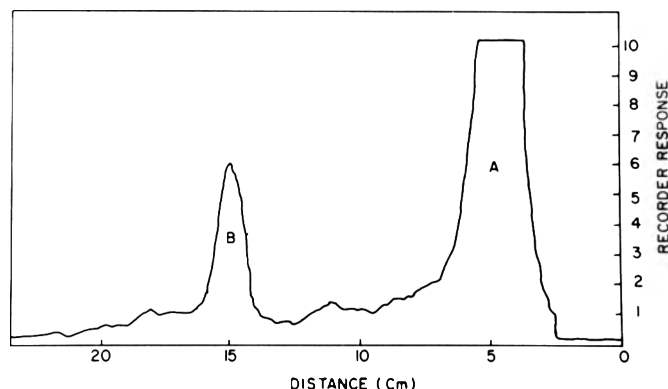

Fig 1—Radiochromatogram scan of a TLC plate from 4 µl thiamine-S^{35} sample at pH 6.0 autoclaved for 15 min. Recorder range = 3,000 cpm, time constant = B.

previously reported data (Dwivedi et al., 1972a). For the calculation of the area of peak A, radiochromatograms with this peak on-scale were obtained by using higher attenuator settings. The total radioactivity of 4-methyl-5-(β-hydroxyethyl) thiazole-S^{35} accounted for most of thiamine-S^{35} destroyed during autoclaving (Table 1). Thiamine-S^{35} samples (pH 6.0) autoclaved for 30 min and 2 hr gave radiochromatograms similar to the sample autoclaved for 15 min. The total radioactivity associated with 4-methyl-5-(β-hydroxyethyl) thiazole peaks in these samples was nearly equal to thiamine-S^{35} destroyed.

Thiamine-S^{35} samples at pH 3.5 and 5.0 autoclaved for 15 and 30 min also gave only two radioactive peaks on radiochromatograms, again corresponding to thiamine-S^{35} and 4-methyl-5-(β-hydroxyethyl) thiazole-S^{35}. 4-Methyl-5-(β-hydroxyethyl) thiazole-S^{35} peaks obtained with autoclaved thiamine-S^{35} samples adjusted to pH 3.5, were smaller compared to corresponding peaks obtained from thiamine-S^{35} samples at pH 5.0 and 6.0, but equal to thiamine-S^{35} destroyed in autoclaving (Table 1).

The radiochromatogram of thiamine-S^{35} sample adjusted to pH 7.0 and autoclaved for 15 min is shown in Figure 2. The total radioactivity of all the peaks in this scan does not equal the total radioactivity of unheated thiamine-S^{35} peak. This difference may be accountable to the production of hydrogen sulfide (Dwivedi and Arnold, 1971) and other highly volatile S^{35} compounds which escape during TLC analysis. The radiochromatogram of the autoclaved pH 7.0 thiamine-S^{35} solutions still shows a peak for 4-methyl-5-(β-hydroxyethyl) thiazole-S^{35}, but this peak accounts for only a small proportion of the thiamine-S^{35} destroyed (Table 1). Two small peaks, C and D, with R_f values higher than 4-methyl-5-(β-hydroxyethyl) thiazole, are present in this scan. These peaks may be elemental sulfur and other relatively nonpolar compounds (Morfee and Liska, 1972). A broad peak near thiamine-S^{35} in this radiochromatogram is possibly due to complexes formed by the condensation of aldehyde groups produced by the opening of the thiazole ring. This peak does not match with any of the sulfur-containing thiamine-related compounds available for comparison. The radiochromatogram of pH 7.0 thiamine-S^{35} sample autoclaved for 30 min gave a pattern similar to Figure 2.

Thiamine-S^{35} samples adjusted to pH 8.0 and autoclaved for 15 and 30 min gave radiochromatograms similar to the pH 7.0 sample, except for the absence of 4-methyl-5-(β-hydroxyethyl) thiazole-S^{35} peak (Fig. 3).

The chemical properties of thiamine in relation to pH have been reviewed by Metzler (1960). Williams and Ruehle (1935) first described the unusual behavior of thiamine chloride hydrochloride and other thiamine salts when titrated with base. The first step in this titration, with pK_1 at about 4.8, represents the titration of the protonated amino pyrimidine group (Structure I > II, Fig. 4). In the second titration step, with a midpoint at pH 9.2, two equivalents of base are taken up in a slow reaction with the formation of pseudo base (III). This intermediate undergoes a ring opening with further dissociation of a proton to give the ionized thiol form (IV) (Clarke and Gurin, 1935; Watanabe and Asahi, 1955).

Based on the above considerations, thiamine under acidic conditions will be present as I and/or II depending on the pH of the solution.

The fact that less thiamine breakdown occurs at pH 3.5 than at pH 5.0 or 6.0 suggests that the protonated form of thiamine (I), which predominates at pH

Table 1—Production of 4-methyl-5-(β-hydroxyethyl) thiazole-S^{35} in relation to thiamine-S^{35} destruction at various pH's and autoclaving times

pH	Time of autoclaving min	% thiamine-S^{35} destroyed	Thiazole-S^{35} derivative as % thiamine-S^{35} destroyed
3.5	15	4.1	100.0
	30	7.8	98.5
5.0	15	8.5	99.6
	30	15.1	99.0
6.0	15	9.6	96.0
	30	18.2	98.6
	120	41.0	97.9
7.0	15	28.0	16.4
	30	46.5	8.9
8.0	15	32.7	–
	30	58.5	–
	120	92.6	–

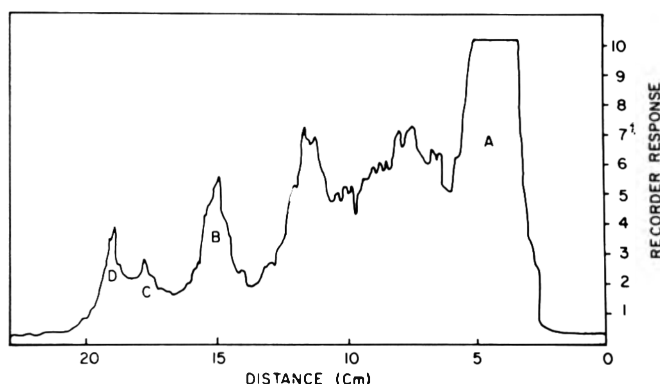

Fig. 2—Radiochromatogram scan of a TLC plate from 4 μl thiamine-S^{35} sample at pH 7.0 autoclaved for 15 min. Recorder range = 3,000 cpm, time constant = B.

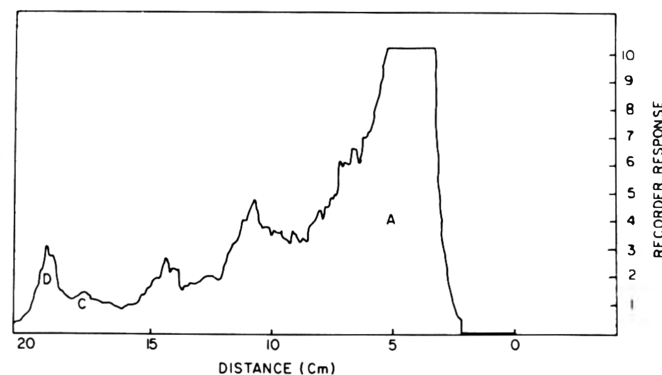

Fig. 3—Radiochromatogram scan of a TLC plate from 6 μl thiamine-S^{35} sample at pH 6.0 autoclaved for 15 min. Recorder range = 3,000 cpm, time constant = B.

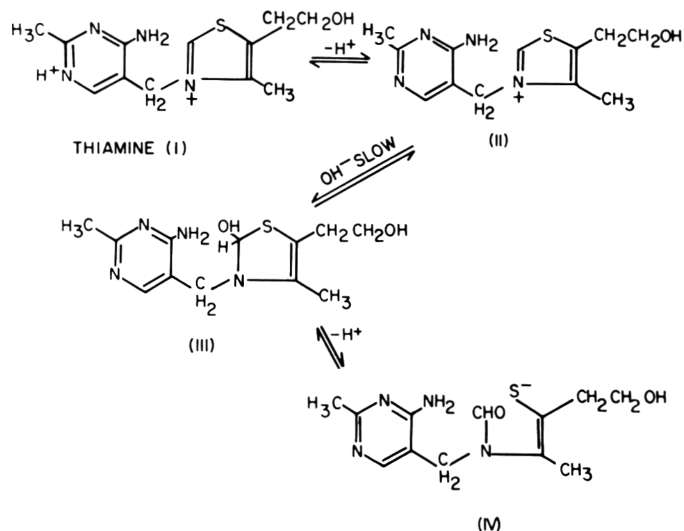

Fig. 4—Acid-base equilibria of thiamine.

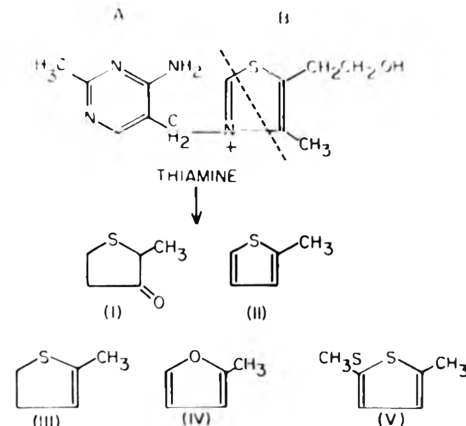

Fig. 5—Volatile compounds from heat degraded thiamine.

3.5, is less prone to thermal destruction than thiamine (II). The mode of thiamine destruction appears to be the same for both forms. This mechanism involves breaking of the C-N bond of the methylene "bridge" between thiazole and pyrimidine moieties of thiamine (Fig. 4). A pyrimidine derivative, probably 2-methyl-4-amino-5-hydroxymethyl pyrimidine, and 4-methyl-5-(β-hydroxyethyl) thiazole result from the breakdown mechanism.

Although at pH 7.0 and 8.0 most of the thiamine should exist as structure II, it will be in equilibrium with small amounts of pseudo base (III) and thiol form (IV). This study indicates that under neutral and slightly alkaline conditions, the mode of thiamine breakdown on heating is governed by the minor forms, (III and IV), of thiamine. Hydrogen sulfide is probably the major degradation product of the thiazole moiety of thiamine under these conditions (Dwivedi and Arnold, 1971). We believe that the energy of activation for the removal of hydrogen sulfide from thiamine at pH 7.0 and 8.0 is smaller than the energy of activation for breakdown of the methylene bridge of thiamine. This would account for the absence of 4-methyl-5-(β-hydroxyethyl) thiazole in the pH 8.0 sample, and its decreased concentration at pH 7.0. When hydrogen sulfide is released from thiamine, the equilibrium between thiamine forms II, III and IV would be disturbed and more thiamine II would be converted to the pseudo base (III) and the thiol form (IV).

In previous work on the volatile degradation products of thiamine (Dwivedi et al., 1972b; Arnold et al., 1969) a number of thiamine degradation products which apparently arise from the 5-carbon fragment B of the thiazole moiety (Fig. 5) were identifed. The reaction sequence of the formation of these compounds is not known. Comparatively few degradation products were obtained from pH 8.0 thiamine samples indicating that energies of activation for the formation of these compounds from thiamine is much higher than that for the main reaction of hydrogen sulfide elimination. We believe that the N-C or the C-S bond is broken as indicated by the broken line, followed by the rearrangement and cyclization of fragment B. In this process one molecule of hydrogen sulfide or water is eliminated and a 5-carbon fragment is cleaved, resulting in the formation of these compounds.

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GAS CHROMATOGRAPHIC ESTIMATION OF THIAMINE

INTRODUCTION

AT THE RECENT White House Conference on Food, Nutrition and Health (1969), nutrient labeling of processed food products was proposed. Effective enforcement of such a policy will require simple, rapid and reliable methods for nutrient analyses. The chemical, biological and microbial assay procedures currently available for vitamin analyses are generally too complex and time consuming for routine quality control applications.

Several chemical and biological methods have been developed for the quantitative determination of thiamine. Chemical assay methods are generally preferred to biological methods since analyses can be performed more rapidly and economically; also, chemical methods are usually more reliable for routine determinations.

The thiochrome method (Assoc. Vitamin Chem., 1966) is the most widely used method for thiamine. However, the necessity for close attention to details makes this method unsuitable for routine control work. Materials which absorb thiamine and those which affect thiochrome fluorescence cause interference in the thiochrome method.

Gas chromatographic methods, in general, offer unique advantages in speed of separation, sensitivity and convenience in quantitation. Gas chromatography may therefore be a useful analytical tool in the implementation of nutrient labeling requirements.

Since thiamine is not volatile and decomposes at high temperatures, it does not lend itself to direct gas-liquid chromatography (GLC). Attempts have been made to prepare organic esters of thiamine, such as o-benzoyl thiamine (Hagiwara et al., 1963) and trimethylsilyl (TMS) derivative (Amos and Neal, 1970). These derivatives are not volatile at low temperatures and pyrolyze above 250°C, rendering them unsuitable for GLC analysis.

Williams et al. (1935) studied the molecular structure of thiamine, and found that thiamine can be cleaved quantitatively into pyrimidine and thiazole moieties (Fig. 1) by allowing it to stand in a concentrated sodium sulfite solution at pH 5. In previous work on the thermal degradation products of thiamine, we found that the thiazole moiety of thiamine, 4-methyl-5-(β-hydroxyethyl) thiazole, is sufficiently volatile as such or as its TMS derivative (Fig. 1) to be quantitated by GLC. Based on this information, a simple GLC method for thiamine estimation has been developed.

EXPERIMENTAL

Reagents

Thiamine hydrochloride or mononitrate, U.S.P. grade
Hydrochloric acid, reagent grade
Sodium sulfite, reagent grade
Sodium bisulfite, reagent grade
Chloroform, reagent grade
Sodium hydroxide, reagent grade
Methanol, reagent grade
BSA (N,O-Bis [trimethylsilyl acetamide]) (Pierce Chemical Co., Rockford, Ill.)
Phosphatase preparation: Takadiastase (Parke, Davis & Co., Detroit, Mich.) or Mylase-P (Wallerstein Labs., Morton Grove, Ill.)

Extraction and derivatization

As with other chemical assay methods for thiamine determination, thiamine must first be extracted from the food or pharmaceutical preparation. Methods described below for extraction are similar to extraction procedures for other thiamine assay procedures.

Food products. A modification of the extraction procedures of Hochberg et al. (1945) and Association of Vitamin Chemists (1966) was used. The sample (about 10g) was weighed into an Erlenmeyer flask and 50 ml 0.1N HCl was added. The sample was blended in a Waring Blendor for 1 min at low speed. Liquids and fine particle solids did not require blending. The suspension was refluxed at 100°C for 30 min. The mixture was allowed to cool and adjusted to pH 6.0 with 5N NaOH. 10g solid sodium bisulfite and 6g sodium sulfite were added and dissolved by shaking. 1g Takadiastase or Mylase-P was added, and the suspension was incubated, preferably overnight at 37°C or 3 hr at 45–50°C. 20 ml distilled water was added and the digested sample was then filtered using Whatman No. 40 filter paper. 50 ml of the filtrate was collected and extracted twice with 100 ml chloroform/methanol: 90/10 solvent. The chloroform/methanol extract was filtered and concentrated to dryness on a rotary evaporator. During concentration, the temperature of the waterbath was maintained at 30–40°C. The trimethylsilyl (TMS) derivative was prepared by adding 1 ml of BSA to the dried extract and allowing it to stand at room temperature for 1 hr with occasional shaking.

A reference thiamine sample was prepared by adding a known quantity (0.1–0.5 mg) of thiamine into the sample. Thiamine extraction and TMS derivative formation of the reference were carried out as described above.

Pharmaceutical preparations. Pharmaceutical preparations containing free thiamine as hydrochloride or mononitrate did not require

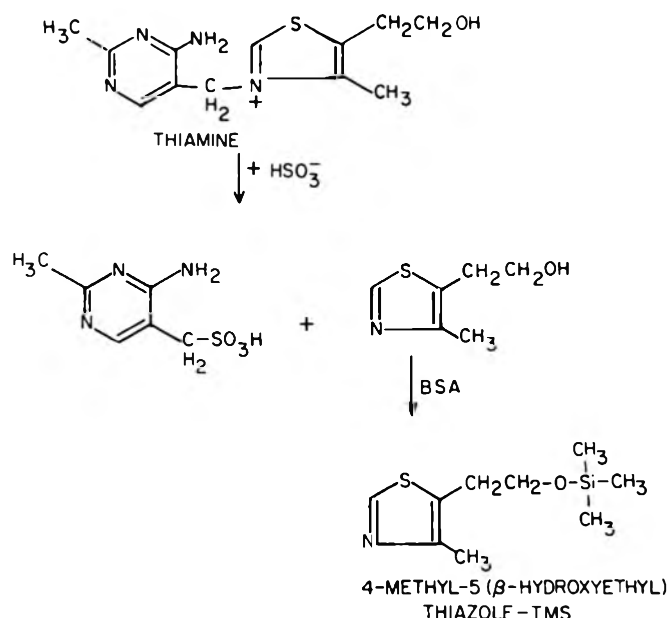

Fig. 1—Sulfite cleavage of thiamine and TMS derivatization of thiazole moiety.

enzyme digestion. Weighed tablets were ground to a fine powder in a mortar. A portion (about 1g) of the powdered tablets was weighed and placed into a 50 ml Erlenmeyer flask. 25 ml deionized distilled water was added. The pH of the sample was adjusted to 6.0 by adding 5.0g of sodium bisulfite and 3.0g sodium sulfite. The solution was mixed thoroughly and allowed to stand overnight at room temperature or warmed to 40–50°C for 3 hr. It was then extracted with chloroform/methanol, dried and derivatized as discussed for food products. A reference thiamine sample was prepared by adding a known quantity (0.1–0.5 mg) of thiamine, and processed as described above.

Gas-liquid chromatography (GLC)

TMS derivatives were analyzed on a Varian Aerograph, Model 1700, gas chromatograph with a flame ionization detector. Operating parameters for GLC were as follows:

Sample volume: 5 μl
Column: 1.7 m × 3.2 mm o.d. stainless steel packed with OV-7 (3.0%) and OV-22 (1.5%) on Chromosorb G (80/100 mesh A/W, DMCS)
Column temperature: 110°C
Injection port temperature: 150°C
Detector temperature: 175°C
Carrier gas flow (N_2): 52 ml/min
Range: 10^{-11} amps/mv

A standard curve (Fig. 2) was prepared by plotting various concentrations of thiamine vs. corresponding peak areas. Areas of the peak corresponding to the TMS derivative of 4-methyl-5-(β-hydroxyethyl) thiazole were calculated for reference and unknown samples. The concentration of thiamine in the unknown sample was calculated as follows:

Area of TMS-thiazole peak (reference) = a
Area of TMS-thiazole peak (unknown) = b
Concentration of thiamine in unknown = x
Amount of thiamine added in reference = s
Total conc. of thiamine in reference = x + s
Correction factor (thiamine conc. corresponding to zero peak area) = c

$$b(x + s - c) = a(x - c)$$
$$bx + bs - bc = ax - ac$$
$$x = \frac{bs + ac - bc}{a - b}$$

These calculations were necessary to correct for extraction efficiency of 4-methyl-5-(β-hydroxyethyl) thiazole.

RESULTS & DISCUSSION

THE QUANTITATIVE aspects of thiamine cleavage into pyrimidine and thiazole moieties, extraction of 4-methyl-5-(β-hydroxyethyl) thiazole, and procedure of TMS derivative formation were studied. The response to various amounts of thiamine assayed as the TMS derivative of its cleavage product, 4-methyl-5-(β-hydroxyethyl) thiazole, has been determined (Fig. 2). It is apparent from this curve that a straight line relationship exists between peak area and thiamine concentration for a wide range.

4-Methyl-5-(β-hydroxyethyl) thiazole can be assayed by GLC without derivatization. However, considerable peak tailing occurs because of the free -OH group in the compound. The presence of the -OH group also limits its volatility. Consequently, only nonpolar stationary phases such as SE-30 may be used, which limits the resolution of 4-methyl-5-(β-hydroxyethyl) thiazole peak from other contaminants. Conversion of the compound to its TMS derivative greatly improves its gas chromatographic behavior. The minimum thiamine concentration which was determined from 10g samples of food products without concentration of the BSA derivatizing solution was 0.05 mg.

Additional sensitivity is provided by allowing the BSA reagent to evaporate at room temperature after derivatization and redissolving the derivative in a smaller

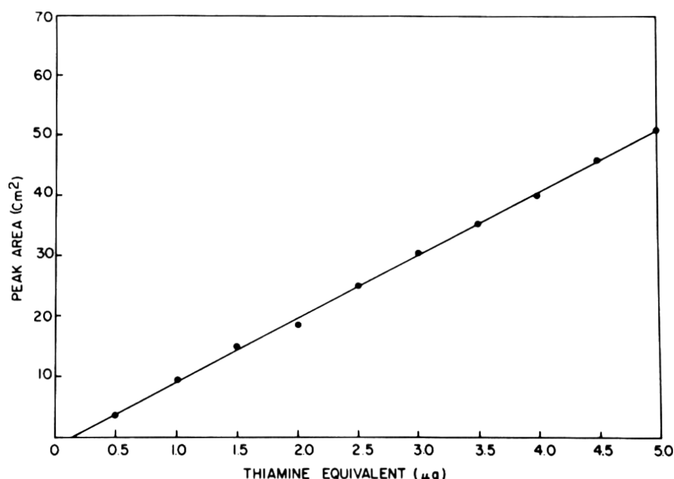

Fig. 2–Standard curve of thiamine concentration vs. peak area of 4-methyl-5-(β-hydroxyethyl) thiazole peak.

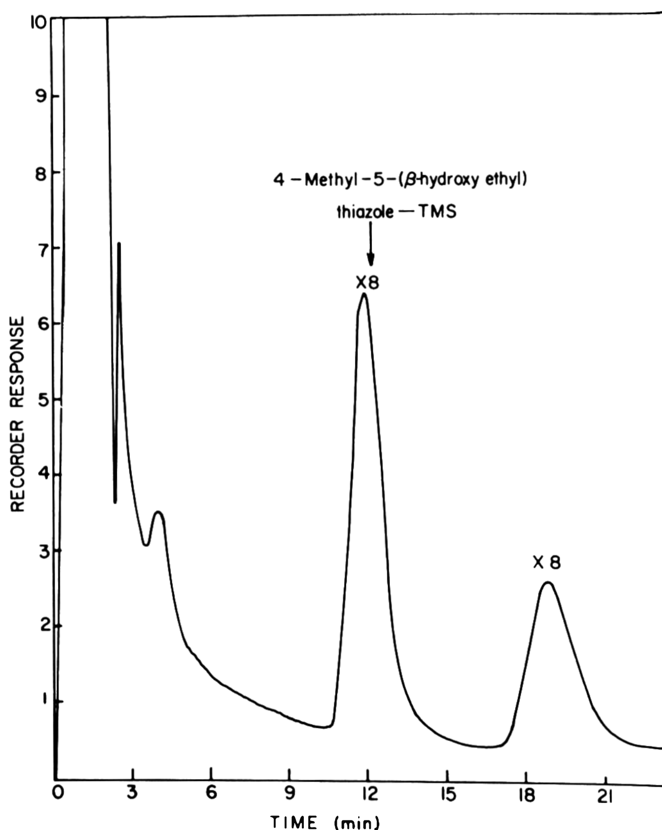

Fig. 3–Gas chromatographic separation of 4-methyl-5-(β-hydroxyethyl) thiazole-TMS derived from a commercial multi-vitamin and mineral preparation.

volume of solvent prior to injection into the gas chromatograph. A ten-fold concentration of the derivative was achieved in this manner.

The possibility of forming the trifluoroacetyl derivative of 4-methyl-5-(β-hydroxyethyl) thiazole using trifluoroacetic anhydride (TFAA) and assaying it by GLC using electron capture detector was studied. Preliminary efforts were not successful in achieving quantitative conversion of 4-methyl-5-(β-hydroxyethyl) thiazole to its TFA derivative. Halogen derivatives of 4-methyl-5-(β-hydroxyethyl) thiazole show promise of improving the sensitivity of thiamine estimation by GLC using an electron capture detector. Additional work is needed on this aspect.

The applicability of the GLC method of thiamine estimation in pharmaceutical preparations was studied. A typical gas chromatographic pattern obtained for a multi-vitamin and mineral tablet product is shown in Figure 3. A few extra peaks were observed in this chromatogram, but these peaks did not interfere with the 4-methyl-5-(β-hydroxyethyl) thiazole-TMS peak. Other pharmaceutical preparations gave patterns different from Figure 3, but no interference by other compounds was noticed. Similarly, no interference by other compounds was observed with food products. Thiamine contents of two breakfast cereals and fortified wheat flour were determined in this study.

Resolution of the compound being studied from interfering constituents is a major advantage of the gas chromatographic method. Gas chromatography is primarily a separation procedure. Resolution can be further improved, if necessary, by manipulation of various GLC parameters.

The proposed GLC procedure provides a simple means of checking for interfering constituents. If sodium sulfite is omitted from the procedure (pH adjusted to 6.0 at this step by sodium hydroxide), thiamine will not be cleaved and 4-methyl-5-(β-hydroxyethyl) thiazole will not be formed. The sample thus serves as a control. Any peaks in the region of that for 4-methyl-5-(β-hydroxyethyl) thiazole would be attributed to interfering constituents. Control samples from food products used in this study produced no peaks in this region of the chromatograms.

Since the GLC method for thiamine estimation developed in this study does not require column cleanup of the digested sample, more samples can be handled in a working day compared to conventional methods.

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MEAT TENDERNESS: AGE RELATED CHANGES IN BOVINE INTRAMUSCULAR COLLAGEN

INTRODUCTION

THE RELATIONSHIP between the structure of muscle and the tenderness of meat has been an area of active investigation for many years. Locker (1960) and later Marsh and Leet (1966) and Herring et al. (1967) convincingly demonstrated that the toughness of meat is related to the contraction of the muscle fibres as reflected by the sarcomere length. However there is always the "background toughness" due to the presence of the connective tissue proteins, particularly the collagen fibres. These fibres possess an extremely high tensile strength and the physical properties change with age. Even after denaturation by cooking, these fibres can still possess a significant tensile strength, hence it is not surprising that it has been implicated in the toughness of meat (for review see Bailey, 1972).

In attempts to correlate the properties of collagen with the texture of meat, age changes in bovine muscle and tendon collagen have been studied by several workers (Tuma et al., 1963; Goll et al., 1964; Hill, 1966; Carmichael and Lawrie, 1967). All these workers showed a decrease in the solubility of collagen in nondenaturing solvents, suggesting an increase in covalent crosslinking. It is well known that changes in collagen occur with increasing age of the various collagenous tissues and this has been attributed to an increase in the number of covalent crosslinks between the collagen molecules in the fibre (Verzar, 1964; Sinex, 1968; Bailey, 1969).

Recent investigations carried out on the covalent crosslinks have led to a better understanding of their nature and biosynthesis (for review see Traub and Piez, 1971). Two types of crosslinks are known to occur: intramolecular crosslinks within the tropocollagen molecule (Bornstein and Piez, 1966) and intermolecular crosslinks between the molecules in the intact fibre. The intermolecular crosslinks are the more important type in the stabilization of the collagen fibres and chemical studies have demonstrated that they are aldimine-type bonds (Bailey, 1969). The three major aldimine type crosslinks have been isolated in a stabilized form from borohydride reduced collagen. One compound was identified as Nϵ-(5-amino-5-carboxypentyl) hydroxylysine, (hydroxylysinonorleucine) and derived from the reaction of allysine and hydroxylysine (Fig. 1).

The second reducible compound was found to be more stable and originally thought to be an aldol (Bailey et al., 1970) but has subsequently been shown to be Δ 6,7 dehydro-Nϵ-(2-hydroxy-5-amino-5-carboxypentyl)-hydroxylysine, (dehydro-hydroxylysinohydroxynorleucine) (Davis and Bailey, 1971; Mechanic et al., 1971) (Fig. 2).

A third component termed Fr. C also possesses properties of an aldimine bond in the nonreduced native fibre but has not yet been characterized. It is a major crosslink in a number of tissues and is probably derived from the intramolecular aldol crosslink.

The present paper deals with studies on these intermolecular crosslinks in bovine muscle and tendon with particular regard to: (a) variation in the type of crosslinkage of intramuscular collagen

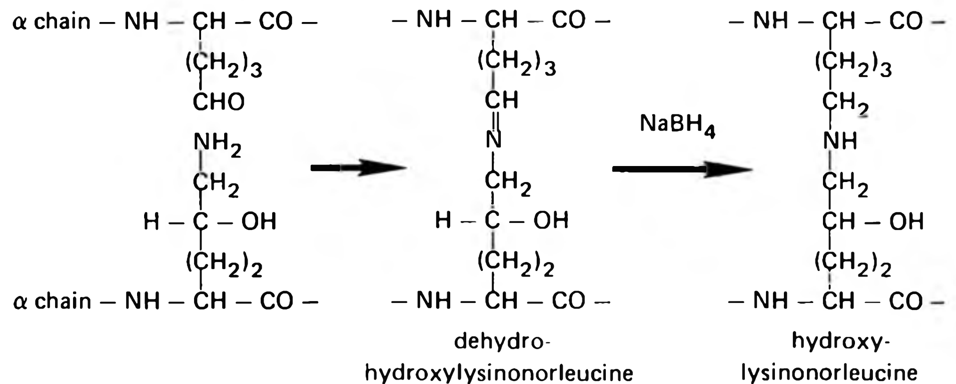

Fig. 1—Biosynthesis and borohydride stabilization of one of the major aldimine-type intermolecular crosslinks in collagen.

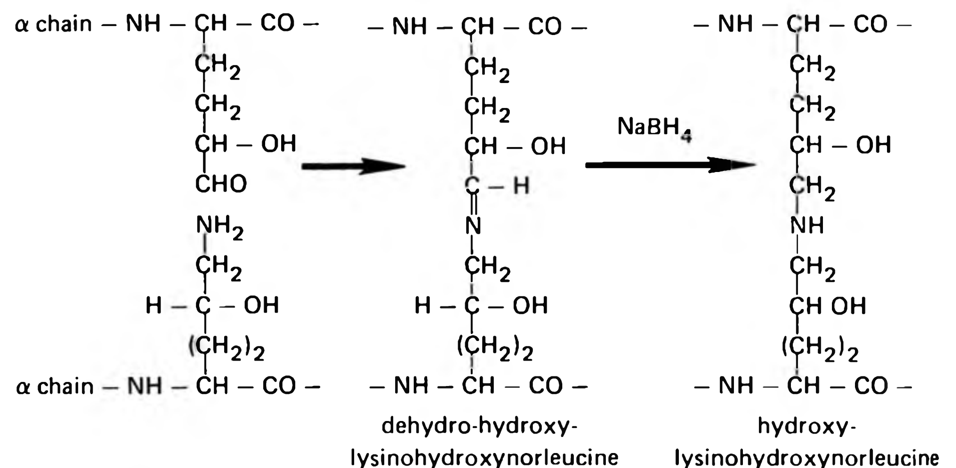

Fig. 2—Biosynthesis and borohydride stabilization of a second major aldimine-type crosslink in collagen.

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(IMC) between various muscles of the same age; (b) variation in the nature of the crosslinks in tendon and IMC of the same muscle; (c) changes in the nature of the crosslinks of IMC with age for a given muscle; and (d) comparison of the rate of aging of different muscles.

EXPERIMENTAL

Materials

Bovine tendon and intramuscular collagen (IMC) were obtained from animals of ages ranging from foetus to 15 yr old. Generally these animals were analyzed at each particular age. The muscles were chosen from different positions in the bovine body to represent some highly active limb muscles (Cassens and Cooper, 1971) which are generally classified as producing tough meat and less active internal body muscles which produce tender meat (Ramsbottom et al., 1945). The muscles used were the neck muscle sternomandibularis, leg muscles (gastrocnemius, extensor carpi radialis and superficial digital flexor) and the body muscles longissimus dorsi, vastus, infra spinatus and psoas major. Tendons were obtained from the gastrocnemius, the extensor carpi radialis and superficial digital flexor.

Tendon collagen was prepared by shredding dissected tendon samples in an MSE Ato Mix homogenizer and washing with several changes of carbonate buffered 0.9% saline (pH 7.4) at ambient temperature (17°C). Homogenized fibres were washed with copious amounts of water to remove salts and then freeze dried.

Intramuscular collagen was prepared from muscle after removal of gross fatty tissue. The sample was then shredded and homogenized in carbonate buffered 0.9% saline at 18°C using a Marsh-Snow (1950) blender. The myofibrillar proteins were removed by disintegration leaving the connective tissue network intact. After extensive washing with saline to remove traces of myofibrillar proteins, the samples were dialyzed against distilled water overnight and freeze dried. Hydroxyproline determinations were carried out to demonstrate the purity of the collagen using the Technicon automatic method (Grant, 1964).

Methods

The samples were analyzed for the presence of reducible crosslinks by amino acid analysis after reduction with tritiated sodium borohydride as previously described in detail (Bailey et al., 1970). About 200 mg of freeze-dried samples were suspended in 30.0 ml of buffered saline solution. Solid sodium borotritide was diluted with nonradioactive borohydride to approximately 10 mCi/mM. The borohydride was then dissolved in carbonate buffered saline at 5°C and equal volumes of the solution were added to the various suspensions of fibres to give a final ratio of 50:1 (w:w) based on the dry weight of the collagen fibre. Reduction was allowed to proceed for 1 hr after which time the excess borohydride was destroyed by the addition of acetic acid lowering the pH to 4.0. The suspension was dialyzed for 24 hr and the reduced fibres were then freeze dried and hydrolyzed.

Hydrolysis was performed by refluxing for 24 hr with freshly redistilled constant boiling

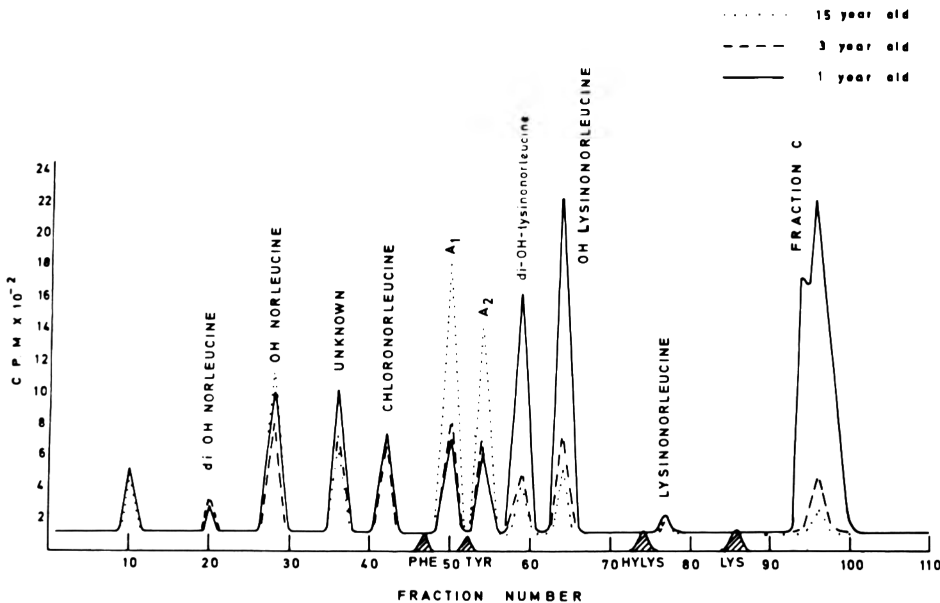

Fig. 3—Typical elution pattern of an acid hydrolysate of tritiated NaB^3H_4 reduced intact collagen, intramuscular or tendon. The position of the known reducible components is shown relative to the usual amino acids using a volatile buffer system. The effect of age is demonstrated by the change in elution pattern at three different ages.

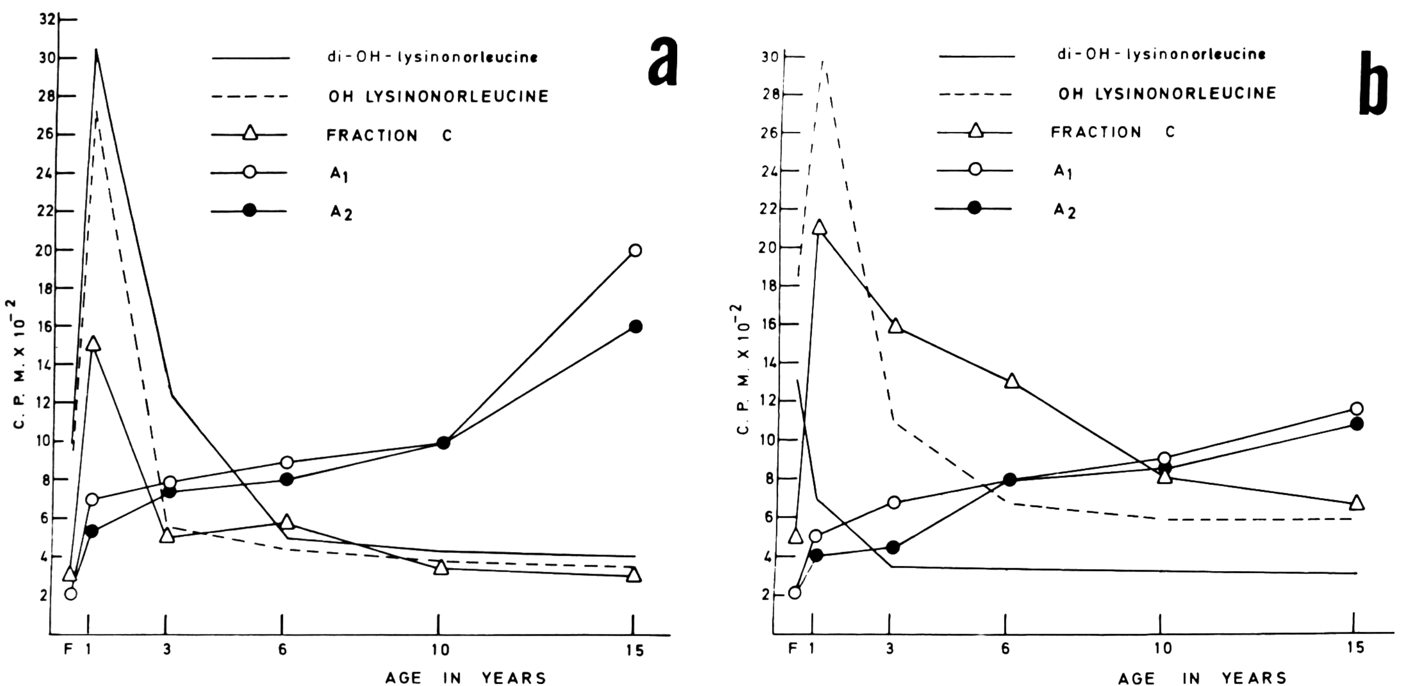

Fig. 4—Effect of age on the relative amounts of the various reducible components in (a) intramuscular collagen from extensor carpi radialis and (b) tendon collagen from extensor carpi radialis.

HCl. The HCl was then removed by evaporation in vacuo at 60°C.

The Technicon Auto-Analyser was adapted for use with volatile buffers (Bailey et al., 1970) based on the system described by Padieu et al. (1965). The amino acids and the radioactive crosslinking compounds were eluted with a gradient formed by running 1.0N pyridine/formic acid (pH 5.0) into a mixing vessel containing 350 ml of 0.1N pyridine/formic acid (pH 2.9). The flow rate was adjusted to 42 ml/hr and 5.0 ml fractions were collected. 0.2 ml of collected fractions were counted using Bray's solution (1960) in a Liquid Scintillation Counter. The identification of the radioactive peaks as intermolecular crosslinks was confirmed by analysis against authentic samples on the Beckman amino acid analyser (Bailey et al., 1970).

RESULTS

Nature of reducible components

A typical elution pattern depicting the position of the borohydride reduced crosslinks hydroxylysinohydroxynorleucine (di-OH-LNL), hydroxylysinonorleucine (OH-LNL) and Fr. C obtained from reduced collagen are shown in Figure 3. Basically similar patterns were obtained from both the IMC and tendon collagen.

Effect of age

Intramuscular collagen (IMC). All the muscles analyzed from the 1-yr old steer contained a higher proportion of reduced crosslinks di-OH-LNL, OH-LNL and Fr. C than the same muscles obtained from the foetal calf. Subsequent to this age there was a steady decrease in all these reducible crosslinks up to about 5 yr when the rate slowed down until the components were virtually absent at 10 yr. The rate of change of these reducible components is summarized in Figure 4, the values being taken from the peak height of each component shown in Figure 3 at a series of different ages. Figure 4 shows the changes for the extensor carpi radialis muscle, very similar curves were obtained for the IMC 4(a) and tendon collagen (4b).

Concomitant with the decrease in these components, two more components designated A1 and A2 increased in proportion until at 5–6 yr they were the major reducible components (Fig. 3). The rate of increase of these components is shown in Figure 4a and b. Since the proportion of the reducible components A1 and A2 increased with age of the tissues, this suggested that they may possibly be related to the crosslinking process; however, recent studies have demonstrated that they are not crosslinks (Robins and Bailey, 1972).

Tendon collagen. Similar elution patterns were obtained for the tendon collagen. Figure 4b summarises the variation with age of the relative amounts of reducible components in tendon collagen from the extensor carpi radialis muscle. Comparison of the rates of decrease in the reducible crosslinks with those of the IMC indicates that the IMC rate is faster than that of tendon. Furthermore, the proportion of di-OH-LNL in reduced IMC appears to be consistently greater than in tendon collagen (Fig. 5).

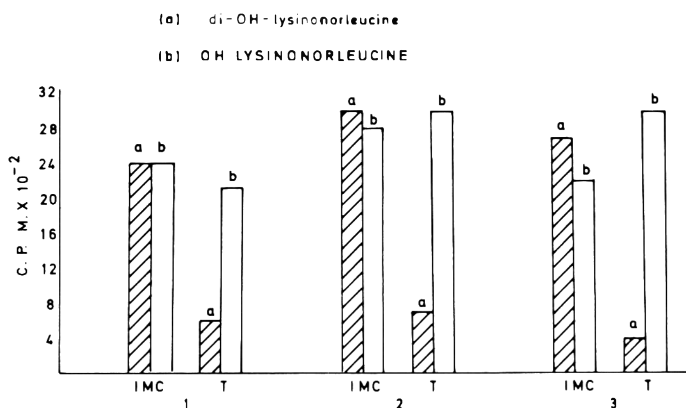

Fig. 5–Relative proportion of hydroxylysinohydroxynorleucine (a) and hydroxylysinonorleucine (b) in the intramuscular and tendon collagen of a number of muscles from a 12-month old steer: 1. superficial digital flexor; 2. extensor carpi radialis; and 3. gastrocnemius.

Effect of thermal denaturation

Intramuscular and tendon collagen from the superficial digital flexor were heated for 10 min at 80°C and the residue reduced with tritiated sodium borohydride in order to observe the thermal stability of the intermolecular crosslinks. The muscle was taken from a 1-yr old steer since the maximum number of reducible crosslinks are present at this age. The elution pattern obtained demonstrated that dehydro-OH-LNL and non-reduced Fr. C are thermally labile since their reduced forms were virtually absent in the reduced denatured collagen. The other aldimine bond dehydro-di-OH-LNL however is more stable and is still present in approximately the same amount as in the native material. Fractions A1 and A2 were also found to be unchanged by thermal denaturation (Fig. 6).

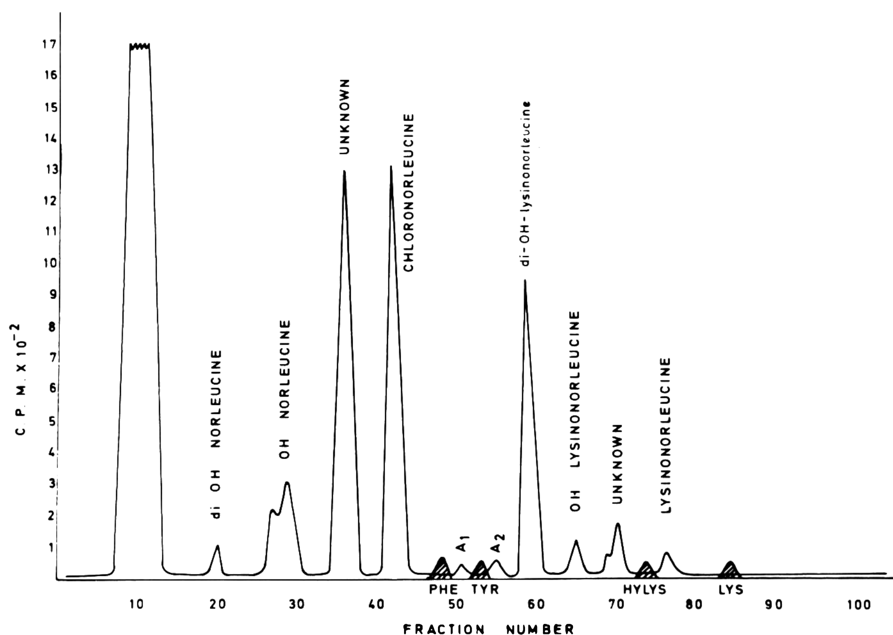

Fig. 6–Effect of thermal denaturation prior to reduction in the elution pattern of intramuscular collagen from the superficial digital flexor. Note the virtual disappearance of OH-LNL and Fr. C compared to Figure 3.

DISCUSSION

ANALYSIS of bovine intramuscular and tendon collagens has shown that both tissues have very similar content of reducible crosslinks. They both contain all the

reducible crosslinks so far known to occur in collagen, in contrast to skin collagen which does not contain dehydro-di-OH-LNL and bone collagen in which the latter is the only major reducible crosslink (Bailey et al., 1970).

In all the muscles and tendons studied there was a gradual decrease in the proportion of all three reducible cross-links until they were virtually absent at maturity. These results confirm the original proposal (Bailey, 1969) that they are intermediates in the crosslinking process and are gradually stabilized with increasing age. Using tissues from species with differing life spans we have recently demonstrated that this process does indeed follow the maturation process rather than the temporal age of the animal (Bailey and Shimokomaki, 1971). The mechanism by which the subsequent stabilization occurs has not yet been elucidated. One possible mechanism is that in vivo reduction of the aldimine bonds could occur. This type of process has been shown to occur in elastin where both dehydro-lysinonorleucine and its reduced form lysinonorleucine are present in the native elastin fibre (Lent and Franzblau, 1967). However, analysis of nonborohydride reduced collagen failed to reveal the presence of any of these reduced crosslinks (Bailey and Peach, 1971), indicating that in vivo reduction as a means of stabilizing the crosslinks is not operative in collagen.

The increase observed in the proportion of the reducible crosslinks from the foetal stage to a maximum at 12–18 months exactly paralleled the change in solubility (Carmichael and Lawrie, 1967). Similar results have been reported for the age-related solubility changes in rat skin (Wirtschafter and Bentley, 1962) and on sheep skin (Bowes and Raistrick, 1965). These changes can now be interpreted as being due to the rapid rate of growth and the formation of labile intermediate crosslinks. These labile bonds are readily cleaved by dilute organic acids and together with the noncovalently-bound neutral salt soluble collagen account for the high solubility during this stage. The subsequent decrease of solubility must be due to the decrease of overall rate of synthesis of new collagen thus giving time for the labile bonds to stabilize themselves by conversion to a nonreducible form. As the rate of synthesis of new collagen slows down, the collagen has time to stabilize and form the permanent, thermally stable crosslinks which render the collagen insoluble.

These results provide a rational basis for the variation in tenderness of meat with age. Although it was always a reasonable first hypothesis to suggest a relationship between the content of the tough collagen fibres and the tenderness of meat, it is now clear why this proposal falls down. The total collagen content is meaningless; rather, it is the relative proportion of the thermally labile and thermally stable intermolecular cross-links. As the proportion of stable cross-links increases with age the heat denatured collagen fibres in the cooked meat become stronger and thus make a greater contribution to the tenderness of the meat. Furthermore, our studies suggests that the tenderest beef should be obtained at about 12–14 months. Texture measurements are not usually measured over this age range but Henricksen and Moore (1965) have reported a maximum in tenderness at this age. Most other workers have compared widely different age groups.

Apart from the variation of texture with age the differences between muscles of the same animal even in immature bovines is considerable. The higher thermal stability of dehydro-di-OH-LNL and its predominance in the very insoluble collagens such as bone and cartilage suggested that it may be an important factor in the difference between muscles. However, no significant differences could be detected in the extent of crosslinking or in the relative proportion of dehydro-di-OH-LNL over the range of muscles analyzed. A clear difference was apparent between the active extensor carpi radialis muscle and the less active psoas major (Fig. 7) and these muscle are known to be tough and tender respectively. However, contrary to expectations, analysis of the extremely tough sternomandibularis muscle gave a lower value for the content of this crosslink than the extensor carpi radialis. The tenderness classification of various muscles is based on taste panels and shear values and it is always possible that some muscles are particularly susceptible to muscular contraction during rigor thus placing them in the incorrect order from a connective tissue point of view. This is particularly important in the case of the sternomandibularis which generally contracts without the restraint of the skeleton after removal of the head at slaughter. These differences in texture between individual muscles are, other things being equal, almost certainly related to the quality of the IMC fibres. Recently McClain et al. (1970) have demonstrated a difference in the solubility of IMC of longissimus dorsi and the semimembranosus of porcine, but the relationship of this finding to the tenderness of the meat was not assessed by these workers.

It is interesting to note that a consistent difference was obtained in the proportion of di-OH-LNL in the IMC and the tendon of the same muscle (Fig. 5). Recently Mohr and Bendall (1969) had shown that the IMC of sternomandibularis was less soluble and apparently more extensively crosslinked than tendon collagen. Our present results offer an explanation of their observation. In addition, it

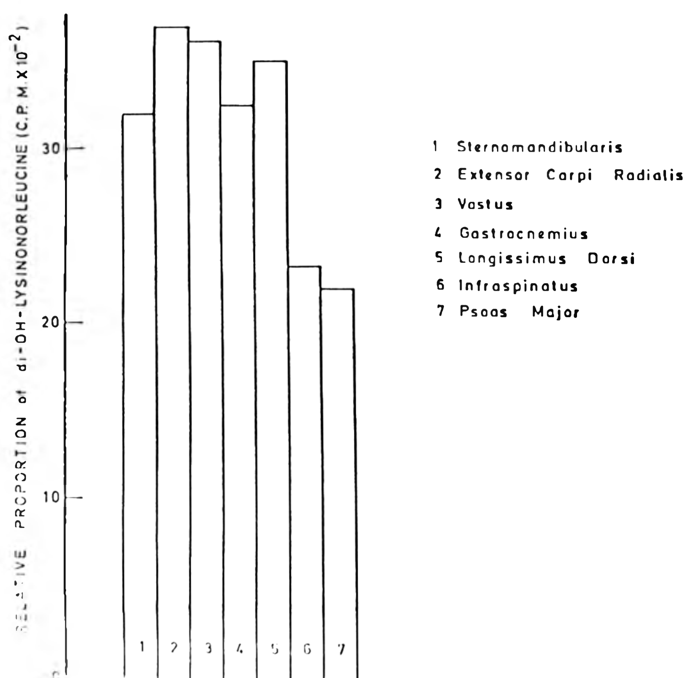

Fig. 7—Comparison of the proportion of the relatively stable crosslink, dehydro-di-OH lysinonorleucine in various muscles from a 12-month old steer classified according to their increasing tenderness from 1 to 7 according to Ramsbottom et al. (1945).

appears that the rate of decrease in the proportion of reducible crosslinks was faster in the case of IMC than tendon (Fig. 4a and 4b), again indicating the greater stability of the IMC.

Although at the outset it was hoped that the physiologically highly active muscles would possess stronger intramuscular collagen fibres, possibly observed as a greater extent of crosslinking, a higher proportion of di-OH-LNL or as a faster rate of stabilization, no convincing differences between the various muscles could be demonstrated. It may be that the differences are smaller than expected. Preliminary studies on partially lathyritic porcine tissues support this suggestion. Clearly further detailed studies along these lines are required.

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EFFECTS OF POST-MORTEM AGING AND STRETCHING ON THE MACROMOLECULAR PROPERTIES OF COLLAGEN

INTRODUCTION

THE EXACT RELATIONSHIP of collagen to meat tenderness has not been elucidated. This is particularly true in regard to the post-mortem tenderization process. Early investigators found no change in the alkali-insoluble portion of collagen during post-mortem aging (Wierbicki et al., 1954, 1955; Khan and van den Berg, 1964; Sayre, 1968; and de Fremery and Streeter, 1969). Other researchers, utilizing salt-soluble collagens, found that solubility decreased during post-mortem aging (Gross, 1958; and McClain et al., 1965). Since the above studies were concerned mainly with gross solubility, they did not consider structural changes which might be occurring at the molecular level. As a result, most researchers tend to discount any possible role for collagen in the post-mortem tenderization process. However, recent ultracentrifugation data by Kruggel and Field (1971) showed that changes in collagen sub-unit composition occurred in stretched and aged bovine tissue. The present study used acrylamide-gel electrophoresis to further investigate covalent cross-linking of collagen from stretched and/or aged muscle. The relationship between post-mortem tenderization and changes in cross-link structure was investigated. In addition, data comparing epimysial and intramuscular collagen are presented.

EXPERIMENTAL

Treatments

Intramuscular and epimysial collagen from the 12th rib region of the longissimus was used in this study. The tissue was obtained from both sides of nine yearling Hereford bulls whose carcasses graded good. The bulls were of similar genetic background and similar management. One side of each carcass served as a control while the longissimus from the other side was stretched. Stretching of the longissimus was accomplished by suspending one side of each carcass by a hook through the obturator foramen immediately after exsanguination, as described by Hostetler et al. (1970). In addition, the thoracic vertebra between the 12th and 13th ribs and all muscles, except the longissimus and a 10 cm portion of tissue on the ventral side, were cut to increase strain upon the longissimus muscle. The control side of each carcass was suspended in the normal manner by a hook between the Achilles tendon and the tibia.

After 24 hr aging at 2°C, two adjacent 20 cm portions of the longissimus which were either posterior or anterior to the cut between the 12th and 13th rib were taken from the control and stretched sides. Adjacent 20 cm segments (those segments remaining that were either anterior or posterior to the 12th and 13th rib cut) of the longissimus from the control and stretched sides were aged on the carcass an additional 20 days at 2°C. Longissimus muscle segments from each side of each carcass were randomized for aging purposes. All longissimus muscle samples were frozen and stored at −20°C after aging until analysis could be completed. Muscle segments from each carcass included control and stretched muscle aged 1 day, and control and stretched muscle aged 21 days.

Sarcomere length

Sarcomere length was determined on each muscle segment after thawing to room temperature (Smith et al., 1969) to give an objective measure of the amount of stretching due to treatment. Six adjacent sarcomeres on 25 myofibrils were measured for each sample, using phase contrast microscopy at 1000× with oil immersion.

Warner-Bratzler shear

Steaks cut from portions of the longissimus adjacent to that taken for isolation of intramuscular collagen were used for tenderness evaluation. The 3 cm thick steaks were roasted in a 177°C oven to an internal temperature of 71°C and cooled overnight to 2°C before evaluation. Three cores, 2.54 cm in diameter, were cut parallel with the muscle fibers. Each core was sheared across the fibers three times. Values were recorded as an average of the nine shears.

Extraction and purification

Portions of the longissimus muscle were trimmed of epimysial tissue and fat, then ground through a 4.8 mm plate. 100g of the chilled, ground meat were placed in a Waring Blendor with 400 ml of cold, isotonic NaCl and blended for 10 sec at full speed. After standing for 2 min, the solution was blended for an additional 5 sec. A layer of cheesecloth was placed over the mouth of the blender. The blender cup was then inverted and agitated, allowing myofibrillar proteins and salt solution to pass through. This procedure was repeated four times. No detectable amounts of salt soluble collagen were found in the salt solution after this short time in NaCl. The crude intramuscular collagen, which remained in the blender cup, was physically removed and centrifuged at 5,500 × G for 5 min to remove any remaining NaCl (Field, 1970). Based upon amount of hydroxyproline in crude intramuscular collagen, 50–60% of the total muscle collagen was recovered by this procedure. All steps were carried out in a 2°C cold room. Epimysial samples were extracted in the same manner, except that they were cut into small pieces and not ground before extraction.

Purification and extraction of the intramuscular and epimysial collagen with 5M guanidine hydrochloride was according to the procedure of Mohr and Bendall (1969), as modified by Kruggel and Field (1971). The purified guanidine hydrochloride-soluble collagen contained 11.5–12.8% hydroxyproline, indicating that this portion was approximately 85% collagen.

Guanidine hydrochloride was utilized because it is a strong denaturing agent capable of extracting high molecular weight aggregates containing intermolecular cross-links, as well as intramolecular links.

Acrylamide-gel electrophoresis

Discontinuous acrylamide-gel electrophoresis was used to determine relative amounts of α, β, and γ components of the guanidine hydrochloride-soluble intramuscular and epimysial collagen. Four gel concentrations were used (Clark and Vies, 1968).

Lyophilized guanidine hydrochloride-soluble collagen was solubilized in 0.01N acetic acid (10 mg collagen/ml acetic acid) and centrifuged at 3,500 × G for 20 min the remove any insoluble material. Sucrose was added (5% by volume) to increase the density of the collagen solution. This prevented the sample from mixing with the buffer after application onto the gel. Immediately before application, the collagen solution was denatured for 15 min in a 48°C water bath to insure separation of the α, β, and γ components as outlined by Clark and Veis (1968). 50 μliters of collagen solution were applied to the top of the gel. A 0.05M sodium acetate buffer (pH 4.8) was used in both the upper and lower electrode baths. Electrophoresis was carried out at a constant current of 4 mA per gel for 9 hr.

The electrophoresis apparatus consisted of a Canalco Model 1200 Bath and a Heathkit Regulated High Voltage Power Supply, Model IP-17. After a 9 hr run, the gels were fixed in 12% trichloroacetic acid and stained with 1.0% naphthol blue-black. Non-protein areas were destained by diffusion over a period of several days with 7% acetic acid. Densitometer readings were made at 615 nm on a Gilford gel scanner attached to a Beckman DU Monochromator and a Gilford Model 222 Photometer. The area of each component was calculated using a planimeter. Alpha, β, and γ component areas were calculated as a percent of the total area recorded by the densitometer.

Heat labile collagen

Crude intramuscular and epimysial collagen samples were isolated from surrounding tissue with isotonic NaCl in a Waring Blendor as described previously.

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Approximately 2g of each collagen sample were heated in 15 ml of Ringer's solution for 8 min at 60°C in a constant temperature water bath. The heat soluble portion was removed by centrifugation at 1,800 × G for 5 min. Washing the residue with 10 ml additional Ringer's solution, and then centrifuging, effectively removed any remaining heat labile collagen from the residue (Hill, 1966).

Samples were then hydrolyzed under pressure in 6N HCl for 15–16 hr at 120°C in teflon-lined, screw-capped 50 ml test tubes. Humin was removed from the residue with activated charcoal G-60, and then filtered through a Büchner funnel using suction. After neutralization with 6N NaOH, the amount of hydroxyproline in each portion was determined according to the method of Woessner (1961). Percent heat labile collagen was calculated by adding the amount of collagen in the soluble portion to the amount in the residue, and then dividing the heat labile by the total.

Gas-liquid chromatography

Samples of purified collagen hydrolysates (0.25 ml) were dried at 100°C under a stream of N_2 and the n-proply N-acetyl esters of the amino acids were prepared using the procedure of Coulter and Hahn (1968).

Statistical analyses

Differences in muscle characteristics for the four treatments were determined by least squares analysis (Steel and Torrie, 1960). In addition, homogeneity of adjacent longissimus muscle segments which were posterior or anterior to the cut between the 12th and 13th rib were tested. This analysis was possible since both anterior and posterior portions were aged 1 or 21 days. A factorial confounded in blocks, as described by Cochran and Cox (1968), was used. No significant effect in shear value, or in collagen sub-unit composition due to the two locations on each longissimus was observed. Therefore, the data presented assumes homogeneity of the segments of the longissimus used for the characteristics studied.

RESULTS & DISCUSSION

WARNER-BRATZLER SHEAR values decreased significantly ($P < 0.01$) as a result of stretching and/or aging (Table 1). The shear value of the control, nonaged muscle was 14.5 kg, as compared to 11.3 kg for the stretched and 11.6 kg for the aged muscle. The muscle which had been stretched and then aged had the lowest shear value (9.3 kg); however, this value was not significantly different when compared to the stretched or aged muscle. Stretching increased sarcomere length from an average of 1.9μ to an average length of 2.8μ, while subsequent aging had no significant ($P < 0.01$) effect upon sarcomere length (Table 1). Sarcomere length was increased approximately 58% by stretching, indicating that intramuscular collagen was stretched as well.

The sub-unit composition of intramuscular guanidine hydrochloride-soluble collagen for each treatment is also shown in Table 1. Acrylamide-gel electrophoresis indicated that the percentage of α component increased from 65.2% in the control, nonaged muscle to 70.7% in the stretched and aged tissue, while the γ component decreased from 6.8 to 4.0%. The amount of β component decreased slightly, from 28.2 to 27.4%. A trend toward increasing α component and decreasing γ component was evident in the stretched and aged muscle. Although the relatively large variation among samples, combined with a low number of observations, may have prevented differences from being statistically significant, similar changes in collagen from stretched and aged tissue have been reported (Kruggel and Field, 1971) using sucrose-density ultracentrifugation. Table 2 represents a comparison of α component obtained with acrylamide-gel electrophoresis and sucrose-density ultracentrifugation. It is important to note that the acrylamide-gel electrophoresis ultilized in the present study gave quite comparable results to the sucrose-density ultracentrifugation results of Kruggel and Field (1971). The apparent changes in collagen sub-unit components suggest that some type of structural change may have occurred in intramuscular collagen as a result of stretching and aging.

It is possible that structural changes, represented by a measurement of sub-unit composition, were actually greater than indicated. Sub-unit composition is used to monitor changes in macromolecular structure, because it is the best tool available. However, this technique has several drawbacks. In the first place, only that collagen which has been solubilized (4–6%) can be categorized as to sub-unit composition. Another disadvantage of this procedure involves the sub-unit migration on acrylamide gels. Although this method was chosen because of the good resolution of α, β, and γ components which can be obtained, higher molecular weight aggregates are not able to migrate into the gel. Miller et al. (1967) have shown that these higher molecular weight aggregates do exist in guanidine hydrochloride-soluble collagen.

Rigby (1964) has shown that extensions of rat tail tendon above 3% lowered its thermal shrinkage temperature, thus supporting the contention that stretching of excised collagen is capable of producing structural changes in collagen. He postulated that this decrease was due to mechanical disruption of the polar, amorphous region of the molecule responsible for covalent bonding. His thought was that, since the polar regions of the molecule are more disordered than the remainder, they would be mechanically weaker, and the first to disrupt.

In the present study, it appears that initial stretching does not have much effect on sub-unit composition. The stretched intramuscular collagen had 66.5% α component, compared to 65.2% for the control (Table 1). The intramuscular collagen which had been stretched and subsequently aged had 70.7% α component. It would appear that, under the conditions of this study, stretching alone is not sufficient to cause breakage of covalent cross-links; the conformational changes in the stretched collagen are probably of a non-covalent nature. The

Table 1–Least squares means for muscle characteristics and intramuscular collagen sub-unit composition of control and stretched muscles aged 1 or 21 days

	Aged 1 day		Aged 21 days		
	Control (n=9)	Stretched (n=9)	Control (n=9)	Stretched (n=9)	Standard error
Shear values, kg	14.5[a]	11.3[b]	11.6[b]	9.3[b]	0.8
Sarcomere length, μ	1.9[b]	2.8[a]	1.9[b]	3.1[a]	0.2
Alpha component, %	65.2	66.5	67.2	70.7	2.1
Beta component, %	28.2	28.0	27.6	27.4	2.2
Gamma component, %	6.8	5.6	5.3	4.0	1.3

[a,b]Means with different superscripts are significantly different ($P < 0.01$).

Table 2–Estimation of intramuscular collagen alpha component by two methods[a]

	Aged 1 day		Aged 21 days		
	Control	Stretched	Control	Stretched	Standard error
Disc-gel electrophoresis (n=36)	65.2	66.5	67.2	70.7	2.1
Sucrose-density ultracentrifugation[b] (n=36)	60.5	62.4	64.3	67.6	2.3

[a]Expressed as percent of sub-unit composition
[b]From Kruggel and Field (1971)

change in conformation brought about by stretching appears to have the effect of allowing forces present during post-mortem aging to act. For instance, conformational changes in the polypeptide chains of collagen may allow H_2O to enter the cross-link region allowing disruption of the aldol link. This is possible because the aldols were originally subject to dehydration, resulting in formation of an α, β-unsaturated system (Fieser and Fieser, 1956). Royals (1954) states that this α, β-unsaturated carbonyl system can be easily reversed to free aldols by the addition of H_2O.

In addition to the model proposed above, it may be that the structural changes resulting from stretching could allow enzymatic activity to occur. The normal helical structure of native collagen is resistant to proteolytic degradation, because of the tight packing of molecules within the fiber. A loosening of the network caused by stretching would allow enzymes to penetrate. In addition, stress occurring as a result of stretching combined with the low post-mortem pH may cause sufficient denaturation to allow some non-specific proteolytic activity.

The fact that the α component tended to increase with stretching and aging indicates that a structural change may have occurred within the collagen network. This change is probably due to cross-link disruption between polypeptide chains, although limited proteolysis cannot be excluded as a possibility.

Correlations in Table 3 relate the structural changes observed in stretched and aged tissue to tenderness. Increasing shear values were negatively correlated to the amount of unlinked α component ($r = -0.46$) and positively correlated to γ component ($r = 0.53$) at ($P < 0.01$). These correlations may be forced since control and stretched tissues were combined. Nevertheless, a similar correlation between α component and tenderness has been reported by Kruggel et al. (1970). These results provide evidence that the molecular configuration of the intramuscular collagen network is involved in meat tenderness.

Alpha component was negatively correlated with both β component ($r = -0.72$) and γ component ($r = -0.54$). This was expected, as it represents a forced correlation obtained by measuring each component as a percent of the total. As percent of α component increases, the percent measured as β or γ must decrease.

The percent heat labile intramuscular and epimysial collagen obtained from heating at 60°C for 10 min in Ringer's solution is given in Table 4. Treatment had no significant ($P < 0.05$) effect upon yield of heat labile collagen. However, tissue which was aged 21 days but not stretched had the highest yield of heat labile collagen. Differences in amount of heat labile collagen between intramuscular and epimysial samples were highly significant ($P < 0.01$). Intramuscular collagen yielded from 2.8–3.4% heat labile collagen, while epimysial tissue yielded from 6.0–7.2%.

It was hypothesized that the structural changes observed with sub-unit composition could also be detected in yield of heat labile collagen. However, it appears that labile collagen yield is not sensitive enough to detect the small changes in molecular structure which have occurred. It is interesting to note that epimysial tissue yielded approximately twice as much heat labile collagen as did intramuscular tissue. The obvious differences in thermal stability indicate structural differences in amount of covalent cross-linking, or differences in primary structure. Preliminary attempts to compare sub-unit composition of guanidine hydrochloride-soluble intramuscular and epimysial collagen did not show any large differences in cross-linking between the two types.

The similarity in sub-unit composition of the soluble collagens seem to indicate that if differences in covalent cross-linking do exist, they must be within the insoluble portion. Solubility studies by McClain et al. (1971) tend to support this contention. They found that intramuscular collagen was less soluble in dilute salt or acid than epimysial collagen, indicating a greater amount of intermolecular cross-linking in the insoluble intramuscular tissue. Field et al. (1970) found that intramuscular collagen also had a higher thermal shrinkage temperature than epimysial collagen. Thermal shrinkage is a measure of stability, and therefore is influenced by amount of covalent cross-linking. Thus the higher thermal shrinkage temperature is another indication that intramuscular collagen is more highly cross-linked than epimysial collagen.

Gas-liquid chromatographic analysis of intramuscular and epimysial collagen did not show any large differences in amino acid content. The imino acid content of the two collagens was not significantly different ($P < 0.05$). Thus the differences in thermal stability probably cannot be attributed to stereochemical properties of the pyrrolidine ring structures of proline and hydroxyproline.

Evidence has been presented which indicates a structural change in intramuscular collagen at the molecular level as a result of stretching with subsequent aging. This structural change is related to increased tenderness. In addition, differences in yield of heat labile collagen between intramuscular and epimysial collagen were apparent.

Table 3—Correlation coefficients for intramuscular collagen and muscle characteristics (n=36)

	Sarcomere length	Heat labile intramuscular collagen	Heat labile epimysial[a] collagen	Intramuscular collagen components		
				Alpha	Beta	Gamma
Warner-Bratzler shear	−0.48*	−0.31	0.03	−0.46*	0.19	0.53*
Sarcomere length	–	−0.06	0.03	0.12	0.23	−0.29
Heat labile, intramuscular	–	–	0.27	0.18	0.08	0.28
Heat labile, epimysial	–	–	–	−0.16	−0.16	0.23
Alpha component	–	–	–	–	−0.72*	−0.54*
Beta component	–	–	–	–	–	0.01

[a](n=28)
*Significant at the 1% level

Table 4—Least squares means of heat labile collagen from intramuscular and epimysial collagen

	Aged 1 day		Aged 21 days		Standard
	Control	Stretched	Control	Stretched	error
Intramuscular collagen, % (n=36)	3.2[a]	2.8[a]	3.4[a]	3.2[a]	0.4
Epimysial collagen, % (n=28)	6.0[b]	6.2[b]	7.2[b]	6.5[b]	0.9

[a,b]Means with different superscripts within columns are significantly different ($P < 0.01$).

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MORAXELLA-ACINETOBACTER AS CONTAMINANTS OF BEEF AND OCCURRENCE IN RADURIZED PRODUCT

INTRODUCTION

GRAM-NEGATIVE diplococci and physiologically closely related diplobacilli have been isolated from foods (Koburger, 1964; Snodgrass and Koburger, 1967; Idziak and Incze, 1968; Silverman and Cohen, 1970; Shewan, 1971). In various reports, these organisms have been placed in the genera *Moraxella, Acinetobacter, Bacterium, Neisseria, Micrococcus, Diplococcus, Alcaligenes, Achromobacter*, and *Pseudomonas* (Baumann et al., 1968a). Early work of DeBord (1942) included three of the more commonly recognized genera in the tribe *Mimeae* and some reports still recognize this classification. More recently, however, closely related species and genera have been recognized (Baumann et al., 1968a,b; Johnson et al., 1970). The suggested genera *Moraxella* and *Acinetobacter* account for most of these organisms. Therefore, for the work reported here, the term *Moraxella-Acinetobacter* (abbreviated M-A) is used.

With the Gram stain most of the M-A cells appear dark red, especially when observing freshly isolated cultures. The cells of many strains, however, resist decolorization with alcohol, therefore tending toward a gram-positive reaction. There may be a tendency to a gram-variable reaction in certain growth phases. The form, coccal or rod, may also be variable accounting for some discrepancies in reports in the literature. The cultures are aerobic, oxidase-positive or oxidase-negative, nonmotile, and highly varied in their nutritional requirements. Some species in this group are considered pathogenic to man and animals, but the exact etiology of the purported disease has not been established.

During a study of the microflora of raw and radurized ground beef, samples were found to contain gram-negative diplococci and diplobacilli. These were studied further and identified as M-A. While the occurrence of these bacteria in meat was expected, there were no data in the literature to indicate the population density in ground beef. Work was therefore undertaken to determine their overall numbers in relation to the total microflora of raw and radurized ground beef. The potential source and comparative growth response were also studied.

MATERIALS & METHODS

Sources of ground beef

Machine dispensed units of ground beef weighing ¼ lb, as prepared for hamburgers, were obtained from a local commissary. This source was selected because it was known to provide a high quality, low microbial count product (Tiwari and Maxcy, 1971). For comparative purposes, samples were obtained from a local supermarket which commonly had products of unusually high microbial count (Tiwari and Maxcy, 1971). To determine the source of M-A, samples from freshly slaughtered animals and from cuts stored for different periods of time at 2°C were obtained by aseptic methods.

Irradiation

A cobalt-60 source providing approximately 17 Krad of gamma radiation per min was used. The physical facility was similar to that described by Teeny and Miyauchi (1970). While radurization was at room temperature (approximately 24°C), the short exposure increased the temperature of the meat no more than 1–2°C. Ground beef was placed in polyethylene pouches for radurization and subsequent storage. Each sample received a two side treatment and the absorbed dose was determined by Fricke dosimetry (Rizzo, 1968). Bacterial cultures were irradiated in the medium in which they were grown in 125 × 16 mm tubes.

Microbial count of beef

General sample preparation, dilution, and plating were according to the procedures outlined in *Microbiological Examination of Foods* (APHA, 1966). For total counts, Trypticase Soy Agar (TSA; Difco) was used with incubation at 32°C for 48 hr. All results were based on a minimum of three individual trials.

Isolation and identification

To determine the incidence of M-A, twenty colonies were picked by random design from countable TSA plates. Identification was based on the following observations: Gram stain reaction, morphology, motility, catalase production, nitrate reduction test, oxidase test, reaction in Triple Sugar Iron (TSI; Difco) Agar, reaction in Phenol Red Agar Base (PRAB; Difco) medium with 1% glucose or 10% lactose, litmus milk reaction, absence of proteolysis on milk agar, growth and reaction in Seller's Agar (SA; Difco) slants, and growth on Nutrient Agar (NA; Difco) slants (Samuels et al., 1969).

Table 1—Moraxella-Acinetobacter in raw and radurized (68 Krad) ground beef

	Raw		Radurized	
Supply	Total count per gram	Percent M-A	Total count per gram	Percent M-A
Retail	5.5×10^7	8	16×10^5	52
Central operation	2.3×10^5	27	3.8×10^4	43

RESULTS

Nature of the isolates and methods of counting

To obtain quantitative data, total counts were made and colonies chosen from countable plates for identification. Frequency of occurrence of the isolates was used in calculation of numbers of various types in the samples being studied.

Numerous isolates from raw and radurized ground beef were identified as M-A. To determine the population density of M-A, colonies were selected by random design from plates that had been used in the determination of the total count. Frequency of occurrence of M-A isolates as related to the total isolates was used to calculate the number of M-A in the samples.

Attempts to select media to estimate the numbers of M-A in ground beef were unsuccessful. For example, media such as Herellea Agar (Mandel et al., 1964) and Eosin Methylene Blue Agar were inadequately selective or excessively inhibitory to M-A.

Isolates grouped as M-A exhibited the following characteristics: Positive nitrate reduction reaction; utilized glucose and lactose with alkaline reaction in PRAB medium; in TSI agar, an alkaline slope was produced but no growth occurred in the butt; litmus milk showed no change during seven days, after which observations were terminated. Approximately 90% of the isolates were oxidase-positive tentatively identifying them as *Moraxella*. The other 10% were *Acinetobacter* (Baumann et al., 1968a). Other criteria such as colony characteristics, Gram stain reac-

tion, morphology, temperature of growth, salt tolerance, etc., were also used for identification. Typical isolates were examined by the Center for Disease Control, Atlanta, Ga., and the National Animal Disease Laboratory, Ames, Iowa. Their reports confirmed our observations that these bacteria are M-A.

M-A in ground beef

Ground beef was obtained from a local retail store, which previously had been observed to supply products with a high total count. For nine samples during a period of approximately six months the average total count was 5.5×10^7 per gram of which 8% were M-A (Table 1).

Samples of ground beef obtained over approximately the same period from a commissary showed an average total count of 2.3×10^5 of which 27% were M-A. Radurization of the above samples reduced the total count, but the residual flora consisted of a larger proportion of M-A.

Comparative growth of M-A and other members of the microflora in ground beef at 5°C

Samples from the retail stores were used to determine the effect of radurization and subsequent storage at 5°C on the population distribution in the microflora. In these samples approximately 3.0% of the total flora was M-A (Table 2). After radurization (68 Krad), however, M-A accounted for 35% of the flora. When raw and radurized samples were stored at 5°C for up to 6 days, M-A accounted for a smaller percentage of the total microflora as other bacteria grew more rapidly.

In samples of ground beef from the commissary, 42% of the total microflora was M-A. After irradiation (68 Krad), 58% were M-A (Table 2). During storage at 5°C bacteria other than M-A grew more rapidly thereby decreasing the percentage of M-A.

Comparative growth of M-A and other members of the microflora in ground beef at 25°C

The high total count in ground beef obtained from a retail store remained essentially constant after radurization at 5°C and storing in ¼ lb units at 25°C for 8 hr (Table 3). The nature of the microflora changed, however, as shown by 12% M-A at zero time and 3% after 8 hr. Irradiated product behaved similarly though containing a higher proportion of M-A at zero time.

Ground beef from the commissary contained fewer microorganisms but showed a similar change in the nature of the microflora during storage at 25°C (Table 3). There was a marked decrease in the frequency of occurrence of M-A in the raw product indicating other organisms were more suited to this environment. In the radurized samples M-A accounted for a high percentage of the microflora at zero time and were overgrown less rapidly than in raw samples.

Effect of various dose levels of radiation on the total microflora and the proportion of M-A in ground beef

Dose levels up to 272 Krad were used to determine the relative effect on M-A and the total microflora. Table 4 shows average results of three trials with ground beef from the commissary. As the radiation dose was increased, there was a reduction in the total microflora but an increase in the proportion of M-A.

Ground beef from a retail source showed the same general pattern of results as those obtained for the product from the commissary (Table 4). The retail samples, however, had a much higher initial total count and a lower initial proportion of M-A.

Sources of M-A in ground beef

Attempts were made to determine the source of M-A by examining cuts from various suppliers to the commissary. The results indicated these bacteria were wide-

Table 2–Growth of total microflora and Moraxella-Acinetovacter in ground beef at 5°C

Storage time at 5°C (hr)	Treatment	Samples from a retail store: Total count per gram	Samples from a retail store: Percent M-A	Samples from central processing: Total count per gram	Samples from central processing: Percent M-A
0	Raw	1.9×10^7	3	1.9×10^5	42
	Radurized[a]	4.4×10^5	35	2.3×10^4	58
72	Raw	1.1×10^9	<1	6.4×10^6	<1
	Radurized	1.4×10^6	6	2.5×10^4	41
144	Raw	5.2×10^9	<1	1.8×10^9	<1
	Radurized	1.1×10^8	<1	1.3×10^6	<1

[a]68 Krad

Table 3–Growth of total microflora and Moraxella-Acinetobacter in ground beef at 25°C

Storage time at 25°C (hr)	Treatment	Samples from a retail store: Total count per gram	Samples from a retail store: Percent M-A	Samples from central processing: Total count per gram	Samples from central processing: Percent M-A
0	Raw	1.3×10^8	12	2.6×10^5	10
	Radurized[a]	2.3×10^6	63	5.6×10^4	35
2	Raw	2.3×10^8	15	2.5×10^5	14
	Radurized	2.3×10^6	73	2.6×10^4	35
4	Raw	1.5×10^8	10	2.6×10^5	17
	Radurized	2.1×10^6	67	1.8×10^4	25
6	Raw	1.6×10^8	3	2.4×10^5	9
	Radurized	2.0×10^6	35	2.0×10^4	40
8	Raw	1.5×10^8	3	1.2×10^5	<1
	Radurized	4.3×10^6	18	1.8×10^4	22

[a]68 Krad

Table 4–The effect of irradiation on the total microflora and proportion of Moraxella-Acinetobacter in ground beef

Irradiation dose (Krad)	Samples from a retail store: Total count per gram	Samples from a retail store: Percent M-A	Samples from central processing: Total count per gram	Samples from central processing: Percent M-A
0	1.5×10^7	8	2.3×10^5	30
68	2.1×10^6	57	3.4×10^4	32
136	6.7×10^5	68	1.6×10^4	63
272	1.0×10^5	73	3.8×10^3	75

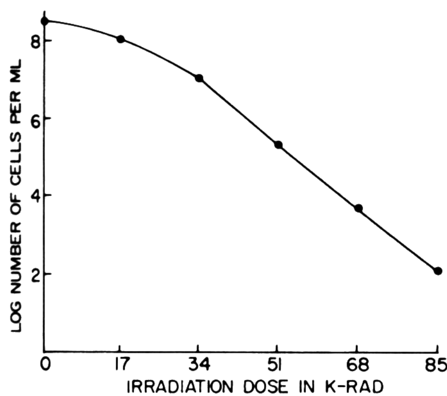

Fig. 1—The effect of radiation on M-A isolate M #2.

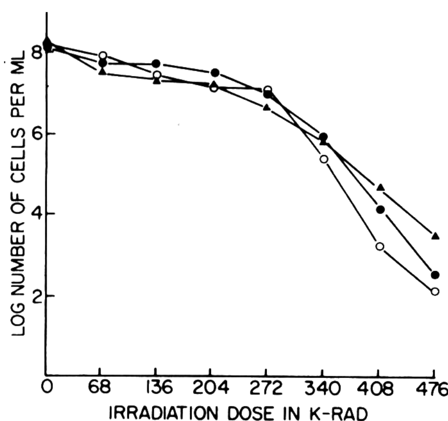

Fig. 2—The effect of gamma radiation on resistant strains of M-A: ●, isolate AM-5; ○, isolate AM-6; ▲, isolate AM-17.

spread because of their presence in samples from all suppliers and all cuts of beef examined. For example, cheeks and plates showed similar levels of contamination.

Susceptibility of pure cultures of M-A to destruction by radiation

Pure cultures of typical isolates of M-A grown in nutrient broth (Difco) were subjected to various dose levels of radiation. Considerable variation in radiation resistance existed among the isolates. The D_{10} values ranged from 15–112 Krad. A typical example of results for a sensitive strain (D_{10} value of 16 Krad) representing *Acinetobacter* is given in Figure 1. Average results for three to four trials with each of three of the most resistant isolates are given in Figure 2. These isolates were tentatively identified as *Moraxella* and had a D_{10} value of 91–112 Krad. The exceedingly resistant strains account for many of the organisms in the residual flora of radurized beef. The radiation resistance of M-A is in harmony with other known characteristics of resistance of these organisms such as their occurrence in freeze-dried beef (Silverman and Cohen, 1970).

DISCUSSION

M-A AS DEFINED in this work covers a rather broad spectrum of microorganisms, which have been isolated and reported from diverse foods. Most reports have dealt with a confirmation of their occurrence without regard to the frequency or to numbers in comparison to the total microbial population (Koburger, 1964; Snodgrass and Koburger, 1967). The magnitude of the population as reported in this work and the high percentage of the population in dried beef as reported by Silverman and Cohen (1970) suggested previous workers had included the M-A in various other genera. Baumann et al. (1968a) drew a similar conclusion about isolates from sources other than foods.

Lack of data on the population density of M-A in food products is likely attributable to unavailability of a specific selective medium. Indirect methods as used in this work are laborious. Furthermore, these organisms are relatively inert and are not associated with economic or recognized public health problems.

There is a sudden concern for these organisms, however, because of their prominence in a potential new technology of radurization. Some species are quite radiation resistant, and M-A constitute a major fraction of the flora of radurized ground beef. They also have been reported to be an important part of the microflora of other food products (Shewan, 1971) and particularly radurized products (Idziak and Incze, 1968; Licciardello et al., 1968).

Though the degree of importance of M-A is not known, our data indicate these organisms decrease in importance during storage of ground beef at 5°C or 25°C. Other members of the microflora in raw or radurized product grow more rapidly than M-A.

The microenvironment which allowed growth of the M-A constituting the inherent high degree of contamination of the beef must have been a specialized one even though these organisms were found in widely different cuts of beef. The generally recognized normal contaminants of beef, however, overgrew the M-A both at 5°C and 25°C. The significance of M-A in raw products is yet to be determined, because neither their origin nor importance in public health is known.

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EMULSIFYING CAPACITY OF MUSCLE PROTEIN: PHASE VOLUMES AT EMULSION COLLAPSE

INTRODUCTION

SWIFT ET AL. (1961) introduced a model system to investigate the emulsifying capacity of various sausage-type meats and meat proteins. The basic method consisted of blending an initial aliquot of oil with a meat slurry or protein extract to form an oil-in-water emulsion. Additional oil was incorporated into the emulsion until the emulsion collapsed or the "break-point" was attained. Slight modifications have been made in similar systems used by Hegarty et al. (1963), Carpenter and Saffle (1964), Borton et al. (1968), Christian and Saffle (1967), Acton and Saffle (1969), Graner et al. (1969) and Webb et al. (1970); however, the principles of the emulsification procedure have remained essentially unchanged.

The emulsifying capacity for the same general type of sausage meat differs between studies (Table 1) and is dependent on the variables of the model system utilized in the evaluation. Saffle (1968) pointed out that equipment design and shape, rpm of blending, rate of oil addition, temperature rise and other factors make comparison between studies difficult. In addition, the emulsifying capacity of various meat slurries and/or protein extracts has been expressed as the total ml of oil emulsified (Carpenter and Saffle, 1964; Webb et al., 1970), the ml of oil emulsified per g of meat (Borton et al., 1968) and the ml of oil emulsified per 100 mg of soluble protein (Carpenter and Saffle, 1964; Borton et al.,1968; Acton and Saffle, 1969; Graner et al., 1969). The report of Borton et al. (1968) showed that the relative rank in emulsifying capacity within a given group of meats will vary depending on the basis that capacity is expressed.

With the present methods of estimating the emulsifying capacity, oil-in-water emulsions have been produced and dispersed to maximum oil volumes. Theoretically, water-in-oil emulsions would occur upon further oil addition. Ostwald's geometric phase volume theory (Becher, 1965) proposed that emulsions would invert from one type to the other type when the internal phase volume exceeded 74% of the total emulsion volume. However, deformation of spherical droplets to the polyhedra form, the occurrence of heterogeneous droplet sizes, variation of the techniques of emulsification and the use of different types of emulsifying agents have allowed the preparation of emulsions containing up to 99% internal phase (Becher, 1965).

Since the basic technique utilized for emulsifying capacity studies has remained essentially unchanged from that initially proposed by Swift et al. (1961), the possibility exists that a phase volume factor may exert some control over the maximum level of oil addition found at the emulsion collapse point. This study was conducted to determine whether such a relationship exists within the basic method currently in use.

EXPERIMENTAL

THE METHOD OF Saffle and Galbreath (1964), as modified by Acton and Saffle (1969), was used for extraction of salt-soluble protein from frozen cow meat. Dilutions of the protein extracts were made prior to oil emulsification to provide solutions ranging in protein concentration from approximately 2.6 mg of protein/ml to 16 mg of protein/ml.

Emulsifying capacities for the salt-soluble protein of the solutions were determined by the procedure of Carpenter and Saffle (1964) as modified by Acton and Saffle (1969). The "emulsifying capacity" was expressed as the total ml of oil emulsified per 100 mg of soluble protein and the "emulsified volume" as the ml of oil emulsified by the 15 ml of the protein solution. The phase volume of oil at which emulsion collapse occurred was determined by calculating the percent oil volume of the total emulsion volume, i.e., ml of added oil phase plus ml of aqueous phase.

Table 1—Emulsifying capacities[a] of salt-soluble protein from various types of sausage meats

Meat type	Literature source: Carpenter & Saffle (1964)	Borton et al. (1968)	Acton (1970)
Cow meat	36.6	–	53.2
Pork trimmings	36.1	261.4	52.2
Beef cheek	32.7	271.3	51.6
Pork cheek	23.4	–	48.1
Beef hearts	22.6	273.2	49.6

[a]Expressed as ml of oil emulsified per 100 mg of protein

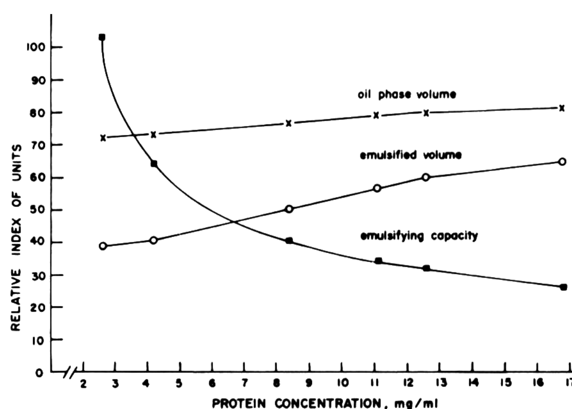

Fig. 1—The effect of protein concentration on emulsifying capacity (ml oil emulsified/100 mg protein), emulsified volume (total ml oil emulsified) and oil phase volume at collapse (percent oil in the total emulsion volume).

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The data of this study were compared to similar data collected by Carpenter (1964). In addition, previous studies (published and unpublished) relating to emulsifying capacities of muscle salt-soluble protein were re-examined to determine oil phase volumes at which emulsion collapse had occurred.

RESULTS & DISCUSSION

THE EMULSIFYING CAPACITY, emulsified volume and oil phase volume at the collapse point are shown in Figure 1 for the protein concentration range of 2.6-16.8 mg/ml. Previous data collected by Carpenter (1964) are shown in Figure 2. In both cases the ability of the salt-soluble protein to emulsify oil per unit of protein (emulsifying capacity) decreased as the concentration of protein in solution increased. Similar results for the oil-emulsifying efficiency were reported for isolated intracellular muscle proteins (Hegarty et al., 1963), soy sodium proteinate, potassium caseinate, and nonfat dry milk (Pearson et al., 1965) and sodium oleate (Becher, 1965).

In the concentration range of 2.6-16.8 mg of protein/ml (Fig. 1), the emulsifying capacity decreased by 75% and the emulsified volume increased by 88% as the concentration of protein in solution increased. However, the oil phase volume at collapse remained within a narrow range, increasing by only 13%. Comparable changes in the concentration range of 2.3-11.3 mg of protein/ml (Fig. 2) are a 68% decrease in emulsifying capacity, a 61% increase in emulsified volume and a 14% increase in the oil phase volume. The percentage variation between the two studies can be attributed to the differences in the protein concentration limits since the same trends were observed for each of the three factors.

An earlier report from this laboratory (Acton and Saffle, 1969) showed the effects of time post-mortem and processing treatments on the emulsifying capacity of salt-soluble protein from boneless chucks. The oil phase volume at emulsion collapse for that study is given in Table 2. The emulsifying capacities for salt-soluble protein from meat in the prerigor-preblended, post-rigor (frozen), and post-rigor (frozen)-preblended treatments were significantly different from each other. However, the oil phase volumes at emulsion collapse were not significantly diffrent between any of these same treatments.

Table 2–Emulsifying capacity[a] and oil phase volume at emulsion collapse[b] from report of Acton and Saffle (1969)[c]

Post-mortem treatment	Emulsifying Capacity	Oil phase vol. at collapse
Prerigor	37.9[a]	78.1[a]
Prerigor-preblended	35.9[a]	77.3[ab]
Post-rigor (Fresh)	23.6[b]	75.1[c]
Post-rigor (Frozen)	22.6[b]	76.4[bc]
Post-rigor (Frozen)-preblended	29.5[c]	75.9[bc]

[a]Expressed as ml of oil emulsified per 100 mg of protein
[b]Percent oil phase of total emulsion volume
[c]Any two means in the same vertical column having one of the same letters are not significantly different at the 5% level of probability.

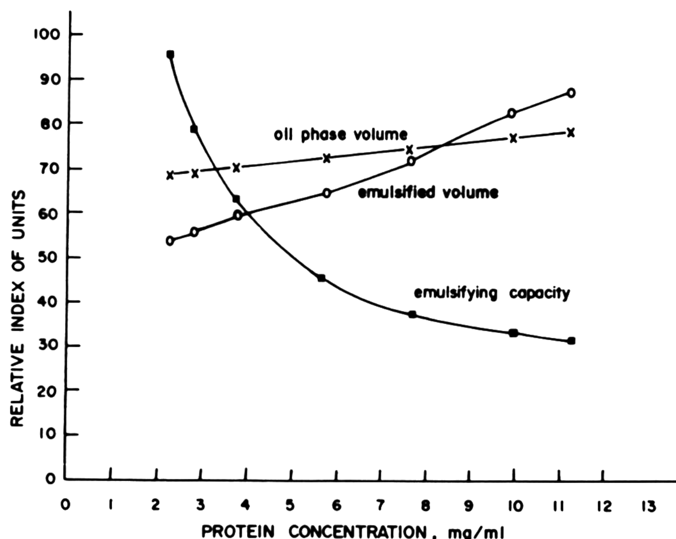

Fig. 2–The effect of protein concentration on emulsifying capacity (ml oil emulsified/100 mg protein), emulsified volume (total ml oil emulsified) and oil phase volume at collapse (percent oil in the total emulsion volume). Data taken from Carpenter, 1964).

The significance of the oil phase volume factor can be demonstrated by comparing the emulsifying capacities and oil phase volumes of the post-rigor (frozen) treatment to that of the prerigor-preblended treatment (Table 2). Although the emulsifying capacity increased by 59%, the emulsions collapsed with approximately the same content of oil (76-77%). Any variation of solution protein concentration in emulsions collapsing at the same oil phase volume would account for the variation observed in emulsifying capacities.

On comparing oil phase volumes in emulsions utilizing salt-soluble protein extracts from fresh muscle tissues of sea bass, pork, and beef (calculated from data of Webb et al., 1970), a range of 88.1-89.6% oil is obtained. It was assumed in this comparison that 20g of extract was approximately equivalent to 20 ml of aqueous solution. The higher level of oil incorporated in the emulsions probably arises from effects of equipment and method of preparation. No effect due to protein concentration difference between extracts was presented.

It is apparent that external factors, in addition to the effect of protein concentration (Fig. 1 and 2), influence the maximum oil phase volume which is attained at the emulsion break-point. Modification of the current method utilized in emulsion capacity studies might elucidate these factors. Examination of the time course of emulsification during oil addition, successive rather than continuous addition of oil, oil globule size distributions and stability studies of emulsions prepared by the basic method (blending), homogenization, orifice mixing and ultrasonics should be considered in attempts to identify a more definitive procedure for determining emulsifying capacity.

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CERTAIN CHEMICAL AND PHYSICAL PROPERTIES OF HAM MUSCLE PORTIONS AFTER THERMAL PROCESSING

INTRODUCTION

COMMERCIALLY, certain hams are separated into specific muscle sections to take advantage of differences in weight and merchandising characteristics for subsequent processing and marketing as canned hams. Several studies have shown that major differences exist in color and pH values between and within ham muscles (Topel et al., 1965; Beecher et al., 1965; and Merkel, 1971). These differences may alter the heat processing characteristics of the muscle sections and directly influence the percentage purge (quantity of fluid released from muscles into container after heat processing) from canned hams. The specific influence of muscle groups on the percentage of purge or its chemical and physical characteristics has not been thoroughly investigated. This study was designed to compare some physical and chemical properties of specific groups of fresh ham muscles after they were subjected to a commercial canning operation.

MATERIALS & METHODS

FIVE normal-to-dark-colored hams (3 to 4 on the Iowa State University color scale, Iowa State University, 1969, and a M. gluteus medius pH of 5.85) and five, pale, soft, watery hams (1 on the Iowa State University Color scale and a M. gluteus medius pH of 5.14) were boned and separated into three major muscle portions: (I) quadriceps femoris, rectus femoris, vastus intermedius, vastus lateralis, vastus medialis; (II) semimembranosus, gracilis and pectineus; and (III) semitendinosus and biceps femoris. The fresh ham portions were weighed and canned without further treatment through the Armour ham canning operation at the Mason City, Iowa plant.

The purge contents obtained from the three muscle portions were weighed, filtered through cheese cloth, and samples collected for specific gravity and sodium and potassium determinations. Specific gravity was determined with a total-solids meter (T/C Refractometer), and the sodium and potassium contents were determined with an Auto Analyzer according to the procedure reported by Technicon Corp. (1965).

The muscle portions were ground three times through an electric grinder, and an aliquot was analyzed for fat, moisture and nitrogen according to AOAC (1965) procedures. A least-squares analysis of variance was used for the statistical analysis (Snedecor and Cochran, 1967).

RESULTS & DISCUSSION

THE OVER-ALL means and standard deviations for percentage protein, fat and moisture of the three ham muscle portions are shown in Table 1. Least-square constant estimates of the effects of color and firmness score and muscle portion of hams on certain properties are presented in Table 2. The effect of color and firmness score grouping (pale and watery vs. normal color and firm), on percentage protein, moisture and fat of the three muscle portions was nonsignificant. This implies that color and firmness score grouping is not a major factor in affecting the amount of protein, moisture and fat in hams. Muscle portion, however, had a significant effect on chemical and physical properties. Portion II (semimembranosus, gracilis and pectineus muscles) was highest in percentage moisture and protein and lowest in percentage fat. Conversely, portion III (semitendinosus and biceps femoris) was lowest in percentage moisture and percentage protein and highest in percentage fat, and muscle portion I (quadriceps femoris) was intermediate for each property.

The data presented in Table 3 indicate that greater variation in percentage protein, fat and moisture exists among ham portions than between individual hams selected for color and firmness characteristics from the exposed, cut surface.

The over-all means and least-squares constant estimates for percentage purge, specific gravity and sodium and potassium content of the purge from the ham muscle portions are shown in Tables 2 and 4. Purge refers to the quantity of fluid obtained from the can after the heating process. The effects of color and firmness score and muscle portion of the ham on percentage purge were highly significant (Table 5), with the muscle

Table 2—Least-squares constant estimates of the effects of color score and muscle portion of hams on some physical and chemical properties

	Color and firmness score		Muscle portion[a]		
Item	Normal	Pale and watery	I	II	III
Percentage moisture	0.4160	−0.4160	0.1657	3.0817	−3.2473
Percentage fat	0.6030	−0.6030	0.8087	−4.6903	3.8817
Percentage protein	−0.1379	0.1379	−0.5019	2.1548	−1.6529
Percentage purge	−3.7428	3.7428	1.1752	3.7727	−4.9478
Specific gravity, purge	−0.0009	0.0009	−0.0005	0.0003	0.0002
Potassium, purge	5.6133	−5.6133	15.0167	−32.0833	17.0667
Sodium, purge	1.3000	−1.3000	2.7000	−6.5000	3.8000

[a](I) Quadriceps femoris; (II) Semimembranosus, gracilis, pectineus; and (III) Biceps femoris, semitendinosus

Table 3—Analysis of variance presenting mean squares for percentage moisture, fat and protein from the three muscle portions

Source	d.f.	Percentage moisture	Percentage fat	Percentage protein
Color group	1	4.984	10.472	0.548
Muscle group	2	94.868**	175.911**	35.458**
Color × muscle	2	3.459	21.598	0.512
Remainder	23	2.187	8.582	1.825

**Significant ($P < 0.01$)

Table 1—Over-all means and standard deviations for percentage moisture, fat and protein from three ham muscle groups

Property	Over-all mean	Std dev
Percentage moisture	60.98	3.01
Percentage fat	16.21	4.64
Percentage protein	20.91	2.01

Table 4–Over-all means and standard deviations for characteristics of the purge from ham muscle portions subjected to the canning process

Item	Over-all mean	Std dev
Percentage purge	16.10	5.82
Specific gravity	1.024	0.001
Potassium (meg/1)	122.15	43.47
Sodium (meg/1)	33.79	8.96

Table 5–Analysis of variance presenting mean squares for percentage purge, specific gravity, potassium and sodium content of the purge

Item	d.f.	Percentage purge	Specific gravity	Sodium	Potassium
Color and firmness group	1	403.453**	0.000026**	909.47	48.67
Muscle portion	2	192.264**	0.00002	7136.71*	295.53*
Color × muscle	2	9.410	0.0001	708.16	4.02
Remainder	23	8.015	0.000001	1622.41	70.71

*Significant ($P < 0.05$)
**Significant ($P < 0.01$)

Table 6–Correlation coefficients among qualitative characteristics of ham muscle groups

Item	Percentage moisture	Percentage fat	Percentage protein	Percentage purge	Purge specific gravity
Percentage fat	−0.82**				
Percentage protein	0.72**	−0.66**			
Percentage purge	0.39*	−0.41	0.59**		
Purge					
Sp gr	−0.08	−0.21	0.33	0.53**	
Sodium	−0.42	0.49	−0.63**	−0.42*	−0.34
Potassium	−0.37	0.34	−0.57**	−0.49**	−0.28

*Significant ($P < 0.05$)
**Significant ($P < 0.01$)

portion from the pale and watery hams having a higher percentage purge than the normal hams. These data support the work of Wismer-Pedersen (1960), Karmas and Thompson (1964) and Merkel (1971) who reported a higher percentage purge from pale, watery hams than normal hams. These results also support the study of Logtestijn (1969) who reported a higher percentage purge from canned, pale, watery loins when compared with percentage purge from normal colored, canned loins. Of the muscle portions, portion II was highest in percentage purge, whereas muscle portion III was lowest in percentage purge. Major differences obviously exist in these two portions of the ham, and since they include the two largest muscle portions studied, the economic importance of these differences is evident when consumer yields and processing characteristics are considered.

The specific gravity of the purge from the three muscle portions was significantly different between the firm, normal colored hams and the soft, watery, pale colored hams (Table 5). The readings for specific gravity for the purge from the soft, pale hams were higher than those for the purge from the normal hams (Table 2). Difference in specific gravity reflects a greater amount of total solids in purge from the pale, watery ham muscle portions, in addition to greater quantities of total purge.

Potassium and sodium levels in the purge from the pale, watery and the firm, normal colored hams were determined because differences between groups in potassium levels may reflect the degree of intracellular release of potassium, and because differences in sodium levels may reflect the extracellular release into the water fluid. Wierbicki et al., (1957) reported that, between 55 and 70°C, dynamic shifts in cations (Na, K, Ca) occur between the muscle and its expressed juice. These shifts may promote hydration of meat proteins to counteract the dehydration caused by denaturation of the proteins during heating to these temperatures. The data reported in Table 5 show that no significant difference existed for sodium and potassium levels in the purge from the firm normal colored and pale, watery hams.

A significant difference ($P < 0.5$) was obtained for sodium and potassium levels of the purge from the three muscle portions, which concurs with the previous conclusion that the variability is greater among ham portions than between ham firmness and color groups. The purge from portion II was lowest in potassium and sodium content and that from muscle portion III was highest in both cations. Ham portion II also had the highest percentage purge, and portion III, the lowest percentage purge. A significant and negative relationship existed between sodium ($P < 0.5$) and potassium content ($P < 0.01$) of the purge and percentage purge from the muscle portions (Table 6). A highly significant ($P < 0.1$) and negative relationship also existed between the percentage protein of the muscle portions and the sodium and potassium content of the purge from those portions. Furthermore, a highly significant ($P < 0.01$) and positive relationship was obtained between the percentage protein of the muscle portions and the percentage purge and total moisture content of the muscle portions.

It is evident from the correlation coefficients that these characteristics are highly related. These differences may be related to the physiological functions of specific muscle groups, and they seem to have economic significance when individual ham muscle sections are used for processing.

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PORCINE AND OVINE MYOGLOBIN: ISOLATION, PURIFICATION, CHARACTERIZATION AND STABILITY

INTRODUCTION

THERE IS MUCH information available which describes the properties of myoglobin from bovine muscle. Snyder and Ayres (1961) prepared crystalline bovine myoglobin which was then used to characterize the stability of bovine oxymyoglobin (MbO_2). Quinn et al. (1964) used both carboxymethyl cellulose (CMC) chromatography and starch gel electrophoresis to demonstrate that bovine metmyoglobin was microheterogeneous. DuFresne (1964) noted that the muscle myoglobin from individual beef animals did not differ. It was also noted that the myoglobin from cardiac and skeletal muscle was identical. The use of isoelectric fractionation by Satterlee and Snyder (1969) to separate all nine of the microheterogeneous bovine myoglobins indicated that the major component comprised 96.8% of the total muscle pigment.

The bovine muscle pigment has also been studied in the intact muscle. Snyder (1964) utilized the Gardner automatic color-difference meter to follow the discoloration of beef at temperatures ranging from −2° to 6°C. Stewart et al. (1965) used reflectance spectrophotometry to measure the total amount of myoglobin and the amounts of metmyoglobin (MetMb) and MbO_2 in meat samples. Franke and Solberg (1971) described a simplified method using reflectance spectrophotometry to quantitatively determine the concentration of MetMb and total pigment in beef and pork muscle.

Other investigators have studied the stability of bovine myoglobin in the intact muscle, as it is affected by packaging and storage conditions. Kraft and Ayres (1954) described the ability of both soft-white fluorescent and ultraviolet light to cause discoloration of beef. Lentz (1971) demonstrated that frozen beef would discolor in 1−3 days when held in a freezer display case at either -20°C or −7°C, whereas storage at the same temperatures in the absence of light caused no discoloration. Solberg and Franke (1971) studied the effect of visible light of six wavelengths varying from 420 nm to 632.8 nm on the color of beef held at both 34° and 41°F. They concluded that visible light at each wavelength resulted in a small but statistically significant increase in the rate of color deterioration, but that the small amount of color change was of no practical significance in fresh meat storage.

Because the fresh meat industry does have color stability problems, several groups of investigators have studied the autoxidation of purfied bovine MbO_2 solutions. Snyder and Ayres (1961) demonstrated that the autoxidation of bovine MbO_2 is temperature dependent. Temperatures just above freezing resulted in the lowest rate of autoxidation. Brown and Dolev (1963b) stated that freezing buffered oxymyoglobin solutions to temperatures of −15°C caused an increase in autoxidation rate. Snyder and Skrdlant (1966) showed that the autoxidation of bovine oxymyoglobin is accelerated by copper ions, whereas iron, zinc and aluminum ions are less active in accelerating the autoxidation rate. Brown and Mebine (1969) in a series of experiments established the correct stoichiometry for the process with respect to oxygen utilization. They indicated that during autoxidation of 1M of MbO_2, ¼ of 1M of oxygen is utilized and ¾ of 1M is evolved.

As observed from the preceding discussion, a vast amount of information is available on bovine muscle myoglobin. The muscle myoglobins from other species which are important economically have also been studied. The properties of tuna myoglobin have been well characterized (Brown, 1961; Brown and Dolev, 1963a, b; Hirs and Olcott, 1964; Brown and Mebine, 1969). The properties of chicken and turkey myoglobin are presently under investigation and thus far have been partially characterized (Froning et al., 1968; Froning, 1972; Janky and Froning, 1972; Goldbloom and Brown, 1971).

Yet with much information available on the before mentioned species, little information is available concerning the myoglobins obtained from porcine and ovine muscle. Bernard et al. (1961a) ran an extract of porcine muscle on gel electrophoresis and indicated that porcine myoglobin migrated faster toward the positive pole, in a pH 8.9 buffer, than did the myoglobins from bovine, equine, or ovine muscle. Atassi et al. (1970) determined the amino acid composition of ovine myoglobin and found it to be similar to the composition of bovine myoglobin. Bernard et al. (1961b) hydrolyzed bovine, porcine, ovine and equine myoglobin and found that the peptide patterns for each differed significantly. Hoyen and Thorson (1970) examined the myoglobins from ovine, porcine, bovine and equine muscle on polyacrylamide gel electrophoresis, in an effort to develop a technique in which the muscle constituents of any meat product could be detected.

Since there is a definite void in information concerning the characteristics and stability of both porcine and ovine myoglobin, the present study was initiated.

EXPERIMENTAL

Isolation of the myoglobins

Ovine myoglobin was obtained by first removing all of the leg muscles and freeing them of extraneous fat and connective tissue. The muscles were ground and then homogenized in a Waring Blendor with an equal volume of cold distilled water. The myoglobin was then extracted from the muscle according to the technique that was used for bovine myoglobin (Satterlee et al., 1969). The resultant crude ovine myoglobin was freeze-dried and stored in the freezer as a powder.

Porcine myoglobin was obtained in a manner which involved removal of all muscles from the ham and freeing each muscle of extraneous fat and connective tissue. After grinding the muscle and homogenizing it with an equal volume of cold distilled water, further extraction and isolation was accomplished using a modification of the technique of Satterlee et al. (1969). The modifications were: (1) the myoglobin was precipitated between 50 and 100% ammonium sulfate saturation; (2) the crude myoglobin was stored in the freezer as the ammonium sulfate paste. Precautions were taken to ensure the porcine muscle used was not from an animal exhibiting the PSE syndrome. Secondly, the before mentioned research cited on bovine, ovine, porcine and poultry muscle myoglobins indicated that the myoglobins are not affected by breed differences. Therefore this manuscript describes the myoglobins from normal bovine, ovine and porcine muscles, without regard to breed.

The bovine myoglobin used in this study was isolated and purified according to the procedure of Satterlee et al. (1969).

Both the porcine and the ovine myoglobins were purified on DEAE cellulose column chromatography. Approximately 300 mg of ovine or porcine metmyoglobin in 10 mM pH 8.6 Tris buffer was placed on a column containing 60g of moist DEAE which had been equilibrated with the same buffer. Cytochrome C and nonheme proteins were eluted with the same buffer. The metmyoglobin was then eluted with 50

mM Tris buffer, pH 8.0.

The purified porcine and ovine myoglobins were kept frozen until needed for further study.

Amino acid analysis

The amino acid composition of purified porcine myoglobin was determined by initially removing the heme group from the myoglobin molecule according to the procedure described by Lewis (1954). The resulting apoprotein was hydrolyzed using constant boiling HCl, in an evacuated tube for both 24 and 72 hr. The amino acid composition of the hydrolyzates was determined on a Beckman 120C amino acid analyzer. The tryptophan content was determined by the method of Bencze and Schmid (1957).

Isoelectric fractionation

The isoelectric points of both the ovine and porcine myoglobins were determined by using a modification of the procedure described by Satterlee and Snyder (1969). The modifications were: (1) fractionation was performed at 11°C; (2) a pH 6–8 ampholyte was used to establish the pH gradient; and (3) the isoelectric point was determined by measuring the pH of each fraction at 23°C with the aid of a Corning model 10 pH meter.

To ensure that the fraction being studied was myoglobin and not hemoglobin, the approximate molecular weight was determined with the aid of a 0.9 × 55 cm Sephadex G-100 column. The Sephadex column had been previously calibrated for molecular weight determination using purified bovine serum albumin, bovine chymotrypsin, bovine metmyoglobin, egg white lysozyme and bovine cytochrome C.

Autoxidation rates

The autoxidation rates of the bovine, porcine and ovine MbO_2 were determined according to the procedure of Brown and Mebine (1969). The myoglobin solutions were passed through ion exchange resin (AG 50LX8) to free them from any oxidation products due to the sodium dithionite treatment. Buffers used in the trials were 10 and 100 mM phosphate adjusted to a pH of 5.5, 6.0 or 6.5. The autoxidation rates were determined at 37°C, 23–24°C, 11–12°C, 5°C, –2°C, –12°C and –19°C.

Autoxidation rates were also determined in the absence of light and in intense fluorescent light (1100 ft-c). Light intensity was measured with the aid of a General Electric type 213 light meter.

Meat and acid denaturation

Heat denaturation curves were obtained for the bovine, ovine and porcine metmyoglobins according to the procedure of Satterlee et al. (1972). The heat denaturation study was performed using a 10 mM phosphate buffer at a pH of 5.5, 6.0 and 6.5.

The acid denaturation curves obtained on all three metmyoglobins were according to the procedure of Satterlee et al. (1972). The metmyoglobins used were dissolved in glass distilled water.

RESULTS & DISCUSSION

Isolation of the myoglobins

The isolation and purification of the ovine myoglobin was very straightforward since this myoglobin is very similar to bovine myoglobin. The isolation and purification of porcine myoglobin was found to be much more difficult. Initially problems were encountered when it was found that the porcine myoglobin would not only precipitate at high levels of ammonium sulfate saturation (75–100% saturation) but began precipitating just above 50% ammonium sulfate saturation and continued to do so until complete saturation (100%) was obtained. The purity of the various porcine and ovine ammonium sulfate fractions is given in Table 1. In addition crude porcine myoglobin cannot be successfully freeze-dried since the freeze-dried powder is only slightly soluble in water or buffer.

DEAE cellulose chromatography works well in the final purification of both ovine and porcine myoglobins. The myoglobins, after final purification on DEAE, were homogeneous, as was evidenced by polyacrylamide gel electrophoresis.

Amino acid analysis

The amino acid composition of bovine and ovine myoglobin (Atassi et al., 1970) is reported in Table 2. The amino acid composition of these two myoglobins is identical except for the amino acids alanine and leucine. Even though they are similar in amino acid composition, Atassi et al. (1970), Bernard et al. (1961b) and Han et al. (1970, 1971) found that many of the peptides obtained from the myoglobins differed both in amino acid content and in immunochemical cross reaction. These results indicate that bovine and ovine myoglobin have similar amino acid compositions but significantly different amino acid sequences.

Only a few amino acids of porcine myoglobin, also reported in Table 2, were found to be similar to those of ovine and bovine myoglobin. Glycine and methionine are found in equal amounts in all three myoglobins. The calculated molecular weight of 17,142 is only slightly less than the molecular weights given for bovine and ovine in Table 2.

Isoelectric fractionation

The isoelectric points (pI) of bovine and ovine metmyoglobin are very similar, as is shown in Table 3. Porcine MetMb is a more negatively charged molecule, when compared to bovine and ovine MetMb, since it possesses a much lower pI. Possessing a lower pI would definitely allow the porcine myoglobin to migrate faster on pH 8.6 gel electrophoresis, as was shown previously by Bernard et al. (1961a).

Table 2—Amino acid composition of bovine, ovine and porcine myoglobin

	Residues/molecule		
Amino acid	Bovine[a]	Ovine[a]	Porcine
Aspartic acid	12	12	13
Threonine	5	5	7
Serine	6	6	7
Glutamic acid	18	18	14
Proline	4	4	8
Glycine	13	13	13
Alanine	17	16	12
Valine	8	8	10
Methionine	3	3	3
Isoleucine	6	6	12
Leucine	16	17	11
Tyrosine	2	2	3
Phenylalanine	7	7	5
Lysine	19	19	11
Histidine	12	12	5
Arginine	2	2	6
Tryptophan	3	3	6
NH_3	–	8	12
Molecular wt	17,693	17,735	17,142

[a]Atassi et al. (1970)

Table 1—Purity of the various ovine and porcine ammonium sulfate fractions

Myoglobin	Ammonium sulfate fraction (% saturation)	Protein content (mg heme protein/mg total protein)
Ovine	50	1/14.9
	75	1/5.0
	100	1/1.2
Porcine	50	1/37.5
	75	1/13.8
	100	1/12.4

Table 3—Isoelectric points of the various myoglobins

Myoglobin	Isoelectric point	Avg dev	Trials
Bovine[a]	7.10	.03	6
Ovine	7.21	.03	2
Porcine	6.82	.02	2

[a]Satterlee and Snyder (1969)

Autoxidation rates

The autoxidation rates for all the three oxymyoglobins were determined within the temperature range of −19° to 37°C. Figure 1 illustrates the effect of temperature on the autoxidation rates. At temperatures ranging from −2° to 37°C, bovine MbO_2 possessed the highest rate constants, followed by ovine MbO_2. Porcine MbO_2 autoxidized the slowest of the three when observed at above freezing temperatures. Below the freezing point of the MbO_2 solution (−12°C and −19°C), there was a noticeable increase in the autoxidation rate. This same phenomenon was observed by Brown and Dolev (1963b) for both bovine and tuna MbO_2. The maximum rate increase was noted at −12°C for all three myoglobins.

The data presented in Table 4 indicate that high intensity soft-white fluorescent light does affect the autoxidation of MbO_2 from the three myoglobins studied. At 4°C the increase in autoxidation rate (K) due to light, is less than the increase seen at 24°C. This experiment was run at a pH 5.5, where the effect of light seems to be less than that reported at pH 6.0 by Satterlee et al. (1972). It should be emphasized that the level of illumination used in this experiment is approximately 10 times greater than the level found in most fresh meat display cases.

Heat denaturation

Figure 2 illustrates the heat denaturation curves for all the three metmyoglobins at a pH of 5.5. There were no significant differences between any of the metmyoglobins in their heat stability properties.

Similar runs were performed at a pH of 6.0 and 6.5. As the pH was increased by 0.5 unit, the heat stability curve for each metmyoglobin moved to the right on the average of 1.8°C (toward a higher temperature).

These data are in agreement with findings of Bernofsky et al. (1959) who found that the myoglobins in ground beef and in ground pork were equally resistant to heat denaturation. The data on bovine metmyoglobin denaturation is in agreement with the data reported by Draudt (1969).

Acid denaturation

Acid denaturation curves were also obtained for each metmyoglobin. The stability of both bovine and ovine MetMb to acid denaturation is identical. As shown in Figure 3, both myoglobins were stable in the pH range of 6.5 to 4.0, then began to denature rapidly as the pH was lowered. Porcine MetMb is much more sensitive to acid denaturation than either bovine or ovine MetMb. Porcine MetMb began denaturating at a pH of 4.85 and the rate increased as the pH was lowered.

CONCLUSION

THE TWO MYOGLOBINS of primary concern in this study were the ovine and porcine myoglobins. Throughout the study these two myoglobins were compared to bovine myoglobin. It became readily evident that the ovine and bovine myoglobins were similar by their behavior during purification and in their stability to both heat and acid. The autoxidation of all three MbO_2 at temperatures above freezing and in normal room light (77 ft-c) was measured. Results indicated that bovine MbO_2 autoxidized the fastest, followed by ovine MbO_2 and the slowest to be porcine MbO_2. Temperatures below freezing increased the autoxidation rate for all three MbO_2 studied. High intensity fluorescent light also increased in the autoxidation rates of the bovine, ovine and porcine MbO_2.

Porcine myoglobin is definitely different from the other two, first in its behavior during isolation and purification. Secondly, porcine myoglobin was found to be very susceptible to acid denaturation, when compared to the other myoglobins. Thirdly, porcine myoglobin has an isoelectric point and an amino acid composition which differs

Table 4–Effect of light on autoxidation rates

	Effect of light K(100 ft-c)/K(0 ft-c)[a]	
Oxymyoglobin[b]	4[b]	24[b]
Bovine	1.25	1.81
Ovine	1.33	1.25
Porcine	1.28	1.75

[a] K = the autoxidation rate constant
[b] The MbO_2 was in 10 mM phosphate buffer, pH 5.5.

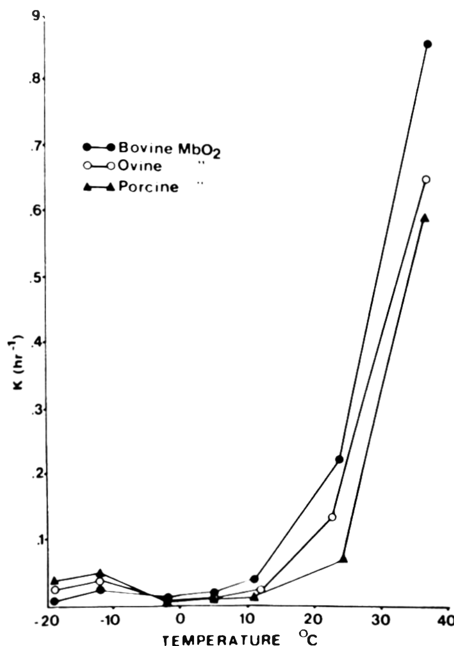

Fig. 1–Effect of temperature on the autoxidation rate constants (K) for bovine, ovine and porcine MbO_2.

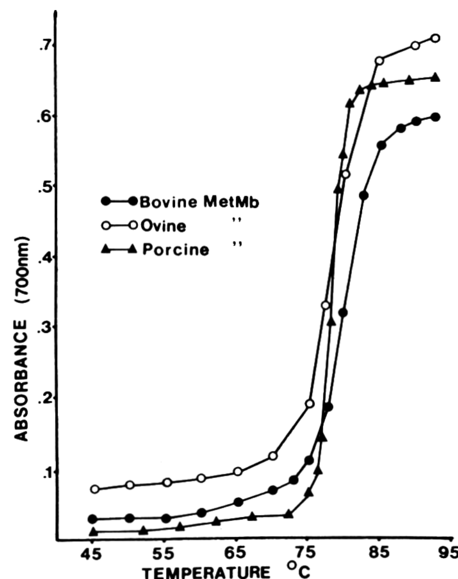

Fig. 2–Heat denaturation curves for bovine, ovine and porcine MetMb.

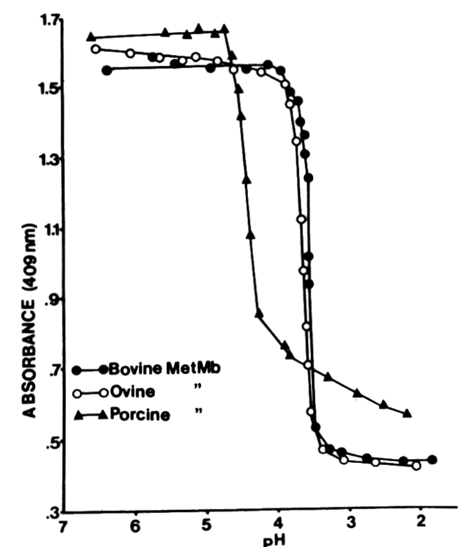

Fig. 3–Acid denaturation curves for bovine, ovine and porcine MetMb.

significantly from the other two myoglobins.

Because bovine and ovine myoglobin are so similar, much of the information available on bovine myoglobin can possibly be directly applied to ovine myoglobin. This study has shown that porcine myoglobin has several unusual properties and that this pigment protein merits further investigation.

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EFFECT OF CERTAIN PHYSICAL AND CHEMICAL TREATMENTS ON THE MICROSTRUCTURE OF EGG YOLK

INTRODUCTION

EGG YOLK consists of alternate layers of white and yellow yolk surrounded by the vitelline membrane. This layering effect is thought to be due to the diurnal rhythm in the formation of the yolk (Riddle, 1911). The white yolk layers are approximately 0.25–0.40 mm in thickness, while the thickness of the yellow yolk layers averages approximately 2.0 mm (Romanoff and Romanoff, 1949). Moran (1925) observed yolk spheres with diameters ranging from 25–150μ, the larger of which were located near the center of the yolk. Microscopic examination of yolk by Grodzinski (1951) revealed that yolk consisted of an emulsion with a variety of particles dispersed in a protein solution. Three major types of particles were noted: yolk spheres, free-floating droplets, and round profiles which contained protein. Two types of spheres were noted in yolk: yellow and white yolk spheres with surface semipermeable membranes (Grodzinski, 1951). It was observed that within the yellow yolk spheres were numerous, tightly packed nonrefractile droplets, while the white yolk spheres contained only a few droplets suspended in a protein fluid. Bellairs (1961), using phase contrast and electron microscopy, found that the yellow yolk spheres had diameters ranging from 25–150μ, while the diameters of the white yolk spheres ranged from 4–75μ. Bellairs (1961) confirmed the appearance of droplets within the spheres as reported by Grodzinski (1951). In addition, Bellairs (1961) also reported that the surfaces of the yolk spheres were mainly of the naked type (void of a surface membrane), but that the laminated capsule and unit membrane type of structure were also present. Numerous free-floating, highly refractile, lipid droplets with diameters of about 2μ were also found in yolk (Bellairs, 1961). This worker also noted that the free droplets, as well as the droplets within the yolk spheres, had dark and light regions which were thought to be due to particles ranging in size from 30–60Å. The small round profiles, 250Å, were found to exist as a ring or in chains in the continuous phase and were thought to be composed of low-density lipoprotein micelles. Sugano and Watanabe (1961) and Martin et al. (1964) reported that the diameter of hydrated low-density lipoproteins ranged from 117–480Å. Powrie (1968) felt that the round profiles were low-density lipoproteins.

This study was undertaken to observe the effects of dilution, concentration, sodium chloride, urea and freezing on the microstructure of yolk in order to further understand the mechanism of yolk gelation upon freezing and thawing.

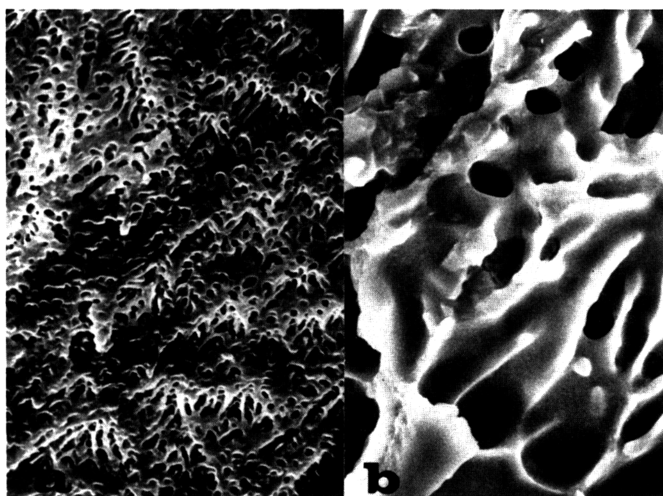

Fig. 1—Scanning electron photomicrographs (750×) of control yolk: (a) unfrozen; and (b) frozen-thawed.

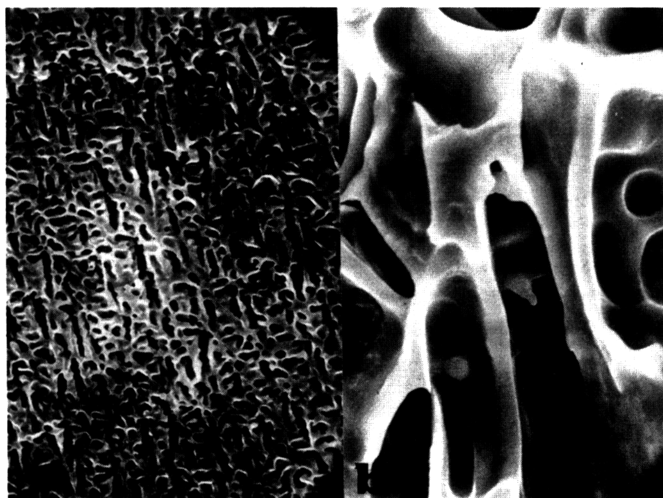

Fig. 2—Scanning electron photomicrographs (750×) of diluted yolk: (a) unfrozen; and (b) frozen-thawed.

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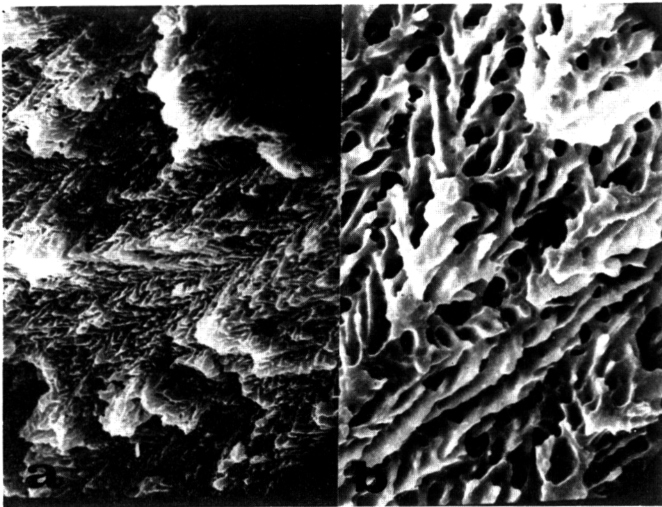


Fig. 3—Scanning electron photomicrographs (750×) of concentrated yolk: (a) unfrozen; and (b) frozen-thawed.

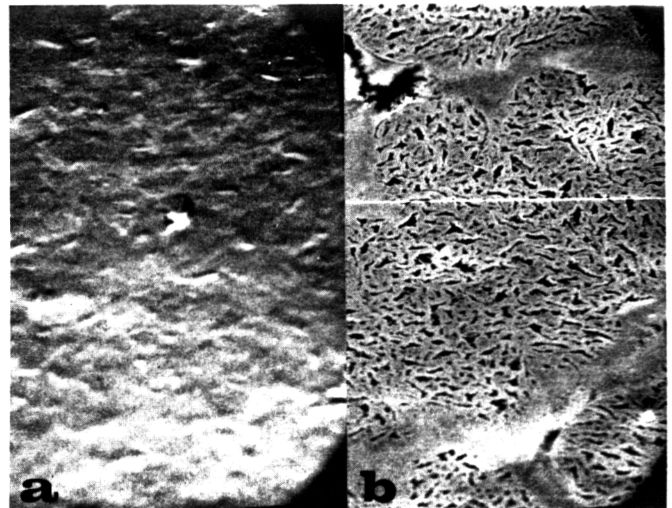


Fig. 5—Scanning electron photomicrographs (750×) of the LDF of yolk: (a) unfrozen; and (b) frozen-thawed.

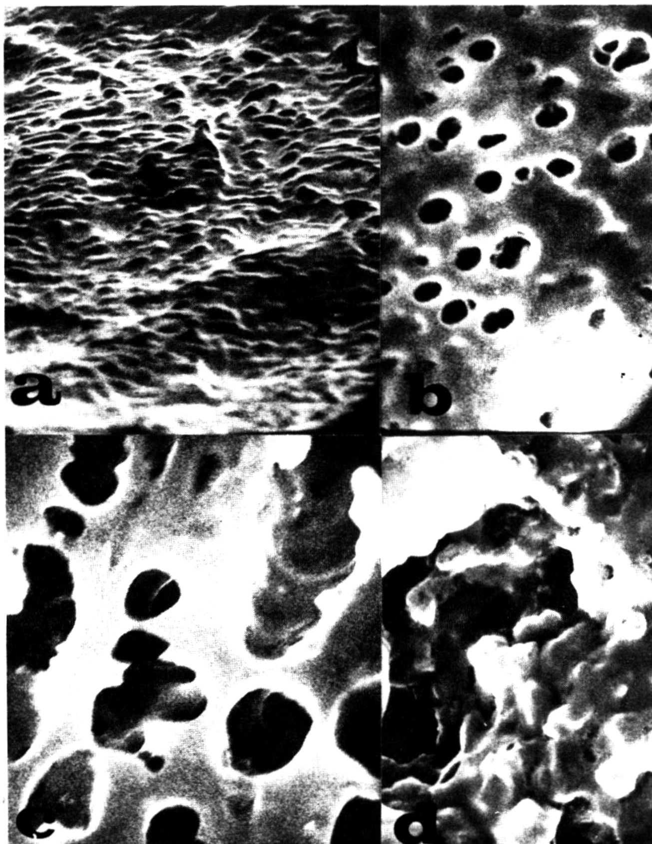


Fig. 4—Scanning electron photomicrographs (1,000X) of urea-treated yolk: reaction times of (a) 0 hr; (b) 12 hr; (c) 24 hr; and (d) 72 hr.

MATERIALS & METHODS

Preparation of yolk

Day-old eggs from single comb White Leghorn hens were used in this study. The yolks were separated from the albumen and the residual albumen removed from the yolks with flowing tap water. The yolks were rolled on thin tissue paper to remove adhering water, pooled, and mixed with a propeller mixer at 200 rpm for 15 min. The yolk was filtered through cheese cloth to remove the vitelline membrane and chalazae.

Dilution and concentration

A moisture level of 67% was obtained by diluting yolk with distilled water (50 ml/100g yolk) and mixing the yolk-water mixture for 5 min with a propeller mixer (200 rpm). Water was removed from yolk by dialysis with polyvinylpyrrolidone (PVP-40T, Sigma Chemical Co.) to a moisture level of 42%. The actual moisture content of the yolk samples was determined by drying to constant weight at 90°C.

Chemical treatments

NaCl was dissolved in 10 ml of distilled water and added to 100g of yolk at a level of 10%. The salt-yolk mixture was mixed for 5 min with a propeller mixer (200 rpm). Urea was added to yolk at a final concentration of 4 molal and mixed as previously mentioned.

Freezing treatment

Samples of other treatments were frozen in glass jars at −26°C for 72 hr. Thawing of the samples was done at 10°C for 16 hr and the samples equilibrated at room temperature (25 ± 2°C) before examination.

Fractionation

Yolk was fractionated into a low-density fraction (LDF) and high-density fraction (HDF) by centrifugation at 25,000 rpm for 24 hr at 2°C on a Beckman preparative ultracentrifuge (SW-27 rotor). The supernatant (LDF) was decanted and residual LDF on the sediment (HDF) surface removed by scraping.

Examination procedures

Scanning electron microscopy. Yolk samples, in 25 ml glass vials, were quick-frozen in liquid nitrogen for 45 min and immediately freeze dried. Sample pieces (2.5–5.0 mm) were mounted on stubs using electrical silver paint, and gold coated. Samples were examined with a scanning electron microscope (Model SEM-900, Advanced Metal Research Corp.).

Transmission electron microscopy. Approximately 1 mm diam spheres of yolk samples were fixed in 2% osmium tetroxide for 1 hr at 0°C. After dehydration in graded ethanol, the samples were embedded in EPON epoxy resin.

Sections were stained with 2% uranyl acetate, followed by 0.002% lead citrate (Hasiak, 1972). Sections were examined with a transmission electron microscope (EM-300, Philips Electronic Instruments).

RESULTS

Surface structure

The photomicrographs showing the influence of freezing and thawing on the surface structures of control yolk are reproduced in Figure 1a and b. The unfrozen sample (Fig. 1a) had a rather close-knit structure containing numerous small voids. However, the freezing process changed the structure extensively, giving the yolk a rather coarse network appearance (Fig. 1b). Dilution of unfrozen yolk had very little effect on the surface structure (Fig. 2a), while the frozen-thawed structure of diluted yolk (Fig. 2b) was more open than that found in the frozen-thawed undiluted sample (Fig. 1b). However, concentration of yolk was found to give both the unfrozen and frozen-thawed yolk a more compacted structure which was rigid in appearance (Fig. 3a and b).

The effect of 4 molal urea on the structure of yolk after reaction times of 0, 12, 24, or 72 hr at 10°C is shown in Figure 4a, b, c and d, respectively. The structure of the urea-treated yolk became progressively more open with increasing reaction time.

The surface structure of unfrozen LDF (Fig. 5a) was rather smooth and lacked the voids observed in the unfrozen control yolk. However, the structure of the frozen-thawed LDF was open and had a network appearance (Fig. 5b). Unfrozen HDF (Fig. 6a) had a surface structure which was rather smooth in appearance, while that of the frozen-thawed HDF (Fig. 6b) was rough and lacked the voids noticed in other samples.

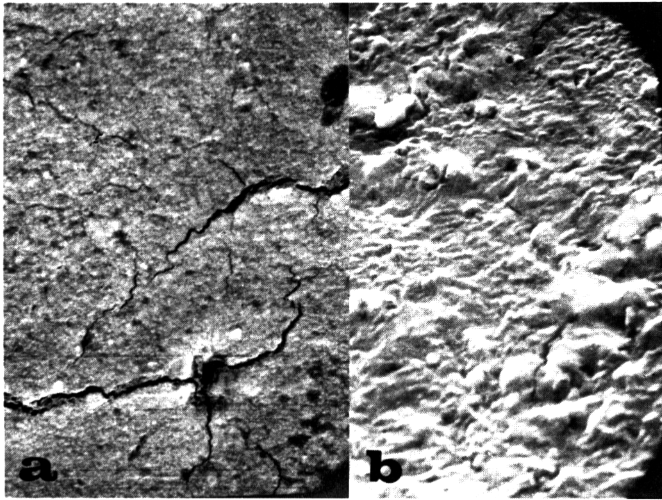


Fig. 6—Scanning electron photomicrographs (750×) of the HDF of yolk: (a) unfrozen; and (b) frozen-thawed.

Ultrastructure

Transmission electron microscopy was used to investigate the effects of various treatments on the ultrastructure of unfrozen and frozen-thawed yolk. It was noticed that unfrozen LDF (Fig. 7a) consisted of small particles (20 mμ) evenly dispersed throughout a smooth continuous matrix. However, the matrix in the frozen-thawed LDF was disrupted by unstained areas, probably lipid, which ranged in size from 60–120 mμ (Fig. 7b). The structure of unfrozen HDF (Fig. 8a) consisted of large irregular-shaped particles (0.5–9μ) dispersed in a continuous matrix similar to that of LDF. Bellairs (1961) identified particles with a similar appearance as free-floating lipid droplets. However, it is believed that these particles are high-density lipoproteins (HDL). The large HDL particles in the frozen-thawed HDF (Fig. 8b) were more spherical in shape, while the matrix was disrupted as was noticed in the frozen-thawed LDF.

The structure of unfrozen control yolk (Fig. 9a) was similar to that observed in the unfrozen HDF, except that the large irregular HDL particles were fewer in number and generally smaller in size (0.2–8μ). However, in the frozen-thawed yolk (Fig. 9b), the HDL particles were more spherical in shape and the continuous matrix disrupted.

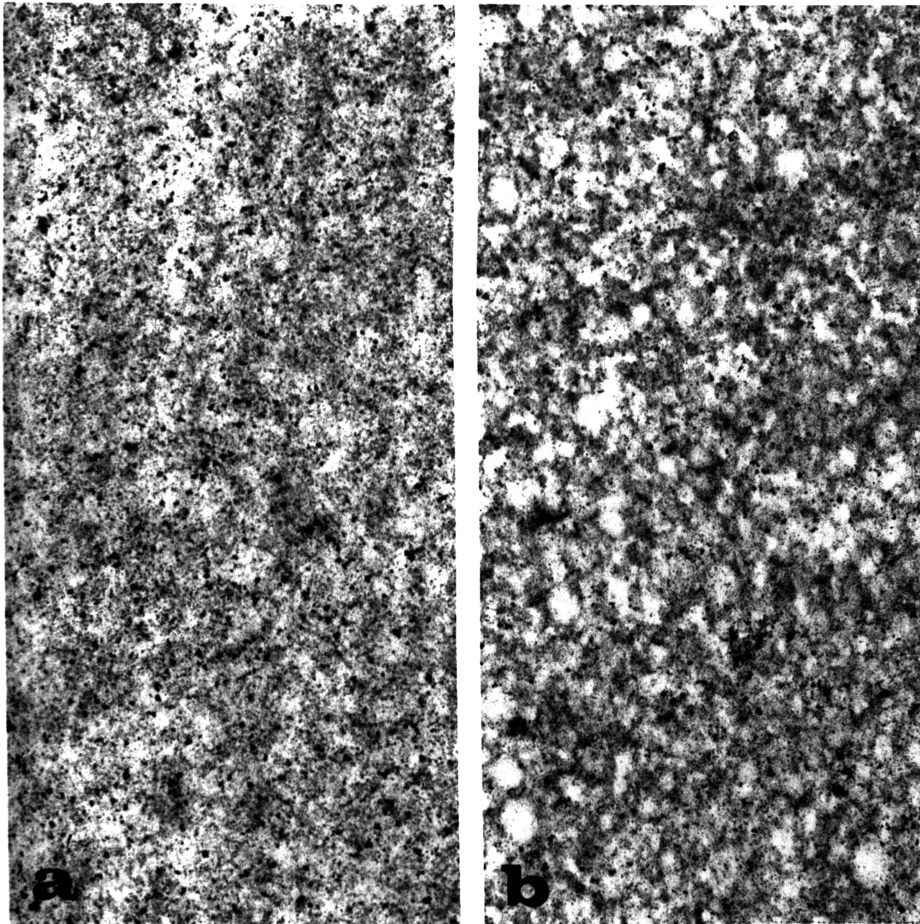


Fig. 7—Transmission electron photomicrographs (53,000×) of the LDF of yolk: (a) unfrozen; and (b) frozen-thawed.

Initially, urea treatment resulted in spherical HDL particles dispersed in a somewhat disrupted matrix (Fig. 10a). However, after storage for 72 hr the matrix of the urea treated yolk sample (Fig. 10b) was extensively disrupted, having the appearance of numerous particles ranging in size from $0.1-1\mu$.

NaCl (10%) disrupted the HDL particles in both the unfrozen and frozen-thawed samples (Fig. 11a and b). The appearance of both the unfrozen and frozen-thawed preparations (Fig. 11a and b) were similar to that observed in the unfrozen LDF sample (Fig. 7a).

DISCUSSION

SCANNING electron microscopy indicated that the structure of frozen-thawed yolk was more open than that of unfrozen yolk. The large open spaces observed in the frozen-thawed sample would indicate that large "droplets" of water were

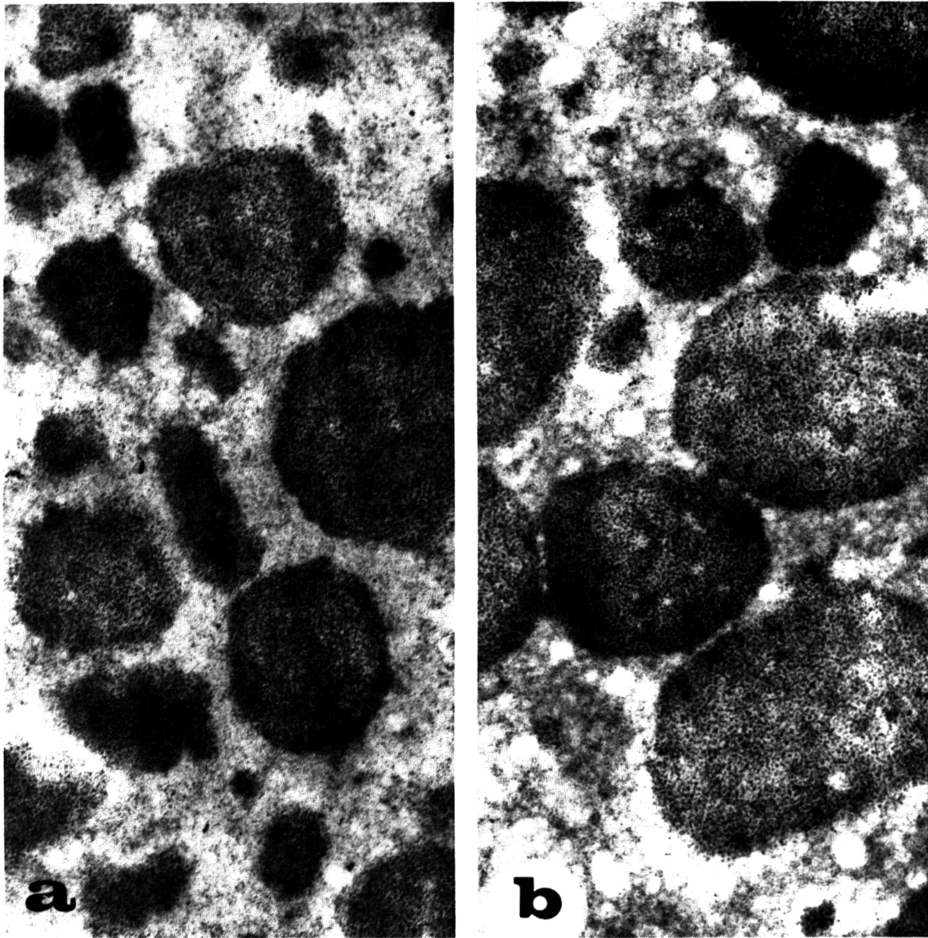


Fig. 8–Transmission electron photomicrographs (53,000×) of the HDF of yolk: (a) unfrozen; and (b) frozen-thawed.

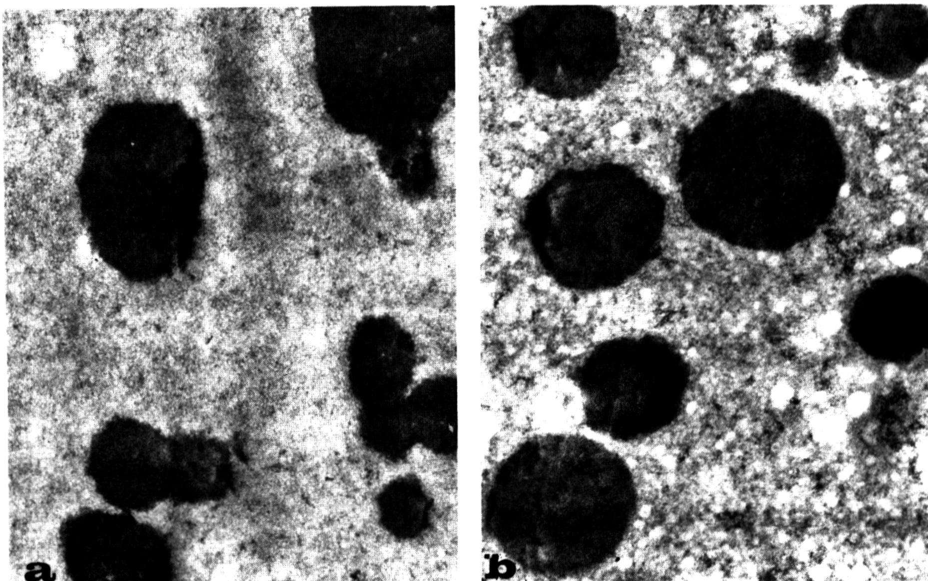
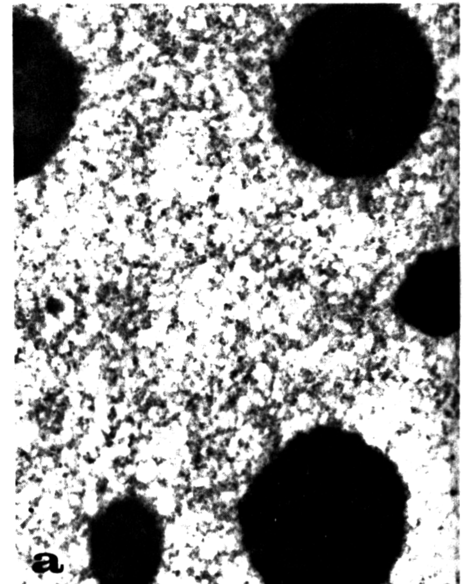


Fig. 9–Transmission electron photomicrographs (32,800×) of control yolk: (a) unfrozen; and (b) frozen-thawed.



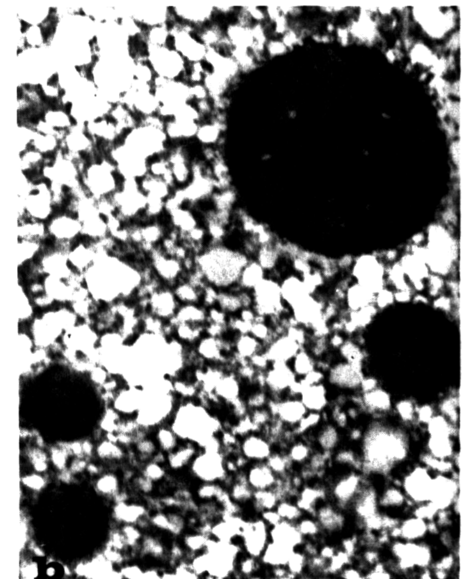


Fig. 10 – Transmission electron photomicrographs (32,800×) of urea-treated yolk (4 molal) after a 0 hr (a) and 72 hr (b) reaction time.

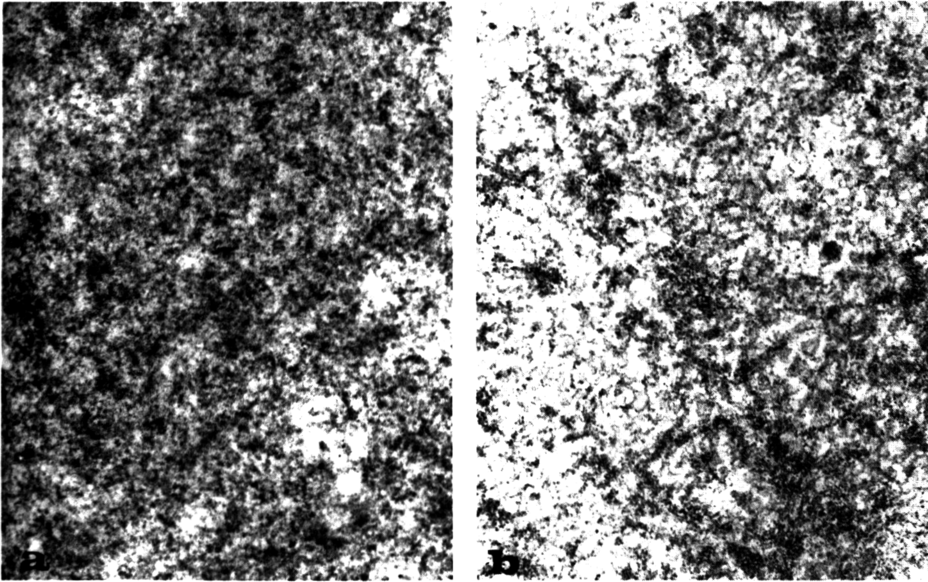


Fig. 11—Transmission electron photomicrographs (32,800×) of NaCl (10%) treated yolk: (a) unfrozen; and (b) frozen-thawed.

trapped in a three-dimensional structure. Further evidence of this was observed when dilution increased the size of the open spaces, while concentration had the reverse effect. Powrie et al. (1963) noted that the viscosity of urea-treated yolk increased with time. The photomicrographs of urea-treated yolk and native yolk would tend to indicate that the increase in yolk viscosity was related to the degree of openness of structure. Thus the results with urea indicate the possible involvement of water in the gelation of yolk since urea has a high affinity for water.

The changes in the surface structure appear to be related to alterations in the continuous matrix and the HDL particles. Transmission electron microscopy of yolk indicated that both the LDF and HDF fractions of yolk were altered by freezing. It was also noted that freezing of control yolk, as well as treatment with urea, drastically altered the low-density continuous phase. The HDL particles were also altered by these processes and appeared to interact with the continuous phase. Sodium chloride, which inhibits freeze-induced increases in yolk viscosity (Marion and Stadelman, 1958), was found to completely disrupt the HDL particles and drastically reduce changes in the continuous LDF phase resulting from freezing and thawing.

CONCLUSIONS

POSSIBLE alteration of the structure of water or the removal of water may result in lipoprotein destabilization and aggregation. The photomicrographs suggest that the freezing process induces aggregation of the low- and high-density yolk lipoproteins through an alteration in the structure of water. This aggregation results in the formation of a three-dimensional structure which entraps large quantities of water, and may result in an increased yolk viscosity.

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THE RELATIONSHIP OF LYSOZYME CONTENT OF EGG WHITE TO VOLUME AND STABILITY OF FOAMS

INTRODUCTION

MANY FOOD USES of egg white depend on its foaming power and the stability of the foams produced. McLaren and Stadelman (1954) used a modification of the method proposed by Barmore (1936) to study the foaming characteristics of egg white from eggs stored for various times and at different temperatures. They found that foam drainage as a measure of foam stability was not related to egg storage time. This was true for both room temperature and cold storage treatments. Garibaldi et al. (1968) reported after extensive study that damage to whipping properties of egg white due to heat denaturation of the ovomucin-lysozyme complex was a first order reaction for both ovomucin and lysozyme and that an increase of 0,33 in the ionic strength of egg white resulted in a 10-fold decrease in damage of whipping properties. Damage to whipping properties appeared to result from an irreversibly denatured lysozyme ovomucin aggregate. Whipping aids compensated for loss of whipping properties but did not reverse the reaction which produced damage to whipping properties. Gandhi et al. (1968) using 3,3-dimethylglutaric anhydride for chemical modification of egg white, found that low levels seriously reduced foaming ability of egg white. Morgan et al. (1970) compared frozen, foam-spray, spray and freeze-dried egg white for use in meringues. Foam drainage was not significantly different in these studies. In contrast, Funk et al. (1971) reported that foams made from freeze-dried egg albumen were significantly less stable than those from either foam-spray or spray-dried egg white. Maesso et al. (1970) reported that foam stability was significantly affected by age of egg and strain of birds. Strain of birds also significantly affected foam volume in this study. Rolfes et al. (1955) reported satisfactory foaming properties for freeze-dried egg white but not for freeze-dried whole egg.

Sauter et al. (1966) reported that eggs having a high lysozyme content in thick albumen lost interior quality at a slower rate than eggs having less lysozyme. This study was conducted to determine the effect of lysozyme content of egg white on foaming characteristics.

EXPERIMENTAL

EGGS FROM INDIVIDUAL hens previously indexed for high or low lysozyme content of egg white were used to study foam volume and stability. Ten hens having the highest and ten having the lowest relative percentage of lysozyme were used to supply eggs for foam studies. Eggs were identified by lysozyme level and date of production, then stored at 3.3°C, 75% RH, for up to 8 wk for comparison of volume and stability of foams.

All eggs were allowed to equilibrate to room temperature for 2 hr, then weighed to the nearest 0.1g and broken out on a flat glass plate. Height of the thick white was measured with an Ames model S-6428 tripod micrometer and Haugh Units (H.U.) were computed. Yolk and white were then separated and egg white used for foam studies. For each foam, the egg white from two eggs from the same hen was blended to uniform consistency without foaming at speed 1 (70 rpm) in a Kitchen Aid Model K 45 mixer (10 sec). 60 ml of the blended egg white were then beaten at speed 6 (336 rpm), using a wire whip for 90 sec. Foam was then placed in funnels of known volume (125 mm × 100 mm × 10 mm diam stem), covered to prevent evaporation and allowed to drain into 100 ml graduate cylinder for 60 min. Drainage volume was determined at 15 min intervals.

5 μliter samples of blended egg white prior to whipping were placed on cellulose polyacetate strips and subjected to electrophoretic separation at 400 volts for 40 min using a Tris-barbital sodium buffer at pH 8.6. At completion of electrophoresis the strips were stained with Ponceau S (500mg/100ml trichloroacetic acid) for 5 min then decolorized by successive rinses of 5% acetic acid. Strips were then dried at room temperature.

After drying, individual protein bands were cut from the strips and eluted with 0.1N NaOH until all color was removed from polyacetate. Optical density was determined with a Spectronic 20 spectrophotometer at a wave length of 540 mμ. Relative percentages of individual protein bands were computed from this data. Similar electrophoretic separations were conducted on the first ml of drainage from each foam; however, only albumins and lysozyme gave distinct protein bands from foam drainage.

The study was replicated three times using 180 foams/replicate. Data were analyzed using analysis of variance Snedecor (1956) and Duncan's (1955) multiple range test. Correlation analysis was used to determine relationships between foam volume and 15 min drainage.

RESULTS & DISCUSSION

INTERIOR QUALITY as measured by Haugh Units (H.U.) of eggs used for foam comparisons is shown in Table 1. Eggs having a low relative percent of lysozyme lost quality somewhat more rapidly than eggs containing more lysozyme. This difference was particularly noticeable during the first 2 wk of storage. High lysozyme eggs lost 5 H.U. compared to 12 H.U. for low lysozyme eggs during this period.

Average relative percentages of various egg white fractions from fresh eggs, as indicated by electrophoretic separations are shown in Table 2. These determinations were made just prior to the start of egg storage for foam studies. Protein bands obtained by electrophoresis were quite consistent and clear for nine protein fractions of egg white from fresh eggs. As storage time increased, protein separations became less distinct; and after 4 wk only albumins, conalbumin and lysozyme were consistently discernible as precise

Table 1—Interior quality of eggs from hens indexed for lysozyme content of egg white and used in foam volume and stability studies after storage at 7°C, 75% RH

Storage time	High lysozyme (H.U.)	Low lysozyme (H.U.)
1 day	91	92
1 wk	87	86
2 wk	86	80
3 wk	83	77
4 wk	79	76
5 wk	77	72
6 wk	74	70
7 wk	72	67
8 wk	70	64

Table 2—Relative percentage of protein fractions in whites of high and low lysozyme eggs

	Relative % protein[a]	
	High lysozyme	Low lysozyme
Albumins	58.19[a]	61.53[b]
Lysozyme	8.37[a]	5.31[b]
Conalbumin	13.51[a]	13.57[a]
Other fractions	19.93[a]	19.59[a]

[a]Values for the same fraction having different superscripts are significantly different ($P < 0.05$).

Table 3–Relative percentages of albumins and lysozyme in egg white and in foam drainage of the same high lysozyme eggs

Storage time	Egg white Albumin (%)	Egg white Lysozyme (%)	Foam drainage Albumin (%)	Foam drainage Lysozyme (%)
1 day	58.2	8.37	70.4	3.02
1 wk	58.3	8.38	65.8	4.84
2 wk	58.4	8.40	64.9	5.25
3 wk	58.3	8.21	69.2	4.30
4 wk	58.5	8.29	69.3	3.36
5 wk	58.3	8.41	69.8	2.57
6 wk	58.2	8.36	69.6	3.82
7 wk	58.3	8.38	67.8	4.81
8 wk	58.3	8.35	71.6	2.72

Table 4–Relative percentages of albumins and lysozyme in egg white and foam drainage of the same low lysozyme eggs

Storage time	Egg white Albumin (%)	Egg white Lysozyme (%)	Foam drainage Albumin (%)	Foam drainage Lysozyme (%)
1 day	61.5	5.31	72.2	2.98
1 wk	61.3	5.30	66.1	4.88
2 wk	61.2	5.16	70.0	3.62
3 wk	61.4	5.26	70.6	3.50
4 wk	61.6	5.17	71.5	2.58
5 wk	61.5	5.26	73.0	3.57
6 wk	61.5	5.31	68.9	4.55
7 wk	61.3	5.29	67.9	3.50
8 wk	61.3	5.28	67.4	4.30

Table 5–Foam volume and drainage after 15 and 60 min, from 60 ml of egg white

Storage time	High lysozyme Volume (ml)	High lysozyme Drain 15 min (ml)	High lysozyme Drain 60 (ml)	Low lysozyme Volume (ml)	Low lysozyme Drain 15 min (ml)	Low lysozyme Drain 60 (ml)
1 day	199	2.2	8.1	219	2.2	6.5
1 wk	179	3.0	11.4	238	2.6	10.2
2 wk	206	2.9	16.0	251	2.4	12.2
3 wk	228	2.7	11.5	274	2.3	11.5
4 wk	270	2.2	10.0	299	1.9	9.8
5 wk	369	1.7	12.0	369	1.7	12.0
6 wk	380	2.0	10.3	382	1.8	10.6
7 wk	396	1.7	12.7	388	1.8	10.6
8 wk	362	2.6	11.0	360	2.6	13.8

bands. At this time the three albumin fractions appeared as a single wide band. Egg white from eggs stored for 7 wk or longer often show distinct separation only of albumins and lysozyme. A continuous lightly stained band may extend from the starting point almost to the albumin band indicating an overlapping of migration rates.

Albumin and lysozyme fractions of high lysozyme eggs used for foam, and the same two fractions from initial foam drainage of the same eggs are compared in Table 3. Similar data for low lysozyme egg white are shown in Table 4. The relative percentage of albumin in foam drainage increased considerably as compared to egg white prior to whipping with both high and low lysozyme egg white. Albumins in the drainage increased 17.8% over the amount in unwhipped egg white from the high lysozyme eggs and by 13.5% with egg white from low lysozyme eggs. Lysozyme was decreased in foam drainage from an average relative percentage of 8.35 to 3.85 for high lysozyme eggs and from 5.26 to 3.83 for low lysozyme eggs. This represents a loss of over 53 and 27% for egg white from high and low lysozyme eggs respectively.

Total relative percentage of albumins and lysozyme combined increased from 66.65 to 72.55 with high lysozyme eggs and from 66.66 to 73.53 with low lysozyme eggs with a corresponding decrease in the relative percentage of other fractions. If any specific fractions accounted for all or most of the reduction in relative percentage of protein, it was not identifiable with the technique used due to overlapping of protein bands.

Average foam volumes from 60 ml of egg white are shown in Table 5. Foam volume increased with storage for both high and low lysozyme eggs; however, volumes produced by high lysozyme eggs were consistently less until after 4 wk of storage. Differences in foam volume during the early weeks of storage may have been due to more thick egg white from high lysozyme eggs. A whipping time of 1.5 min would not have produced foams of maximum volume. If thick egg white required more beating than thin white to achieve maximum volume, then 1.5 min would more nearly achieve maximum volume for thin than for thick egg white. Differences in the ratio of thin to thick egg white could then account for the larger volume of foams from low lysozyme eggs during the first 4 wk of storage. Foam volumes were not significantly different after 5 wk of storage.

Average foam drainage values after 15 and 60 min are shown also in Table 5. Drainage from foams of high lysozyme eggs averaged from 1.7–3.0 ml after 15 min and from 8.1–16.0 ml after 60 min. Comparable drainage from foams of low lysozyme eggs were 1.7–2.6 ml and 6.5–13.8 ml respectively after 15 and 60 min. Drainage from high and low lysozyme foams was not significantly different at any time. Volume and 15 min drainage data were used to compute a correlation coefficient for these factors using all foams from each egg storage time. Values ranged from −0.321 to −0.931 for the different storage times without apparent relationship to storage time. The correlation coefficient for all foams and drainage was −0.517, indicating a highly significant negative relationship between foam volume and drainage.

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FUNGAL DECAFFEINATION OF ROAST COFFEE INFUSIONS

INTRODUCTION

ACCORDING TO Sivetz (1963) some 10% of the coffee consumed in North America and Europe was decaffeinated. Organic solvents, especially trichloroethylene, were used to extract the caffeine, mostly from the green bean. Persistent appearance of new patents (e.g., Size, 1969) and analytical studies of chloroalkane residues in coffee (Brandenberg et al., 1969; Schilling and Gal, 1971) suggest that such solvents are still being used. We considered both chemical and biological alternatives to present decaffeination practices. To this end we have isolated and studied the metabolism of a strain of *Penicillium* which efficiently utilized caffeine as sole source of nitrogen in clearly defined growth media, containing nitrogen-free Hoagland salts and 0.088M sucrose (Kurtzman and Schwimmer, 1971; Schwimmer et al., 1971). There was no assurance the fungus would remove caffeine from coffee as well. In the present communication we report that in the presence of sugar this strain of *Penicillium* can indeed remove caffeine from infusions of commercial roast coffee as effectively as on synthetic growth media to yield a product which preliminary feeding trials indicate is sub-acutely harmless to rats.

EXPERIMENTAL

Caffeine analysis

Caffeine in coffee infusions was determined by a slight modification of the chromatographic-spectrophotometric AOAC (1971) method for the determination of caffeine in decaffeinated coffee. To 1 ml of infusion was added 0.5 ml of reagent NH_4OH and 6g of Celite 545. The mixture was transferred to the basic Celite column described in the AOAC method and the analysis proceeded as previously described except that a total of 25 ml instead of 50 ml of $CHCl_3$ was collected. Our tests showed that no caffeine was extracted after the first 25 ml. The results have been routinely expressed as mg of caffeine per ml of coffee infusion. Caffeine in synthetic growth media was determined by measuring the absorbance of these media at 273 nm after removal of insolubles by centrifugation and appropriate dilution.

Routine maintenance of fungal cultures

The fungus, identified by Dorothy I. Fennell and accessioned as *Penicillium crustosum*, strain NRRL 5452, was grown and maintained in a medium containing a mixture of nitrogen-free salts ($CaCl_2$ and KCl, 0.01M; $MgSO_4$, 0.04M; K_2HPO_4, 0.02M), 0.088M sucrose and 0.01M caffeine. 50 ml aliquots of the culture were shaken (200 rpm) in 250-ml Erlenmeyer flasks at 20°C and transferred every 2 wk.

Standard procedure for fungal fermentation of coffee infusions

Coffee brew was prepared from commercial ground coffee by the "filter paper" method usually employed for home consumption using Whatman No. 1 filter paper at the rate fo 46g per liter. After the addition of a 1–3% autoclaved sucrose solution, the sucrose-containing coffee infusion was inoculated by adding 5g (wet weight) of the washed pellet from a routine fungal culture to 50 ml of infusion and was incubated under the above-described conditions. Samples were periodically withdrawn for caffeine analysis. The initial caffeine content of these standard infusions varied from 0.45–0.59 mg per ml.

Large scale fermentations

To coffee infusion (4–10 liters) in a Humfeld fermentor (Humfeld, 1947) was added, separately, autoclaved sucrose and washed inoculum in approximately the same proportion as used in the standard infusions (excepting B, Table 3, in which case the coffee infusion was added to the fermentor which already contained the culture from experiment E. In the latter experiment the fungus was grown on a synthetic caffeine-containing medium). The culture was maintained at 24°C and aerated at the rate of 1 liter of air per min per liter of medium. The impeller ran at 460 rpm.

Table 1—Effect of sugar on decaffeination of coffee[a]

Sugar	% Caffeine left after: 9 hr	12 hr	15 hr
None	73	54	40
Sucrose, 0.03M	57	17	1
Sucrose, 0.06M	53	19	5
Glucose, 0.06M	55	29	6

[a]See "Experimental" for details.

RESULTS

Effect of sugars on the rate of decaffeination

The fungus used in the present investigation was capable of removing caffeine from coffee infusion in the absence of any additives other than inoculum. In a typical experiment, shown in Table 1, almost half of the caffeine was removed in the absence of sugar in 12 hr. However, the rate of caffeine removal was sharply stimulated by the presence of either sucrose or glucose during the early phases of growth. As shown in Table 2, the stimulating effect of sucrose was not so great in the presence of citrate buffer. This inhibiting effect also appeared to hold for weakly decaffeinating conditions as well. Thus in another experiment 50 ml aliquots of 0.1M citrate buffered infusions (pHs 4.0, 4.5, 5.0) containing 0.03M sucrose were inoculated with a low level (1–2g) of standard *P. crustosum* culture. The percentages of caffeine remaining after 24 hr were 46, 35 and 28 respectively. This is to be compared with coffee containing no buffer in the presence (23%) and in the absence (65%) of 0.03M sucrose.

Buffer and pH effects

The limited data on the effect of buffers and of pH in conjunction with sugars suggest that these variables might exert appreciable influence on the rate of decaffeination. The influence of pH on the caffeine content of inoculated coffee containing lactate buffer is shown in Figure 1. Optimal decaffeination at pH 4.8 was close but not identical with pH

Table 2—Effect of buffers on the sucrose-stimulated decaffeination of coffee

	% Caffeine left after:					
	8 hr		24 hr		32 hr	
Buffer	–S[a]	+S	–S	+S	–S	+S
None, pH 5.1	–	66	–	31	–	8
0.1M Lactate, pH 5.0	95	94	46	39	30	14
0.1M Citrate, pH 4.7	97	91	60	48	43	17

[a]–S and +S indicate the absence and presence of 0.088M sucrose respectively.

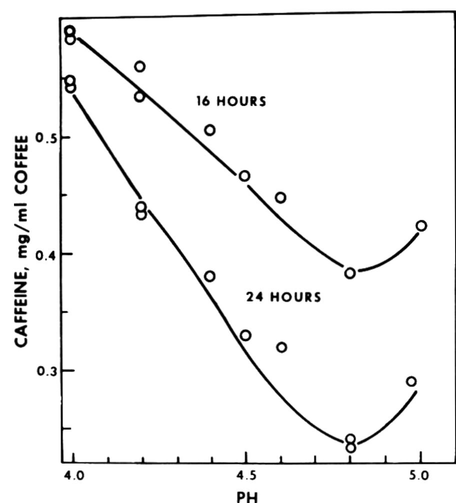

Fig. 1–Effect of pH on the caffeine content of coffee in the presence of 0.1M lactic acid–sodium lactate buffers after 16 and 24 hr fermentation.

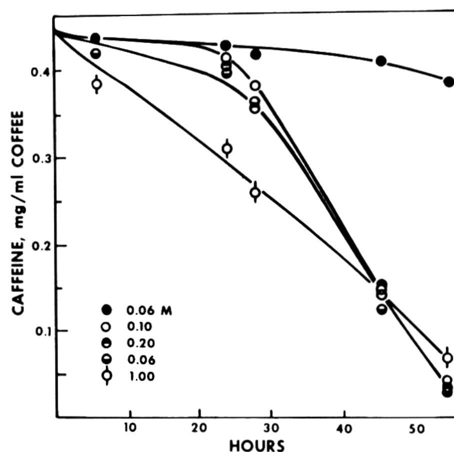

Fig. 2–Effect of pH 4.0 citric acid–sodium citrate buffer concentation on the caffeine content of fungally inoculated coffee.

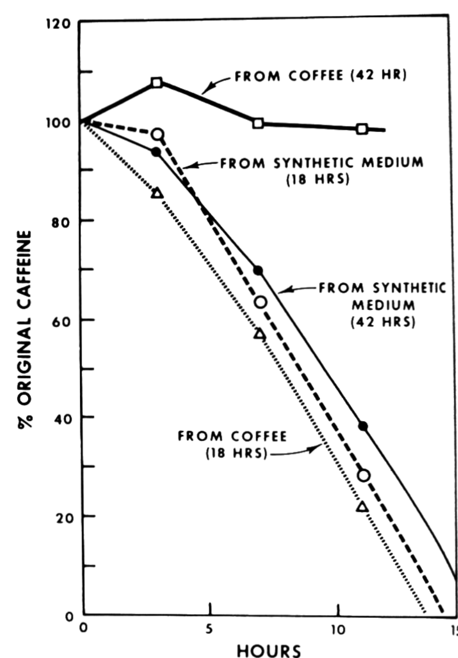

Fig. 3–Effect of inoculum on fungal decaffeination of coffee.

(5.0–5.1) of unbuffered coffee infusions. The pH increased in each sample from 0.05–0.10 units in 24 hr. The influence of variable buffer concentration at constant pH 4.0 is shown in Figure 2. Increasing concentration tended to diminish the initial lag in caffeine utilization. Although the pH rose in the samples, in no instance did the pH exceed 4.5, even after 54 hr.

Other experiments with citrate, isophthalate and cyclopentadiacetate buffers, all 0.1M, consistently showed decreased caffeine-removing activity at pH 4.0 as compared with pH from 4.2–5.0.

Effect of culture conditions

Although no systematic study was made of the effect of inoculum size, our experience indicated that, as expected, heavier inocula resulted in faster decaffeination. For instance, in the experiment on the effect of variable pH with citrate buffers alluded to above, only 77% of the caffeine was utilized as contrasted with complete consumption in 24 hr with a heavier inoculum.

In general, it was found that the age of the inoculum grown on the synthetic medium did not greatly influence the subsequent rate of coffee decaffeination, provided the culture had been transferred within 7 days prior to inoculation. However, this rather nonstringent time requirement did not extend to coffee cultures used as inocula, as graphically depicted in Figure 3. Decaffeination of coffee containing 1% sucrose inoculated with cultures grown on standard synthetic medium for 18 and 42 hr (as well as an inoculum from coffee grown for 18 hr prior to inoculation) proceeded vigorously and at about the same maximal rate of 8% per hr. By contrast, no caffeine was consumed in coffee inoculated with a culture grown on coffee for an additional day (total of 42 hr) prior to inoculation. In spite of the lack of caffeine utilization, the fungus (in a separate repeat experiment, results of which are shown in Table 3) grew equally well in coffee inoculated with either an 18- or 42-hr coffee-grown culture.

The data of Figure 3 and Table 3 suggest that using a fresh coffee culture as inoculum may be somewhat more efficient, due to less lag time, than using an inoculum from the standard synthetic medium. In the repeat experiment, it was found that 50% of the caffeine could remain in 6 hr from coffee inoculated with a coffee-derived culture (18 hr old) whereas coffee inoculated with the "standard" synthetic medium still retained 62% of its caffeine in 24 hr.

Table 3– Effect of inoculum on growth of P. crustosum in coffee

Inoculum grown on	Synthetic medium		Coffee	
Time prior to transfer to fresh coffee, hr	18	42	18	42
Caffeine left after 6 hr on fresh coffee %	60.3	62.8	50.0	93.7
Dry weight of mycelia,[a] 10 days, mg	168	235	255	247

[a]Dry weight of inocula 65 to 85 mg

Scale-up and effect of initial caffeine level and volume of culture

The data hitherto presented were obtained with 50 ml aliquots of brewed coffee containing ca. 0.5 mg/ml of caffeine. We scaled up the fermentation primarily to obtain sufficient amounts of the fermented product for feeding tests but also to examine the kinetic characteristics of decaffeination when performed in a laboratory fermentor at variable volumes and initial caffeine concentrations. The results of three scaled-up runs in a Humfeld fermentor are displayed in Table 4 and Figure 4. In addition to one run with coffee brewed from ground beans and two with instant freeze-dried

Table 4–Large scale coffee fermentation[a]

Designation	A	B	C	D	E
Culture medium (caffeine source)	Brewed coffee	Soluble coffee	Soluble coffee	Brewed coffee	Synthetic medium
Volume, liters	0.05	4	6	10	4
Sucrose, %	1	2	3	1	3
Initial caffeine, mg/ml	0.46	0.60	0.91	1.65	2.00
Final pH (days)	5.3(1)	7.3(3)	6.7(4)[b]	7.5(6)	–
Maximum rate, mg caffeine/ ml/day	0.24	0.25	0.27	0.28	0.28

[a]See "Experimental" for details.
[b]pH adjusted to 6.0 at 3 days.

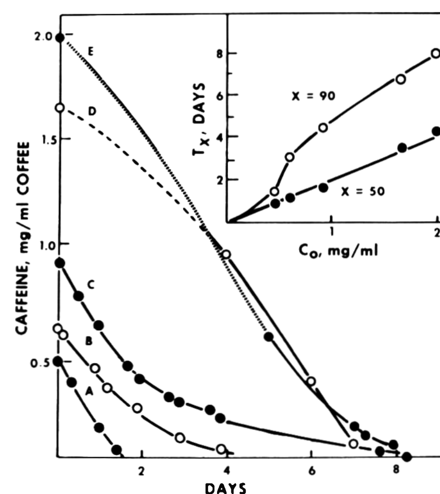

Fig. 4–Decaffeination of coffee in Humfeld fermentor. (See "Experimental" and Table 3 for details.) Inset: c_o = initial caffeine concentration; T_x = days required to achieve x percent decaffeination.

coffee, included are a run with a synthetic caffeine-containing medium and, for comparison, a routine small-scale run.

One of the most striking correlations observed is the relative lack of dependence of the rate of decaffeination on both initial caffeine concentration and on volume as well as on conditions of culture, i.e., fermentor vs. shake flask. The rate of caffeine removal was estimated to vary between 0.24 and 0.28 mg/ml/day in all five fermentations. This range of values is probably well within the error of graphic estimation.

Whereas in the small-scale control, where the caffeine disappeared at a comparatively linear rate to completion, the caffeine vs. time profiles for the larger scale experiments are characterized by a more or less diminishing rate, at least after more than half of the caffeine has been removed. As shown in the inset of Figure 4, there is a rough parallelism between the initial concentration and the time required to remove half of the caffeine, quite independent of initial volume thus indicating that the inocula were present in excess. This parallelism (excluding the small scale experiment) also holds for the time to remove 90% of the caffeine.

DISCUSSION

THE PRESENT INVESTIGATION has established that caffeine can be removed from coffee infusions as well as from synthetic growth media by a caffeine-utilizing strain of *Penicillium crustosum*. We have further delineated the effects of some of the parameters affecting the rate of decaffeination. Sugars improve whereas buffers tend to diminish the efficiency of decaffeination in the presence of sucrose. This stimulating effect of sugar is probably due to additional growth.

Observations that (a) the slight acidification of the coffee did increase the rate of decaffeination; (b) the rate in the presence of citrate was somewhat greater in its presence than in absence (without sucrose); and (c) the rate increased with increasing citrate concentration suggest that citrate was being utilized for growth of the fungus, albeit not as well as sugars.

The observation that the fungus previously cultured in coffee for prolonged periods loses the ability to metabolize caffeine but can still grow in coffee suggests that upon the exhaustion of caffeine repression of the caffeine metabolizing genome occurs concommitantly with adaptation to an alternative nitrogen source. Such a nitrogen source could be trigonelline, also an N-methyl derivative (the methyl betaine of nicotinic acid) present in coffee in amounts approximately equimolar to caffeine (Thaler, 1963).

While scale-up did not decrease the efficiency of caffeine utilization as measured by the rate of decrease of caffeine concentration, it would appear that batch operation is not the route to the increased fermentation efficiency required. According to Pictet (1971), a maximum fermentation time of 6 hr is imposed upon any process which would be compatible with preservation of desirable beverage attributes. We feel that adaptation to continuous fermentation processing, and applying some of the recent innovative bioengineering reactor designs, a more intense study of environmental factors, and avenues of approach using genetic manipulation such as mutation and gene dosage should increase the efficiency of decaffeination by at least one order of magnitude.

Previous studies (Kurtzman and Schwimmer, 1971; Schwimmer et al., 1971) established that absorbance decreased throughout the UV spectrum during fungal growth. Hence at the end of the growth neither purine nor large amounts of other high-UV absorbing material accumulated in either the medium or in the fungi. The metabolism was shown to proceed through progressive demethylation of the purine moiety of caffeine with appearance of theophylline (1,3-dimehtyl xanthine) during the first few hours after inoculation. This is in comparison with the accumulation of xanthine and its methylated derivatives in shake cultures of a caffeine-utilizing strain of *Aspergillus niger* after 15 days at 30°C (Ina, 1971) and also in contrast with formation of methylated catabolites of xanthine from methylxanthines by *Pseudomonas aeruginosa* (Franke and Hahn, 1955; Bergmann et al., 1962).

Preliminary results of feeding trials on rats fed the fungally decaffeinated coffee equivalent to 22 cups of coffee per 150-lb man per day, has revealed no adverse effects after 90 days. Details of this and expanded feeding tests will be presented in a subsequent publication.

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THE NATURE AND CONFORMATION OF THE CAFFEINE-CHLOROGENATE COMPLEX OF COFFEE

INTRODUCTION

THE CAFFEINE-CHLOROGENATE complex, first described by Gorter (1907, 1908) has received little attention since that date, especially with regard to its physicochemical properties. Sondheimer et al. (1961) in a partition study considered in a semiquantitative manner the influence on complex strength of the various parts of the chlorogenate ion and of caffeine. They observed little difference in the complexing ability of the chlorogenate ion and of free chlorogenic acid. However, their work, and other recent investigations of caffeine complexes in aqueous and nonaqueous media (Pullman et al., 1965; Kristiansen et al., 1970; Moriguchi et al., 1969) have failed to produce a consistent explanation of the nature of the complexes formed.

The present study attempts to clarify this situation, particularly as regards the caffeine-chlorogenate complex, of considerable interest in the chemistry and physiology of coffee.

EXPERIMENTAL

Materials

Potassium chlorogenate was obtained by exhaustive chloroform extraction of a 50% aqueous ethanol solution of the chlorogenate-caffeine complex (Fluka-Buchs), and by subsequent evaporation of the residual aqueous layer. The purity was verified by TLC and NMR. The remaining organic salts were prepared by the addition of a slight excess of the acid (Fluka-Buchs) to a 0.5M sodium carbonate solution, followed by precipitation with acetone. After filtration and drying to constant weight, the salts were stored over P_2O_5. Residual water in the salts estimated by the Karl-Fisher method was in general less than 5% by weight.

Evaluation of K_r^{CS}

The association constant for the formation of a 1:1 complex, K_r^{CS}, is represented by the expression (CS)/(C) · (S), where (CS), (C) and (S) are the equilibrium concentrations of the 1:1 caffeine-salt complex, free caffeine and free salt respectively. For solutions where $[S]_o \gg [C]_o$ (initial concentrations), the complex induced displacement Δ_T of a caffeine methyl ^{1}H-absorption is given by Eq. 1, where the term $m \cdot [S]_o$ has been added to the equation derived by Foster and Fyfe (1965) for the estimation of association constants of organic charge-transfer complexes. In Eq. 1, Δ_o^{CS} is the theoretical displacement which would be observed in the pure complex relative to pure caffeine and m is a molar displacement coefficient which accounts for changes in the bulk solvent environment as $[S]_o$ is increased.

$$\Delta_T = \frac{K_r^{CS} \cdot \Delta_o^{CS} \cdot [S]_o}{1 + K_r^{CS} \cdot [S]_o} + m \cdot [S]_o \quad (1)$$

A series of solutions in which $[S]_o$ ranged from 0.05–0.5 mol·kg^{-1} solution (concentrations adjusted to allow for residual water) was prepared for each salt by dissolving a weighed amount of the salt in a weighed quantity of a 0.005 mol·kg^{-1} caffeine solution (prepared by

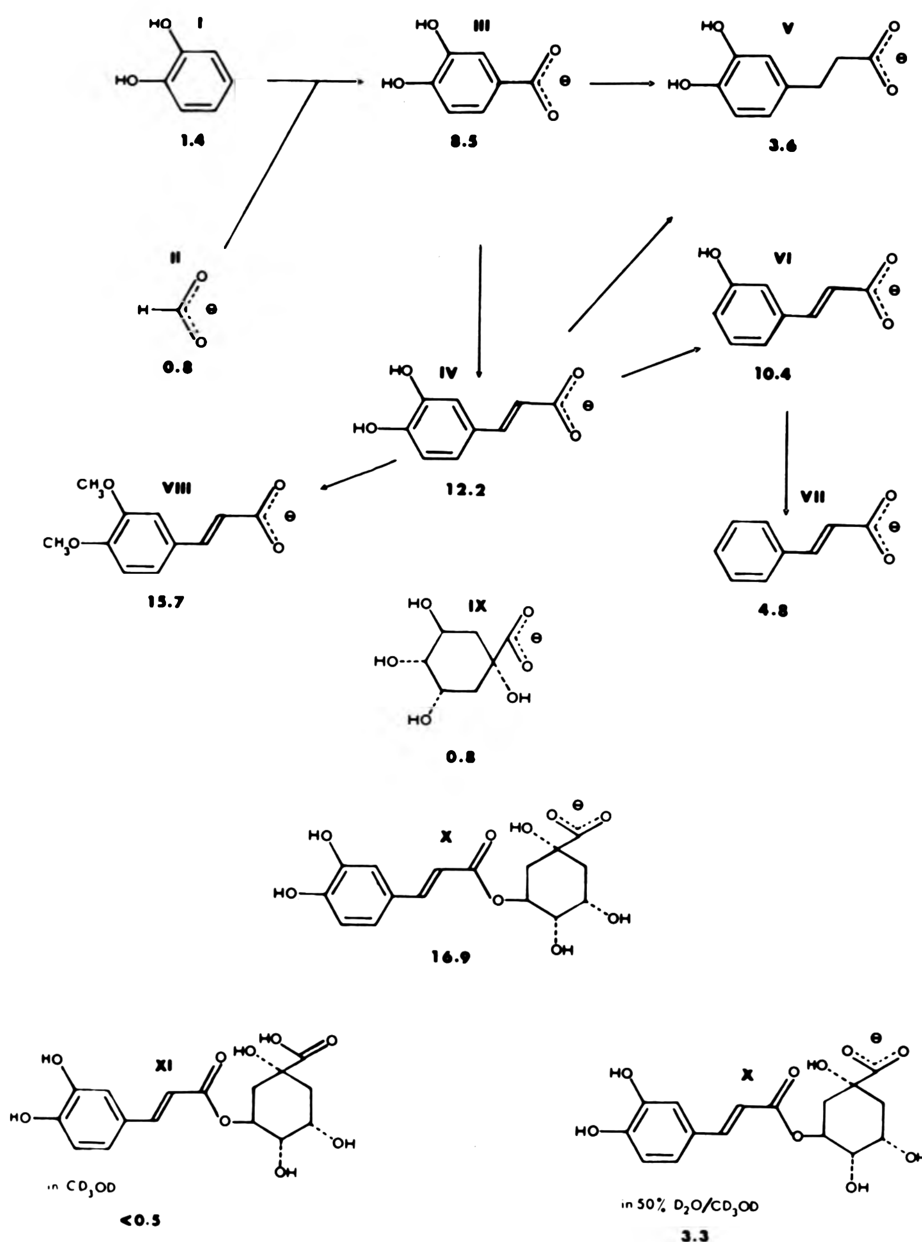

Fig. 1—Values of association constants, K_r^{CS}(kg·mol^{-1}), at 40°C in D_2O solution (unless otherwise stated) for the compounds studied. Arrows indicate series of compounds discussed in the text, and not chemical reactions.

dissolving caffeine in D_2O, (Fluka-Buchs) and/or methanol-d_4 (Merck). The ^1H line positions of the caffeine methyl absorptions were determined in the pure caffeine solution and in each complex solution relative to a capillary standard (0.1M MCl solution absorbing at 4.7δ) as the average of ten scans. Displacements, Δ_T, for each concentration of $[S]_0$ were measured, and K_f^{CS} (along with Δ_0^{CS} and m) was determined from Eq. 1 according to the Gauss-Newton method of least squares on an IBM 360/40 computer.

All NMR spectra were run at 40°C on a Varian Associates A60-D instrument fitted with a variable temperature probe at an oscillator frequency of 60 MHz.

RESULTS & DISCUSSION

Association constants

The values of K_f^{CS} determined at 40°C in D_2O solution for the formation of 1:1 complexes of caffeine with the various species studied are presented in Figure 1, from which the contributions to complex formation of the different chemical functions of the chlorogenate ion may be inferred.

The importance of an extended π-system is indicated by the marked increase in complexing ability observed when pyrocatechin (I) and the formate ion (II), both of which complex weakly, are combined to give the pyrocatechuate ion (III); by the further increase in complex strength resulting from the insertion in (III) of a conjugated double bond to give the caffeate ion (IV); and by the subsequent decrease observed when the double bond is reduced to form the dihydrocaffeate ion (V).

The influence of the phenolic hydroxyl groups is shown by the decrease in complexing ability when first one then two OH-groups are removed from V to give the m-coumarate (VI) and cinnamate (VII) ions. Methylation at the phenolic hydroxyl groups to give the 3,4-dimethoxycinnamate ion (VIII) results in an increased complex strength.

The influence of the quinate moiety, itself complexing only weakly, is indicated by the increased complexing ability of the chlorogenate ion (IX) relative to the caffeate ion (IV), whilst the effect of solvent is shown by the marked decrease in K_f^{CS} for (X) when 50% perdeuteromethanol is employed. Pure perdeuteromethanol induces a further decrease in K_f^{CS} although this could be partially due to the use (owing to solubility reasons) of free chlorogenic acid (XI). Similar observations of solvent effects have been reported for caffeine-methylnaphthoquinone complexes in aqueous dioxane and aqueous glycol by Kristiansen et al. (1970).

The nature of the complex

The increased values of K_f^{CS} observed on extension of the π-system and from hydroxylation or methylation of the aromatic ring of the salt indicate the formation of a π-molecular complex. Calculated values of the enthalpy and entropy of complex formation (Horman, 1972), which lie in the range usually associated with this type of weakly bound complex (Foster, 1969), support this conclusion.

The reduced values of K_f^{CS} in particular for the caffeine-chlorogenate system

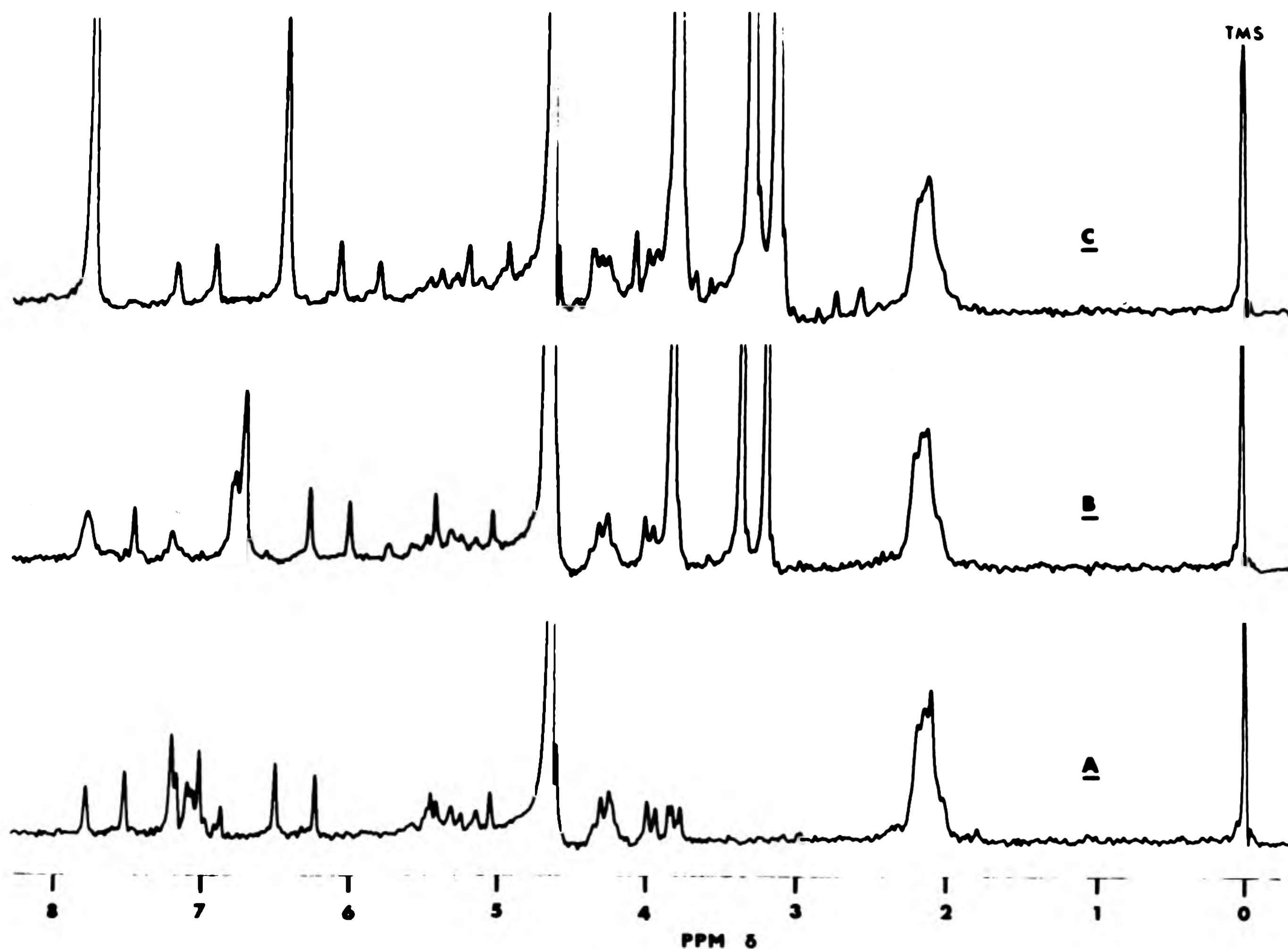

Fig. 2–The NMR spectra of (A) free chlorogenate ion (0.01 mol·kg⁻¹ solution) in D_2O; (B) partially complexed chlorogenate (caffeine concentration 0.02 mol·kg⁻¹); (C) of chlorogenate in the presence of excess caffeine.

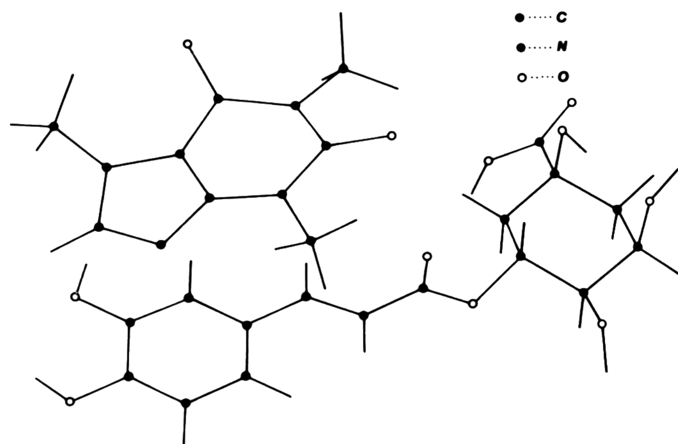

Fig. 3–The suggested conformation of the caffeine-chlorogenate complex in aqueous solution.

three aromatic protons become equivalent. The near equal upfield shifts observed for the three caffeine methyl groups in the presence of excess chlorogenate suggest that in the complex, the plane of caffeine is parallel to the plane of the caffeoyl aromatic ring. In addition the five- and six-membered rings of caffeine are equally involved in complex formation.

The proposed conformation of the complex based on the chemical shift data discussed above is shown in Figure 3. It is considered that caffeine exists as a near planar species. The equivalence of the aromatic protons and of the caffeine methyl shifts in the complex indicates that the conformation is not absolutely fixed, but represents the time-average of many other conformations involving relative twisting, sliding and rocking of the two components.

on using basic conditions (Sondheimer, 1961) or perdeuteromethanol arise from increased solvent competition. In neutral aqueous solution, it might be expected that solvation would occur in the peripheral polar sites of the caffeate or chlorogenate ions, thus leaving the π-systems of caffeine and the salt free to overlap in the form of a π-complex. The formation of such a complex would be energetically favorable because of the hydrophobic nature of the π-system, as seen in the increased solubility of aromatic hydrocarbons, themselves hydrophobic species, in aqueous solutions containing caffeine (Eisenbrand and Baumann, 1970).

The use of methanol, an organic solvent, or basic conditions where the charge on the ionized phenolic hydroxyl groups is delocalized into the aromatic π-electron system, would lead to increased solvation of the ring, and, ipso facto, to a decrease in complex formation owing to competitive interference from the solvent. The enhancement of K_r^{CS} on going from the caffeate ion (IV) to the chlorogenate ion (X) is thus explained: the bulky quinate system helps to shield the caffeine-chlorogenate complex from solvent interference.

Classical donor-acceptor interactions can be eliminated as playing a major role in the binding. For this, caffeine would have to act as an electron acceptor. This has been demonstrated in a nonpolar medium (Hanna and Sandoval, 1968), but the absence of complex formation between caffeine and the iodide ion, itself a powerful electron donor, suggests that in aqueous solution this is not the case. Also, the enhanced K_r^{CS} values induced by the phenolic hydroxyl groups, and on adding the quinate moiety to the caffeoyl group might indicate a contribution to the binding from hydrogen bonding. However, the observed increase in K_r^{CS} on methylation of the phenolic hydroxyl groups, and the low value for quinate itself indicate that the contribution is very small or zero.

Thus in summary, the caffeine-chlorogenate complex may be described as a hydrophobically-bound π-molecular complex.

The conformation of the complex

When the complex is formed, the components experience mutual magnetic shielding (or deshielding) effects. The degree of shielding of a proton within the complex depends on its position relative to the center of complexation. Ring currents, chiefly in π-electron systems, give rise to magnetic shielding in a direction perpendicular to the plane of the π-system, and deshielding parallel to the plane. Figure 2 shows the effect of complexation on the peak positions of the chlorogenate ion. Addition of excess caffeine results in an upfield shift of the entire chlorogenate spectrum, the shift being smallest for the protons of the quinate moiety (2.12δ in A $\rightarrow$ 2.14δ in C), intermediate for the ethylenic protons (6.38δ $\rightarrow$ 5.98δ and 7.65δ $\rightarrow$ 7.08δ), and greatest for the caffeoyl aromatic protons (7.06δ $\rightarrow$ 6.46δ), indicating that complexation occurs in the region of the aromatic ring system. Further evidence for this position is that the aromatic multiplet of chlorogenate (Fig. 2A) is reduced to a singlet in the complex (Fig. 2C), i.e., the

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THERMAL DETECTION OF SPOILAGE IN CANNED FOODS

INTRODUCTION

SMALL LOTS of processed foods are sometimes stored, after processing, for periods of weeks or even months, in order to identify and remove cans that spoil as a result of improper processing. This is particularly prevalent in the rapidly expanding field of "aseptic canning." The storage period is expensive, and there is no general nondestructive method for identifying flat-sour spoilage. Cans are also stored for spoilage detection in establishing process schedules for new products.

It is well known that bacterial growth is accompanied by the evolution of heat. The experiments described below establish the possibility of detecting the heat liberated during spoilage of commercially canned goods. This finding offers a basis for the development of a nondestructive technique for the identification of spoilage at the time of occurrence.

The major problem is to detect temperature changes much smaller than the normal fluctuations of the environmental temperature. We were able to accomplish this by using an uninoculated can as a reference. In commercial practice, each can's neighbor could serve as its reference can. Since the probability of simultaneous initiation of spoilage is insignificant, any temperature increase of a can with reference to its neighbor would be presumptive evidence of spoilage.

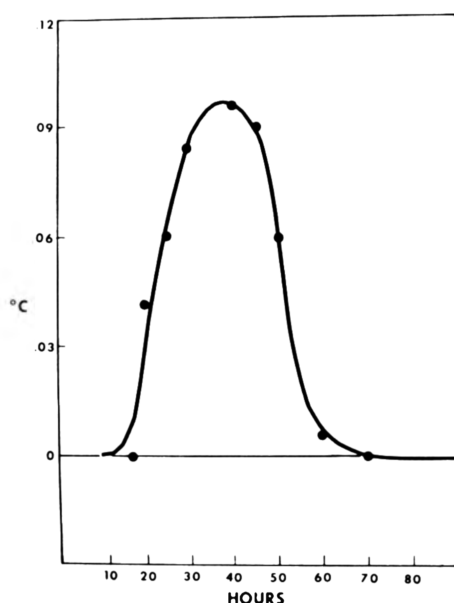

Fig. 1—Heat evolution from can (1 lb) of cream style corn, inoculated with 0.2 ml (1.1 mg) of spores of B. macerans 7X1. Spores were activated at 70°C for 1 hr. Cans were placed in Dewars, temperature 35°C, thermocouples used for heat detection. Data replotted from recorder trace.

EXPERIMENTAL

Bacterial cultures

B. macerans NCA strain 7X1 was obtained from the National Canner's Association. Putrefactive anaerobe 3679 was obtained from a stock collection in the Western Regional Research Lab.

Spore formation

Spores of *B. macerans* were formed and purified as previously described (Sacks, 1969). Spores of PA 3679 were formed on the following medium: Beef extract, 10g; pork infusion, 400 ml; pea infusion, 100 ml; egg albumin, 1.0g; NZ amine, 10g; Proteose Peptose, 10g; Tryptone, 2g; yeast extract, 2g; soluble starch, 1g; K_2HPO_4, 3.97g; KH_2PO_4, 2.40g; sodium thioglycollate, 0.1g; water to 1 liter. The cultures were incubated for 3 wk at room temperature (ca. 25°C) in volumetric flasks. Vegetative cells and sporangia were washed three times in water and used without further purification.

Heat detection in inoculated cans

Heat-processed cans were punctured, and 0.1–0.2 ml of a thick bacterial suspension was inoculated into the can from a syringe. Relatively heavy inocula were employed to reduce the lag period and insure complete spoilage within 48 hr. The puncture hole was covered with a small piece of tape and sealed with fast-setting epoxy resin. Thermistors (or thermocouples) were taped to the top of the inoculated can and an identical uninoculated reference can. The thermistors were connected through a Wheatstone bridge, balanced and temperature difference monitored as a voltage change on a strip chart recorder.

In some cases, jars with twist-seal lids were used, rather than cans. In such cases, the lid was removed, the inoculum added and the lid replaced.

All experiments reported here were conducted at 35°C. All cans, inocula and equipment were temperature-equilibrated before inoculating the cans.

Verification of spoilage

Microscopic examination, as well as odor, viscosity and pH were used to verify microbial spoilage at the termination of experiments.

Thermistors

Initially, measurements were made using copper-constantan thermocouple pairs. Later, banjo-type surface thermistors of 2000 ohms resistance were used for both reference and sample measurements.

RESULTS

FIGURE 1 shows the results of an early experiment in which thermocouples were used to detect temperature changes induced in commercially processed cream-style corn inoculated with spores of *B. macerans* 7X1. In this experiment the cans were placed in separate Dewar jars packed with fiber glass to minimize heat loss. Figure 1 shows that a maximum temperature difference of about 0.1°C was obtained, and this difference persisted for about 10 hr. Microscopic examination of the opened can indicated about 10^8 cells/ml.

Figure 2 shows a similar experiment in which cans were not insulated in any

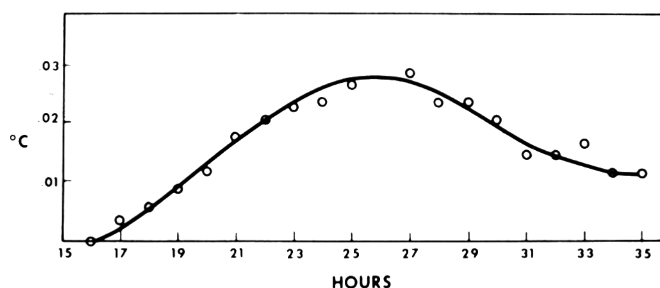

Fig. 2—Heat evolution from can (1 lb) of cream style corn, inoculated with 0.5 ml (0.5 mg) of spores of B. macerans 7X1. Spores were activated at 70°C for 1 hr. Temperature 35°C. No insulation; cans not shielded. Data replotted from recorder trace.

way. In this, and all subsequent experiments, thermistors rather than thermocouples were used to monitor temperature. Here a maximum temperature difference of 0.03°C was obtained; a difference of more than 0.02°C persisted for over 9 hr.

Figure 3 shows the temperature increase occurring in a nonviscous liquid, clam juice, inoculated with a suspension of PA 3679, mixed spores and nonviable vegetative cells. This shows that the method is not limited to viscous food products.

Glass jar containers with twist-off metal lids may be monitored in similar fashion. The spoilage of cream of chicken soup in a baby food jar, inoculated with a PA 3679 suspension is shown in Figure 4. The jars were uninsulated but shielded from drafts and light by a large black box with a black curtain entrance. A maximum temperature increase of more than 0.06°C was obtained; over 30% of the maximum temperature difference persisted for more than 7 hr. Microscopic examination of the opened jar indicated more than 5 x 10^8 cells/ml.

A second experiment was performed, identical in all respects, except that the inoculum was diluted 1:1000. A peak similar in all respects to that described in Figure 4 was observed, but occurring 3 hr later. If one assumes a generation time of 18 min (reasonable at this temperature) this is precisely the delay one would anticipate, assuming the lag to be roughly the same in both experiments.

Our equipment is relatively primitive and in some cases heat evolution is barely detectable. However, the results seem to be quite reproducible, and seemingly abnormal fluctuations have been traced back to various unusual conditions. For example, a sharp temperature drop at 40 hr after inoculation of cream style corn with *B. macerans* spores was found to result from the dislodgement of the thermistor from a can bulging because of gas formed by the spoilage organism. Anomalous temperature decreases which sometimes occurred in late spoilage were usually found to be due to evaporative cooling when liquid was forced out under the seal.

Time-temperature curves may sometimes show more than one peak; this may reflect diauxic growth in a complex medium, or the development of a contaminant introduced with the inoculum.

DISCUSSION

BACTERIAL GROWTH is always accompanied by the liberation of heat. The experiments reported above demonstrate clearly that abundant growth at rapid rates in small commercial size cans or jars is readily detectable. Rapid growth was possible at the incubation temperature of 35°C used in all experiments. Such rapid growth might be anticipated under conditions of "accelerated storage" where temperatures of 35–50°C are commonly employed. However, under conditions of ordinary warehouse storage lower temperatures and lower levels of heat evolution must be expected. The temperature characteristic (activation energy) for bacterial growth has been estimated at levels approximating 15,000 (Hanus and Morita, 1968). Using this as a basis, it is possible to estimate the maximum temperature increase in a particular can spoiling with a

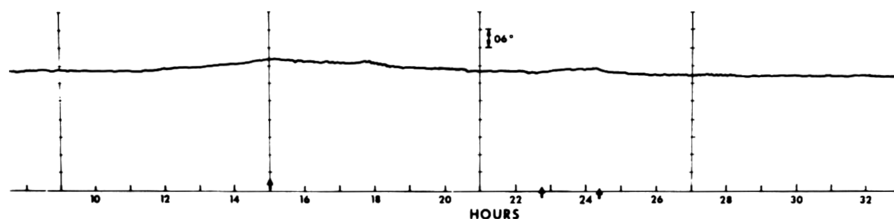

Fig. 3—Heat evolution from can (10-½ oz) of clam juice inoculated with 0.2 ml suspension (ca. 10^8 viable cells) of washed PA 3679. Cans held at 35°C without insulation. Original recorder trace.

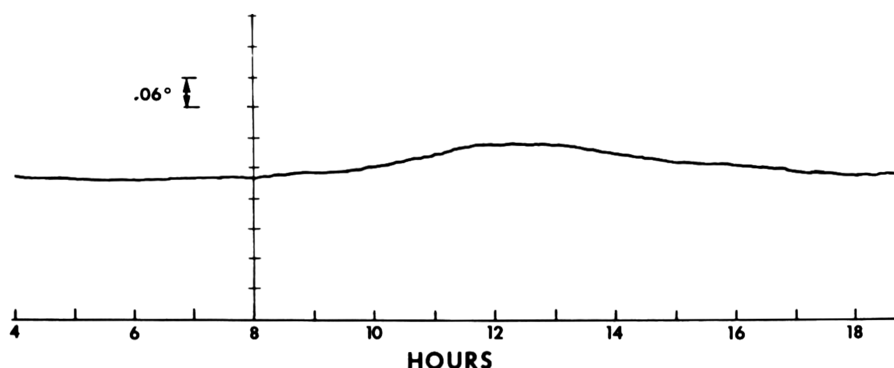

Fig. 4—Heat evolution from baby food jar of cream of chicken soup, inoculated with 0.2 ml (ca. 10^8 viable cells) suspension of washed PA 3679. Jars held at 35°C without insulation, but shielded from light and drafts in a large, black wooden box, with a black curtain entrance. Original recorder trace.

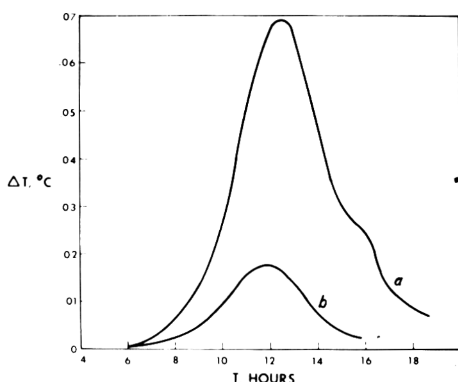

Fig. 5—Curve a: Observed temperature rise of cream of chicken soup in twist-seal jar, after inoculation with PA 3679 at 35°C (replotted data of Fig. 4). Curve b: Calculated temperature rise of cream of chicken soup after inoculation with PA 3679, at 25°C. (See text for method of calculation.)

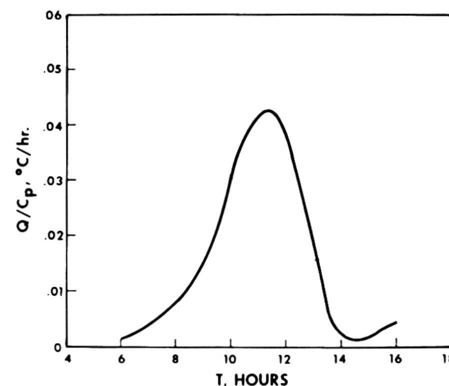

Fig. 6—Calculated heat production of cream of chicken soup in twist-seal jar after inoculation with PA 3679 at 35°C, assuming no heat loss. (See text for method of calculation.)

particular microorganism, if the ambient temperature is 25°C rather than 35°C.

Heat evolution during exponential growth of bacteria is generally proportional to the growth rate (Forrest, 1969). Hence, we assume that the temperature difference between a can in which spoilage occurs and an identical but unspoiled can is an indication of the rate and extent of bacterial growth. The net rate of heat production due to spoilage will be, approximately

$$\frac{dQ}{dt} = \dot{q} - K\Delta T \qquad (1)$$

where Q is the heat from spoilage in the sample can at time t, $\dot{q}$ is the rate of heat production by spoilage at time t, and ΔT is the (positive) difference in temperature between the sample can and an identical but nonspoiling reference can. K is a composite heat loss coefficient following from the assumption that the total rate of heat loss, occurring in several ways, is linearly proportional to the temperature difference ΔT.

K, the so-called Newton cooling coefficient, can be found from the cooling curve of a can that has been warmed slightly with no spoilage organisms present. In this case $\dot{q} = 0$, so that

$$\frac{dQ}{dt} = -K\Delta T \qquad (2)$$

Q may be written as $Cp\Delta T$, where Cp is the heat capacity at constant pressure. Integrating equation (2), we have

$$\Delta T = (\Delta T)_o \exp\left(-\frac{K}{C_p} t\right) \qquad (3)$$

Figure 5a shows the temperature rise of a glass container of cream of chicken soup kept in a 35°C room after inoculation at t = 0 with PA 3679 (Fig. 4). K is found from the long time portion of this curve by using equation (3); it is approximately 0.43 hr^{-1}. Figure 6 shows the heat production by spoilage, obtained by writing equation (1) in the form

$$\frac{\dot{q}}{C_p} = \frac{d\Delta T}{dt} + \frac{K}{C_p} \Delta T \qquad (4)$$

and differentiating the curve of Figure 5a, using a five point quadratic approximation for the first derivative (Savitzky and Golay, 1964).

The total heat produced can be found by integrating equation (4):

$$Q_T = \int_{-\infty}^{\infty} \dot{q} dt =$$

$$C_p \int_{-\infty}^{\infty} \frac{d\Delta T}{dt} dt + K \int_{-\infty}^{\infty} \Delta T\, dt \qquad (5)$$

The first integral on the right side vanishes, and if a C_p of 1 cal/g-°C is assumed, Q_T is found after integration of the curve of Figure 5a to be about 0.36 cal/g. Thus under perfect insulation conditions the temperature rise would be about 0.36°C. The actual rise of about 0.07°C reflects the rapid heat loss from the uninsulated container, but is still easily within range of measurement.

The temperature rise curve to be expected at 25°C ambient can be estimated from the 35°C example by using the assumption that Arrhenius kinetics can be applied to $\dot{q}$, with an activation energy E of about 15 k-cal/mole:

$$\dot{q}\,(25°C) = \dot{q}\,(35°C) \exp \frac{E}{R}\left(\frac{1}{308} - \frac{1}{298}\right)$$

$$= 0.434\, \dot{q}\,(35°C) \qquad (6)$$

Approximate numerical solution of equation (4) using equation (6) to determine $\dot{q}/C_p$ at 25°C as a function of time yields the ΔT curve shown in Figure 5b. The maximum temperature rise in this case is only about 0.02°C, indicating that testing using this method could be conducted at 25°C although optimal bacterial growth conditions are desirable for accurate detection.

These values are quite consistent with previous findings. Rubner (1906a, b) filled thermos bottles with 250 ml of various culture media and measured the temperature increase after inoculation with different bacteria. Increases of about 0.2–1.0°C were detected, with peaks occurring 1–3 days after inoculation. Bayne-Jones and Rhees (1929) calculated the amount of heat liberated per individual bacterial cell to be 0.84×10^{-9} cal/cell. Assuming the maximum density achieved to be 10^8 bacteria/ml, the total heat liberated on reaching maximum density would be

$$0.84 \times 10^{-9} \times 10^8 = 0.084 \text{ cal/ml}$$

Assuming a generation time of 1 hr, half of the above calculated heat should be liberated in the last hour, or 0.042 cal/hr. If the specific heat of the can contents are assumed to be 1.0, this should result in a temperature increase of 0.042°C/hr, assuming no heat loss. The above calculations assume a moderate growth rate and moderate ultimate cell density, such as might be anticipated in the spoilage of corn by *B. macerans* (Fig. 1 and 2). However, the spoilage of richer foods, such as cream of chicken soup (Fig. 4), may show generation times of approximately 18 min and a maximum cell density of perhaps 5×10^8 cells/ml (microscopic cell density estimate, experiment of Fig. 4). In such cases, the total heat evolved would be 0.42 cal/ml, and a temperature rise of 0.37°C could be anticipated in the last hour before maximum cell density was achieved (cf. Fig. 6). The volume of the can contents will influence the cooling rate only.

Partial growth, or arrested growth, might be difficult to detect. If, for example, growth of the spoilage organism should cease because of inadequate N (e.g., starchy foods) before dense bacterial growth was achieved, the heat liberated would be proportionately diminished. However, this type of spoilage may be difficult to detect by conventional means.

We have considered the practical possibilities of temperature sensing devices for the detection of spoilage of processed foods at the time of spoilage. In some commercial operations, it is common practice to take a specified number of cans from each day's run and incubate them at elevated temperatures for several weeks ("accelerated storage") after which the cans are opened and inspected for signs of spoilage. Such an operation might be suitable to the temperature difference detection method.

One possibility might be to install permanent racks in the accelerated storage room, fitted with positional thermistors. The cans would be positioned in contact with the thermistor. All thermistors would be connected to a sequential switch and Wheatstone bridge, arranged to detect any temperature difference between successive pairs of cans at fixed intervals (e.g., 60 min). In this way, each can would be compared with its neighbors on either side. Such a device would be practical only for a limited number of cans. 250 cans per day, stored for 2 wk would require a capacity of approximately 5,000 cans. This is not an uncommon figure for accelerated storage, and might be within the realm of practical possibility.

At the present time it does not seem possible to detect spoilage under ordinary warehouse conditions by this method. Here the cans are stacked in contact with one another, and heat loss would be rapid. Moreover, unheated warehouses would not support rapid growth during cold winter months, and the low rate of heat evolution accompanying slow growth at low temperature is probably undetectable by any means presently known.

Other temperature sensing mechanisms may offer special potentialities, based on either cost or scale. Optical pyrometry, or infra-red scanning, is a rapidly developing technology. Liquid crystals painted on the cans or incorporated in the label ink is another possibility. Any of these systems will still depend on a comparison technique where pairs of containers are measured.

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Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Dept. of Agric. to the exclusion of others that may be suitable.

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QUANTITATIVE ANALYSIS OF BETACYANINS IN RED TABLE BEETS (Beta vulgaris)

INTRODUCTION

COLOR is one of the most important factors determining the consumer's acceptance of canned beet products. Variation in color is great because of differences in beet varieties and in horticultural and processing practices. In addition, there is a need to explore use of natural pigments as food colorants: the red beet is a rich source of red and yellow pigments. To investigate varietal differences in a plant breeding program, or to study the feasibility of the beet pigment as a food colorant, a quantitative method to analyze for the pigments is needed. The purpose of this study was to develop such a method to analyze for betanin as well as total betacyanin content.

Betacyanins (red) and betaxanthins (yellow) are the pigments responsible for the color in beets. They are collectively known as betalains (Mabry and Dreiding, 1968). The major betacyanin in beets, betanin, was crystallized by Wyler and Dreiding (1957) and simultaneously by Schmidt and Schoenleben (1957). The detailed structure of betanidin, the aglycone of betanin, was established by Wyler et al. (1963). Other betacyanins in beets are isobetanin, isobetanidin, prebetanin and isoprebetanin. The structure of isobetanin was found to be the C-15 epimer of betanidin (Wilcox et al., 1965a). Piatelli et al. (1964) and Wilcox et al. (1965b) reported that betanin and isobetanin were the 5-0-β-glucosides of betanidin and isobetanidin, respectively. Prebetanin and isoprebetanin are the sulfate monoesters of betanin and isobetanin, respectively (Wyler et al., 1967).

Concentrations of pigment in beets have been estimated and reported by several authors (Pucher et al., 1938; Manunta, 1963; Piatelli and Imperato, 1969). Methods used by these investigators involved spectrophotometry, and results were obtained either by measuring relative changes in absorbance at the maximum wavelength or by separation and elution of the pigment with subsequent calculation of the concentration using reported extinction coefficients.

The spectrophotometric method developed by Nilsson (1970) directly determines betacyanin and betaxanthin pigments in beets without initial separation. Betanin has an A_{max} at 535–540 nm, but also absorbs at the A_{max} of vulgaxanthin I (476–478 nm), the major betaxanthin in beets. Since the absorbance varies with concentration, calculation of the ratio of A_{538}/A_{476} leads to the determination of the measurable concentration of betanin. Vulgaxanthin I does not absorb at the A_{max} of betanin, and therefore, the absorbance of a mixture at that wavelength results from betanin alone. Results are expressed in terms of betacyanin and betaxanthin concentration. This method yields results which include all minor betacyanins, since no separation is made.

EXPERIMENTAL

Raw material

Fresh beets were obtained from the University of Wisconsin Experimental Station. They were blanched, lyophilized and ground in a Wiley Mill to pass through a 20-mesh screen. The powders were stored under an atmosphere of nitrogen in cans at −23°C.

Preparation of betanin

Pure betanin was prepared from an aqueous extract of beet powder by blending 200g with several 800 ml portions of distilled water. The pulp was extracted until clear. The extract was centrifuged, adjusted to pH 3 with 0.1N HCl, and placed onto Dowex 50W-X2 columns (H^+ form, 5 × 30 cm). Columns were washed with 0.1% HCl and the washings were discarded. Pigments were then eluted with water and concentrated. Seven Polyclar-AT (polyvinylpyrrolidone GAF Corp. New York) columns (5 × 30 cm) were used to further purify and to separate the pigments. The Polyclar-AT was prepared by washing a 1-kg lot several times with 5-liter portions of water. The mixture was allowed to stand for 5 hr and the supernatant decanted. Washing was repeated until the supernatant was clear. Elution with water gave several fractions but only the betanin fraction was collected. This fraction was lyophilized, dissolved in 0.1% HCl and allowed to crystallize overnight at 4°C.

Electrophoresis

The Spinco Model R electrophoresis system was used in this study. Each electrophoretic cell was equipped with eight paper strips, Beckman No. 319328. Electrophoresis was carried out by a method similar to that of Powrie and Fennema (1963) using a 0.15M pyridine-citric acid buffer, a voltage gradient of 5.6 volts/cm and a temperature of 4°C. Aliquots of 0.01 ml of the solution to be analyzed were applied to strips by a sample applicator. After electrophoresis, strips were dried under a nitrogen atmosphere in the presence of calcium chloride. Strips were then scanned with a densitometer, (Beckman Model RB Analytrol) equipped with a B-2 cam at 550 nm and a slit width of 4 mm.

Preparation of beet extracts

12g of lyophilized beet powder were extracted with 150 ml of distilled water in a Waring Blendor for 1 min. The mixture was centrifuged and filtered through Whatman No. 1 filter paper using reduced pressure. The extraction was repeated several times until the extract was colorless. All extractions were carried out under a nitrogen atmosphere to prevent degradation of the pigments. Extracts were combined and made to a known volume.

Analysis

Concentration of betacyanins were calculated in terms of betanin and total betacyanins (betanin + minor betacyanins). Results of all determinations were expressed in terms of betanin concentration. A standard curve was prepared from known betanin concentrations. After electrophoresis the peak areas (cm^2) as determined by densitometry were plotted against betanin concentration. Each point resulted from the average of 16 determinations. Standard deviations were determined. Betanin

Table 1—Electrophoretic migration and band width of beet pigments

Band	Migration (cm)[a]	Band width (cm)[a]
P_1 (light pink)	1.5	0.6
Y_1 (yellow orange)	3.2	0.8
R (red)	5.7	1.5
P (light pink)	7.3	0.4
P_2 (pink)	7.9	0.6
Y_2 (yellow)	10.4	0.8
Y_3 (light yellow)	11.7	0.9
Y_4 (yellow green)	13.0	0.9

[a]Average of seven determinations

and total betacyanin concentrations of each beet sample were expressed as mg per 100g fresh weight. For each sample, eight determinations were made. Concentrations were determined by comparing peak areas obtained against the standard curve, and the amount of pigment was calculated using appropriate dilution factors and moisture content. The moisture content of each sample was determined from a 10-g sample dried to constant weight under vacuum at 70°C.

Experiments to determine percent of recovery were conducted by adding known amounts of pure betanin to beet extracts. The amount of pigments in the extract as well as the mixture were determined under similar conditions.

RESULTS & DISCUSSION

THE BETALAINS are more soluble in water than in alcohol (Nilsson, 1970), and therefore all extractions were made with water. No attempt was made to remove other constituents, i.e., sucrose or proteins, since they do not interfere with the electrophoretic separation of the pigments. Sucrose does not migrate under the experimental conditions used (Powrie and Fennema, 1963). Proteins, though they migrate, will not interfere in the determination since they do not absorb at 550 nm, and degradation products formed before extraction remain at the origin during electrophoresis. This made it possible to determine the pigments in absence of interfering substances which might contribute to the absorbance at the λ_{max} and thus corrections for interfering substances were not needed.

Optimal electrophoretic separation of beet extract occurred at pH 4.5 using 0.15M pyridine-citric acid buffer at 4°C (Powrie and Fennema, 1963). Bands and their widths as observed in the electrophoretic migration are shown in Table 1. When paper strips were compared, it was found that the thicker Beckman No. 319328 gave better separation than did the S&S No. 2043A used by Powrie and Fennema (1963). The electrophoretograms exhibited satisfactory resolution of the pigments with no overlapping of bands. Band R was shown to be betanin by co-electrophoresis with crystallized betanin. Band P_1, P, and P_2 were considered minor betacyanins. The identity of crystalline betanin was established by comparing IR, visible and UV spectra with those reported in the literature (Wyler et al., 1967; Piatelli and Minale, 1964).

The color intensity of the separated bands was determined with a densitometer and expressed as peak area in cm^2. A 550 nm filter was chosen since the absorption maximum of betanin is known to be at 538 nm (Nilsson, 1970). The area (cm^2) was measured with the aid of an integrator after correcting for the average background noise and baseline. Background noise was determined from the analytrol traces of strips without sample but was treated similarly as the strips with sample and was found to be 0.4 cm^2.

The standard curve as well as the standard deviations of each betanin concentration is shown in Figure 1. Each point represents the average of 16 determinations. Values within the range of 2–9 μg had a standard deviation of 0.4 μg.

To calculate the betanin or total betacyanin concentration, the peak area obtained was related to betanin content by the use of the standard curve. The pigment concentration in the raw beet was calculated by using appropriate dilution factors and corrections for moisture contents.

When pure pigment was added to a sample, an average recovery of 98% was obtained (Table 2).

Results obtained when the method was used to analyze for differences in breeding lines are presented in Table 3. These data support the previous observations (Lusas et al., 1960; Manunta, 1963) that the variety will affect the color of both fresh and canned beets. Betacyanin contents for the breeding lines ranged between 35–135 mg per 100g fresh weight.

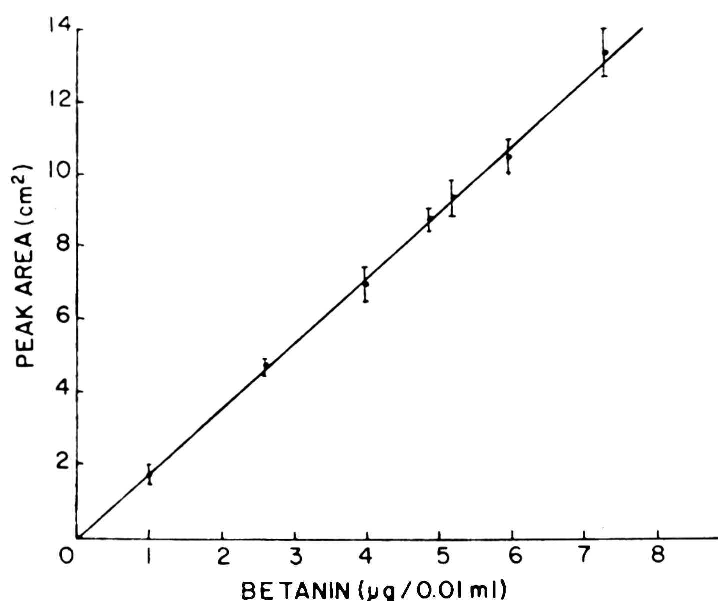

Fig. 1—Betanin standard curve.

Table 2—Recovery data of betanin in raw beet extract[a]

	Peak area (cm^2)	Concentration μg/0.01 ml	Recovery %
Pure betanin	4.6	2.6	–
Beet extract	4.3	2.4	–
Betanin + extract	8.9	4.9	98

[a]Average of three determinations, seven paper strips each

Table 3—Concentration of betanin and total betacyanins in four breeding lines of beets (Beta vulgaris)

Breeding line	Total solids	Concentration[a] mg/100g fresh weight	
		Betanin	Total betacyanin
1382	16.5	27 ± 3	36 ± 3
1372	16.4	105 ± 4	135 ± 4
1356	17.9	68 ± 3	88 ± 3
Parma globe	15.4	85 ± 3	108 ± 4

[a]Average of eight determinations

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INFLUENCE OF SELECTED 5'-NUCLEOTIDES ON FLAVOR THRESHOLD OF OCTANAL

INTRODUCTION

OVER 200 VOLATILE components have been shown to be present in orange juice from several varieties, and more than 100 of these have been identified. Aldehydes, long known for their flavor and aroma qualities, have been found in relatively high concentrations in orange juice. Historically, octanal has been described as possessing orange-like flavor and aroma. Naves (1947) described octanal as the principal aldehyde of orange essence. Octanal reportedly is present in orange juice at 60 ppb, greater than other aldehydes (Kirchner and Miller, 1957). In addition, octanal has a relatively low flavor threshold of approximately 1 ppb (Schinneller, 1972).

5'-Nucleotides have been implicated as flavor modifiers (Kuninaka, 1960). Recently it was determined that oranges contain relatively large quantities of some 5'-nucleotides (Barmore, 1972). The adenosine and guanosine nucleotides were present in greater quantities than the uridine, cytidine and other nucleotides. Barmore (1972) also reported that in fresh juice the di- and triphosphorylated nucleotides dominated; however, upon storage of the juice, the mono- and diphosphates increased markedly.

This study was designed to detect the influence, if any, of adenosine 5'-monophosphate (AMP), adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), guanosine 5'-monophosphate (GMP), guanosine 5'-diphosphate (GDP) and guanosine 5'-triphosphate (GTP) on the apparent flavor threshold of octanal.

EXPERIMENTAL

FOR THE PURPOSE of this study, flavor was defined as the overall sensory response to a material taken orally including odor, taste and other mouth sensations. Threshold was defined as that concentration at which 50% of the panelists indicated a difference between a sample containing a given concentration of octanal and a reference blank.

Sample preparation

Nucleotides used throughout this experiment were purchased from P.L. Biochemicals, Inc., Milwaukee, Wisc., and were stored under refrigeration and desiccation due to their instabilities. Octanal was purchased from Aldrich Chemical Co., Milwaukee, Wisc., and was shown to be greater than 99% pure by gas-liquid chromatography. Water used in preparing the various concentrations was doubly glass distilled and then boiled for at least 1 hr prior to use.

2-oz, wide-mouth amber-glass bottles were used in presenting samples to panelists. The bottles were individually capped with a 2-in. square piece of Parafilm (American Can Co., Neenah, Wisc.). Various plastic screw caps were tested, but all imparted a readily detectable flavor and/or odor to distilled water. This film was flavorless, odorless and relatively impermeable to water and water vapor.

All nucleotides were presented in aqueous solutions at concentrations of 10 ppm. Each solution was prepared fresh daily by adding 50 mg of 5'-nucleotide to 5 liters of distilled water. The di- and triphosphate nucleotides were relatively unstable in the unbuffered solutions; therefore, initial dilutions were made no more than 2 hr before presentation to the panelists.

Testing procedure

Ten screened and trained panelists, six females and four males, were selected for determining threshold differences. Kramer et al. (1961) recommended the use of screened and trained panelists when determining differences. Their ages ranged from 19–47 yr and averaged 27.6 yr. Panelists were trained and screened for selected qualities during previous panels. Selection was based primarily upon the following: (1) consistency of results between replications of previous panels; (2) dependability in attendance; and (3) ability to detect octanal.

A multiple paired comparison test was used in presenting samples to the panelists. Five pairs were presented per session, enabling a sufficient number of concentrations to be presented while keeping fatigue and carry over effects at a minimum. Several screening panels were conducted to select the five octanal concentrations to be presented, based upon the following requirements: (1) the log of the concentration had to be equally spaced for purposes of statistical analysis; (2) the concentrations had to be centered about the threshold of octanal; (3) the differences between concentrations had to be large enough to allow for the large variation between panelists; and (4) the differences between the weakest and strongest concentrations had to be small enough so that a linear response existed. The concentrations 0.1, 0.3, 1.0, 3.0 and 10.0 ppb were chosen. Samples (25 ml each) were presented to the panelists in ascending order to further reduce carry over effects. Each sample concentration was at least twice the concentration of the preceding samples as recommended by Gregson (1962).

Numbered samples arranged in five pairs were presented to the panelists. Panelists were instructed to taste each pair in numerical order and indicate if the samples within each pair were alike or different. Swallowing was allowed at the discretion of each panelist, and oral rinsing was strongly encouraged after each sample. If a pair was judged to be different, that member of the pair possessing the stronger flavor was so indicated. Panelists were not informed of the panel design until the conclusion of the experiment. They were requested not to discuss their results with fellow panelists, as an attempt to control bias.

Panel sessions were held once each day between 11:45 A.M. and 12:00 Noon, five days per week for seven consecutive weeks. Each day of the first week a replication consisting of five concentrations of octanal plus five H_2O references was presented to each panelist. During the following weeks the flavor thresholds of octanal in the six 5'-nucleotide solutions were determined. All panelists participated in each test session without fail. Refreshments were served to encourage attendance.

Statistical analysis

A sample pair received a score of one if a panelist (1) indicated that the two samples within the pair had different flavors, and (2) correctly indicated the sample containing nucleotide plus octanal as having the stronger fla-

Table 1—Flavor thresholds of octanal determined in aqueous solutions of selected 5'-nucleotides at 10 ppm

	Concentrations of octanal in ppb (wt/vol)		
5'-Nucleotide	Lower confidence limit[a]	Threshold	Upper confidence limit[a]
Control (none)	1.20	1.38	1.59
ATP	0.91	1.22	1.65
GTP	1.00	1.21	1.47
AMP	0.87	1.20	1.64
GDP	0.51	0.99	1.68
GMP	0.57	0.86[b]	1.29
ADP	0.76	0.85[b]	0.95

[a]95% confidence limits
[b]Significantly different from control at 95% level as determined by t-test

vor. If both of these conditions were not met, the sample pair received a score of zero. The scores for each of the five concentrations of octanal were summed for all ten panelists and five replications giving a total of 50 responses per concentration per nucleotide from which the percent of detection was determined. The regression of Y (percent of detection) on X (log of concentration) was used to determine thresholds. The "t-test" (Snedecor and Cochran, 1967) was used to determine significant differences between the various thresholds.

A 5 × 7 factorial analysis of variance with five blocks (replications) was used to analyze for differences in total detection of octanal in each of the six nucleotides plus the control, differences in detection of the five concentrations, and nucleotide × concentration interaction. The responses were summed for each of the panelists per concentration per replication per nucleotide. Arcsine transformation was made to assure validity of the analysis of variance for the data (Snedecor and Cochran, 1967).

RESULTS & DISCUSSION

Thresholds of octanal

Flavor thresholds of octanal were determined in the six 5′-nucleotide solutions and in distilled water (Table 1). The flavor thresholds of octanal in GMP and AMP were significantly lower than that of the control as determined by t-tests (Snedecor and Cochran, 1967). The flavor enhancing potential of GMP has been well-publicized (Wagner et al., 1963; Shimazono, 1964; Kuninaka et al., 1964; Luh and Chen, 1969). Thus, it was not unexpected that octanal in 10 ppm solutions of GMP had a significantly lower threshold than the control. However, octanal in a 10 ppm solution of ADP also had a significantly lower threshold than the control. This threshold, 0.85 ppb, was similar to that established for GMP, which was 0.86 ppb. ADP has been reported by Kuninaka (1960), Shimazono (1964, 1965), Kuninaka et al. (1964), and Luh and Chen (1969) to possess little, if any, flavor enhancing or modifying potential when added to various foods.

Flavor enhancement

Variation in the detection of octanal among replications, 5′-nucleotide solutions, concentrations and nucleotide × concentration interaction was analyzed by analysis of variance (Table 2).

The variation between nucleotide solutions was significant. Table 3 lists the number of times octanal was detected in each of the six 5′-nucleotide solutions and the control. Duncan's Multiple Range Test revealed that the variation between the number of times octanal was detected in distilled water was significantly lower than the number of times it was detected in the 5′-nucleotide solutions containing GDP, GMP, or ADP. Dunnett's Test, designed to compare a control with all the treatments, also showed a significant variation between the control and ADP, GDP and GMP. Thus, identical concentrations of octanal were designated as having a stronger flavor when sampled in solutions containing GMP, GDP, or ADP, than when sampled in distilled water.

Variations among concentrations was significant (Table 2). The total number of detections of octanal for the five increasing concentrations were, respectively, 28, 78, 184, 254 and 300 for the 350 observations of each concentration. The Duncan Multiple Range Test indicated the number of detections per concentration was significantly higher than the preceding concentration. The majority of the variation between concentrations was due to a linear relationship between percent of detection and concentration as opposed to quadratic, cubic, etc. The sum of squares due to the different concentration presented was 22.97. Of this, 22.47 (over 99%) was due to a linear relationship between concentrations and percent detected. The results shown in Figure 1 are an example of the linear relationship observed throughout this experiment. Such linear response indicated that a sufficiently narrow range of concentrations were presented and that little, if any, carry over effects existed. Although the variance due to cubic effect was significant, it accounted for less than 1% of the concentration's sum of squares. The cubic effect is a measure of the sigmoid curve which is observed when a wide range of concentrations are presented.

No significant variation (difference) was found among replications. Thus, no increase in the ability to detect octanal between days of the week or replications was observed with a trained panel since replications and days of the week were synonymous. The number of detections of octanal from Monday through Friday were 181, 162, 162, 167 and 172, respectively, of a possible 350 detections.

The flavor enhancing ability of the 5′-nucleotides was influenced by the number of phosphate groups attached; i.e., mono-, di- or triphosphates. All of

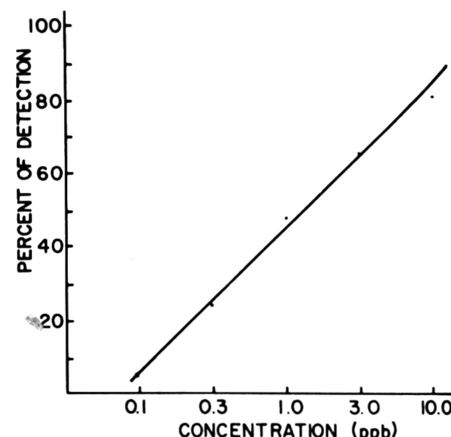

Fig. 1–Semilogarithmic relationship between percent of detection and concentration of octanal for the actual concentrations utilized (j = 50).

Table 2–Analysis of variation in the detection of octanal between replications, nucleotide solutions and octanal concentrations

Source of variation	Degrees freedom	Sum of squares	Mean square	F-test
Total	174	26.4710		
Replications (Blocks)	4	0.1350	0.0338	1.73
Treatments	34	23.6818	0.6965	35.69**
Nucleotides	6	0.3463	0.0577	2.96*
Concentrations	4	22.9657	5.7414	294.19**
Linear	1	22.7357	22.7357	1164.99**
Quadratic	1	0.0265	0.0265	1.36
Cubic	1	0.1630	0.1630	8.35**
Quartic	1	0.0399	0.0399	2.04
Nucleotide × Conc.	24	0.3699	0.0154	0.79
Error	136	2.6541	0.0195	

*$p < 0.05$
*$p < 0.01$

Table 3–Variation in the detection of octanal between nucleotide solutions and control (250 possible detections)

5′-Nucleotide in solution at 10 ppm	Number of detection	Arcsin transformation values[a]	Dunnett's test[b]
Control	111	12.2 x	
ATP	115	12.9 x,y	NS
GTP	115	13.0 x,y	NS
AMP	116	13.1 x,y	NS
GDP	125	14.6 y,z	S
GMP	131	15.0 y,z	S
ADP	131	14.4 z	S

[a]Based on analysis of variance in Table 2. Values followed by the same letter are not significantly different at 95% level.
[b]NS–not significantly different from control; S–significantly different from control at 95% level

the thresholds determined in the nucleotide solutions appeared lower than that of the control. The thresholds of octanal in the triphosphate solutions, GTP and ATP, appeared to be only slightly lower than that of the control. Thus, the triphosphates did little or nothing to enhance the flavor of octanal. The diphosphates, on the other hand, had two of the three lowest thresholds. The threshold of octanal in GDP was approximately 30% lower than that of the control, while the threshold of octanal in ADP was approximately 40% lower.

According to Beidler's theory (Beidler, 1966), nucleotides may enhance flavors by unmasking certain flavor receptor sites, allowing them to contribute to stimulus adsorption and taste receptor stimulation. Perhaps the triphosphate molecules are too large and in the process of unmasking the receptor sites, partially conceal them. The diphosphates possess three partially negative charges in their ionized state as opposed to two for the monophosphates. This additional charge may enable the diphosphates to unmask the receptor sites to a greater degree than do the monophosphates.

Kuninaka (1966) and Kuninaka et al. (1964), along with several other authors, have stated the following to be requirements for flavor enhancement by nucleotides: (1) the base moiety should be purine; (2) a hydroxy group on the number 6 carbon; (3) the group on the number 2 carbon is optional and has no effect on flavor; and (4) the 5'-position of ribose must be esterified with phosphoric acid. Based upon results of this experiment, an amino group may be present on the number 6 carbon and ribose esterified with a phyrophosphate group, as in the case of ADP.

Woskow (1969) raised the question as to whether nucleotides favorably alter the preference for foods by enhancing the flavor of the food or by the supression of undesirable "off-flavors." Results of this experiment indicate that the nucleotides ADP, GDP and GMP enhanced the flavor of octanal since theoretically there were no other flavors present to suppress.

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CHARACTERIZATION OF LIPIDS FROM SEEDS OF THE ROSACEA FAMILY

INTRODUCTION

APRICOT AND PEACH kernels are by-products in the canning and fruit drying industry. The oil from almond kernel is used in various cosmetic products and pharmaceuticals; also oils extracted from kernels of apricot and peach are sometimes used for the same purposes (Cruess, 1958; Ben Gera and Kramer, 1969; Vaughan, 1970).

Data are available on the fatty acid composition of the different Prunus species (Hilditch and Williams, 1964; Gupta and Garg, 1968; Lotti and Anelli, 1969), but no data are available on the fatty acid composition of the Prunus species cultivated in Israel. Sterol and tocopherol composition was reported only for almond oil (Wolff, 1968; Slover, 1971); no data are available on sterol and tocopherol composition of the apricot and peach oils.

Recently the canning of apricots and peaches in this country increased very appreciably (Har'el and Rapaport, 1969). It was of interest therefore to find possible uses for oils from kernels of apricots and peaches (also as substitutes for almond oil). An investigation of compositions of all three oils was thus undertaken. It is also worth mentioning that the prices of apricot and peach seeds (kernels) are much lower than those of almond seeds.

EXPERIMENTAL

Materials and reagents

The kernels investigated were: apricot—*Prunus armeniaca* (variety Raanana); peach—*Amygdalus perisca* (variety L-198-12); and almond—*Prunus amygdalus* (variety Ne-plus-ultra).

All solvents and chemicals were of analytical grade. Standards of fatty acid methyl esters and of sterols were purchased from Applied Science Laboratories; α-tocopherol and squalene were purchased from Fluka AG. Standards of γ- and δ-tocopherols were obtained from soybean oil by TLC.

Extraction of the lipids

Pits were broken in a hammer mill and the kernels separated manually from the shells. Lipids were extracted by a method similar to that suggested by Folch et al. (1957). A mixture of 100g kernels was blended in a Waring Blendor with 500 ml chloroform:methanol (2:1) for 2 min. The homogenate was filtered through a No. 1 Whatman filter paper in a Büchner funnel with suction. The residue was returned to the Blendor and the oil reextracted twice, as described above. The combined filtrates of the extracts were placed in a separator funnel and water was slowly added with shaking, till two phases were obtained. The chloroform phase was separated and dried over anhydrous sodium sulfate. The lipids were obtained after evaporation of the solvent at 50°C in a vacuum rotary evaporator. The weight of the oil was determined in order to calculate its content in the kernel.

Fatty acid methyl esters were prepared by methylation of the lipids according to the Ce-2-66 method of AOCS (1969). The unsaponifiable matter was obtained according to Ames (1971), and redissolved in chloroform.

Thin layer chromatography

The sterol and tocopherol fractions were separated from the rest of the unsaponifiables by TLC. The separation was carried out on 20 × 20 cm plates, covered with 0.5 mm layer of silica gel G (Merck Catalog, No. 7731), containing 2′–7′dichlorofluorescein (Stahl, 1969). The unsaponifiable matter in chloroform, 150 μl, corresponding to 1g oil, was applied to the plate as a stripe 14 cm long. At both the edges of the stripe a control solution in chloroform of a mixture of β-sitosterol and of α-, γ- and δ-tocopherols was spotted. The developing solvent was petrol ether (60–80°C):ethyl ether: acetic acid (50:50:1) (Thorpe et al., 1969); the solvent front was allowed to advance 17 cm. After developing, the plates were dried and viewed under light of 366 nm. The sterols appeared as a single white stripe (Rf = 0.30–0.37), and the tocopherols as dark-purple overlapping stripes (Rf = 0.56–0.72). The stripes corresponding to sterols and to the tocopherols were scraped off with a sharp spatula. The scrapings were transferred to a filter paper, on an ordinary funnel, and the sterols were then eluted with three successive 10 ml portions of chloroform. The tocopherols were also eluted from the respective stripes in the same way, but absolute ethanol was used instead of chloroform. The solvents were removed at 50°C from the recovered fractions with a stream of nitrogen. Each fraction was redissolved in chloroform and subsequently examined by GLC.

Absolute amounts of tocopherols in the respective oils were determined according to the modified Emmerie-Engel procedure of the Analytical Methods Committee (1959).

To confirm the results of GLC determination, the tocopherols were separated (as individual spots) from the unsaponifiables by TLC, using chloroform as developing solvent, and then determined by the above mentioned Emmerie-Engel procedure.

Gas liquid chromatography

The apparatus was a Packard Gas Chromatograph, Model 7821, equipped with a hydrogen flame-ionization detector. Gas chromatographic analyses were conducted on a 6-ft by ¼ in. (OD) glass column. Carrier gas was Argon, at a flow rate of 30 ml/min.

10% EGGS-X on 100–120 mesh Gas Chrom P (Applied Science Labs) was used for determination of fatty acid methyl esters. Column temperature was 160°C; the temperatures of the inlet, outlet and of the detector were 200°C.

Compositions of the sterols and tocopherols were determined with 3% SE-30 on 80–100 mesh Gas Chrom Q (Applied Science Labs). Column temperature was 230°C; the temperatures of the inlet, outlet and of the detector were 280°C. The same column was also used for the determination of squalene in the unsaponifiables; calculations were performed with the aid of a calibration curve of peak heights vs. amounts of injected squalene.

Identification of the peaks of fatty acid methyl esters, squalene, sterols and tocopherols was carried out by comparing their retention times with those of standards. The relative amounts of the examined components were determined from their peak areas; the latter were measured by the triangulation method.

RESULTS & DISCUSSION

LIPID CONTENTS in kernels of apricot, peach and almond were 30, 23 and 35%, respectively.

Fatty acid compositions of the investigated oils are given in Table 1. The fatty

Table 1—Fatty acid compositions of lipid extracts from kernels of apricot, peach and almond

	Fatty acid[a] (%)				
Oil	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic
Apricot	6.2	0.6	0.8	72.9	19.5
Peach	4.6	0.2	0.9	80.4	13.9
Almond	5.8	0.8	0.6	62.7	30.1

[a]Retention times (min) of plamitic, palmitoleic, stearic, oleic and linoleic acids were 4.9, 5.8, 9.7, 11.2 and 14.2, respectively.

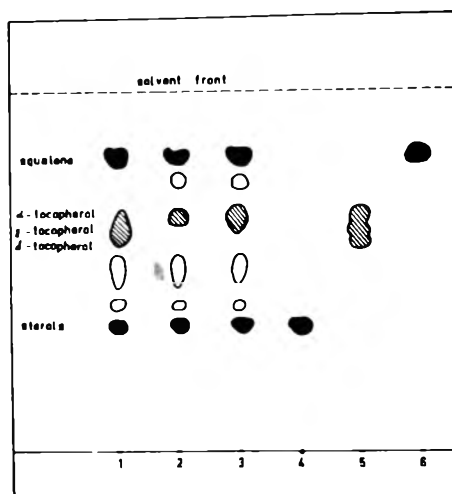

Fig. 1—Thin layer chromatogram of the unsaponifiables from apricot, peach and almond oils. Developing solvent, petrol ether (60–80°C):ethyl ether:acetic acid (50:50:1); detecting reagent, 50% sulfuric acid spray, followed by heating 5 min at 110°C. 1, 2 and 3, unsaponifiable matter from apricot, peach and almond oils, respectively; 4, β-sitosterol; 5, α-, γ- and δ-tocopherols; and 6, squalene.

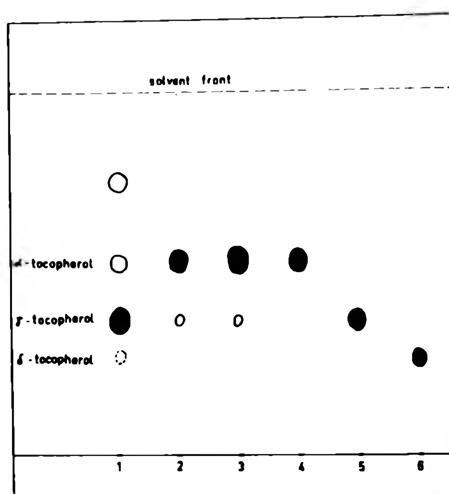

Fig. 2—Thin layer chromatogram of the tocopherols from apricot, peach and almond oils. Developing solvent, chloroform; detecting reagent, Emmerie-Engel spray, 1, 2 and 3, unsaponifiables from apricot, peach and almond oils, respectively; 4, 5 and 6, standards of α-, γ- and δ-tocopherols, respectively.

acid compositions of the three oils were similar. The examined oils are rich in unsaturated acids, in particular in oleic (62–80%) and in linoleic (14–30%) acids. The obtained figures are in good agreement with those reported by Hilditch and Williams (1964) and by others (Gupta and Garg, 1968; Lotti and Anelli, 1969).

In order to avoid oxidation of the tocopherols, the time of saponification according to Ames (1971) is only 10 min instead of the 30 min required by the AOAC method (1970). It was shown (in a preliminary experiment, using sterol acetate as standard and TLC separation techniques), that the saponification according to Ames (1971) was sufficient to hydrolyze all the sterol esters. Therefore, the saponification procedure (Ames, 1971) which has been used originally as a preliminary step in analysis of tocopherols, was used for the recovery of both sterols and tocopherols. The solvent used in the separation of sterols and tocopherols by TLC was that recommended by Thorpe et al. (1969), with the addition of 1% acetic acid (to minimize the spreading of the separated spots). Typical separation according to the above method is shown in Figure 1.

Table 2—Sterol and tocopherol compositions of lipid extracts from kernels of apricot, peach and almond

Composition of	Oil		
	Apricot	Peach	Almond
Sterols[a] (%)			
Campesterol	4.7	2.4	2.0
Stigmasterol	–	0.6	0.6
β-sitosterol	95.3	97.0	97.4
Tocopherols[a,b]			
α-tocopherol	6	89	96
γ-tocopherol	91	11	4
δ-tocopherol	3	–	–

[a]Retention times (min) of δ-tocopherol, γ-tocopherol, α-tocopherol, campesterol, stigmasterol and β-sitosterol were 9.1, 12.0, 14.8, 17.8, 19.5 and 22.2, respectively.
[b]Apricot, peach and almond oils contained 630, 150 and 440 ppm tocopherols, respectively.

Gas chromatographic separations of the whole unsaponifiable matter showed that squalene (retention time: 6.9 min) was present in all three oils in about the same amount (0.02%).

The compositions of sterols and tocopherols in the unsaponifiables from the three lipid extracts (as obtained by gas-chromatography) are summarized in Table 2.

There is no significant difference in the composition of the sterols in the three examined oils. β-sitosterol was found to be the dominant compound in each oil, accounting for over 95% of the total content of the sterols. Campesterol was present at 2.0–4.7%, but traces of stigmasterol were found only in peach and almond oils. The results for almond oil are in agreement with the data of Wolff (1968).

The results for tocopherols were obtained by GLC analyses as described in Experimental (see Table 2); they were also confirmed by TLC using chloroform as the developing solvent (see Fig. 2) and the Emmerie-Engel spray as the detecting reagent (Stahl, 1969). α-tocopherol was the main component in peach and almond oils (about 90%), while γ-tocopherol was the principal component in the oil from apricot kernels (also about 90%). α- and γ-tocopherols were present in all three oils, while δ-tocopherol was detected only in the oil from apricots. Slover (1971) also reported that α-tocopherol was the principal component in almond oil.

It may be concluded that apricot, peach and almond oils closely resemble each other in compositions of fatty acids and of sterols; differences exist however in the composition of tocopherols. Apricot oil was found to have the highest concentration of tocopherols (630 μg/g; see Table 2); its tocopherol fraction consisted largely of γ-tocopherol (over 90%) which is considered a better antioxidant than α-tocopherol (Parkhurst et al., 1968), the main tocopherol in peach and almond oils. Almond oil contained 440 μg/g, and peach oil (poorest in tocopherols content) 150 μg/g (0.015%) of tocopherols.

Tocopherols are regarded as natural antioxidants, and all three oils contain sufficient amounts of these to be protected against autoxidation (Lundberg, 1962). As all three oils in question are also similar in composition of their fatty acids, it may be expected that they should also be similarly resistant to oxidation. Also the physical characteristics of the three oils are similar (Pearson, 1970), and therefore in many cases substitution of the relatively expensive almond oil with apricot and peach oils seems possible.

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ABSORPTION OF AQUEOUS BISULFITE BY APRICOTS

INTRODUCTION

GASEOUS SULFURING of apricots before sun drying has been an established practice in California since the late nineteenth century. Sulfur dioxide serves several purposes: it reduces or prevents microbial spoilage, preserves ascorbic acid and carotene, and inhibits browning during drying and storage.

In present commercial sulfuring practice, apricot halves are spread cups up on wooden slatted trays and placed in a sulfur house where they are exposed to gaseous sulfur dioxide, produced by burning sulfur. After sulfuring, the trays of apricots are placed in the sun to dry until the moisture level reaches approximately 18%. The sulfur dioxide level in dried apricots processed by the traditional method can vary from 1000 ppm to over 6000 ppm (McBean et al., 1964). This wide variation is caused by factors which affect the absorption and retention of sulfur dioxide, such as sulfur house design, sulfur dioxide exposure concentration and time, and desorption losses during drying which are greatly affected by weather conditions.

The sulfur dioxide level in dried apricots influences their marketability, since darkening will occur rapidly in the distribution channels if the sulfur dioxide level is too low. Correcting the final sulfur dioxide level is time consuming and costly.

Potential advantages in using a bisulfite solution to sulfur apricots would be: (1) decreased air pollution; (2) better control of the sulfuring process; (3) greatly shortened sulfuring time; and (4) decreased desorption losses during drying.

MATERIALS & METHODS

FRESH TILTON and Blenheim apricots were obtained from a local produce market and held in cold storage until used. The fruit was removed from cold storage a day before its use. Apricots were sulfured by dipping approximately 2 lb of pitted fruit halves into a gal of bisulfite solution of known concentration and pH for 0–45 min. The bisulfite solutions contained 1, 3, 4, 5 and 6% titratable SO_2 and were made by adding sodium bisulfite or sulfur dioxide gas to water until the desired concentration was obtained. Sodium hydroxide or hydrochloric acid was added as required to obtain dip solutions of pH 2.5, 3.0, 3.5 and 4.5. Effects of dipping time, concentration and pH on the absorption and penetration rate of the bisulfite into the apricots was followed by measuring the amount of polyphenol oxidase inactivation (Ponting, 1944) and sulfur dioxide content of the apricots (Ponting and Johnson,

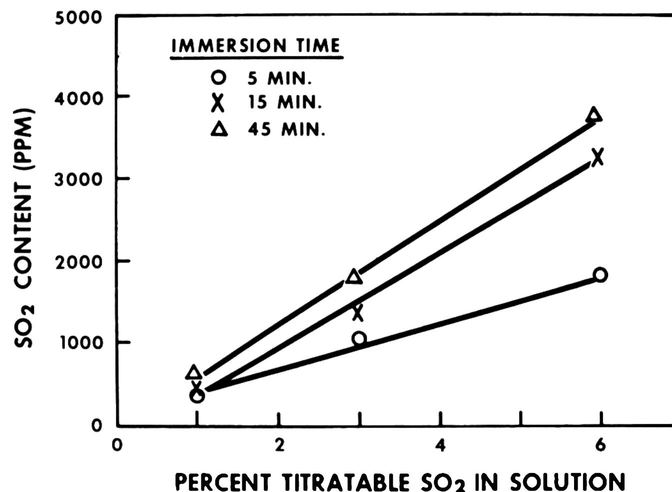

Fig. 1—The effect of concentration of the dip solution and time of immersion in bisulfite solutions at pH 3.5 on the SO_2 content of bisulfite treated fruit.

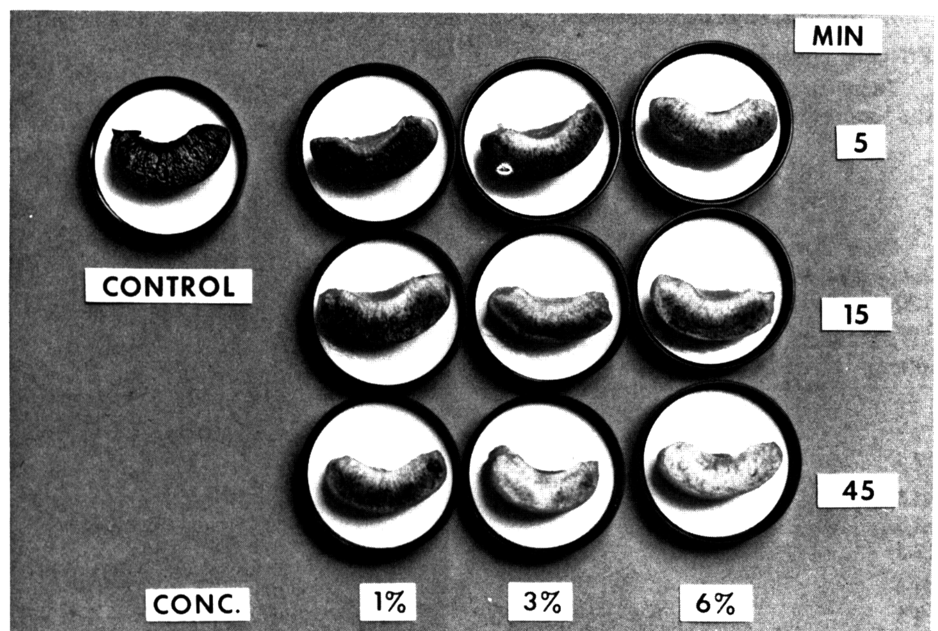

Fig. 2—Penetration of bisulfite into apricots halves after dipping in sodium bisulfite solutions of 1, 3 and 6% (SO_2) at pH 3.5

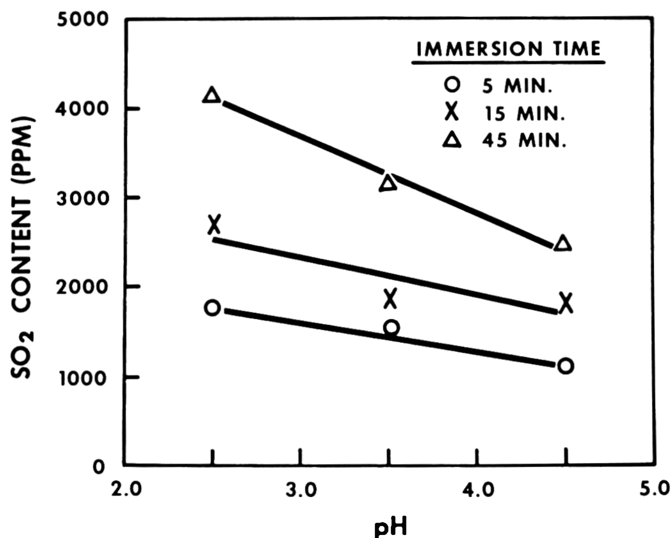

Fig. 3–The effect of pH of the dip solution and time of immersion in 4% (SO_2) bisulfite solutions on the SO_2 content of bisulfite treated fruit.

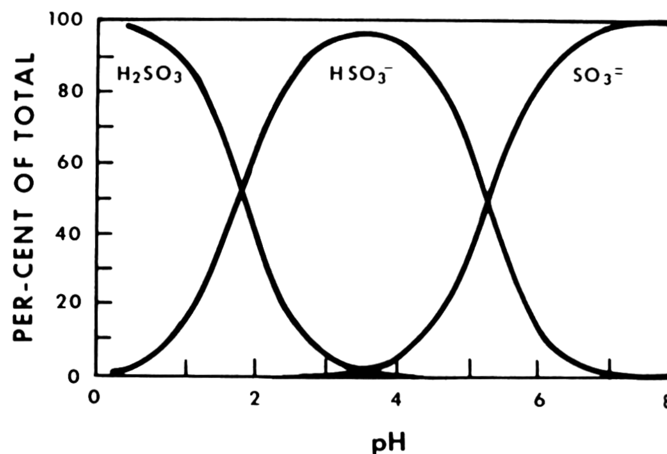

Fig. 4–Effect of pH on the distribution of (H_2SO_3), (HSO_3), and ($SO_3^{=}$) in a bisulfite solution (Vas and Ingram, 1949).

1945). The enzyme test consisted of cutting the apricot halves and spreading a 1% solution of catechol on the rinsed cut surface of the apricot as quickly as possible after the halves are removed from the dip solution. Catechol-treated fruit surfaces will darken within 5 min in the areas of the fruit not penetrated by the bisulfite. For the sulfur dioxide analyses 20- and 100-g samples were used with dried and fresh fruit, respectively. Sulfur dioxide was titrated with 0.02N or 0.01N iodine solution depending on the amount in the fruit. Due to unsuitable sun drying weather at our laboratory, a tunnel-type dehydrator was used to dry the treated apricots at 160°F and 980 ft per second air velocity.

RESULTS & DISCUSSION

Effect of concentration

There is a linear relationship between the concentration of the bisulfite dip solution and the sulfur dioxide content of the treated fresh apricots (Fig. 1), for the dip times shown. These curves were drawn from a linear regression analysis.

The undarkened areas on each apricot piece (Fig. 2) illustrate the extent of the bisulfite penetration into the apricot tissue at the three dip concentrations and immersion times studied. Penetration of the bisulfite into the fruit continues during drying.

Effect of pH

There is an increasing absorption rate of bisulfite into apricots that are dipped into progressively lower pH solutions (Fig. 3). The effect is due to an increase in the concentration of sulfurous acid (H_2SO_3) in the bisulfite solution (Fig. 4). At pH 2.5 approximately 20% of the bisulfite is present as sulfurous acid, which is in equilibrium with sulfur dioxide and water. While at pH 4.5 there is

Table 1–Ratio's dry apricot/fresh apricot sulfur dioxide content of apricots dipped in 4% (SO_2) sodium bisulfite solutions at pH 2.5, 3.5 and 4.5

pH[a]	Time (min)	Ratio[a]
2.5	5	2.49
2.5	10	2.34
2.5	15	2.57
3.5	5	3.33
3.5	10	2.92
3.5	15	3.13
4.5	5	3.16
4.5	10	3.30
4.5	15	3.56

[a]Correlation between pH and ratio: $r = 0.892$, $r_{.01} = 0.798$.

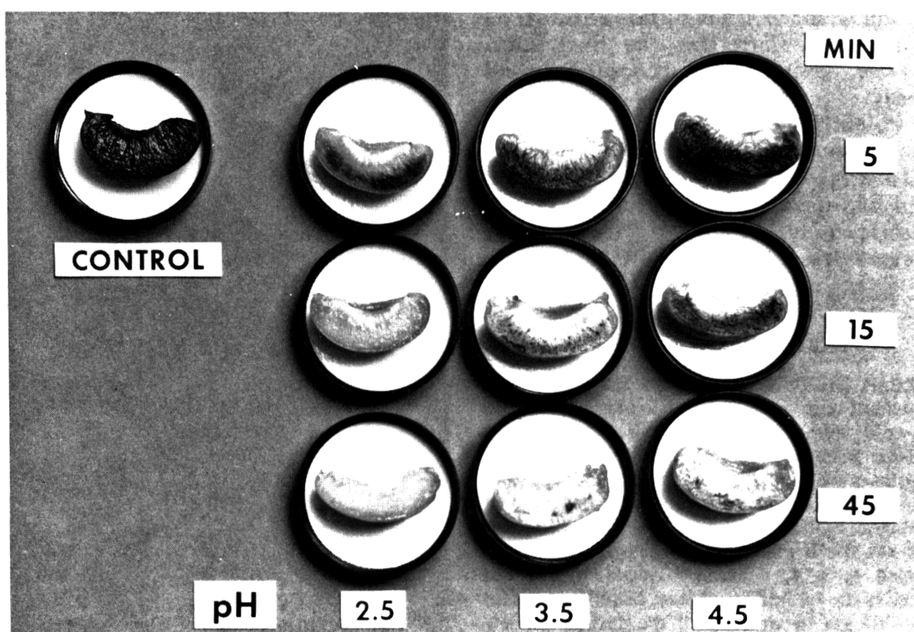

Fig. 5–Penetration of bisulfite into apricots halves after dipping in 4% (SO_2) sodium bisulfite solutions at pH 2.5, 3.5 and 4.5.

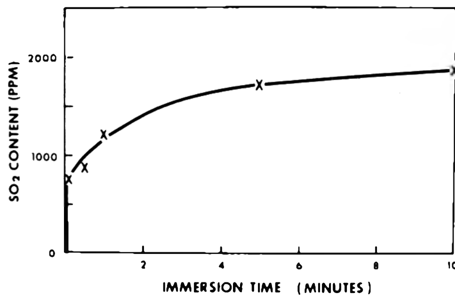

Fig. 6—The effect of time on the SO_2 content of apricots dipped in 5% (SO_2) sodium bisulfite solution at pH 3.0.

essentially none. The absorption rate of sulfurous acid would be expected to be greater than the bisulfite ion and is confirmed by the greater sulfur dioxide content of the fruit treated with lower pH solutions. The pH effect is also shown in Figure 5 where increasing the concentration of sulfurous acid increases the amount of polyphenol oxidase inactivation, especially in the skin area of the fruit. Most of the bisulfite absorption at all pH values is through the pit cavity and cut surface.

While there is an increasing absorption rate as the pH of the bisulfite solution is lowered from pH 4.5, there is also a significant increase in the amount of sulfur dioxide lost to the atmosphere from the solution. In addition, an increasing amount of sulfur dioxide is lost during drying as the pH of the bisulfite dip solution is lowered and this is shown in Table 1 by the significant positive correlation between the dry apricot/fresh apricot sulfur dioxide content ratio and the pH of the dip solution.

Effect of time

The effect of treatment time on the absorption of sodium bisulfite is shown in Figures 1, 2, 3 and 5. Figure 6 illustrates a rapid surface absorption during the first few seconds of immersion and a rapidly diminishing rate thereafter.

These studies have shown the effect of dip solution concentration, pH and time of immersion on the absorption of bisulfite into apricots and how the pH of the dip solution affects desorption losses during drying. Field studies are needed to determine whether the use of bisulfite solutions to sulfur apricots, with its many potential advantages, is now commercially feasible.

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FREE AMINO ACIDS IN RAW AND PROCESSED TOMATO JUICES BY ION EXCHANGE CHROMATOGRAPHY WITH A LITHIUM CITRATE COLUMN FOR SEPARATION OF GLUTAMINE AND ASPARAGINE FROM THREONINE AND SERINE

INTRODUCTION

STUDIES of the amino acid composition of the tomato fruit and its products have been made by a number of investigators. Paper chromatography was used by: Safina (1953) to determine the amino acid composition of tomato puree; Ito (1954) to determine amino acids in tomato juice; Hamdy and Gould (1962) to evaluate the amino acid content in freshly extracted and processed juices from eight different tomato varieties; and Kim et al. (1962) to compare amino acid composition in canned tomato juice before and after heat sterilization.

Trinitrophenyl derivatives prepared from some of the amino acids found in tomatoes by two-dimensional thin-layer chromatography were determined spectrophotometrically by De Bruyn et al. (1971).

After Moore and Stein (1951) developed the technique of ion exchange chromatography of amino acids, Carañgal (1954) and Saravacos et al. (1958) used manually operated ion exchange columns to study the effects of different fertilizer treatments on the amino acid composition of tomatoes.

Automatic ion exchange chromatography was used by Luh and El-Tinay (1966) to determine amino acids in tomato pastes from five different tomato varieties; by Luh and Daoud (1968) for amino acid analysis of juice from four tomato varieties; and by El Miladi et al. (1969) to determine differences in amino acid composition between raw and processed tomato juices.

The author has studied the amino acid composition of tomatoes both before and after heat processing, giving special attention to changes in glutamine and glutamic acid. Many amino acid chromatograms of tomato sera were run using a Technicon single-column automatic amino acid analyzer. Conventional sodium citrate buffer eluants were used for most of the chromatograms run for this study. In spite of the general excellence of the chromatograms, it was not possible to resolve separate peaks for glutamine and asparagine. They either did not separate from each other or were eluted together with serine or threonine. Benson et al. (1967) recommended the use of lithium equilibrated ion exchange columns with lithium citrate buffers as eluants for separating glutamine, asparagine, threonine and serine in biological fluids. Several investigators (Peters et al., 1968; Sowden and Ivarson, 1968; Perry et al., 1968; Mondino, 1969; Nunn and Vega, 1968; Vega and Nunn, 1969; Kedenburg, 1971; and Blohm, 1970) have since used chromatography with lithium citrate buffer eluants for amino acid analysis of animal fluids.

Lithium citrate columns should prove especially useful for studying the physiological fluids of plants where concentrations of glutamine, asparagine, and glutamic acid are often very high relative to the other amino acids present. Accurate assay for these amino acids has been difficult. This report presents the analyses of amino acids in tomato juices and describes the use of ion exchange chromatography with lithium citrate buffer for isolating glutamine, asparagine and serine from tomato juices before and after heat processing.

There are many discrepancies in the various data reported in the literature on the amino acid composition of tomatoes and tomato products; a critical review of published works on this subject is offered as part of the discussion section of this paper.

EXPERIMENTAL

TOMATO JUICE for this study was prepared on the day of harvest from a field lot of tomatoes (variety VF 145-F5) grown near Stockton, Calif.

Raw (cold break) juice

A representative sample (∿ 50 lb) of fruit was pulped in a Food Processing Equipment Co. laboratory pulper using a 0.027 in. screen. The cold break juice was mixed well, and samples sealed in # 2-1/2 cans, without vacuum, were placed in freezing storage immediately.

Heated (hot break) juice

The heated juice was also prepared on the day of harvest from the same field lot of fruit used for the raw juice. The washed fruit, after the culls and green fruit were removed, was macerated in a Reitz disintegrator and heated by steam injection to 220°F at a flow rate of 14 lb/min. It was held at this temperature for 15–23 sec in 1-in. diam holding tubes. The heated juice was separated from seeds and skin tissues with a Brown extractor equipped with a 0.027-in. diam screen. The extrusion screw was operating at 800 rpm against 8 lb pressure on the discharge orifice. The juice was then cooled in Cherry Burrell and Creamery Package Co. heat exchangers. Samples in # 2-1/2 cans, sealed without vacuum, were frozen immediately and kept frozen until needed.

Reheated sample of raw juice

In order for these results to be compared with other published results for heated juices, one can of raw juice and one can of hot break juice were removed from cold storage, thawed and allowed to come to room temperature. The cans were then placed in a retort and heated at 220°F for 20 min.

Preparation of samples for chromatography

Since amino acid amides are somewhat labile (glutamine especially is subject to hydrolysis under elevated temperatures and high pH), conditions that might cause chemical changes were avoided. A sample of juice from each treatment was centrifuged at 10,000 rpm for 30 min. The serum was decanted from the residue. The slightly turbid serum was then filtered under pressure through a Type E Gelman glass fiber filter. After filtration, the raw juice serum was crystal clear and the heated juice sera was very slightly turbid. Amino acid analyses of 0.02–0.5 ml aliquots were made chromatographically on a 0.6 × 125 cm column of Technicon Chromo-Bead Type B resin. The gradient elution system recommended by Technicon Corp. (1962) using three different sodium citrate buffers, was used.

To avoid possible acid hydrolysis of glutamine, the serum samples were not adjusted to pH 1–2 before chromatographing. Samples with pH in the natural range (4–4.5) gave good separations with sharp peaks and narrow band widths.

Separation of glutamine and asparagine without interference from other amino acids was accomplished on a similar column equilibrated with lithium citrate buffer as eluant. Although several investigators (Sowden and Ivarson, 1968; Perry et al., 1968; Nunn and Vega, 1968; Vega and Nunn, 1969) have employed the Technicon single-column automatic amino acid analyzer with lithium citrate elution techniques to separate amino acids, the procedure reported here was developed independently.

In tomatoes the concentrations of glutamine, glutamic acid, asparagine and aspartic acid are so high relative to the other amino acids that it is necessary to run chromatograms at two or more concentrations to resolve all of the amino acids present. It was convenient therefore to determine the neutral and basic amino acids on a column set up for routine analysis of amino acids using sodium citrate buffers as eluants, and to use a much more dilute sample on a second column with a lithium citrate buffer eluant to determine aspartic acid, asparagine, threonine, serine, glutamine and glutamic acid.

The lithium citrate column consisted of a Chromo-Bead Type B column prepared in the lithium form according to the procedure described by Benson et al. (1967). A single buffer (pH 2.7) was used to separate the amino acids of interest. It was prepared according to Benson et al. (1967) and modified slightly by the addition of 10 ml. of Brij 35 per liter.

To improve the separation of threonine and serine, a two solution gradient elution was used. The first solution consisted of 80 ml of buffer and 5 ml of methyl alcohol, the second was 85 ml of buffer. Increasing the alcohol-to-buffer ratio in the first solution to 8:77 ml, improved the separation of threonine and serine but glutamine eluted with asparagine. Column temperature was 40°C and buffer flow rate 0.5 ml per min resulting in a column back pressure of 400 psi. All of the amino acids through glutamine were eluted in 5-1/2 hr.

A standard mixture of amino acids containing 0.20 μmoles each of six acidic amino acids (aspartic acid, asparagine, glutamine, glutamic acid, threonine and serine) was run before each tomato serum was analyzed. The column was not regenerated between standard and sample runs, but it was always regenerated with 0.3N lithium hydroxide followed by pH 2.7 buffer after each sample.

RESULTS

IN FIGURE 1 a typical chromatogram of aspartic acid, threonine, serine, asparagine, glutamic acid and glutamine on an ion-exchange column equilibrated and eluted with lithium citrate buffer at pH 2.7 is compared with an ion-exchange chromatogram of the same amino acids separated on a column eluted with sodium citrate buffer.

The amino acid composition of the juices from tomato variety VF-145-F5 as determined by automatic ion exchange chromatography is reported in Table 1. The amino acid composition of raw (cold break) juice is compared with that of processed (hot break) juice prepared by steam injection heating and with juice given a retort processing at 220°F for 20 min. For the raw juice, 31 compounds giving blue color reactions with ninhydrin were separated and are reported in Table 1 in the order of their elution from the column. They include ammonia and 20 identified amino acids. The six most abundant, in order of decreasing concentration are glutamine, glutamic acid, γ amino butyric acid, asparagine, aspartic acid and alanine. Serine, threonine and phenylalanine occur in smaller amounts. The other amino acids are present in relatively low concentrations. Only one of the 10 unknown compounds (eluted after 1128 min as peak No. 28) occurred in an appreciable concentration. Cystine was not found in heated juice, and unknown (# 22) was not detected in the steam injected juice.

Changes in amino acid composition in tomato juice after heat treatment are also shown in Table 1. Amino acid concentrations in Table 1 are expressed as millimoles per 100 grams of tomato solids to permit direct comparison of raw and steam injection processed juice samples. Units based on juice volume i.e., mg/100 ml juice, could not be used because some dilution by steam condensate occurs in steam injection processing. Many changes are slight, and probably fall within the limits of variation for the method. When the juice was prepared by steam injection

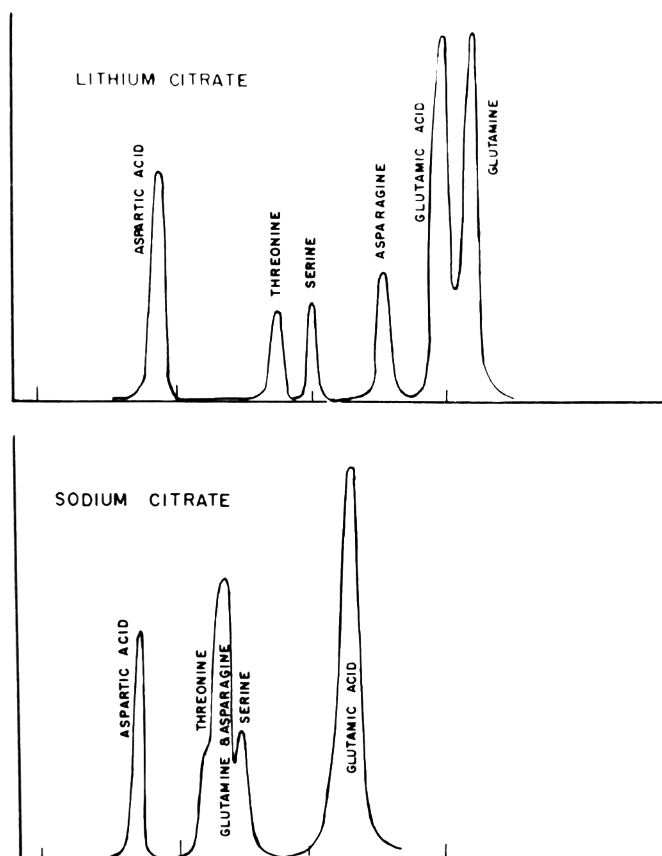

Fig. 1—Comparison of ion exchange chromatograms of acidic amino acids with sodium citrate buffers and lithium citrate buffer as eluants.

Table 1—Amino acids in fresh and processed tomato juices (millimoles per 100g solids)

	Retention time (min)	Raw (cold break) juice	Steam injection line juice	Retort processed raw juice (220°F 20 min)[a]
1. Unidentified	261	+	+	+
2. Unidentified	277	+	+	+
3. Aspartic acid	176[b]	5.30	4.88	4.75
4. Threonine	235[b]	1.78	1.69	1.57
5. Serine	240[b]	1.90	1.63	1.73
6. Asparagine	271[b]	6.21	5.95	5.51
7. Glutamic acid	298[b]	17.02	17.92	16.34
8. Glutamine	310[b]	21.93	21.23	17.67
9. Proline	411	.49	.47	.41
10. Unidentified	428	+	+	+
11. Unidentified	479	+	+	+
12. Glycine	495	.28	.39	.26
13. Alanine	524	3.15	2.38	2.50
14. Unidentified	579	+	+	+
15. Valine	622	.38	.30	.31
16. Cystine	670	Trace	–	–
17. Methionine	704	.15	.06	.11
18. Isoleucine	773	.66	.06	.11
19. Leucine	799	.47	.39	.44
20. Tyrosine	888	.23	.21	.21
21. O-Alanine	890	1.37	1.20	1.15
22. Unidentified	958	+	–	+
23. γ-Amino butyric acid	980	13.79	9.51	10.08
24. Ammonia	1014	4.54	2.94	6.24
25. Unidentified	1076	+	+	+
26. Unidentified	1090	+	+	+
27. Unidentified	1100	+	+	+
28. Unidentified	1128	++	++	++
29. Lysine	1138	.71	.71	.74
30. Histidine	1203	.76	.83	.73
31. Arginine	1419	.57	.54	.52

[a] There were no significant differences between the amino content of raw juice and steam injection line juice after retort processing.
[b] Retention times on lithium citrate column

heating, concentration values increased for only glutamic acid, glycine and histidine. Except for glycine, the increases are within the limits of experimental variations. Concentrations decreased for all amino acids in juice processed at 220°F for 20 min. In most instances, the observed decreases under prolonged heating were only slightly greater than those found for the sample prepared by steam injection heating.

Changes in the concentration ratios between the various amino acids occurred after processing. Significant differences between the raw juice and the heat treated juices were observed for the concentrations of γ-amino-butyric acid which decreased by 31% in steam injection heated juice and 27% in the retort processed juice. A significant concentration decrease of 17.8% was recorded for glutamine in juice processed at 220°F for 20 min, but under the less drastic heating conditions of steam injections, only 3.2% of the glutamine disappeared.

The ammonia increase in juice heated at 220°F for 20 min reflects decreases for asparagine and glutamine, since these compounds hydrolize to release ammonia upon heating. Glutamine, especially is easily converted to pyrrolidonecarboxylic acid with release of ammonia when heated in aqueous solution. With steam injection processing there was a contrasting 35.2% decrease in ammonia. This may reflect a loss of volatile ammonia compounds by distillation during steam injection. Juices heated in sealed cans under retort conditions are never exposed to the atmosphere to let ammonia escape.

DISCUSSION

THE AMINO ACID composition of the tomato as reported by various investigators differs a great deal. Tables 2a, 2b and 2c, in order to show more clearly differences that exist, present results reported in this paper on a comparable basis with data reported by Safina, 1953; Ito, 1954; Hamdy and Gould, 1962; Carañgal et al., 1954; Saravacos et al., 1958; Luh and El-Tinay, 1966; Luh and Daoud, 1968; El Miladi et al., 1969. Only data from papers reporting general assays of amino acids in tomatoes and tomato products have been included. Results of analyses reported by Kim et al. (1962) will be referred to from time to time in the discussion.

Wherever possible, and sufficient published data permitted it, the reported values have been converted to the same concentration units (mg/100g of fresh fruit or juice). Data based on whole fruit could not, in most cases, be converted to a fruit juice basis and vice versa; however, under standard extracting procedures the weight of skins and seeds only comprises about 3% of the fresh fruit weight which is within the limits of experimental error.

Table 2a–A comparison of reported results for the free amino acid composition of tomatoes as determined by paper chromatography[a]

	Safina (1953) mg/100g (Dry Wt)	Ito (1954)	Hamdy and Gould (1962)																		
			Rutgers			Wisconsin 55		KC 135		KC 146		Glamour			Early Bird-F2		Moreton Hybred		Cardinal Hybred	
			Fresh	Ext[a]	Proc[b]	Ext	Proc	Ext	Proc	Ext	Proc	Fresh	Ext	Proc	Ext	Proc	Ext	Proc	Ext	Proc
Aspartic acid	824(2)	+++	++	+	+	++	++	++	++	+	+	+	++	+	++	++	++	++	++	++
Threonine	(10)[b]	++	+	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+	+	+
Serine	(5)	++++	+	++	++	+	+	++	++	+	+	+	+	+	+	+	+	+	+	+
Asparagine	(8)	++	+	+	+	++	++	++	++	+	+	+	+	+	+	+	+	+	+	+
Glutamine			+++	++++	++++	+++	+++	+++	+++	+++	+++	+++	+++	++	+++	++	++++	+++	+++	++
Glutamic acid	1228(1)	+++	++++	++++	+++++	++++	+++++	++++	++++	++++	+++++	++++	++++	+++++	+++	++++	++++	++++	++++	+++++
Proline			++	++	+	++	+	+	+	...	+	+	...	++	...	++	++	++	+	++
Glycine	(7)		trace	trace	trace	trace	trace	trace	trace	trace	trace	...	trace	trace	trace	...	trace	...	trace	trace
Alanine	560(4)	+++	+	+	++	++	++	++	++	+	++	+	+	++	+	+	++	+	++	+
Valine	740(3)	++++	++	+++	+++	++	++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
Cystine																				
Methionine																				
Isoleucine			trace	trace	trace	+	trace	trace	trace	trace	trace	trace	trace	...	trace	trace	trace	...	trace	trace
Leucine	(9)	++	trace	+	+	+	trace	++	++	trace	trace	trace	+	+	+	+	+	+	+	trace
Norleucine																				
Tyrosine			+	trace	trace	trace	...	trace	trace	trace	trace	...	...	...	trace	trace	trace	trace	trace	trace
φ-Alanine			trace	trace	trace	+	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace	trace	...
γ-Amino butyric																				
Lysine			+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tryptophan		+++																		
Histidine			+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	trace	...
Arginine	(6)																			

[a]Ext.–Extractor juice; Proc.–processed juice
[b]Number in parentheses designates order of concentration

Table 2b—A comparison of reported results for the effect of nutrients on the free amino acid composition of tomatoes

	Carañgal et al. (1954)								Saravacos et al. (1958)							
	K 200 ppm P 100 ppm					K 400 ppm P 200 ppm		K 200 ppm P 100 ppm	1955 Crop			1956 Crop				
	NH_4^+ 50 ppm	NH_4^+ 100 ppm	NO_3^- 50 ppm	NO_3^- 100 ppm	NO_3^- 200 ppm	NO_3^- 100 ppm	NO_3^- 200 ppm	NO_3^- 100 ppm	Control	NH_4NO_3 130 lb. N per Acre	NH_3 130 lb. N per Acre	Control	NH_4NO_3 120 lb. N per Acre	NH_4NO_3:TSP K_2SO_4 120 lb. N: P_2O_5 K_2O per Acre	NH_3 60 lb. N per Acre	NH_3 120 lb. N per Acre
	(amino acid concentration mg/100g fresh fruit)								(amino acid concentration mg/100 ml juice)							
Aspartic acid	15.2	8.8	12.3	25.6	2.3	17.6	8.8	23.0	36.2	60.3	90.5	27.5	50.6	49.5	22.7	63.6
Threonine									2.8	7.7	6.2	6.6	34.6	11.4	8.2	18.0
Serine	17.7	7.3	8.0	14.2	21.2	17.6	13.0	11.2	5.7	8.4	6.6	16.4	14.1	12.8	15.6	15.9
Asparagine } Glutamine									16.5	22.8	15.5	23.2	62.2	62.5	37.0	60.6
Glutamic acid	83.3	8.7	28.3	83.2	124.8	107.1	113.1	96.0	150.0	173.0	243.0	172.0	213.0	214.8	176.0	201.7
Proline																
Glycine	some samples appeared to contain traces								20.5	6.9	13.9	18.2	11.5	14.6	19.0	15.8
Alanine	5.8	3.0	3.3	3.0	2.8	3.7	7.2	4.2	7.1	26.6	72.0	13.9	39.7	40.3	38.1	51.4
Valine	7.3	trace	6.7	trace	trace	6.0	trace	trace	24.5	11.0	34.0	15.0	6.5	9.7	26.5	21.5
Cystine																
Methionine	some samples appeared to contain traces															
Isoleucine	some samples appeared to contain traces															
Leucine	8.2	9.4	5.0		5.7	0.9		6.0	22.5	11.2	13.0	5.9	6.1	11.9	8.2	4.1
Norleucine																
Tyrosine } φ-Alanine	18.2	9.9	10.7	11.7	13.8	15.1	6.4	16.4								
γ-Amino butyric A.																
Lysine	8.4	1.6	6.4	10.4	9.9	6.8	16.1	10.4								
Tryptophan	56.2	9.9	1.2	8.7	25.7	6.8	44.2	21.7								
Histidine	9.6	3.3	8.8	20.3	11.3	44.4	6.4	9.4								
Arginine	7.2	7.8	4.4	trace	trace	8.9	9.0	trace								

Data presented by Safina (1953), Ito (1954) and Hamdy and Gould (1962) can only be compared qualitatively. Safina did present some quantitative data reported as mg/100 g dry wt rather than on a wet weight basis. The data of Carañgal et al. (1954) are presented in this comparison, but they must be considered with the knowledge that these investigators did not report on analysis of the whole juice of the tomato. They made separate determinations for the amino acid composition of the "pulpy" locuole content of the fruit, and the fleshy portion of the fruit. The ratio of the locuole contents to the flesh portion of the fruit was not given, so it was not possible to calculate whole juice values from their data. For this comparison, it was assumed that the fleshy portion of the fruit comprises the major part of the juice, so values for this fraction were used. The data from Luh and El-Tinay (1966), reported as mg/100g of paste were converted to mg/100g juice using concentration factors determined from the total juice solids and the total paste solids values presented in their paper.

Data in Table 2 show how certain variables affect the amino acid content of tomato juice and tomato products as reported in the literature.

Effects of processing temperature

Heat is known to accelerate chemical changes such as the hydrolysis of glutamine and/or glutamic acid to pyrrolidonecarboxylic acid, and carbonyl-amino acid browning reactions. These reactions can also occur at lower temperatures under long storage conditions.

Kim et al. (1962) studied the effects of processing on amino acids in tomatoes by unidirectional ascending paper chromatography. They found that fresh tomato juice contained aspartic acid, asparagine, glutamic acid, glutamine, lysine and an unknown amino acid. After processing at 212°F for 25 min., they reported that both the glutamine and glutamic acid has been converted to pyrrolidonecarboxylic acid but that the other amino acids found in the fresh juice were still present. Minor changes, if any, could not be detected under the conditions of their experiments.

Results reported in this paper show that amino acids in juice from tomato variety VF-145-F5 decrease after processing. Similar results have been observed by the author for juices from two separate lots from variety VF-145-7879 processed in 1970, two lots from variety VFN8 processed in 1963 and one lot from variety VF-145-7879 processed in 1967.

Results reported by El Miladi et al. (1969) (reference Table 2) are in direct opposition to these. They reported four- to ten-fold increases for amino acids found in Heinz variety 1370. It is difficult to reconcile such large amino acid

Table 2c–A comparison of reported results for the free amino acid composition of tomatoes as determined by ion exchange chromatography

	Luh and El-Tinay (1966) (Paste) as mg/100 ml juice Variety					Luh and Daoud (1968) (Juice) mg/100 ml Variety				El Miladi et al. (1970) (Juice) mg/100 ml		Stadtman (This Paper) (Juice) mg/100 ml		
	Ace	Roma	VFN-8	VF-36	VF-145	VF-145 -11-38	VF-13L -34	VF-145 -21-4	Coml.	Fresh	Proc	Fresh	Steam injection proc	Retort proc
Aspartic acid	70.44	63.16	44.00	72.37	45.12	58.7	34.6	38.6	37.3	5.5	51.6	43.5	34.6	39.0
Threonine	31.15	33.54	38.39	60.44	37.85	58.4	70.3	76.2	25.0	1.0	9.0	13.1	10.7	11.9
Serine	2.64	1.23	2.87	.67	1.57	21.0	12.6	10.5	10.5	2.3	12.7	12.3	9.1	11.2
Asparagine						Included with threonine				7.8		50.6	41.8	44.9
Glutamine												197.8	165.7	151.0
Glutamic acid	295.79	257.18	213.93	379.83	184.17	225.0	215.0	250.0	110.0	21.9	212.5	154.5	140.5	139.3
Proline										0.1	0.4	3.7	2.9	2.9
Glycine	1.72	1.61	1.73	1.65	1.82	3.0	2.3	1.5	1.5	0.3	1.2	1.4	1.6	1.2
Alanine	9.14	9.09	6.71	9.68	12.54	39.2	14.3	12.5	16.9	1.0	9.0	20.0	11.3	13.7
Valine	1.69	2.51	2.45	3.26	2.00	4.7	3.5	2.3	1.2	0.4	1.7	2.7	1.9	2.2
Cystine												0.2		
Methionine	1.89	2.00	1.25	2.54	1.27	trace	trace	trace	trace	0.2	0.9	1.5	.5	1.0
Isoleucine	4.48	4.58	4.66	6.99	4.20	13.1	5.3	5.3	3.9	0.6	3.8	5.7	3.7	4.6
Leucine	3.37	3.51	3.38	4.75	3.07	6.6	3.9	3.9	2.6	0.6	3.0	3.8	2.7	3.5
Norleucine						32.8	27.6	36.7	34.1					
Tyrosine	3.79	3.89	2.66	5.03	2.32	5.4	3.6	3.6	3.6	0.5	3.4	2.6	2.0	2.4
ϕ-Alanine	19.83	22.60	22.11	50.72	16.55	14.9	11.6	13.2	8.3	1.4	10.8	16.7	10.6	11.7
γ-Amino butyric						124.9	78.4	62.9	69.1			87.7	52.3	64.2
Lysine	7.03	7.83	7.34	7.48	5.93	8.8	7.3	7.3	4.4	0.9	5.1	6.2	6.4	6.7
Tryptophan						4.1	2.0	trace	4.1					
Histidine	8.12	8.31	8.43	10.26	4.97	9.3	7.8	7.8	4.7	0.9	7.5	7.0	6.8	7.0
Arginine	5.84	7.93	5.46	9.91	2.60	6.9	12.2	10.5	3.5	0.7	4.4	6.2	5.0	5.6

increases with new data presented in this paper. Chemical differences between varieties of tomatoes are not sufficiently great to account for such large discrepancies. One would not expect the acidity of tomatoes (pH 4–4.5) to be high enough to cause extensive hydrolysis of proteins in 20 min even at temperatures as high as 220°F.

The results reported by El Miladi et al. (1969) are also not consistent with results reported by Hamdy and Gould (1962) who evaluated the amino acid concentrations of eight varieties of tomatoes (Table 2) by visually comparing the color intensity of spots on paper chromatograms. They reported 89 instances where no differences could be detected between spots from samples of extractor juice and those of processed juice. They indicated 25 instances where spot intensity decreased and only 14 where the color increased. Six of the increases indicated were for glutamic acid where increases would be expected if glutamine is hydrolized to glutamic acid as well as to pyrrolidonecarboxylic acid. Concentration increases as great as seven- to ten-fold as reported by El Miladi et al. (1969), should also have been detected by Hamdy and Gould (1962) even with the qualitative method they used.

El Miladi et al. (1969) suggested that amino acid increases they reported could possibly be accounted for by hydrolysis of proteins during heat treatment. Other reported results do not support this conclusion. The extent of hydrolysis of proteins during processing is not great as evidenced by the amino nitrogen values for tomatoes before and after processing as reported by Hamdy and Gould. They reported less than 15% amino nitrogen increases as a result of processing for six of the eight varieties studied. Seven- to ten-fold increases in amino acid concentration would be reflected in the amino nitrogen analyses. It could be argued that large amino acid increases reported by El Miladi et al. (1969) is a special characteristic of the Heinz 1370 tomato variety they studied; however, as will be indicated later varietal differences do not appear to account for such large chemical differences.

It seems probable that the El Miladi (1969) data reflect a ten-fold error in calculating the amino acid concentration values for the fresh fruit sample. It would certainly seem desirable to re-evaluate the processing changes for amino acids occurring in the Heinz 1370 tomato to clarify this matter.

Effect of mineral and organic nutrients on amino acid composition of tomato products

Effects of nitrogen and mineral nutrients on the amino acid composition of tomatoes is shown in the data presented

by Carañgal et al. (1954) and by Saravacos et al. (1958). The experimental conditions for these studies were different; therefore, it is difficult to determine how well the results agree. One can conclude, however, that the quality and quantity of nutrients the growing tomato plant receives can affect the concentrations of free amino acids in the mature fruit. From Possingham's (1956) study on the availability of mineral nutrients on the free amino acids in tomato plants, one finds that deficiencies in some minerals such as Cu, Mn, Fe and Mo can result in the disappearance of some amino acids such as histidine and lysine. The effect of these minerals on the amino acid content of the mature tomato fruit has not been studied. One might expect that certain mineral deficiencies could cause differences in the amino acid composition of the fruit as well as in the green plant.

Effect of varietal differences on the amino acid composition of tomato products

Hamdy and Gould (1962), Luh and El-Tinay (1966), Luh and Daoud (1968) and De Bruyn et al. (1971) have all shown that varietal differences can affect the amino acid composition of tomato products. It is significant, however, that essentially the same free amino acids are found in all varieties in approximately the same relative concentrations. In comparing the data from the various published sources one must keep in mind that amino acid composition is influenced by varietal characteristics of the tomato studied, yet it is important not to confuse varietal differences with differences due to cultural practices. Data presented by both Luh and El-Tinay (1966) and Luh and Daoud (1968) are subject to some criticism in this respect. Luh and El-Tinay (1966) presented data for tomato paste made from five different varieties of tomatoes. Three of the varieties were grown with one set of conditions and two with another. The authors noted this discrepancy in their discussion but did not indicate, in connection with Table 5 of their report, that direct comparison of all of the data should not be made. The data presented by Luh and Daoud (1968) are more reliable since all varieties studied were grown under the same field conditions. The inclusion in their study of an analysis of commercially packed tomato juice would appear to have little significance. For valid varietal studies, all varieties should be grown at the same time with the same climatic and soil conditions. Preferably they should be grown under greenhouse conditions in inert culture beds where exact nutrient controls can be maintained.

Effects of different analytical procedures

Many differences between the results reported by the various investigators can be attributed to limitations inherent in the experimental methods used. Even though valuable qualitative data are obtained, it is difficult to get quantitative results from paper chromatograms. It is also difficult to increase the sensitivity of this method without complicated purification procedures which might change the chemical composition of the samples. For tomatoes the sensitivity of this technique is further limited by other complications. High concentrations of aspartic acid, glutamic acid, glutamine and asparagine produce very large often streaked spots that merge with and mask out other spots. This is particularly true for unidirectional paper chromatograms. Unidirectional paper chromatography by Kim et al. (1962) may explain why they only found two amino acids besides glutamine, glutamic acid, aspartic acid and asparagine in tomatoes.

Reduction of sample size to get "readable" paper chromatograms limits one's ability to assay for amino acids that occur in low concentrations. This might explain why so few amino acids were reported by Safina (1953) and of Ito (1954), and could also account for the differences in amino acid composition reported by these investigators. Safina (1953) detected only ten amino acids in tomato juice, the most abundant being glutamic acid followed by aspartic acid, valine and alanine. Ito (1954) only reported nine. He did not find glycine or arginine, reported by Safina (1953), but found tryptophan which had not been reported before. Hamdy and Gould (1962) were more successful in their use of paper chromatography, for they detected, qualitatively, up to 16 amino acids in some of the tomato juices they analyzed. Their lists include glutamine, lysine, histidine, isoleucine, tyrosine and phenyl alanine which were not reported before.

Ion exchange chromatography techniques for separation of amino acids permits more reliable quantitative analysis of amino acid in biological fluids. Due to the high concentrations of some amino acid components of tomato, this technique is also limited in its "single run" sensitivity, but the less prominent amino compounds can be detected if several runs are made with different sample concentrations.

Using a manually operated ion exchange column, Carañgal et al. (1954) were able to detect as many as 16 amino acids in tomato juices and measured 9–12 of them quantitatively in the eight samples they analyzed. Saravacos et al. (1958) also employed a manually operated ion exchange column. They only reported the nine amino acids that emerged before tyrosine since they discontinued their runs before any of the basic amino acids emerged.

Automated ion exchange chromatography has greatly simplified the assay of biological fluids for amino acids. As a result of this technique, Luh and El-Tinay (1966) were able to detect 15 amino acids in tomato pastes. Luh and Daoud (1968) found 18, and El Miladi et al. (1969) 19 in tomato juice. This paper reports the presence of 31 ninhydrin positive compounds in raw tomato juice, 20 of which have been identified.

Discussion of specific discrepancies in reported results

Differences in concentration values for most of the amino acids in tomatoes fall within the range of certain reasonable limits of variation, dependent on variables discussed above. There are a few major discrepancies in concentration values reported for certain amino acids. Most of these involve the amino acids that cannot be separated with sodium citrate buffer systems by ion exchange chromatography. Each of these amino acids will be discussed in detail.

Glutamine and asparagine. With sodium citrate buffers as eluants, depending upon the temperature, eluant flow rate, physical characteristics of the resin, and nature of the eluant, glutamine and asparagine will either: (1) elute together as a single peak (Moore and Stein, 1954; Stein and Moore, 1954; Tallan et al., 1954); (2) elute together with threonine (Boulter, 1966); or (3) elute together with serine (Hamilton, 1963; Benson et al., 1967). Results of the first two of these conditions are evident in the data presented in Table 2.

Both El Miladi et al. (1969) and Saravacos et al. (1958) reported that glutamine and asparagine eluted as a single peak. The relative concentration cannot be determined under these circumstances since glutamine and asparagine have distinctly different color yields upon reaction with ninhydrin.

Luh and El-Tinay (1966) and Luh and Daoud (1968) have reported unusually high values for threonine in all of their samples, as compared with those reported by other investigators. This suggests that the peak reported as threonine in their chromatograms, was actually a coincidence of peaks, and probably represented asparagine and possibly glutamine as well as threonine. The extent to which glutamine might have been present is dependent upon the amount of hydrolysis that occurred during processing.

Luh and Daoud (1968) have actually reported that asparagine is part of the so called threonine bands of their chromatograms indicating that some sort of independent check was made to establish this. The data suggest that the earlier work reported by Luh and El-Tinay (1966) was not checked to confirm the purity of the threonine fraction of their samples.

From most of the data available at the

present time it can be argued that asparagine is present in tomatoes in relatively large amounts. It has been reported by all investigators except Carañgal et al. (1954) and Luh and El-Tinay (1966). In the latter case it might have been present but was reported as threonine. Kim et al. (1962) and Hamdy and Gould (1962) as well as the author have shown that asparagine did not disappear from tomato juice with heat processing. Through the use of lithium citrate columns, the author has been able to determine relatively high concentrations of asparagine in tomato juice both before and after processing. 50.6 mg/100 ml were found in the fresh juice and from 41.8–44.9 mg/100 ml in processed juices. Asparagine is reasonably stable under normal processing temperatures at the pH of tomato juice.

El Miladi et al. (1969), however, have reported that asparagine disappeared in their samples after processing. Conditions under which asparagine might disappear completely from processed juice is a subject for more study. In making such a study, however, one should be aware of the limitations of certain ion exchange techniques, and that the amides glutamine and asparagine can be hydrolized under some of the conditions that are often used for sample preparation, seriously affecting results.

The wide range of discrepancies in the reported concentrations for glutamine in tomatoes and tomato products is not surprising. It is heat labile. It can be readily detected qualitatively by paper chromatography, but, as pointed out above, it has, until recently, been difficult to isolate chromatographically by ion exchange techniques.

The author has found slightly greater amounts of glutamine than glutamic acid in the tomato juice samples he has analyzed. These results do not agree with the results of other investigators. Relatively large amounts of glutamine but less than those of glutamic acid have been reported (De Bruyn et al., 1971; Hamdy and Gould, 1962; Kim et al., 1962). For processed samples amounts varying from relatively high concentrations to none at all have been reported. Due to limitations of ion exchange chromatography systems used, Saravacos et al. (1958) and El Miladi et al. (1969) were not able to separate glutamine from asparagine.

Some investigators (Kim et al., 1962; El Miladi et al., 1969) have concluded that the glutamine in raw tomato juice is almost completely converted to pyrrolidonecarboxylic acid (PCS) and/or glutamic acid with heat processing. The author has analyzed only two lots of juice with results that support this conclusion. Complete conversion of glutamine to PCA and/or glutamic acid does not always occur. The author has found that all juices prepared from tomatoes harvested in 1970 still contained relatively large amounts of glutamine after processing. Hamdy and Gould (1962) did not find a decrease in glutamine after processing. Mahdi et al. (1959) followed the changes in glutamine and PCA in tomatoes during processing and storage. They reported a relatively high concentration of glutamine still in the tomatoes immediately after processing which gradually decreased from 114 mg/100g (dry wt) to 7 mg/100g at the end of 24 months' storage.

It is generally agreed in the literature (Hamdy and Gould, 1962; Mahdi et al., 1959; and Mahdi et al., 1961) that the ultimate fate of glutamine in processed tomato products is its conversion to PCA. It may not yet be possible to predict how rapidly this conversion will take place, but among the contributing factors are: (1) pH–Glutamine is converted to PCA more rapidly at higher pH; (2) Processing temperature; (3) Processing time; (4) Cooling rate after processing; (5) Storage temperature; and (6) Time in storage.

In pastes analyzed by Luh and El-Tinay (1966) one might expect all of the glutamine to be hydrolyzed due to the longer heating required to concentrate the juice. This cannot be stated unequivocally, however. The author has analyzed immediately after concentration a paste made from juice of the 1971 crop of tomatoes prepared by concentration in a wiped-surface evaporator and found large amounts of glutamine. Glutamine did disappear on storage. After storage at ambient temperatures for 1 yr, only traces of glutamine remained in tomato paste made from VF-145-F5 tomatoes processed in 1970. This fruit originally contained a high concentration of glutamine (see Table 1).

Ito (1954) reported no glutamine in his samples. He used a sample purification technique involving an alkaline precipitation with Na_2CO_3 which might have caused the hydrolysis of glutamine.

Threonine. Table 2 shows that Carañgal et al. (1954) are the only investigators failing to report the presence of threonine in tomatoes. An analysis of the results reported indicates that threonine is present in concentrations ranging between 2.8 and 13.1 mg/100 ml of juice. Saravacos et al. (1958) however, have reported as much as 34.6 mg/100 ml of tomato juice from fruit that was grown with high nitrogen fertilization. This value is 2.6 times the maximum value for the range indicated above. This high value seems to be out of line because another sample of juice analyzed by the same investigators which received the same level of nitrogen fertilization only contained 11.4 mg/100 ml of threonine. This second sample of juice came from tomatoes that were also fertilized by high levels of TSP and K_2SO_4 which makes any valid comparison of the results difficult, however.

The data presented by Luh and Daoud (1968) and by Luh and El-Tinay (1966) were not considered in arriving at the range of concentrations reported above for threonine. It has already been pointed out that it is not possible to separate threonine, glutamine and asparagine under the experimental conditions used by these investigators.

Norleucine. It is generally agreed norleucine is absent from plant materials (Hunt, 1959); therefore, it is commonly used as an internal standard for ion exchange chromatograms. In this Department it has been a common practice to add 0.25 μmoles of norleucine as an internal standard for every amino acid analysis determined with the Technicon Amino Acid Analyzer. Both the author and Luh and Daoud (1968) followed this procedure when determining amino acids in tomato products. Luh and Daoud (1968) are the only investigators who have reported norleucine as a constituent of tomato juice; it seems reasonable to assume that they inadvertently included values for their internal standard as a constituent of tomato juice. The values they have reported for this amino acid are relatively high, and approximate the amount expected if 0.25 μmoles had been added to the sample.

Gamma amino butyric acid. This amino acid is common to many plant materials and abundant in many fruits. Shinano et al. (1956) have described a method for isolating it from tomatoes and grapes. It is strange that it has only been reported for two of the investigations compared in Table 2. Its presence should have been detected by paper chromatographic analysis, for γ-amino butyric acid separates without interference on most paper chromatograms. Kim et al. (1962) found an unknown amino acid by unidirectional paper chromatography which might have been γ-amino butyric acid. Both the author and De Bruyn et al. (1971) found γ-amino butyric acid to be the third most abundant amino acid in the tomato. Luh and Daoud found it to be the second most abundant amino acid, but they did not report the presence of glutamine.

Only Shinano et al. (1956) have reported γ-amino butyric acid in tomatoes as a result of paper chromatographic analyses. Under the conditions they used, it gave a spot very close to that of valine. Perhaps other investigators have mistaken γ-amino butyric acid for valine. Safina (1953), Ito (1954), and Hamdy and Gould (1962) all reported relatively large amounts of valine, yet investigators who used ion exchange chromatographic techniques have found only small amounts of valine in tomatoes. Only Saravacos et al. (1958) found valine in amounts as high as

15–35 mg/100 ml juice in a few of their samples. Valine is widely separated from γ-amino butyric acid by ion exchange chromatography.

The author has always found this amino acid in relatively high concentration in all tomato samples he has analyzed. On a number of occasions it has failed to separate from ammonia, due to variations in column temperature or buffer composition. It seems reasonable to suggest, therefore, that those investigators who did not observe γ-amino butyric acid on ion exchange chromatograms of amino acids in tomatoes failed to do so because it co-chromatographed with ammonia under the conditions they used for their separations. Saravacos et al. (1958) did not continue their chromatograms long enough for γ-amino butyric acid to be eluted.

Tryptophan. Tryptophan has been reported in tomatoes by Safina (1953), Carañgal et al. (1954) and Luh and Daoud (1968). Tryptophan is best determined by independent analytical procedures. It has not been reported by the author, but may be represented by one of the unknown peaks found in the chromatograms. More work on the amount of tryptophan present in tomatoes under various conditions is desirable.

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EFFECTS OF SUB-ATMOSPHERIC PRESSURE STORAGE ON RIPENING OF TOMATO FRUITS

INTRODUCTION

STUDIES ON physiology and biochemistry of tomato ripening have been conducted in our laboratories for the past 10 yr in an attempt to elucidate the ripening process and biogenesis of flavor compounds during the physiological stages of development, maturation, physicochemical treatments and artificial ripening (Boe and Salunkhe, 1963, 1967; Boe et al., 1967, 1968; Dalal et al., 1965, 1966a, 1966b; Shah et al., 1969; Wu and Salunkhe, 1972; Yu et al., 1967, 1968a, 1968b, 1968c).

The storage life of several fruits and vegetables is increased from 20 to 92% when stored in a refrigerator with a pressure of 658–709 mm Hg (Hummel and Stoddard, 1957). The storage life of fruits such as tomato, avocado, mango, sweet cherry, lime and guava can also be extended when stored at pressures from 125–200 mm Hg (Burg and Burg, 1966; Tolle, 1969). The refrigerated life of apricots was also extended when stored under sub-atmospheric pressures (Wu and Salunkhe, 1972).

The purpose of this study was to determine the effects of various levels of sub-atmospheric pressures on the ripening behavior and related biochemical and flavor changes in tomato fruits during storage and subsequent ripening under normal atmospheric conditions.

MATERIALS & METHODS

THERE WERE THREE sub-atmospheric pressure treatments (471 mm Hg, 278 mm Hg, and 102 mm Hg) and one control (646 mm Hg, the atmospheric pressure at Utah State University, Logan, Utah). Both control and treated tomatoes in triplicate, each replication containing 60 fruits, were stored in 19 liter fruit chambers, maintained at 55 ± 2°F and continuously evacuated by means of a vented-exhaust, oil-seal pump. Constant ventilation was achieved by admitting air to each chamber through a vacuum regulator (Matheson Model 49) which maintained the selected vacuum (none in the control) by allowing air to be bled into the system at the proper rate. Incoming air was saturated with moisture (containing a volatile fungicide-sec. butylamine) by passing through a humidifier. The air flow through the apparatus (Fig. 1) was regulated with a valve at the rate of 30 ml per min. By so doing, relative humidity was maintained at 90–95%.

The experiments were conducted three different times during the year 1971–1972. Greenhouse tomatoes (*Lycopersicon esculentum* Mill., c.v. Michigan–Ohio hybrid) were used for the first and second experiment. Field tomatoes (c.v. DX-54) were used for the third experiment. They were selected for weight and size uniformity and absence of mechanical and pathological injury. The 'green-wrap' tomatoes of uniform maturity which sank in 35% ethanol and floated in 25% ethanol were used for the experiments.

Tomato fruits were sampled periodically for measuring ripening coefficient and chemical analyses. These included chlorophyll, lycopene, β-carotene, starch, sugars and flavor compounds. After storage for 100 days, tomatoes originally stored at 102 mm Hg were then ripened at 646 mm Hg, 55°F and 90–95% RH. A numerical value for color (1 = green, 2 = breaker, 3 = pink, 4 = red and 5 = red ripe) was assigned for fruit in a given sample at each

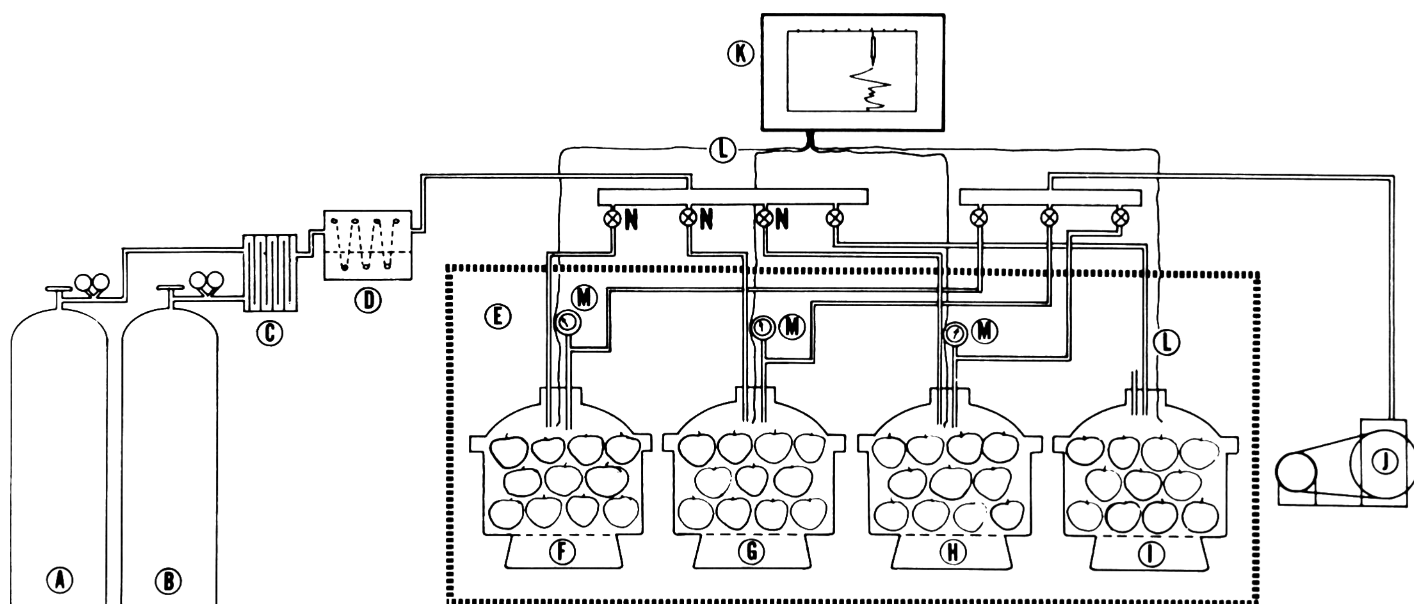

Fig. 1—Schematic diagram of the apparatus for storage of fruits at sub-atmospheric pressures. (A) Nitrogen; (B) Oxygen; (C) Mixer; (D) Humidifier containing volatile fungicide-sec. butylamine in humidifying water; (E) Constant temperature chamber; (F) Storage container with 471 mm Hg pressure; (G) Storage container with 278 mm Hg pressure; (H) Storage container with 102 mm Hg pressure; (I) Storage container with 646 mm Hg (atmospheric pressure at USU); (J) Vacuum pump; (K) Temperature recorder; (L) Thermocouple; (M) Vacuum gauge; (N) Vacuum regulator.

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observation period. The average value for color is termed the ripening coefficient of the sample. Chlorophyll was analyzed according to the method of AOAC (1960). Lycopene and β-carotene were determined as outlined by McCollum (1953). Soluble sugar was measured by Somogyi-Shaffer method (AOAC, 1960). Starch was extracted and determined by the method of McCready et al. (1950).

Volatile flavor compounds were extracted according to a method of Shah et al. (1969). The ether extract of the steam distillate (700 ml) was concentrated to 200–225 μl. A 1 μl sample was injected into a glass column (1.8 m × 6 mm OD, 10% SE-30 on Chromosorb W. acid washed 60/80, program rate 4°c/min.) fixed to a Packard 7400 gas chromatograph with a single flame ionization detector. Nitrogen was the carrier gas at a flow rate of 30 ml/min. The peaks were identified on the basis of enrichment retention techniques. Analysis of variance was made and the means were compared according to Tukey's ω-procedure (Steel and Torrie, 1960).

RESULTS & DISCUSSION

THE DATA presented in this paper were that of the third experiment using field tomatoes. However, all three experiments gave similar results.

Ripening behavior

The ripening of 'green-wrap' tomato fruits was retarded and the storage life extended by sub-atmospheric pressures (Fig. 2). The ripening coefficients of treated tomatoes are significantly (0.01 level) different from that of control. Under normal conditions (control) 'green-wrap' tomatoes ripened in 35 days at 55°F; storage at 471 mm Hg tomatoes ripened in 65 days, and at 278 mm Hg in 87 days. Tomato fruits never ripened and deteriorated if stored at 102 mm Hg beyond 100 days. As shown in Figure 3,

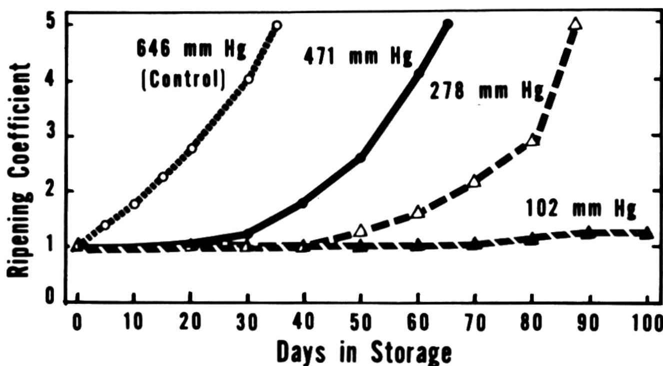

Fig. 2—Effect of sub-atmospheric pressure storage on the ripening behavior of tomatoes at 55°F and 90–95% RH.

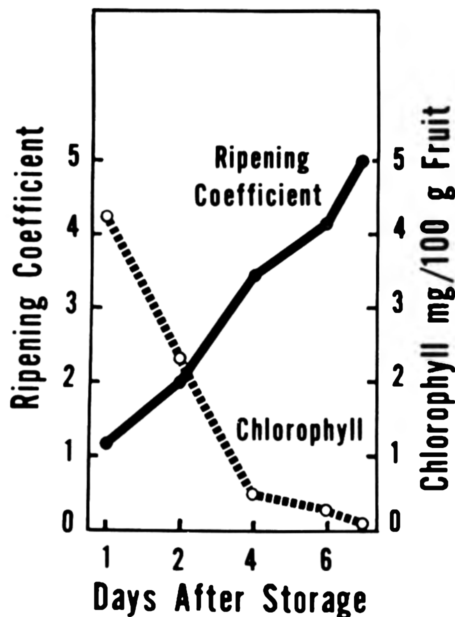

Fig. 3—Ripening behavior and degradation of chlorophyll of tomatoes at 646 mm Hg after storage at 102 mm Hg for 100 days at 55°F and 90–95% RH.

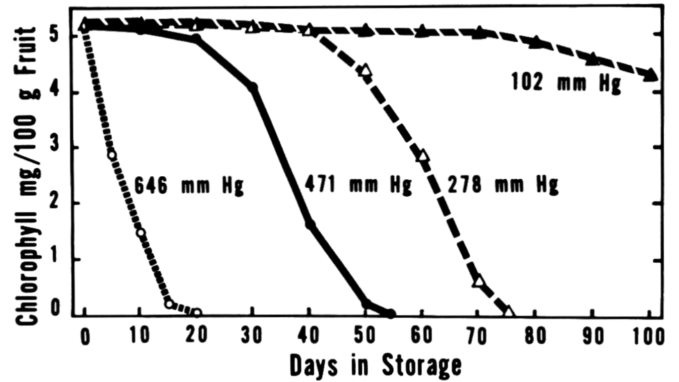

Fig. 4—Effect of sub-atmospheric pressure storage on the degradation of chlorophyll of tomatoes at 55°F and 90–95% RH.

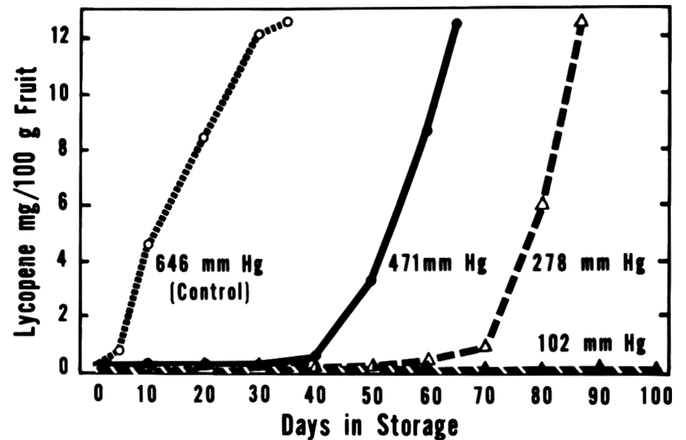

Fig. 5—Effect of sub-atmospheric pressure storage on the formation of lycopene of tomatoes at 55°F and 90–95% RH.

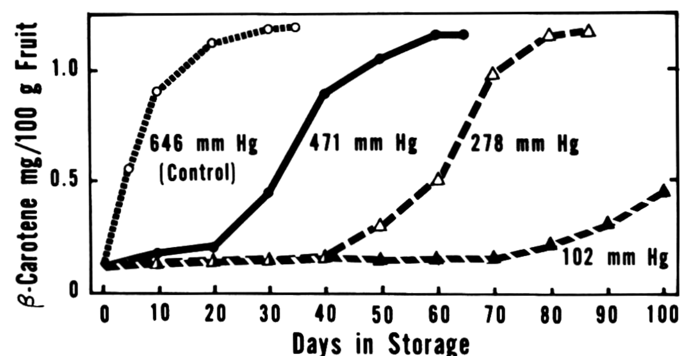

Fig. 6—Effect of sub-atmospheric pressure storage on the formation of β-carotene of tomatoes at 55°F and 90–95% RH.

tomato fruits previously stored at 102 mm Hg for 100 days ripened normally in 7 days when transferred to 646 mm Hg (atmospheric pressure at Utah State University, Logan, Utah) at 55°F and 90–95% RH.

Chlorophyll

The loss of chlorophyll from the fruit was delayed by storage at sub-atmospheric pressures (Fig. 4). The lower the pressure the greater the delay. Loss of chlorophyll proceeded normally when fruits stored at 102 mm Hg were placed at 646 mm Hg.

Lycopene and β-carotene

Synthesis of lycopene and β-carotene of tomato fruits was inhibited by sub-atmospheric pressures (Fig. 5 and 6). The lower the pressure the longer the inhibition, especially lycopene. The formation of lycopene is negligible in the early stage of storage. It becomes significant in the late storage. With storage at 102 mm Hg, lycopene formation was completely inhibited for 100 days (Fig. 7). After transfer to 646 mm Hg, lycopene formation was stimulated. The formation of β-carotene is less inhibited by sub-atmospheric pressure when compared with that of lycopene.

Starch and sugars

Sub-atmospheric pressure storage inhibited starch degradation and sugar formation (Fig. 8 and 9). Under normal conditions, starch was decreased and sugar was increased during the ripening of tomato fruits. The inhibition of this process was more distinct in the early stages than in later stages. At 102 mm Hg, this inhibition prolonged for 90 days. After 100-day storage at 102 mm Hg and then 7 days at 646 mm Hg (Fig. 10), the sugar content of the red ripe fruit was significantly (at 0.05 level) less than that of fruit stored at 646 mm Hg for 35 days. However, the fruits were still marketable.

As suggested by Burg and Burg (1966), lowering the air pressure reduces the amount of oxygen available for fruits while ethylene and carbon dioxide were eliminated by continued evacuation. Ripening was thus delayed. The same effect

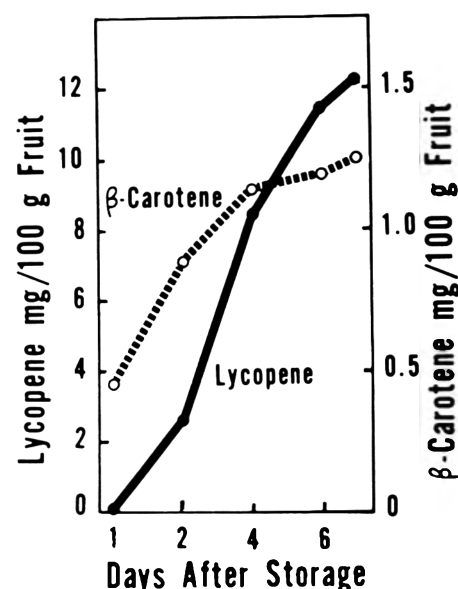

Fig. 7—Formation of lycopene and β-carotene of tomatoes at 646 mm Hg after storage at 102 mm Hg for 100 days at 55°F and 90–95% RH.

Table 1—Effect of sub-atmospheric pressure storage on the concentration of tomato volatiles

Peak no.	Compound[b]	Peak areas mm²[a] 646 mm Hg	471 mm Hg	278 mm Hg	102 mm Hg
1	Acetaldehyde	32	14	6	1
6	2-Methyl propanal	99	29	2	1
7	Methanol	60	34	11	2
8	Butanal	28	6	2	–
10	3-Methyl butanal	99	42	9	3.6
13	Hexanal	60	19	2	1
14	Butanol	3	2	1	–
15	Isopentyl acetate	9	6	2.4	1
17	2-Methyl butanol	5	3	1.6	1
20	2-Methyl-3-hexanol	201	84	26	6
26	Hexanol	6	4	1.8	–
32	Linalool	72	42	18	2

[a]Average of duplicate results
[b]Identified on the basis of enrichment retention techniques

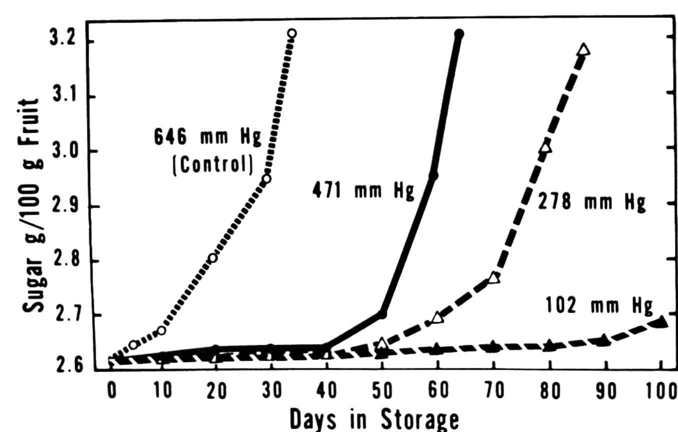

Fig. 8—Effect of sub-atmospheric pressure storage on the sugar content of tomatoes at 55°F and 90–95% RH.

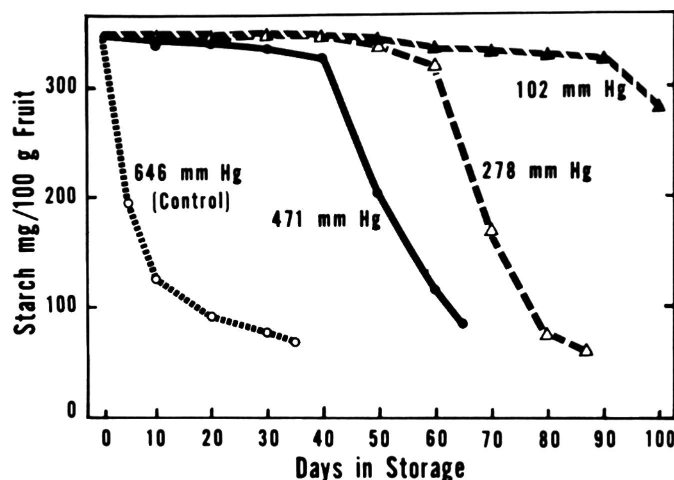

Fig. 9—Effect of sub-atmospheric pressure storage on the starch content of tomatoes at 55°F and 90–95% RH.

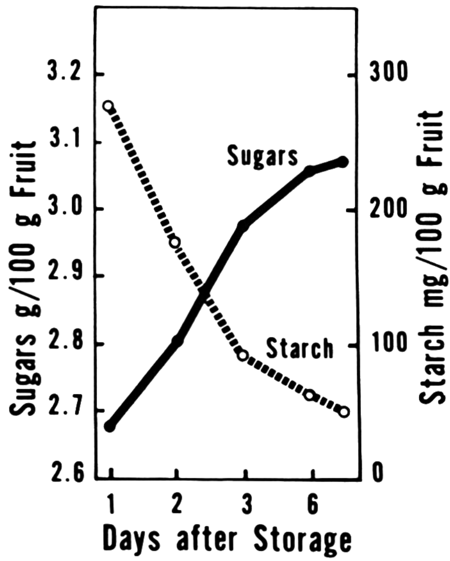

Fig. 10—Changes of sugar and starch of tomatoes at 646 mm Hg after storage at 102 mm Hg for 100 days at 55°F and 90–95% RH.

was produced when the oxygen content of air was reduced (Young et al., 1962). However, at the same oxygen partial pressure, fruits ripened slower in 180 mm Hg than in 760 mm Hg.

Flavor

The patterns of the flavor development under the conditions of low pressures are presented in Figure 11 and the analytical data of major volatiles are expressed in Table 1. The inverse relation between the amount of volatiles and pressure is attributed to a continuous evacuation of flavors in the gaseous state. Compared to control, the fruits under high vacuum for 100 days and subsequent ripening at atmospheric pressure had the least amount of aroma components. This may be due to the removal of flavor compounds and their precursors under vacuum.

As shown in this study, tomato fruits can be stored at 102 mm Hg for 100 days. Beyond that, fruits never ripened and started to deteriorate. However, if tomato fruits were transferred to 646 mm Hg at 55°F and 90–95% RH after 100 days of storage at 102 mm Hg, they ripened normally within the context of the objective measurements used in this paper.

In tomato fruits, decreases in chlorophyll and starch and increases in lycopene, β-carotene, sugar, and flavor accompany the ripening process. Therefore, the inhibition of these changes under the sub-atmospheric pressure can be attributed to the inhibition of the ripening processes of the fruits.

Fig. 11—Gas chromatograms (SE-30 column) of the volatiles from tomatoes subjected to different levels of pressures.

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EFFECTS OF FUNGICIDES IN COMBINATION WITH HOT WATER AND WAX ON THE SHELF LIFE OF TOMATO FRUIT

INTRODUCTION

MAKING AVAILABLE good quality fresh produce at overseas installations is an objective of the Military. From the standpoint of cost, tomatoes are the second most important vegetable item. Experience has shown that current handling practices do not dependably insure that tomatoes arriving in Asia will meet consumer standards. The guidance for Military procurement of fresh tomatoes for overseas shipment specified the use of mature green tomatoes (Anonymous, 1968). Green tomatoes pose particular problems due to their susceptibility to physical, chemical or environmental damage. Damage is manifested by increased susceptibility to infection or by irregular ripening. The only requirement for the handling of tomatoes stipulates that an in-transit temperature of 55°F be maintained (Anonymous, 1968). Tomatoes are purchased without regard to place or origin, post harvest treatment, packaging containers, handling practices or holding conditions. The lack of uniform practices makes it impossible to predict the loss on any one shipment, but losses as high as 70% have occurred. A great deal of research has been conducted on tomato storage requirements (Magoon, 1969; McColloch et al., 1968). The commercial market demands a shelf life of 1–3 wk. The chemical and physical post harvest treatments have been exhaustively reviewed elsewhere (Eckert, 1969; Eckert and Sommer, 1967; Tolle, 1969). Overseas shipments may require a shelf life of as long as 5–8 wk.

The criteria used in the selection of treatments for this research included availability, cost, Food and Drug Administration (FDA) approval for use on tomatoes and proven effectiveness. Each of the selected chemicals has a specific approved residue tolerance on tomatoes with no FDA time limit for application prior to harvest: Maneb (manganese ethylene bisdithiocarbamate), Dithane M45 (coordination of zinc and manganese ethylene bisdithiocarbamate), Thiram (tetramethylthiuramdisulfide; bis (dimethylthiocarbamoyl, disulfide), Botran (dichloronitroanaline), Ziram (zinc dimethyldithiocarbamate), Captan (N-[(trichloromethyl) thio]-4-cyclohexene 1,2-dicarboximide), Sodium O-Phenyl Phenate (OPP), and Difolitan (cis-N-[(1,1,2,2, tetrachloroethyl) thio]-4-cyclohexene-1,2- dicarboximide). In addition, two new chemicals meeting all the above criteria except FDA approval were tested: Benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate) and a 2% solution of chlorine dioxide.

The genera of tomato pathogens exhibits differing sensitivities to each chemical. Their spectrum of control for each chemical has been documented by other researchers working with fresh produce. The following control can be expected in short term storage by each chemical. Meneb, ziram and dithane M45 control *Colletotrichum, Phytopthora, Rhizopus* and *Alternaria* (Mitchell et al., 1968; Chandler, 1965; Harrison and Lockhart, 1963; Crossan and Morehart, 1962). Botran controls some species of *Rhizopus* (Ogawa et al., 1963); thiram controls *Botrytis* (Lockhart and Forsyth, 1964); difolitan controls *Collectotrichum, Alternaria,* and *Stemphyllium* (Chandler, 1965); OPP controls *Penicillium, Rhizopus* and other citrus pathogens (McCornack and Hopkins, 1964; McClure and Smith, 1959); Captan is a "fair to good" fungicide showing slight activity against *Rhizopus* and tomato storage rots (Eckert and Sommer, 1967; Ogawa et al., 1963; Lockhart, 1969); and Benomyl controls *Cladosporium, Botrytis* and *Penicillium* (Ogawa et al., 1968; Gutter, 1969).

Benomyl and OPP have been shown to retain their effectiveness when applied in combination with wax (Gutter, 1969; Hopkins and McCornack, 1959). Addition of fungicide to wax applied on fruits was once widely practiced on citrus and has shown promising results when applied on other crops (Heiligman, 1956). The process offers several advantages when applied on tomatoes. The surface coating by wax produces a lustrous and appealing fruit while helping reduce water loss. The wax also acts as a spreader and sticker giving a maximum uniform deposit of fungicide with a minimum of material. Mixtures of chemicals were shown to give a direct benefit by reducing the concentration of each chemical needed by enlarging the control spectrum and by prolonging their effectiveness (Harrison and Lockhart, 1963; Ogawa et al., 1964).

Dipping fruit in heated fungicide solutions is an area of recent interest. Botran applied in hot water gave a better control of *Rhizopus* and *Monilinia* on peaches than with either treatment alone (Wells and Harvey, 1970). Captan in hot water showed increase control of *Alternaria* and *Rhizopus* (Stewart and Wells, 1970). Singh et al. (1968) studied the temperature tolerance of tomatoes by dipping in hot water. He reported that a temperature of 41–42°C for 10 min had optimum effect on tomato physiology. Higher or lower temperatures had lesser or detrimental effects as indicated by ripening characteristics. He indicated that 41–42°C had no effect on inhibition of storage pathogens.

Table 1 – Reduction in the frequency of occurrence of selected organisms by fungicides[a]

Chemical	Alternaria	Fusarium	Cladosporium	Rhizopus	Botrytis	Penicillium
Maneb	NS	NS	S↓	NS	NS	NS
Dithane	NS	NS	NS	S↓	NS	S↓
Ziram	NS	NS	NS	S↓	NS	NS
Thiram	NS	NS	NS	NS	NS	NS
Captan	NS	NS	NS	NS	NS	NS
Botran	NS	NS	NS	NS	NS	S↑
Benomyl	S↑	NS	S↓	NS	S↓	S↓
Difolitan	NS	NS	NS	S↓	NS	S↓
OPP	NS	NS	NS	NS	S↓	NS
Chlorine dioxide	NS	NS	NS	NS	NS	NS

[a]Information presented represents the results of analysis on data gathered at the end of 8 weeks.
S↑ = significant increase in the frequency of occurrence over the untreated control (5% level)
S↓ = significant reduction in the frequency of occurrence over the untreated control (1% level)
NS = no significant reduction in the frequency of occurrence

EXPERIMENTAL

IN ORDER to comply with Military practices, mature green tomatoes were procured. Although mature green was specified upon purchase, each box invariably contained fruits of various stages of maturity. All fruits were Los Angeles Lug size 6 x 7 (2-4/16 in. – 2-10/16 in.). Fruits were then contained in 10 lb monolayer cardboard cartons with lids. Each fruit was held in a separate paper cup to prevent any contact between neighboring fruits.

The treatments used for this work were adaptations of dip applications. The treatments used were: (1) a room temperature (75°F) solution of fungicide in water; (2) a solution of fungicide in water at 105°F; (3) a room temperature solution of fungicide in a concentrate water soluble wax (Sta Fresh 920C manufactured by FMC Corp.); (4) water at room temperature (control); (5) concentrate water soluble wax at room temperature (wax control). Dipping was accomplished in 3-gal capacity plastic tanks. This container filled with 1½ gal of solution accommodated one 10 lb box of fruit. The room temperature treatments were equilibrated to 75°F for 30 min. The 105° solution was held in a large volume automatic water bath. The concentration of active ingredients for each treatment was tailored to leave a residue within the FDA tolerance.

Eight boxes of fruit were used in each test. The fruits in all eight boxes were selected so that all boxes contained the same number of fruit (40 per box) of similar size and maturity (based on external color). The treatments involving fungicide were each replicated on two boxes of fruit. The weight of the treated fruit was recorded. Preparation of the fruit for storage was completed within 8 hr from the time the tomatoes were received. The storage temperature for the 8 weeks period was 50–55°F. This is the lowest temperature at which tomatoes can be stored without causing chilling injury. Mature green tomatoes stored below 50°F are more susceptible to infection by *Alternaria* (McCulloch and Worthington, 1952), *Geotrichum* (Butler, 1960) and fungi. The quantity of fruits utilized in this research made it necessary to evaluate the effectiveness of each treatment during storage under normal atmospheric conditions. The storage temperature and normal atmospheric environment simulates current Military shipping practices. The tests were analyzed at 7–10 day intervals to assess the number of infected fruit in each box. At the conclusion of the experiment a determination of storage rot pathogens was made by direct analyses and by isolation of nonsporulating lesions onto potato dextrose agar (Table 1). The usable yield percentage was determined by noting the weight of the fruits either showing no infection or with no more than two superficial infections and dividing by the initial weight of all the fruit in that box (Table 2). All results were statistically evaluated by analyses of variance.

RESULTS & DISCUSSION

FIGURES 1–4 show interesting parallels in the results of the general treatments and the controls. Almost all the treatments delayed the spread of infections within the boxes. Treated and untreated fruits showed a low incidence of infection for 2–3 wk. The number of infections showed the greatest rise between 3 wk and 6 wk. After 6 wk a plateau was reached and nearly all fruits in a box became infected.

The benefits expected by combining chemicals with wax were borne out by results of this work. The wax formulations in all tests were significantly better than both 75°F or 105°F water solutions of the fungicides (Fig. 1–4). Wax without fungicide gave significant extension of shelf life in four of nine experiments. Only thiram, benomyl, difolitan, maneb and dithane applied at 75°F and/or at 105°F showed significant improvement over wax without fungicide; captan showed marginal improvement and chlorine dioxide, ziram and botran showed no improvement. There were no benefits derived from applying fungicide at the higher temperature. A difference was seen

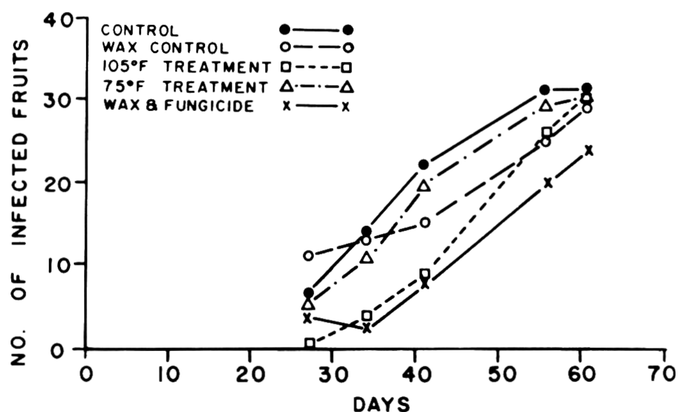

Fig. 1–Tomato infections as affected by physical and chemical (Dithane) pretreatments.

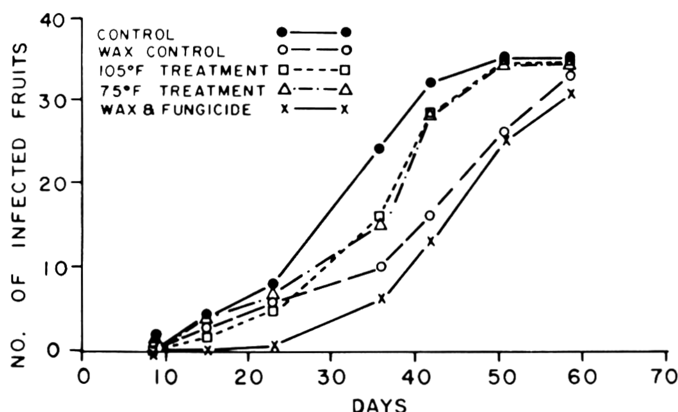

Fig. 3–Tomato infections as affected by physical and chemical (OPP) (O-phenyl phenate) pretreatments.

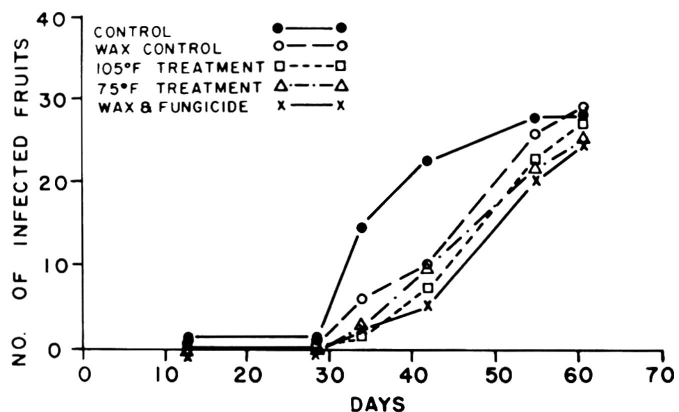

Fig. 2–Tomato infections as affected by physical and chemical (Captan) pretreatments.

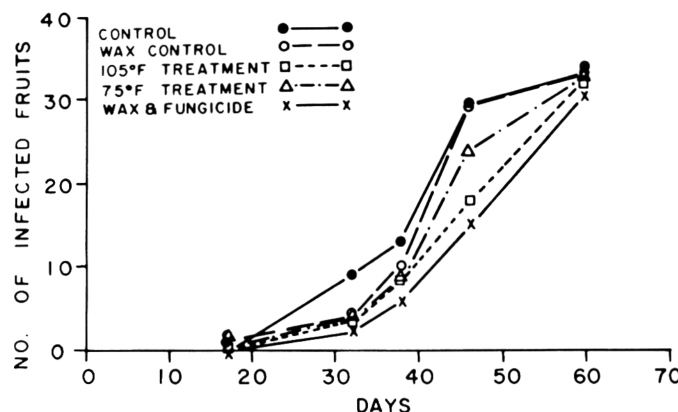

Fig. 4–Tomato infections as affected by physical and chemical (Thiram) pretreatments.

Table 2–Usable yield percentage determined at the end of 60 days storage[a]

	Maneb %	Dithane %	Ziram %	Thiram %	Captan %	Botran %	Benomyl %	Difolitan %	OPP %	Chlorine %	Avg of all fungicides for each treatment (across) %
Control	0	0	0	0	3.1	0	0	0	0	0	0.34
Wax control	0	7.5	5.9	5.8	6.4	0	0	0	20.3	6.8	5.856
75° water	0	4.5	1.8	6.4	18.4	0	0	5.1	1.5	0	4.011
105 Water	0	7.7	1.45	7.7	19.9	0	0	0	0	3.1	3.97
Wax plus fungicide	0.45	31.2	11.8	14.6	24.9	1.8	8.9	1.8	17.8	6.3	11.955
Avg of all treatments for each fungicide (down)	0.15%[a]	14.4%	5.05%	9.55%	21.83%	0.583%	2.96%	2.28%	6.40%	3.10%	

[a]The statistical analysis was performed on the average of all three methods of application for each chemical and comparison made against the control and wax control. The significant level used was the 5% level.

in the ability of each chemical to provide benefit by one or another of the treatments.

The usable yield of all treatments was greater than the untreated controls (Table 2).

Statistical analysis of the average of all fungicides for each treatment showed that wax plus fungicides gave a significantly greater yield than the control or wax control. The analysis performed on the average of all treatments for each chemical showed that only captan gave significantly higher yield when compared against its control and the wax control.

The only fungicide that showed any visually evident effect on ripening was difolitan. The fruit surface was spotty in appearance, covered with light pink spots. The appearance was considered unsatisfactory. With all the wettable powder or flowable chemicals an unsightly deposit remained on the surface of the tomato. Since the tomatoes will not be resold, this is considered a minor drawback. The susceptibility of tomatoes to infections is a direct reflection of the physiologic condition of the fruit (Morris, 1972). The period of rapid infection spread probably corresponds to a period of physiologic aging and breakdown. The chemical and physical changes that take place during storage of tomatoes are discussed in several papers by Hall (1963, 1964, 1965, 1966, 1968).

The effectiveness of the chemicals in inhibiting the infection and growth of the fungi was inconclusive. The authors were interested in determining whether or not a chemical that is reported as controlling an organism for 1–3 wk would still control that organism at 8 wk. Table 1 shows, for example, that *Fusarium* was not controlled by any of these chemicals.

Penicillium (ssp), were controlled by dithane, benomyl and difolitan. Benomyl seemed to continue expected control up to 8 wk, better than any other material. It controlled *Botrytis, Penicillium* and *Cladosporium*. As expected, benomyl showed no control of *Alternaria*. Probably the reduced competition from the other organisms allowed *Alternaria* to show the marked increase in infection. Ziram and dithane controlled *Rhizopus*. Maneb controlled *Cladosporium*, but not *Alternaria*. *Botrytis* was controlled by OPP; *Penicillium* was not. Botran, thiram and captan did not show significant control of any organisms.

The amount of loss that occurs with all chemical treatments after 60 days rules out the use of chemicals with cold storage as a practice for that length of time. The authors, therefore, refrain from trying to establish a "one best chemical" in this study. The aim was to evaluate these chemicals for future work.

In making an evaluation as to the value of a chemical one must consider the interaction of many factors. For example, the types of decay caused by the various pathogens. *Botrytis, Rhizopus* and *Fusarium* are fast growing soft-rot causing fungi, that engulf the entire fruit. A low incidence of infection by these organisms will cause a rapid decay of all fruit in a box giving a very low yield. Due to their competitive advantage, they cause a low incidence of infection by other slow growing pathogens. *Alternaria*, *Cladosporium* and *Penicillium* cause a slow-spreading lesion on the fruit surface. This type of lesion, when small, can be removed and the rest of the tomato used. A high frequency of occurrence of these fungi can still give a high yield. It is evident that the type of organisms a chemical will control is directly related to the yield and can be irrespective of the frequency of occurrence. A chemical-like captan that caused a marginal reduction of all organisms and concurrently showed insignificant reduction in incidence of infection can still show a high yield. This is probably explained by the fact that no organism got out of hand, and the rapid rise in fruit infections was retarded until about 35 days.

CONCLUSIONS

BASED on the interpretation of the interaction among all factors in the experiment, it is concluded that captan, dithane, OPP and thiram showed the greatest potential as fungicide treatments on tomatoes. The best method of application was to apply fungicides in the wax solution. The chemicals selected showed the greatest usable yield which ultimately is the most important criteria. Benomyl did an outstanding job preventing growth by the most damaging pathogens. For this reason the authors consider benomyl to be a very promising fungicide, to be used in combination with other fungicides. Further work is underway to explore the usefulness of the selected method and chemicals for use with other storage practices, such as (CA) controlled atmosphere.

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RESEARCH NOTES

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A Research Note

FATTY ACID CONTENT OF FRANCHISE CHICKEN DINNERS

INTRODUCTION

FRANCHISE FOOD OUTLETS have shown rapid growth in recent years accounting for over 30,000 of the 145,000 restaurants in the U.S. (Anon., 1970). Of the major franchise chicken operations, Kentucky Fried Chicken has over 3700 outlets (Anon., 1971). Public concern over the nutritional quality of food served at franchise outlets has also increased, and kinds and amounts of fat present in the food are of special interest. The Food and Nutrition Board (1968) recommends unsaturated fatty acids should replace saturated fatty acids. Essential fatty acids appear to be necessary for fat utilization, thus the ratio of unsaturated to saturated fatty acids in the diet may be important (Alfin-Slater and Aftergood, 1964; Jolliffe, 1961).

The kinds and amounts of fatty acids in institutional meals have been investigated by several authors. Osborn and Ohlson (1963) employed gas-liquid chromatography in the fatty acid analyses of meals served at a hospital cafeteria. Cafeteria meals served over an 84-day period contained an average of 5.6% myristic, 26.2% palmitic, 26.3% stearic, 38.6% oleic and 8.8% linoleic acid. The ratio of unsaturated to saturated fatty acids was approximately 5 to 6. Using gas-liquid chromatography Murphy et al. (1970) reported a similar pattern of fatty acids in Type A school lunches. Five fatty acids accounted for approximately 90% of the total fatty acid content. The lunches contained an average of 5% myristic, 27% palmitic, 14% stearic, 33% oleic and 10% linoleic. The ratio of unsaturated to saturated fatty acids was 1 to 1.

The objective of the present study was to evaluate franchise chicken dinners for fatty acid content.

MATERIALS & METHODS

CHICKEN DINNERS were purchased from six franchise food outlets in Gainesville. Franchises were coded by randomly assigning a letter to each of the six franchises. Five dinners were purchased from each franchise with a time of 1–4 wk elapsing between individual purchases from the same franchise.

Bones were removed from the fried chicken and total dinners were homogenized in a Waring Blendor and dried in a Virtis freeze dryer. 3g of dried sample were extracted for 30 min with petroleum ether at the rate of 6–8 drops of ether per second using a Goldfisch extractor (AOAC, 1970). This extraction gave approximately 1g of crude fat which was then saponified to free the fatty acids in a manner similar to that recommended by AOCS (1970).

250 mg of the free fatty acids were then methylated using BF_3-MeOH according to Metcalfe and Schmitz (1961). Fatty acid methyl esters were identified and quantified by use of a Varian Aeorgraph gas chromatograph equipped with a Honeywell recorder. The separations were effected on a 6ft × ¼ in. copper tube column packed with 15% diethylene glycol succinate on chromasorb W as solid support. The column was operated at 185°C with a flow rate of 40 ml of helium per minute. Temperatures of the injection port and thermal conductivity detector were 205°C and 125°C respectively. A 3–5 μl injection of fatty acid methyl esters gave satisfactory peak height after concentration was adjusted by evaporation of petroleum ether. Fatty acid methyl esters were identified by matching their peaks with those of standard fatty acid methyl esters purchased from Applied Science Labs. A Technicon Integrator/Calculator was used to determine peak areas. The relative percent of each fatty acid methyl ester in the sample was determined by dividing area under the individual peak by total peak area.

RESULTS & DISCUSSION

TABLE 1 shows mean and standard deviation for relative percent fatty acid content of the chicken dinners. Fatty acid peaks were well separated on the chromatogram. Analyzed fatty acids would not be expected to contain foreign substances since fats were extracted with petroleum ether, saponified, and fatty acids extracted with diethyl ether. Five fatty acids constituted approximately 98% of the total fatty acids present in the extracted fat. Fatty acids not listed include myristic and linolenic acid which were present in trace amounts.

The ratio of unsaturated to saturated fatty acids in the dinners averaged 2.5/1.

Table 1—Percent fatty acid content of total edible dinner—mean ± standard deviation for relative percents (average of five dinners from each franchise)

Franchise	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic	Other
A	17.0	1.4	9.3	53.5	18.5	0.3
	± 0.2	±0.3	±0.5	± 0.6	± 0.7	
B	23.2	2.6	6.8	44.8	22.1	0.5
	± 0.9	±0.5	±0.4	± 1.5	± 1.3	
C	19.4	2.4	6.6	45.7	24.1	1.8
	± 0.8	±0.2	±0.3	± 2.2	± 2.9	
D	19.2	2.8	6.3	46.9	23.0	1.8
	± 1.2	±0.3	±0.4	± 1.2	± 1.2	
E	23.4	2.6	9.6	44.2	19.1	1.1
	± 0.7	±0.5	±0.5	± 1.0	± 1.0	
F	18.7	2.3	4.9	44.1	28.0	2.0
	± 0.6	±0.6	±0.4	± 1.9	± 1.5	
Mean	20.2	2.4	7.3	46.5	22.5	1.3

Although this ratio represents fatty acids in the entire fried chicken dinner, it corresponds well with the unsaturated to saturated fatty acid ratio of 2.6/1 reported for fried chicken alone in USDA Handbook No. 8 (Watt and Merrill, 1963).

Differences in dinner components and cooking methods contribute to differences in fatty acid content. Prolonged heating of frying oils could cause oxidation of the unsaturated fatty acids (Kaunitz, 1967).

Although the Food and Nutrition Board makes no recommendation for dietary fatty acids, Holman (1960) suggests that linoleate should comprise 1% of the dietary calories. Linoleate comprises approximately 22.5% of the extracted fatty acids (Table 1). Assuming total fat to contain 90% triglycerides, the average amount of linoleate in fat would be 0.90 × 22.5% or 20.3%. Fat contributes an average of 50% of the total caloric content of the dinner therefore 0.50 × 20.3% or approximately 10% of the total calories would be provided by linoleic acid. Based on Holman's observations, the dinners would contain adequate amounts of linoleic acid.

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A Research Note

THE EFFECT OF FLAVOR ENHANCERS ON DIRECT HEADSPACE GAS-LIQUID CHROMATOGRAPHY PROFILES OF BEEF BROTH

INTRODUCTION

THE USE OF various flavor enhancers has continually been increasing in foods (Kuninaka et al., 1964; Shimazono, 1964; Connell, undated). Their relative importance has also merited several symposia specifically organized to discuss their properties (Qm.F&CI, 1948, 1955; Arthur D. Little, 1964). Numerous reports have appeared on the flavor modifying properties of these products in various food systems (Sjostrom and Crocker, 1948; Caul and Rockwood, 1953; Caul and Raymond, 1964, Kurtzman and, Sjostrom, 1964; Hashida et al., 1966; Sawyer, undated; Stier et al., undated; Woskow, 1969). However, the exact mechanism by which enhancers modify flavor is not known nor do many theories exist postulating a plausible explanation. Aside from modifying taste perception (Woskow, 1969) several reports have appeared indicating that enhancers also can affect aroma (Cairncross and Sjostrom, 1948; Sjostrom et al., 1955).

Therefore, this study was undertaken to measure by analytical means any possible difference in odor resulting from the additions of various common enhancers and to postulate possible methods of reaction if a difference were found.

EXPERIMENTAL

THE BEEF BROTH used in this study was prepared by simmering at atmospheric conditions for 1 hr 1000g of beef bones in 1 liter of tap water. Upon cooling, the mixture was filtered and the filtrate divided into four equal portions. No enhancer was added to one portion while to the other portions 0.05% of the following enhancers were added: monosodium glutamate (MSG), equal protions of disodium 5'-inosinate and disodium 5'-guanylate, and equal portions of the above two enhancers. The samples were stirred and then refrigerated for 24 hr. The following day all samples were permitted to equilibrate to room temperature and the vapor from 2 ml of each sample was analyzed using the same techniques and GLC conditions as reported by Lorenz and Maga (1971) except in this study a Hewlett Packard Model 3370 B Automatic Integrator was used to measure individual peak areas.

RESULTS & DISCUSSION

IN ALL CASES the addition of enhancers altered peak areas although no new peaks appeared nor did existing peaks disappear through the incorporation of enhancers in beef broth. The headspace evaluation of water and enhancer combinations revealed no measurable peaks. As can be seen in Table 1, the areas of eight specific peaks were compared as well as total peak areas.

It was found that the addition of 0.05% MSG to the broth increased total peak area 1.66 times over that of the control broth containing no enhancer addition. The enhancement action of MSG appeared to be fairly consistent among all eight peaks and ranged from an increase in peak area of from 1.39 times for peak 4 to 1.81 times for peak 8.

With the incorporation of the 5'-ribonucleotide mixture total peak area was increased substantially over the control to 2.30 times its total area. Individual peak variations ranged from a low of 1.78 times for peak 4 to 2.68 times for peak 7.

Interestingly both enhancers had their minimum effect on the same peak, thus indicating that as with flavor (Woskow, 1969) enhancers affect the odor of different compounds to varying degrees. An interesting study would be to measure the effect of enhancers on the odor properties of individual pure compounds representing various classes of compounds associated with foods. This would demonstrate which classes or specific compounds are most susceptible to enhancer modification.

Table 1—Effect of flavor enhancers on GLC headspace peak area ratios

Peak	Area ratio[a]	Area ratio[b]	Area ratio[c]
1	1.51	2.09	2.17
2	1.53	2.13	2.31
3	1.48	2.30	3.52
4	1.39	1.78	2.79
5	1.58	2.27	3.00
6	1.57	2.20	2.02
7	1.64	2.68	2.47
8	1.81	2.22	2.13
Total area	1.66	2.30	2.35

[a]Control vs. 0.05% MSG
[b]Control vs. 0.05% total of disodium 5'-inosinate and disodium 5'-guanylate
[c]Control vs. 0.05% total of above two enhancers

The synergistic effect of enhancer combinations was apparent since the MSG – 5'-ribonucleotide mixture increased total peak area the most. Also, the combination of enhancers resulted in the greatest individual peak area increase since peak 3 had an area increase of 3.52 over that of the control peak 3.

The above observations could theoretically be explained in several ways. The most obvious explanation would be that the incorporation of the enhancers had altered the vapor pressure of the volatile compounds in the system thus resulting in increased volatility. However, at the concentration used in this study (0.05%), this theory is not probable. Thus one is led to believe that a chemical reaction or bonding had occurred upon the addition of the enhancers. A chemical reaction theory would support the observation reported by Caul and Raymond (1964) that enhancers increase the apparent viscosity of liquid products.

CONCLUSION

BY RELATIVELY SIMPLE MEANS, the data reported demonstrate that not only can flavor be modified through the incorporation of enhancers but as suggested by earlier investigators they also modify odor profiles.

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A Research Note
WATER BINDING OF SOME PURIFIED OILSEED PROTEINS

INTRODUCTION

THE AMOUNT OF water adsorbed by a mixture of components, as in a food system, to a first approximation is the sum of the water bound by the component parts, principally the salts and protein (Berlin et al., 1968). Therefore the amount of water bound by proteins is of some concern to those interested in equilibrium moisture content of protein foods. This note reports on the quantitative measurement of water binding of some food proteins, with emphasis on the oilseed proteins. By "water binding" is meant the water vapor adsorbed by a dried protein powder, after equilibration against water vapor of known relative humidity, which in this case was 84%.

EXPERIMENTAL

Protein samples

Casein, bovin serum albumin and dried egg whites were purchased from Sigma. Soybean isolates were obtained from Central Soya ("Promine D") and Grain Processing Corp. ("Pro Fam 90 H/S"). Cottonseed isolates were prepared by the two-step method of Berardi et al. (1969) from glandless cottonseed. Coconut protein was prepared by precipitation at pH 5.0 after extraction of fresh coconuts at pH 8 (Hagenmaier et al., 1972). Peanut protein was precipitated at pH 4.5 after extraction of full-fat peanuts at pH 8.0 (Rhee et al., 1972). Fish protein concentrate was prepared from deboned hake by hot isopropyl alcohol extraction (Aberdeen Pilot Plant). Sunflower protein was prepared by the method of Gheyasuddin et al. (1970) by extraction at pH 10.5 and precipitation at pH 5.0, without the alcohol wash.

The peanut and coconut samples were initially rather high in oil content (8% and 25%), and were consequently hexane extracted to achieve oil contents of less than 1%. All samples were subjected to extended dialysis to remove hydrophilic salts. The pH values of the liquid suspensions were adjusted to the desired value and the entire suspension freeze dried in each case. Total nitrogen and amide nitrogen contents of all freeze-dried samples were measured and the results are included in Table 1.

Methods

Water binding was measured as weight uptake after exposure of the dry protein sample to water vapor of 84% relative humidity (over saturated KCl), after the method of Mellon et al. (1947). Our measurements showed that equilibrium was achieved within 24 hr. Measurements of weight uptake were consequently made after 24–48 hr of equilibration. The reported results are the averages of measurements made on at least three different samples of each protein. All protein samples remained in the solid state after equilibration: they did not form glasses or solutions.

Protein solubility was measured in separate experiments. To measure solubility the freeze-dried protein sample was added to water to make a 1% suspension, the pH maintained at the desired value for 45 min (with intermittent stirring), then the suspensions centrifuged, filtered, and the dissolved protein determined from Kjeldahl analysis of the filtrate. Each reported solubility is the average of at least two measurements.

Amide nitrogen was determined by the method of Chibnall et al. (1958).

The amino acid contents used in calculations were from the literature, with the exception of peanut and coconut, which were measured in this laboratory. The sunflower analysis was from Gheyasuddin et al. (1970); the cottonseed analyses were from Martinez et al. (1970); the soybean was averaged from several sources: company literature, Circle and Johnson (1958) and FAO (1970). The eggwhite, casein and fish analyses were from FAO (1970), and the serum albumin results were from Spahr and Edsall (1964).

RESULTS

THE DATA in Table 1 give the measured values for amide nitrogen and total nitrogen. Also shown are the values for water adsorbed by protein samples adjusted to pH 6.0 before freeze drying, and amount of protein dissolved at pH 6.0. The results are arranged in Table 1 so that the first seven entries are oilseed proteins and the last four are animal proteins.

The data in Table 2 reflect the influence of pH on water binding and solubility. For the water binding results, the pH values pertain to the aqueous suspensions that were freeze dried to give the dry protein samples. For the solubility experiments, the pH values pertain to aque-

Table 2—Effect of pH on water binding and solubility

	Bound water (g/16g N)[a]			% of Protein dissolved[b]		
Protein sample	pH 4.5	pH 6.0	pH 7.5	pH 4.5	pH 6.0	pH 7.5
Cottonseed II	15.6	16.0	16.4	20	5	30
Soybean (Promine D)	18.9	19.2	21.6	5	22	57
Casein	21.1	21.6	26.4	5	90	97

[a]At 84% relative humidity, standard deviation is 0.7.
[b]In 1% aqueous suspension, standard deviation is 3%.

Table 1—Water bound at 84% relative humidity, nitrogen analyses of dry samples and protein solubility in 1% aqueous suspension

Protein sample	Bound water $\left(\frac{\text{g water}}{\text{16g N}}\right)$[a]	% N of dry sample[b]	% of N which is amide N[c]	% of protein dissolved at pH 6.0[d]
Cottonseed isolate I	22.4	15.7	11.9	84
Coconut	21.6	15.8	6.5	37
Soybean				
"Profam 90/HS"	20.3	15.5	8.7	40
"Promine D"	19.2	16.2	9.7	22
Peanut	17.4	17.1	10.1	48
Sunflower	17.3	16.5	11.4	8
Cottonseed isolate II	16.0	18.0	9.2	5
Serum albumin	29.5	15.7	4.1	100
Egg white	24.0	15.4	7.0	94
Casein	21.6	15.5	8.6	90
Fish (FPC)	21.0	16.4	5.5	6

[a]Standard deviation is 0.7.
[b]Standard deviation is 0.1.
[c]Standard deviation is 0.2.
[d]Standard deviation is 2.5.

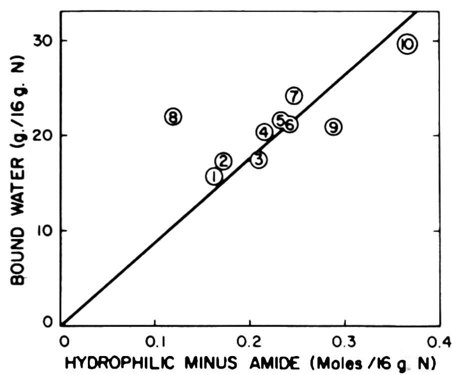

Fig. 1—Dependence of water binding of proteins on moles of hydrophilic groups minus moles of amide groups for (1) Cottonseed II; (2) Sunflower; (3) Peanut; (4) Soybean (Pro Fam 90/HS); (5) Casein; (6) Coconut; (7) Egg white; (8) Cottonseed I; (9) Fish and (10) Serum albumin.

ous suspensions in which solubility was measured. All pH values are accurate to ± 0.2 pH units.

DISCUSSION

THE RESULTS in Table 1 indicate that the animal proteins are generally lower in amide nitrogen and bind more water than the oilseed proteins. The animal proteins are also generally more soluble—with the exception of the fish sample, which is considered to be a denatured protein. The ranking of the oilseed proteins is in order of decreasing water binding. This ranking is for 84% relative humidity, but should hold at all high values of relative humidity. Literature values for water binding of proteins (Bull, 1944) at different water activities indicate, that for relative humidities not greatly different, proteins may be ordered according to water binding capacity at one relative humidity, and that the same order holds at other relative humidities.

The data in Table 2 indicate that pH (of an aqueous suspension of the protein sample) has little effect on water adsorption. The protein solubility, on the other hand, shows the expectedly large dependence on pH. This contrasting dependence on pH indicates that there is not a good correlation between water binding and protein solubility.

The amino acid contents and amide nitrogen values were used to calculate the amount of hydrophilic groups of the different proteins. The hydrophilic groups were taken as hydroxyl plus carboxyl plus basic groups. It should be pointed out that protein carboxyl groups are not merely the sum of glutamic acid and aspartic acid values as normally reported in amino acid analyses, because these values normally include their amides, glutamine and asparagine. The amide nitrogen must be subtracted out to give the true aspartic and glutamic acid contents. For the data in Figure 1 the number of amide groups is subtracted out a second time so that "hydrophilic minus amide groups" is doubly dependent on the amount of amide nitrogen.

The data in Figure 1 indicate that the trend is for increased water binding with larger values of hydrophilic minus amide groups. This relationship was first noted by Bull and Breese (1968). This dependence on amide nitrogen is especially significant for the oilseed proteins, because of their generally large amount of amide nitrogen. The data suggest that deamidation of the oilseed proteins might substantially increase their water binding potential, and consequently render these proteins more valuable for food applications that demand a more hygroscopic protein.

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A Research Note
IMPLICATION OF Bacillus subtilis IN THE SYNTHESIS OF TETRAMETHYLPYRAZINE DURING FERMENTATION OF COCOA BEANS

INTRODUCTION

ALKYLPYRAZINES are important contributors to flavor in many food products. Although major emphasis has been given to pyrazines thermally generated in roasted or fried foods, pyrazine homologs have been reported in nonheated foods. As an example, 2-methoxy-3-isobutylpyrazine was identified as a natural constituent of bell peppers (Buttery et al., 1969) and tetramethylpyrazine (TMP) was found in several fermented foods including Natto (fermented soybeans), vinegar, wine and beer (Kosuge et al., 1969). A strain of *Bacillus* was responsible for the TMP in Natto. Other genera notably *Aspergillus, Acetobacter* and *Saccharomyces,* may be important to TMP synthesis in other products (Kosuge and Kamiya, 1962; Kosuge et al., 1969).

B. subtilis is of particular interest in cocoa bean fermentation since in the latter stages of fermentation, it and other bacilli dominate the microbial population (Ostovar, 1971). Quantitative studies of Reineccius et al. (1972) showed TMP as the only pyrazine in unroasted cocoa beans and then only in samples from countries which traditionally export a well-fermented crop. Roasting studies revealed that TMP was the most easily heat-generated pyrazine in both fermented and unfermented samples of cocoa beans.

Although chocolate flavor does not become evident until the beans are roasted, fermentation after harvesting and opening of the pods is considered necessary for the formation of the flavor precursors. The study, reported herein, on TMP formation in cocoa beans was undertaken to contribute a more thorough understanding of this important process.

EXPERIMENTAL

TO DETERMINE if TMP concentration in cocoa beans could be related to the *B. subtilis* population samples taken at various stages during the fermentation of cocoa beans in Brazil and Trinidad were obtained. The Brazilian beans were dried prior to shipment, but the samples from Trinidad were transported via air freight to the laboratory in an undried state so that *Bacillus* populations could be approximated and compared. The unavoidable time delay involved in the Trinidad shipment was recognized as a complicating factor in the interpretation of data. Nevertheless, it was assumed that relative comparisons would be meaningful.

TMP analysis was according to the GLC method of Reineccius et al. (1972) except that the size of sample was increased from 30g to 100g. Samples were extracted with diethyl ether in a column or with a continuous extractor. An internal standard was then added and the ether extracted with pH 1 water. The aqueous phase was then adjusted to pH 8.3 to make the pyrazines insoluble in water. Dichloromethane was used to extract the pyrazines from the water. The extract was dried over anhydrous Na_2SO_4. Samples were chromatographed on a 10 ft × 1/8 in. Carbowax 20M column programmed from 60 to 190°C at 4°C per minute.

RESULTS & DISCUSSION

RESULTS of the analyses for TMP concentration in cocoa beans and *B. subtilis* population at various stages of fermentation are presented graphically in Figure 1. TMP increased almost linearly with fermentation time in both the Brazilian and Trinidad samples. The plot of log number *B. subtilis* cells showed a similar linear increase which suggested the possibility of microbial synthesis of the TMP found in fermented cocoa beans.

Evidence which implicates *B. subtilis* in the synthesis of TMP was obtained through in vitro studies. A strain of *B. subtilis* isolated by Ostovar (1971) from a fermenting mass of cocoa beans in Trinidad, was cultured for 4 days at 45°C on the synthetic medium described by Kosuge and Kamiya (1962). Following incubation TMP concentration was determined by GLC after recovery from the aqueous culture by continuous, liquid-liquid extraction wtih diethyl ether.

Quantitation of TMP on duplicate samples of the *Bacillus* culture yielded 141 and 154 μg/ml of media with viable cell counts of 21 and 22 × 10^6 respectively. TMP production was thus 6 × 10^{-7} μg/cell. Microbial synthesis involving *B. subtilis* strain from cocoa was substantiated by the finding that incubation of uninoculated sterile media yielded no detectable TMP.

The greater apparent TMP production in the synthetic media compared to fermenting cocoa beans can be logically attributed to environmental variables. An illustration of this was the lower TMP values obtained with the synthetic media when the pH was adjusted from 6.6 to 4.5 which is more typical of fermenting cocoa beans. After incubation the pH was

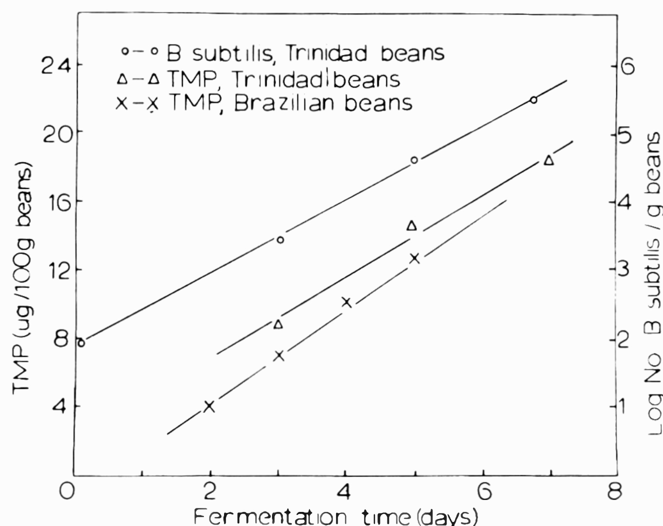

Fig. 1—Changes in B. subtilis population and tetramethylpyrazine concentration during fermentation of cocoa beans. No detectable TMP at 0 days fermentation in either Trinidad or Brazilian beans.

again 6.6; however, TMP production was reduced to 73 μg/ml.

Tracer techniques utilizing uniformly labeled carbon-14 glucose added to unfermented beans during an in vitro fermentation in our laboratory showed labeled TMP only when cell growth occurred during incubation. Addition of 100,000 units of penicillin resulted in no viable cells or TMP after incubation. This is consistent with the data of Koehler et al. (1969) which suggested that the carbon atoms of the thermally-generated pyrazines come from sugar moieties. Also van Pragg et al. (1968) showed that sugars are fragmented to form intermediates such as acetoin, 2,3-butanediol and pyruvaldehyde which then condense with amino acids and/or ammonia to form methyl-substituted pyrazines. It is well known that *B. subtilis* and other microorganisms are able to produce large amounts of acetoin and ammonia which may be the precursors of TMP as proposed by Kosuge et al. (1969).

Conversion of these intermediates to TMP could be enzymatically or thermally induced. The center of a fermenting mass of cocoa beans may exceed 50°C.

The level of TMP in unroasted beans may be of practical importance to the chocolate manufacturer in that it could be used as an index of the degree of fermentation and the potential quality of the beans prior to roasting.

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A Research Note
EFFECTS OF LIGHT AND TEMPERATURE ON THE FORMATION OF SOLANINE IN POTATO SLICES

INTRODUCTION

SOLANINE, a glycoalkaloid, occurs in potato tubers in variable amounts depending upon the variety, stage of development and the environmental conditions. An increased amount of solanine in tubers results from light exposures (Conner, 1937; Gull and Isenberg, 1958, 1960; Liljemark and Widoff, 1960; Patil et al., 1971). The peeling of such tubers removes about one-third of the total glycoalkaloid but residual amount is still high enough to render potatoes unpalatable and unsafe for food consumption (Zitnak, 1961). In recent years, intensification of bitter taste and solanine poisoning of potatoes have been associated with technological developments. In most supermarkets, potatoes are displayed under artificial light to appeal to customers or, in many instances, potato slices are held for hours in the processing plants that are flooded with high intensity lights. These can stimulate the formation of solanine in potatoes and raw potato products.

Since little is known about the effects of light and temperature on solanine formation in potato slices, this study was undertaken.

EXPERIMENTAL

RUSSET BURBANK potato tubers of uniform size were washed, peeled and then cut into 0.7 × 0.7 × 5 cm slices. Such slices were divided into two groups: one was stored in the dark and the other was exposed to 200 ft-c (18.5 lux) fluorescent light. Each was subjected to four temperature treatments: 0°, 8°, 15° and 24°C at 90–95% R.H. There were three replications for each treatment and each replication consisted of 400g of slices. A sample was taken every 12 hr and the experiment ended in 48 hr. The method of solanine extraction and determination was that of Gull and Isenberg (1960). 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, temperature affected the level of solanine in potato slices. At low temperatures (0° and 8°C) there was a slow but significant increase in solanine content during a 48-hr period in the dark while the storage temperatures of 15° and 24°C vigorously stimulated the formation of solanine. After 48 hr at 24°C in the dark, the solanine content reached a concentration of 2.05 mg per 100g slices. This is seven times as much as that in the original (zero-time) sample. Another fact to be noted from Figure 1 is the increasing rate of solanine synthesis in the later stage. It could, therefore, be assumed that potato slices require an induction period for solanine synthesis.

A considerable difference in solanine content between potato slices in cold (0° and 8°C) and warm (15° and 24°C) storage under light is illustrated in Figure 2. The latter showed a relatively high

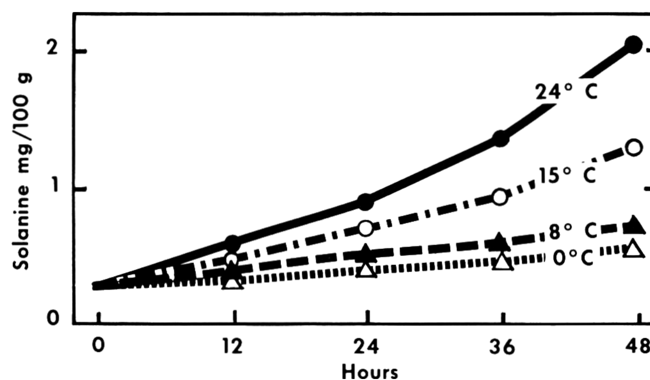

Fig. 1—Effect of temperature on solanine formation in potato slices stored in dark.

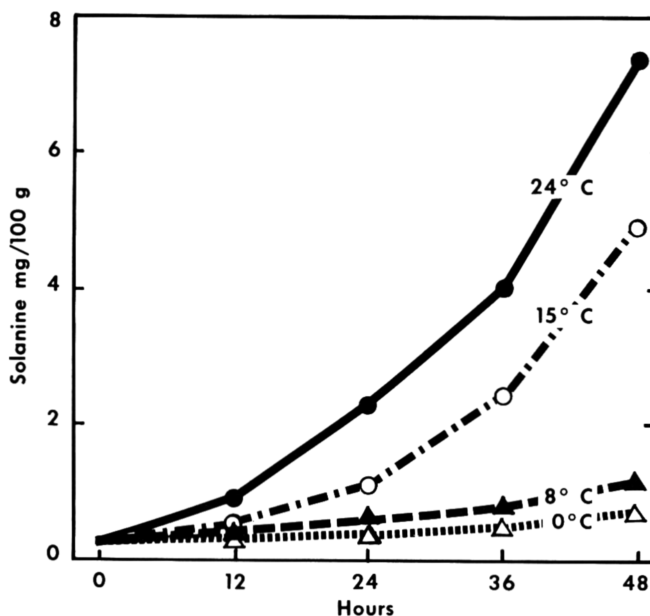

Fig. 2—Effect of light exposure (200 ft-c) on solanine formation in potato slices at different temperatures.

amount compared to the former. In the 48-hr exposure to 200 ft-c light at 24°C, the solanine concentration increased up to 7.4 mg per 100g slices. In general, light increased the rate of synthesis of solanine nearly three to four times more than dark. However, the amount of solanine in the early and late stages followed a similar correlation that was observed in the dark storage.

The experimental evidence presented in this work establishes the fact that light and relatively high storage temperatures stimulate solanine biosynthesis in potato slices, a basically wounded tissue. The production of solanine has been reported by McKee (1955) in wounded potatoes. Perhaps this may be a form of a physiological defense mechanism in tubers or in slices when exposed to stress such as high light intensity in grocery stores or wounding as in the case of slices or strips prepared for chips and French fries. In many instances, in potato processing plants, slices, cubes, mash, strings, strips, shreds and others are stored or held at relatively high light intensity and temperature for some time before cooking or dehydration. This may cause synthesis and subsequent accumulation of solanine which cannot be destroyed during cooking, baking or frying. Therefore, the only effective means of avoiding high concentrations of solanine in potato products is to process slices, strips or other products as soon as they are prepared.

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A Research Note

EFFECT OF VARIOUS SUGARS AND THEIR DERIVATIVES UPON THE GERMINATION OF Bacillus SPORES IN THE PRESENCE OF NISIN

INTRODUCTION

DURING THE PROCESS of spore germination there is very little utilization of exogenous substrates (Gould, 1969). However, the inhibition of spore germination has been shown to be influenced by a number of metabolites. Among various metabolites having such quality, the role of amino acids (Hermier, 1962; Martin and Harper, 1963; Gould and King, 1966; Seregin, 1968) and sugars (Thorley and Wolf, 1961; Hyatt and Levinson, 1964; Foerster and Foster, 1966) is well documented. Since some kinds of amino acids and sugars are always present in most foods, the use of nisin as a food preservative (Hall, 1966) must take into account the influence of such metabolites on the germination of bacterial spores. A preliminary report on the effect of some carbohydrates on the germination of *Bacillus* spores both in the presence and absence of nisin is presented.

MATERIALS & METHODS

Organisms, media and materials

The strains of *Bacillus stearothermophilus*, *Bacillus cereus* and *Bacillus megaterium*, nutrient agar and nisin were the same as used previously (Gupta et al., 1971).

Preparation and counting of spores

The method of spore preparation was identical to that of Thorpe (1960) and Tramer and Fowler (1964). The number of spores was determined microscopically after staining as follows: The spore suspension was diluted and 0.01 ml suspension was spread evenly on 1 sq cm area on the microscope slide. This preparation was stained with malachite green. The number of spores per field was determined by calculating the number of spores in 1 sq cm and multiplying by the dilution factor. The initial spore concentration was 10^8/ml.

Germination of spores

The minimal medium used for spore germination was the same given by Gupta et al. (1971). The sugars and their derivatives used in this study are given in Tables 1 and 2. To 9.0 ml of minimal medium containing 0.1M of sugar, 1.0 ml of spore suspension was added. The tubes were incubated for 24 hr at 30°C for *B. cereus* and *B. megaterium*, and at 37°C for *B. stearothermophilus*. The ungerminated spores present at the end of the incubation period were counted and the percent spore germination determined.

RESULTS & DISCUSSION

Effect of monosaccharides and their derivatives

The germinative response of *Bacillus* spores to monosaccharides and their derivatives is shown in Table 1. In the absence of nisin, sugars stimulated about 20–47% spore germination. Glucose was invariably the best stimulant, but other sugars had similar effects on the germination of spores of *Bacillus* species. Arabinose, mannitol and fructose were the best stimulants for the germination of spores of *B. spearothermophilus*; ribose, sorbose, arabinose and fructose were best for *B. cereus*; and arbutin and fructose were best stimulants for spores of *B. megaterium*.

In the presence of 100 μg/ml of nisin, only 3–20% germination was observed. The sugars which were powerful stimulants in the absence of nisin were also highly effective in the presence of nisin. Sorbose, arabinose and glucose stimulated spore germination about 15–20%. Other sugars were less effective. The minimal medium did not support any spore germination in the presence of nisin.

Effect of di, tri and polysaccharides

As shown in Table 2, all sugars tested induced spore germination in the absence of nisin. Sucrose was the best inducer (about 50% germination); other sugars appeared to act specifically: melibiose, inulin and glycogen were good for *B. stearothermophilus*; lactose, inulin and glycogen for *B. cereus*; and lactose, glycogen and melibiose for *B. megaterium* spore germination.

Germination response of all the test organisms was significantly lower in the presence of nisin (100 μg/ml). The only

Table 1—Effect of monosaccharides and their derivatives on the germination of Bacillus spores both in the presence and absence of nisin

Sugar (0.1M)	Nisin[a] (μg/ml)	Percent spore germination after 24 hr[b]		
		B. stearothermophilus[c]	*B. cereus*[d]	*B. megaterium*[d]
Basal medium[e]	0	3.2	4.0	3.5
	100	0.0	0.0	0.0
Ribose	0	23.3	30.0	25.3
	100	3.3	5.0	3.0
Mannose	0	26.3	22.3	28.3
	100	9.6	15.8	8.2
Sorbose	0	41.1	33.0	29.2
	100	17.5	15.1	20.3
Arabinose	0	32.3	31.3	25.0
	100	18.8	18.1	15.8
Glucose	0	47.3	46.0	47.0
	100	18.4	21.7	18.7
Mannitol	0	34.3	25.0	28.0
	100	13.4	6.6	17.4
Arbutin	0	23.4	21.3	30.4
	100	10.1	9.2	3.0
Mucic acid	0	13.9	17.6	25.1
	100	7.3	6.2	2.9
Glucuronic acid	0	–	18.3	16.0
	100	–	6.2	3.0

[a] 10^6 units per g
[b] Determined by direct microscopic observation
[c] Incubated at 37°C
[d] Incubated at 30°C
[e] Minimal medium to which various test compounds were added

Table 2–Effect of di, tri and polysaccharides on the germination of Bacillus spores in the presence and absence of nisin

Sugar (0.1M)	Nisin[a] (μg/ml)	Percent spore germination after 24 hr[b]		
		B. stearothermophilus[c]	*B. cereus*[d]	*B. megaterium*[d]
Basal medium[e]	0	3.2	4.0	3.5
	100	0.0	0.0	0.0
Raffinose	0	9.8	25.2	15.3
	100	3.3	6.2	6.2
Sucrose	0	50.0	52.1	50.1
	100	26.2	21.8	21.6
Lactose	0	21.1	42.1	42.7
	100	12.8	15.9	15.0
Melibiose	0	30.4	15.8	38.0
	100	22.3	18.0	11.2
Inulin	0	38.0	36.2	26.6
	100	28.3	31.1	36.1
Glycogen	0	34.5	28.7	33.0
	100	3.6	4.0	3.0

[a] 10^6 units per g
[b] Determined by direct microscopic observation
[c] Incubated at 37°C
[d] Incubated at 30°C
[e] Minimal medium to which various test compounds were added

sugars having a fairly stimulatory effect were sucrose, lactose and inulin (15–20% germination).

From this study, it can be concluded that the efficiency of nisin as a food preservative is significantly affected by two factors: the kinds of *Bacillus* species spores present, and the kinds and nature of metabolites contained by the food. The variation in the spore germination among *Bacillus* species is evident from the study of Stedman (1956). Thorley and Wolf (1961) reported that spores of *B. megaterium* and *B. subtilis* responded to glucose but not *B. cereus* and *B. licheniformis*. On the other hand, *B. cereus* T spores germinated in glucose and gluconate media (Halvorson and Church, 1957). In our study, not only *B. cereus* spores but also *B. stearothermophilus* and *B. megaterium* spores gave as much as 50% spore germination in glucose and sucrose media. The efficacy of the sugars to induce spore germination of *B. megaterium* was studied by Hyatt and Levinson (1964). They found glucose and its derivatives supported appreciable germination at low concentration but other sugars like fructose and sorbose induced spore germination at high concentrations.

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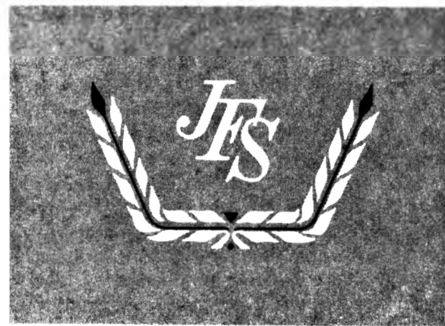

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Institute of Food Technologists

The Institute of Food Technologists is a professional society of scientists, engineers, educators and executives in the field of food technology. Food technologists are professionals who apply science and engineering to the research, production, processing, packaging, distribution, preparation, evaluation and utilization of foods. Individuals who are qualified by education, special training or experience are extended an invitation to join in professional association with the select group of the food industry's scientific and technological personnel who are Institute members. Membership is worth many times its modest cost, reflecting positive benefits, stimulation and opportunities for the individual in his business or profession.

OBJECTIVES

The Institute, as a non-profit, professional, educational society, has several major aims: to stimulate investigations into technological food problems; to present, discuss and publish the results of such investigations, to raise the educational standards of Food Technologists; and to promote recognition of the scientific approach to food and the basic role of the Food Technologists in industry. All of these activities have the ultimate objective to provide the best possible foods for mankind.

ORGANIZATION AND PROGRESS

Organized July 1, 1939, at Cambridge, Mass., with a membership of less than 100, the Institute has grown to more than 11,000. It is worldwide in scope with almost 2,000 of its membership overseas.

QUALIFICATIONS FOR MEMBERSHIP

Professional Members. Any person who meets the following minimum requirements by training and experience in food technology: (1) Bachelor's degree or higher from a college or university in which he has majored in one or more of the sciences or branches of engineering associated with food technology; (2) Five years of professional experience in food technology, for which a master's degree may be presented as the equivalent of one year's experience; a doctor's degree, the equivalent to three years' experience.

Members. Any person who is active in any aspect of the food industry and who evidences interest in supporting the objectives of the Institute.

Student Members. Any person who is registered as a student in an educational institution and who is actively pursuing candidacy for an Associate Degree or higher in one or more of the sciences or branches of engineering associated with food technology shall be eligible for membership as a Student Member. He may remain a Student Member until the end of the calendar year in which he completes his schooling.

Fellows. Any Professional Member who has been active for at least ten years and who has been nominated by the IFT Committee on Fellows for outstanding contributions to the field of food science/technology is eligible to be elected a Fellow of the Institute by the IFT Council.

DUES

Professional Members and Members–$20 a year; includes subscription to *Food Technology* and *IFT World/Directory & Guide;* option to subscribe to the *Journal of Food Science* at members' special rate of $10. Student Members–$5 a year; includes subscription to one IFT journal, *IFT World/Directory & Guide,* and option to subscribe to the other journal at $5. Emeritus Members–no dues; option to subscribe to either journal at $5 each.

PUBLICATIONS

The Institute publishes two journals. *Food Technology,* issued monthly, is the official journal of the Institute. The *Journal of Food Science,* issued bimonthly, is devoted to basic and applied research papers on fundamental food components and processes. In addition, an *IFT World/Directory & Guide* is published annually.

REGIONAL SECTIONS

Where 25 or more members live within commuting distance of a given point, a regional section may be established. Meetings can be held at more frequent intervals by such groups. Presently, there are 43 regional sections.

DIVISIONS

Where 50 or more members of the Institute have a common interest in a particular broad-based discipline of food technology, they may form a division. There are presently five divisions serving the areas of Carbohydrates, Food Service, Quality Assurance, Refrigerated and Frozen Foods, and Sensory Evaluation.

STUDENT CHAPTERS

An IFT Student Chapter certificate may be granted to a group of students enrolled in the food science and technology curriculum in a particular school who have organized to form a student club. There are 15 student chapters.

AFFILIATE ORGANIZATIONS

Affiliate certificates may be granted to food technology organizations outside the U.S.A. There are currently nine chartered affiliate organizations.

ANNUAL MEETINGS

An Annual Meeting of the Institute provides a specially-organized technical program, awards program, and industrial exhibit of equipment, services, processes and ingredients. The program is designed to emphasize current trends and technological developments. Special guest speakers are invited.

AWARDS

The Institute administers the following awards:

NICHOLAS APPERT AWARD. Purpose of this award (Medal furnished by the Chicago Section, and $1,000) is to honor a person for pre-eminence in and contributions to Food Technology.

BABCOCK-HART AWARD. Purpose of this award ($1,000 and Plaque; sponsored by The Nutrition Foundation) is to honor a person for contributions to Food Technology that have improved public health through some aspects of nutrition or more nutritious food.

IFT INTERNATIONAL AWARD. Purpose of this award (Silver Salver sponsored by Australian Institute of Food Science and Technology, and $1,000) is to recognize an IFT Member for promoting international exchange of ideas in Food Technology.

IFT FOOD TECHNOLOGY INDUSTRIAL ACHIEVEMENT AWARD. Purpose of this award (Plaques to company and individuals) is to recognize and honor the developers of an outstanding new food process and/or product representing a significant advance in the application of Food Technology to food production, successfully applied in actual commercial operation.

WM. V. CRUESS AWARD FOR EXCELLENCE IN TEACHING. Purpose of this award ($1,000 and Medal sponsored by the Northern California Section) is to recognize the teacher's role in Food Technology advances.

SAMUEL CATE PRESCOTT AWARD FOR RESEARCH. Purpose of this award ($1,000 and Plaque; sponsored by IFT) is to recognize a research scientist 35 years of age or younger who has demonstrated outstanding ability in Food Science or Technology research.

FELLOWSHIPS

- Florasynth–$2,500 and plaque
- Monsanto–$1,000 and plaque
- Nestlé–Two, each $1,000 and plaque
- Pillsbury–Two, each $1,000 and plaque

Purpose of IFT-administered Fellowships is to encourage graduate work in the field of Food Science and Technology directed to extending or improving knowledge in some phase of food conservation, food production, or food processing. Available to graduate students.

SCHOLARSHIPS

- Coca Cola (donor: Coca Cola Co.)–Five, each $1,000 and plaque
- R.T. French (donor: R.T. French Co.)–Two, each $1,000; accompanied by plaque
- Fritzsche-D&O's F.H. Leonhardt Sr. Memorial (donor: Fritzsche-D&O)–$1,000 and plaque
- Gerber (donor: Gerber Products Co.)–Six, each $1,000; accompanied by plaque
- Alexander E. Katz Memorial (donor: F. Ritter & Co.)–$1,000 and plaque
- Mexico (donor: Mexico Section of IFT)–$1,000 and plaque

Purpose of IFT-administered Scholarships is to focus attention on the need for more young people in Food Science and Technology, and to encourage deserving and outstanding students to take undergraduate work leading to a Bachelor's Degree in Food Science, Food Engineering, or Food Technology. Available to Juniors and Seniors who have completed at least one term of study at the institution from which they expect to earn a bachelor's degree.

IFT SCHOLARSHIPS

- IFT Freshman/Sophomore Scholarships–Thirty, each valued at $500 plus a complimentary subscription to *Food Technology* during the tenure of the scholarship.

Purpose of the IFT Scholarships is to attract and encourage worthy students to enter the fields of Food Technology, Food Engineering, or Food Science. Available to incoming college freshmen, and sophomores.

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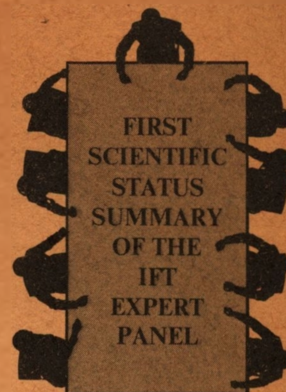
BOTULISM

A Scientific Status Summary by the Institute of Food Technologists' Expert Panel on Food Safety and Nutrition



Botulism is a rare and poorly understood, but currently widely-used term that describes a particularly notorious type of food poisoning. Botulism differs from the other types of bacterial-caused food poisonings, such as those caused by staphylococci, salmonella, and perfringens in that it causes disorders to the human nervous system rather than purely to the digestive tract.

Incidence of Botulism

The total number of people affected by the disease each year is surprisingly low. There are only 10 to 20 outbreaks reported annually in the United States involving only from 20 to 30 people that may result in 2 to 5 deaths. The majority of the limited outbreaks are caused by improperly heated home-canned foods. Canned foods commercially processed in the U.S. have had a remarkably good record during the past 45 years. In this period, about 29 billion cans of food have been processed each year; out of the total of 775 billion food cans produced by commercial firms in the United States only three outbreaks of botulism have resulted. Mushrooms canned in 1941 caused one death, tuna canned in improperly sealed cans caused two deaths in 1963, and in 1971 one person died from eating vichyssoise. These statistics thus indicate that home-canned foods produce the same number of illnesses and deaths **each year** from botulism as have been produced in **45 years** of commercial production of canned foods even though many fewer jars or cans of food are processed in homes. Thus, although there is a small risk in getting botulism from either commercially processed or home preserved foods, there is a substantially higher risk from botulism when home-preserved foods are consumed.

Description of the Microorganism and the Illness

The symptoms of botulism have been described in medical literature dating back to the 1700's. It was accurately described and made a reportable disease in southern Germany in 1854 as a result of fifteen years of study involving more than 230 cases of sausage poisoning. The term botulism was commonly accepted as the descriptive term for the illness due to the pioneering and excellent microbiological studies of the Belgian scientist van Ermengem who first isolated the microorganism after a food poisoning outbreak that occurred in Ellezelles, Belgium in 1896.

The type of bacteria that produces botulism is called *Clostridium botulinum*; it is a large, motile, spore-forming, rod-shaped gram positive bacterium that will not grow in the presence of small quantities of oxygen. Oxygen, in fact, may prevent the growth or even cause the death of *C. botulinum* cells. However, the bacterial cells are capable of growth in suitable foods or can grow just a few millimeters under the surface of foods exposed to the air. Large numbers of bacterial cells can be produced under suitable growth conditions and the potent botulism neurotoxin can be subsequently released into the food. Eating food containing the neurotoxin of botulism permits the toxin to enter the circulatory system via the small intestine. The disease is thus a food-borne intoxication rather than a food-borne infection.

Botulism toxin causes muscle paralysis of susceptible animals and man by blocking nerve terminals at myoneural junctions. Paralysis progresses downward, usually starting with the eyes and face, then to the throat, chest, and eventually to the arms and legs. Finally, if sufficient toxin is present in the blood, the diaphragm and chest muscles become paralyzed and death may result from asphyxiation. The over-all mortality rate has decreased over the past 70 years in the U.S. and Canada from about 60% up to 1945, to about 25% mortality for the diagnosed and reported cases at present. This reduced mortality is probably largely due to prompt administration of the tri-valent A, B, E antitoxin that is widely available across the U.S. through the U.S. Public Health Service.

The early signs of botulism are fatigue, weakness, and vertigo, usually followed by blurred vision and progressive difficulty in speaking and swallowing. Muscle weakness, labored breathing, abdominal discomfort and distention, and constipation are other common symptoms. Early vomiting, nausea and inflammation of the mucosal lining of the throat may be produced by the type of botulism commonly produced in seafoods.

Usually, symptoms develop about 8 to 72 hours after eating food containing preformed botulinum toxin. However, symptoms such as thirst, weakness, throat pain and abdominal swelling may persist for some time. Botulism can be diagnosed due to the clinical symptoms alone, but differentiation from a variety of other diseases is often difficult. Initial misdiagnosis of botulism for bulbar poliomyelitis or other central nervous system disorders has occurred.

Botulism caused by the Different Toxin Types of *C. botulinum*

Six antigenic types of toxin are produced by different members of the species and these form the basis of classification of the organism into toxin types A through F although two subtypes of type C have been reported, C_α and C_β. Types A, B, E, and F have been responsible for human cases; types C and D are generally associated with botulism in animals. However, all types of botulism microorganisms have been reported to have caused the disease in man.

Botulism was thought to be associated exclusively with sausages or meat products until the 1890's, but shortly afterward one outbreak in Germany and another in California were traced to bean salads. These incidents were followed in the United States by a series of outbreaks that were traced to canned vegetables and fruits. Statistics for 1918 through 1922 show 83 outbreaks with 297 cases and 185 deaths. Since the 1920's, the occurrence of botulism toxin in commercially canned foods has been virtually eliminated by improved canning methods that resulted from research sponsored by the canning industry and public health authorities.

All of the outbreaks reported through 1922 were due to organisms producing one or the other of the two above types of toxin, later designated as types A and B. Both of the type C subtypes (C_α and C_β) and type D were reported in 1922. Types C and D have caused heavy losses of chickens, ducks, waterfowl, cattle, horses, and mink, but there is little evidence that they have ever been responsible for human botulism, although there is no evidence to indicate man is not susceptible.

Type E was first identified as a toxigenic type in 1935. This strain, isolated from Russian sturgeon, was found to be identical to two other strains previously isolated in New York. One strain had been isolated from an outbreak in 1932 involving salmon caught and smoked in Labrador and the other from imported canned sprats responsible for an outbreak in 1934. Although one type E outbreak was due to commercially canned mushrooms in 1941, all other outbreaks in the United States have involved fish and seafood. Since 1960, outbreaks traced to fish caught and commercially smoked in the Great Lakes area have been responsible for 21 cases and 9 deaths, but one outbreak responsible for two deaths in 1963 involved canned tunafish. However, this outbreak was due to improper can seaming and recontamination, since the type E spores are readily destroyed by temperatures greater than 190°F. This organism is an important public health hazard in areas of Japan where "Izushi" a dish made of fermented raw fish, rice, koji, and chopped vegetables, is a popular food. It has given rise to morbidity rates from type E botulism of about 50% with fatality rates of 20% to 100%.

Type F was first isolated in 1960 from an outbreak in Denmark in which a liver paste was the vehicle. The only other type F outbreak on record occurred in California in 1966 and was traced to contaminated deer jerky.

The types of foods involved in botulism vary according to food preservation and eating habits in different regions. Almost any type of food with a pH above 4.6 can support growth and toxin formation. Botulinum toxin has been found in a considerable variety of foods, such as canned corn, peppers, green beans, beets, asparagus, mushrooms, ripe olives, spinach, tunafish, chicken, and chicken livers and liver pate, and in luncheon meats, ham, sausage, stuffed eggplant, lobster, and smoked fish.

Distribution of the Microorganism in Nature

The food-borne illness of botulism occurs throughout the world. This is because the organism is widely distributed in nature and occurs in both cultivated and forest soils, bottom sediments of streams, lakes, and coastal waters, the intestinal tracts of fish and mammals, and the gills and viscera of crabs and other shellfish. Canned vegetables, sausages, meat products, and seafood products have been the most frequent vehicle for human botulism. Of the 659 outbreaks recorded in the United States from 1899 through 1969, 21.8% were due to type A, 5.6% to type B, 2.6% to type E, and 0.3% to type F. In 69.5% of the outbreaks the type could not be determined. Outbreaks have been reported in 44 states, but 5 western states account for over half of all reported outbreaks.

Of the 144 type A outbreaks, 92% took place in states west of the Mississippi River, whereas 25 of 37 type B outbreaks, or 67%, occurred in eastern states from 1899 through 1969. Type E outbreaks have been reported in 10 states. The geographic association of this type with Alaska and the Great Lakes area may be more apparent than real.

The regional distribution of outbreaks by toxin type is in keeping with the results of a spore survey of soil samples by K.F. Meyer and B.J. Dubovsky. These investigators found a predominance of type A in soil specimens from the west and of type B in soils of the northeast and central states. Other investigators have shown that type E is the predominant type in fish and sediments from the Great Lakes, in estuarine and coastal waters, and in sediments, fish, and shellfish from the Atlantic, Pacific, and Gulf coasts of North America.

In Europe, type B is the predominant type both in soils and in outbreaks. However, type E has accounted for 46% of the outbreaks in Japan, Canada, and Scandinavia in the Twentieth Century.

Most Botulism Outbreaks due to Home-Preserved Foods

Outbreaks of botulism in the United States between 1899 and 1969 have been shown to be caused mostly by home-preserved foods and have been identified mainly on the basis of clinical symptoms instead of isolation and typing of the organism. These have included: vegetables–395 outbreaks of which 362 involved home-preserved foods; meat–44 outbreaks, 36 home-preserved; milk and milk products–7 outbreaks, 5 home-preserved; fish and seafood–48 outbreaks, 33 home-preserved; and fruit and pickles–35 outbreaks, of which 34 involved

home-preserved foods. In many outbreaks the kind of food involved was not identified.

Most of the outbreaks have been traced to home-canned vegetables, fish, fruits, and meat products, with a very much smaller number involving commercially processed foods. Although many outbreaks were caused by commercially canned products during the 1920's and 1930's, home-canned string beans, corn, beans, spinach, and asparagus account for over half of the total number.

Botulism is usually associated with foods that have been given an inadequate or minimal preservation treatment, held for some time unrefrigerated, and consumed without appropriate heating. The growth of *C. botulinum* in foods frequently, but not always, produces a foul, putrid odor that serves as a warning to the consumer. Signs of spoilage, however, have not prevented botulism because the degree of tolerance to disagreeable odors or off-flavors varies among individuals. Moreover, in green beans or in foods that are smoked, heavily spiced, or fermented, the off-odor may be difficult to recognize.

Determining the Cause of Suspected Botulism Outbreaks

Since botulism is food-borne and results from ingestion of toxin formed in the food following growth of the organism, determination of the source of an outbreak is based on detection and identification of the toxin or cultivation of *C. botulinum* from the food involved. Currently the most widely used method for detecting toxin in suspect food is the injection of extracts of the food into white mice previously immunized with specific antitoxin. Type E toxin is usually activated by treatment with the enzyme trypsin and the enzyme-treated food sample is used to inject mice. The first objective in an epidemiological investigation is usually the detection of toxin in the suspect food by such a test. Rapid identification of the toxin is important for proper treatment of the victims and for determining the source so that the implicated food can be removed from further distribution as quickly as possible. This analysis is followed by culturing all suspect foods in an enrichment medium for the detection and isolation of the causative organism.

The details of these procedures will vary somewhat with the nature of the material to be examined. Products such as meat or fish are ground with gelatin-phosphate buffer to extract the toxin, whereas liquid portions of some products can be injected directly into mice. Suspect foods are cultured for the bacillus because the food is occasionally no longer toxic at the time the mouse test is performed due to growth of other microorganisms, pH changes, or temperature fluctuation. Human illness with symptoms of botulism, demonstration of toxin in the suspect food, and isolation of a toxic strain of *C. botulinum* from the food involved is considered a positive laboratory confirmation for botulism.

Growth of the Microorganism

There are many enrichment media used for the cultivation of the different types of *C. botulinum*. Generally, all of the media contain reducing agents to maintain anaerobic conditions and also contain buffering agents. The usual incubation times and temperatures for maximum toxin production and growth of the various types range from 3 to 7 days, at 78° to 96°F.

Elimination of nonspore-forming contaminants from enrichment cultures is performed in the laboratory by treatment with alcohol. Cultures are then streaked on plating media for the selection of typical colonies of *C. botulinum*. Plating media frequently employed are blood agar, liver veal egg agar, brain heart infusion agar, a variety of beef infusion agars, and modifications of special egg yolk media.

C. botulinum is an obligate anaerobe and atmospheric oxygen has a toxic effect on its vegetative cells. Botulinal growth in food, therefore, is related to the oxidation-reduction (O-R) potential of the food itself. Such potentials are established in many foods by oxygen-reducing systems present in the food such as sulfur-hydrogen molecular groups that bind oxygen in meats, ascorbic acid or vitamin C that binds oxygen in many foods, and other microorganisms that use up oxygen. No special conditions of incubation, therefore, are generally required for *C. botulinum* since frequently the food itself produces a sufficiently low oxygen-reduction potential to permit outgrowth.

The resistance of *C. botulinum* spores to chemical agents is quite high. Complete inhibition of spore germination and outgrowth requires 5% common table salt (sodium chloride) to inhibit type E, while about 10% is needed to inhibit types A, B, and F.

Methods of Commercial Control of the Botulism Problem

Canning procedures have been developed to destroy spores with a sufficient margin of safety to make the probability of any survivors extremely remote. Where there is a question about the safety of a product, cans should be discarded when they show even slightly bulging ends or any other evidence of spoilage such as souring, gas formation, discoloration, or leaks. However, these recommendations do not apply to those commercially canned products that contain pressure such as canned soft drinks, beer, and coffee. Botulism bacteria cannot grow in these three products.

Any other food cans or jars showing bulging lids should be suspected of containing botulism. Never under any circumstance should the contents of these containers be tasted, but they should be discarded where animals cannot gain access to them.

The canning industry, using the information provided by research carried out in the 1920's and 1930's, has adopted standardized processes for treating low-acid foods so that the probability of *C. botulinum* spores surviving is very remote. The spores of *C. botulinum*, although quite resistant, are not the most heat-resistant known, and other more resistant spore-formers are frequently used in time-temperature studies. However, canning processes must be of such lethality that the likelihood of *C. botulinum* spores surviving is negligible. The concentration of spores in a food product is important because the greater the number of spores there are in the product,

the greater the processing time and temperature required to insure their complete destruction. Therefore, the so-called 12 D standardized canning process was designed to reduce a bacterial load of 60 billion spores in each container to less than one spore per container. This assumes that if one spore was present in each of 1000 billion cans, there would be less than one can containing a surviving spore in the 1000 billion cans.

These processes, which take into account among other things the consistency and chemical nature of the product and the size of the can, are now standardized to the point that only through recontamination after heating is it likely that a significant degree of spoilage could occur due to container leakage. An additional safety factor is provided by proper sanitary control, as specified in FDA's Good Manufacturing Practice guidelines, which serves to reduce the original bacterial load to be inactivated during the canning cycle. The proposed FDA-National Canners Association "Better Process Schools" that will ensure better education of retort operators and can seam operators should result in materially increasing the already remarkable safety factor of our commercially processed food supply.

The knowledge concerning the effects of chemicals, such as salt, nitrite, and organic acids, on the germination and outgrowth of *C. botulinum* spores has been utilized by the meat-curing industry to produce safe and acceptable products. The deletion of nitrites from cured meats would impose the microbiological risk of botulism that must be weighed against possibly harmful aspects of the presence of the chemical sodium nitrite in our diet.

Vacuum-packaged, moist, low-acid foods such as uncured meat, fish, and certain vegetables offer a unique environment for the growth of *Clostridum botulinum*. Therefore, these products should always be kept under refrigeration or frozen.

General Procedures to Keep Foods Safe from Botulism

How then should foods be kept safe from botulism? This is accomplished by the destruction of the bacteria and their very heat-resistant spore forms in processed foods. If proper pressure-cooking methods are not used, some spores may survive, germinate, grow, and produce toxin if the food and growth conditions are suitable. The resting spores themselves are not dangerous and many are probably eaten by man under normal conditions. These spores cannot grow and produce toxin in the human intestinal tract and are eliminated intact from the body.

The toxins of *C. botulinum* are heat sensitive and are, therefore, readily destroyed by normal cooking. Boiling for 10 minutes provides a reasonable margin of safety against all types. All home-preserved foods that are to be served warm should be boiled as above shortly before they are to be served.

Freezing will not destroy either toxin or spores but will prevent the germination and outgrowth of spores that could lead to toxin production at temperatures above 38°F. It is most important that frozen food products be kept frozen until they are cooked.

Best control of the botulism hazard is by using proper home and commercial food preservation techniques. Government agencies continue to urge home processors to use pressure-cooker processes which have proven to be safe for low-acid foods. Any home-canned food that appears abnormal should not be tasted. Also, the serving of home-preserved foods in public eating places is prohibited in some localities as a further precaution.

U.S. Department of Agriculture Home and Garden Bulletins on safe methods of preserving and handling foods in the home are for sale by the Superintendent of Documents, Government Printing Office, Washington, D.C. 20402: No. 8–Home Canning of Fruits and Vegetables, No. 40–Freezing Combination Main Dishes, and No. 174–Meat and Poultry-Care Tips for You, all 20 cents each; No. 106–Home Canning of Meat and Poultry and No. 70–Home Freezing of Poultry, 15 cents; No. 93– Freezing Meat and Fish, 25 cents; and No. 162–Keeping Foods Safe to Eat, 10 cents.

Fresh foods, whether eaten raw or cooked, have never been implicated in an outbreak of botulism. Food preservation methods, therefore, are designed to prevent the development of botulinum toxin unless the nature of the food itself is inhibitory to the growth of the organism. These are foods with a high acid, sugar, or salt content. Some of these are canned fruit juices, sauerkraut, many tomato products, heavily salted hams, jellies, and jams. Adequate preservation methods must, in order of preference, either destroy all spores, prevent their germination and the outgrowth of vegetative cells, prevent the development of toxin, or provide conditions under which any toxin that might be formed will be inactivated.

Proper food safety can thus be achieved by following proper procedures exactly, use of food sanitation, and most importantly, good common sense. If food is judged to be spoiled or questionable in quality, the old rule of "when in doubt, throw it out" should be employed.

The above article is a revision and adaptation of an article by D.A. Kautter and R.K. Lynt, Jr., that appeared in the November, 1971 issue of *FDA Papers*.

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In another study, the Agricultural Research Service of the USDA has uncovered trace amounts of dimethyl nitrosamines in three samples of cooked sausage purchased at retail outlets–forty-seven were negative. In laboratory tests this group found that about ten times the normal level of nitrite was required in cooked sausages before nitrosamines could be recovered.

The laboratories of the Food and Drug Administration have developed a multi-detection procedure which will identify 14 nitrosamines. Using this procedure they have found dimethyl nitrosamines in one ham at 5 ppb. In another study, 30-106 ppb of N-nitropyrrolidine was found in cooked bacon. None was found in the raw bacon.

The National Research Council, Division of Agriculture, has released a study on nitrates, nitrites in soil and run-off water entitled, "Accumulation of Nitrate." One section is devoted to nitrosamines and concludes there is no evidence that foods consumed in this country contain nitrosamines at levels which might induce carcinogenic, mutagenic, and teratogenic effects.

Analyzing for Nitrosamines is Difficult and Tedious

As previously pointed out, the analysis for nitrosamines is a difficult and tedious procedure and requires considerable laboratory skill. Current analytical techniques for nitrosamines are highly sophisticated, using gas chromatography followed by mass spectrometry. Interpretation of the resulting data is equally demanding in sophistication.

Much of the early work on nitrosamines using simpler techniques remains suspect and must be repeated, and questions of recoverability and accuracy need to be answered. Fiddler (1972) has additionally reported that sampling for nitrosamines in comminuted sausage products presents difficulties due to uneven distribution of nitrosamines in the product. This is not surprising in an analysis for substances at the ppm and ppb levels.

It is apparent from these results that the synthesis and appearance of nitrosamine reaction products are highly unpredictable, and to date a proposed, useful mechanism of formation does not exist. Wolff (1972) has proposed that localized areas within the meat products may offer optimum conditions for nitrosamine formation, but these conditions remain to be determined. Such studies are underway in his laboratories–USDA's Eastern Regional Research Laboratory. Wolff and Wasserman (1972) have published a summary of the present state of knowledge on nitrosamines.

Why Not Eliminate Amines and Nitrites from Foods?

If, as earlier pointed out, the reaction in question is that between certain amines and nitrites, why not simply rule both substances as unsuitable for use in foods? In fact, by interpretation of the Delaney Amendment to the Food, Drug and Cosmetic Act, such substances should be ruled out for use in food.

This dilemma may be ventilated soon–a suit was recently filed in Federal District Court seeking to prohibit the use of nitrates and nitrites in cured meat products. Amines are impossible to rule from use in foods since they occur naturally as breakdown products from precursors present. Nitrites, on the other hand, while naturally occurring in human saliva and some foods, are also added in meat curing and some fish curing. It is for this reason attention has been focused on cured meats.

It is now quite clear that certain cured meat products–namely those which, in general, are pasteurized and require post-processing refrigeration–could not be manufactured without nitrite. In such products, nitrite acts to inhibit the germination of spores of *Clostridium botulinum* at a minim level of 150 ppm nitrite added. Wit out nitrite, the botulinum haza would proscribe such products as they are presently manufactured. Perhaps a higher level of salt and certainly a greatly increased heat treatment would circumvent this problem. But it would create a new one, that is, is such a product acceptable at all for use by consumers?

Perhaps some of these answers w be forthcoming, since effective Ja ary 1, 1973 the use of nitrate and nitrite as an additive in food products is to be banned in Norway. They may be added, however, by petition to the government based on botulinum hazard. Color improvement is no accepted as a reason for a petition, an colors may not be added to sausa products.

Resolving the Dilemma Requires More Data

Therefore, the dilemma is, shall we follow the dictates of the Delaney Amendment and prohibit the use of nitrites in human foods, as well as amines wherever they may occur; shall we limit the levels of use; or shall we conclude that it is still too early to have enough data to reach a reasonable decision and push vigorously forward to get more information. For example, the mechanism of nitrosamine biological action is not known. The nature of the compound acting at the organ site is not known. More and more of the scientific community is swinging toward the position of acquiring more data as quickly as possible so that we may make a sensible, considered judgment.

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terial action. This was followed soon by work from Lehman in 1899, who established that the pink color of cured meat was due to nitrite and not nitrate. Mechanics of the color reaction were finally clarified by Haldane, the great British physiologist, in 1901 as a combination of nitric oxide with meat pigments. This discovery led to the use of nitrite directly as a meat curing ingredient, rather than waiting for the unpredictable bacterial conversion of nitrates to nitrites.

Amounts Permitted in Foods

Several years ago (1925) a top limit on the use of sodium nitrite in meat curing was established at 200 parts per million (ppm), based on the finished product, by the meat inspection branch of the USDA. Current regulations permit up to 3.5 oz of sodium or potassium nitrate in 100 lb of meat when used as a dry cure, and 2.75 oz per 100 lb when used in chopped meat. The nitrites, sodium and potassium, are permitted at 1 oz per 100 lb meat, dry cure, and 0.25 oz per 100 lb chopped meat. There is a further restriction that 200 ppm nitrite is the top allowable level in any finished cured meat product.

Nitrite and Nitrate Toxicity Studies

Studies on the toxicity of nitrates and nitrites were conducted as early as the beginning 1900's. Tanner and Evans wrote a review in 1934 quoting the toxicity of nitrites as a level of 0.3 g single dose and 1 g daily dose for adult male. The Swiss Pharmacopeia of 1928 gave figures of 0.1 g and 0.3 g for such dosage. Nitrates, on the other hand, have been reported several times to be on the same order of toxicity as sodium chloride. Recent work by London et al. (1967) using potassium nitrite has shown that pigs fed more than 21.3 mg/kg body weight died; however, when fed up to 18.3 mg/kg, there was no effect on weight gain or feed efficiency, but methemoglobinemia was apparent.

A concomitant interest in nitrate ingestion by infants has been reviewed by Fassett (1966). A factor rendering such ingestion of interest is the high gastric pH of infants, which facilitates the reduction of nitrate to nitrite by bacteria. This study followed the recognition of methemoglobinemia in infants after ingestion of high-nitrate spinach puree.

Recent Interest in Nitrosamines

The interest in nitrosamines is recent, since the work of Magee and Barnes (1956) demonstrated liver damage induced by dimethylnitrosamines. Since then, the presence of various nitrosamines has been reported in a variety of substances, including cheese, fishmeal, flour, mushrooms, and alcoholic beverages. The analysis for nitrosamines is a difficult and tedious procedure and requires considerable laboratory skill. The interest in nitrosamines was fanned by a report by Ender et al. (1964) on liver necrosis in sheep that had consumed nitrite-treated fishmeal prepared by adding 630 ppm nitrite followed by high heating. Work in this field has continued and toxicity of nitroso compounds has been reviewed by several authors, including Magee and Barnes (1963) and Druckrey et al. (1967).

Toxicity varies depending on the compound. The LD_{50} for rats is 27-41 mg/kg for N-Nitrosodimethylamine, 216 mg for N-Nitrosodiethylamine, and 1200 mg for N-Nitrosodi-n-butylamine. An explanation of the potential role of nitrosamines has been offered by Lijinsky and Epstein (1970). They postulated that human cancer might be caused by nitrosamines formed in the body from ingested nitrites and secondary amines. The secondary amines could be formed during cooking.

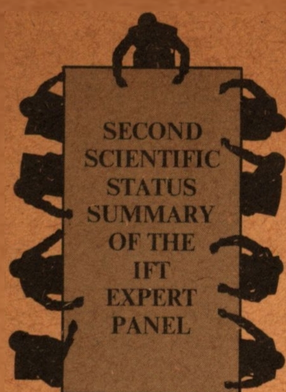
It had earlier also been postulated by Sen et al. (1969) that amines and nitrite might combine in the human stomach to produce nitrosamines. Incubation of a simulated stomach environment with diethylamine and sodium nitrite has been shown to yield diethylnitrosamine. These workers have found similar results when the gastric juices of rabbits and cats were examined after the animals had been fed nitrosamine precursors.

Foreman (1972) has reviewed the current status of nitrosamines. He reports some interesting characteristics, namely, that nitrosamines as carcinogens are dosage-dependent. With some of the nitrosamines, a single dose at the proper level is sufficient to produce carcinogenesis, whereas at lower levels, longer ingestion periods are required. Furthermore, while some nitrosamines are general carcinogens, others are quite organ specific.

Foreman also reiterates the need to employ newer technology to review the older work and points out that since nitrosamine recovery is by steam distillation, little information is available on compounds higher than dioctyl, since this is the limit of steam volatility. He also points out that nitrosamines are quite heat stable.

Search for Nitrosamines Centers on Meat Products

The search for nitrosamines in foods, for obvious reasons, has centered on meat products. USDA's Consumer and Marketing Service has reported levels of 11 to 48 parts per billion (ppb) in two samples of dried beef and one sample of cured pork. Forty-five other samples showed negative results.



Nitrites, Nitrates, and Nitrosamines in Food – a Dilemma

A Scientific Status Summary by the Institute of Food Technologists' Expert Panel on Food Safety and Nutrition

Considerable attention is currently being focused on a group of chemical compounds generally called nitrosamines, which under certain conditions can be formed in food products as a reaction between nitrites and certain amines. Some of these nitrosamines have been shown to be carcinogenic under certain conditions of use. Attention has been directed to the use of nitrites in foods since some scientists have hypothesized that if this practice is discontinued the potentially hazardous reaction will not proceed. Particular attention has been focused on cured meats with the reasoning that this is a major use of nitrite in foods; however, nitrite has been used in other food products, for example, certain cheeses in England.

Origin of Use Lost in History

The origin of the use of nitrate as a meat curing ingredient is lost in history. It was probably originally carried along with salt used to preserve the hunting catch. With the passage of time, the salt from certain areas was considered highly desirable because it produced a pink color in meats. Since a characteristic flavor was introduced as well, this became identified in time with the flavor of cured meats.

Understanding of the role of nitrites in cured meat color is relatively recent. Polenski in 1891 observed that nitrate was reduced to nitrite by bac-

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