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ABSTRACTS:
IN THIS ISSUE

VARIATIONS IN PROXIMATE COMPOSITION OF NORTH CAROLINA SCALLOP MEATS. N. B. WEBB, F. B. THOMAS, F. F. BUSTA, & R. J. MONROE, *J. Food Sci.* **34,** 471–474 (1969)—The proximate composition varied widely among locations and months of harvesting. In general coefficients of variation were relatively low for moisture and protein but high for fat, glycogen and ash. The range for each component was fairly similar among species studied. The percent ranges for bay and calico scallop meats, respectively, were: moisture 74.15–83.66, 76.12–81.86; protein 13.44–21.57, 13.28–17.53; fat 0.23–0.91, 0.23–1.13; glycogen 0.13–3.36, 0.35–3.71 and ash 1.09–2.24, 1.17–1.91. Sea scallop percent ranges were moisture 74.63–80.97; protein 13.87–18.11; fat 0.31–0.96; glycogen 0.27–8.74 and ash 1.28–1.81.

HEDONIC DIFFERENCES AS A FUNCTION OF NUMBER OF SAMPLES EVALUATED. J. M. KAMEN, D. R. PERYAM, D. B. PERYAM & B. J. KROLL. *J. Food Sci.* **34,** 475–479 (1969)—This study sought to answer two questions: (1) Does the preference difference between two food samples vary according to the number of other samples with which they are evaluated? (2) Does the difference depend on whether samples are presented early or late in a testing session? There was no evidence that serving up to 12 samples in a session adversely affected preference discrimination.

A SIMPLIFIED AND RAPID METHOD TO UNIFORMLY SIZE RIB STEAK PIECES FOR TASTE PANEL EVALUATION. K. E. BEERY & J. H. ZIEGLER. *J. Food Sci.* **34,** 480–481 (1969)—Uniformity of size and preparation of steak samples are important prerequisites for accurate taste panel analysis. A system is described to utilize a Broiling and Sectioning Apparatus (BSA) to obtain this uniformity. Frozen beef rib steaks are uniformly sized before broiling and then sectioned into bite-size pieces for presentation to a taste panel.

LIPID IN RIPENING BANANA FRUIT. J. L. GOLDSTEIN & E. L. WICK. *J. Food Sci.* **34,** 482–484 (1969)—Lipid extracts were isolated from unripe and ripe banana pulp and peel and the fatty acid composition of the extracts was determined. Unsaturated acids, particularly linoleic and palmitoleic, decreased about 3-fold in the pulp while more than a 2-fold increase in stearic acid occurred. In general, unsaturated fatty acids decreased in both the pulp and peel during ripening. The peel contained almost 4 times more lipid than the pulp.

PHOTOINDUCED REDUCTION OF NOOTKATONE. K. L. STEVENS. *J. Food Sci.* **34,** 484–485 (1969)—Photolysis of the principal flavoring constituent of grapefruit, nootkatone, in methanol gave rise to a number of compounds. One of them has been isolated and shown to be 1,10-dihydronootkatone.

PALATABILITY AND SELECTED RELATED CHARACTERISTICS OF THREE TYPES OF ROASTED PORCINE MUSCLE. D. SEARCY, D. L. HARRISON & L. L. ANDERSON. *J. Food Sci.* **34,** 486–489 (1969)—There was no significant organoleptic preference for "normal," PSE, or DFD muscle. Significant differences ($P < 0.05$) occurred between PSE and DFD or between "normal" and DFD muscle for roasting losses, total moisture (Brabender), and pH. DFD muscle exhibited the lowest Warner-Bratzler shear value, smallest roasting loss, greatest total moisture and highest pH. PSE muscle had the greatest roasting loss, least total moisture and lowest pH. LD from Durocs was more tender ($P < 0.05$), had a higher ($P < 0.05$) pH, and contained less ($P < 0.01$) total moisture (Brabender) than LD from Poland Chinas. Cooking losses were greater ($P < 0.05$) for roasts from Durocs than for roasts from Poland Chinas.

RELATIVE EFFICIENCY OF PAIRED COMPARISONS AND RANK ORDER IN PREFERENCE DISCRIMINATION AMONG COFFEES. N. F. GIRARDOT, D. R. PERYAM & E. E. LOCKHART. *J. Food Sci.* **34,** 489–492 (1969)—Consumer preference tests were run on four coffees which varied only in strength. The method of two-pair paired comparisons were used with 576 subjects, 3-sample rank order with 384 and 4-sample rank order with 192. All possible combinations of samples were used equally often with each method. Subjects were male and female coffee drinkers whose ages covered a wide range. Rank order data were analyzed like paired comparisons. Preference order was the same by all three methods.

WHEAT FLAVOR COMPONENTS. M. McWILLIAMS & A. C. MACKEY. *J. Food Sci.* **34,** 493–496 (1969)—The volatile flavor components of a lightly milled, whole-grain soft wheat, Moro variety, were isolated and studied by gas-liquid chromatography, mass spectrometry, paper chromatography and/or chemical tests. The 11 following compounds were identified in the wheat essence or in headspace vapors: acetaldehyde, isobutyraldehyde, butyraldehyde, hexanal, heptanal, octanal, crotonaldehyde, 3-methyl-2-butanone, 2,2-dimethyl-3-pentanone, diacetyl and ethyl acetate. Tentative identifications were made of seven additional compounds: butanone, valeraldehyde, isovaleraldehyde, cyclopentanone, phenylacetaldehyde, amyl alcohol and isoamyl alcohol.

IDENTIFICATION AND QUANTITATIVE ANALYSIS OF PHOSPHOLIPIDS IN COCOA BEANS. J. G. PARSONS, P. G. KEENEY, & S. PATTON. *J. Food Sci.* **34,** 497–499 (1969)—The composition of the total lipid extract was 98% neutral lipid and 1 to 2% polar lipid of which approximately 70% was glycolipid and 30% was phospholipid. The relative distribution of the phospholipids was determined by quantitative phosphorus analyses of individual spots scraped from two-dimensional thin-layer plates. The major components were lyso-phosphatidyl choline, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol. Phosphatidyl choline was found to contribute 36 to 40% of the phospholipids of cocoa beans.

BISULFITE EFFECT ON THE CHEMISTRY AND ACTIVITY OF HORSERADISH PEROXIDASE. R. J. EMBS & P. MARKAKIS. *J. Food Sci.* **34,** 500–501 (1969)—Bisulfite retarded the inactivation of horseradish peroxidase by weak acids. Spectral analysis indicated that the retardation was accomplished by the stabilization of the linkage between the iron containing prosthetic group and the protein. Cyanide, azide and fluoride, which form reversible complexes with peroxidase iron, exerted a similar effect; thus, it is inferred that bisulfite also forms a complex with peroxidase iron. A kinetic method was used to calculate a dissociation constant of 0.02*M* for the bisulfite peroxidase complex.

ALDEHYDES, KETONES AND ESTERS IN VALENCIA ORANGE PEEL OIL. M. G. MOSHONAS & E. D. LUND. *J. Food Sci.* **34,** 502–503 (1969)—The aldehydes, ketones and esters in the volatile portion of Valencia orange oil have been isolated and identified. Seventeen aldehydes and ketones and three esters are included, 10 of which have never been identified as constituents of Valencia oil. Cis- and trans-limonene oxide have also been found to occur in the same fraction. This is the first time that these compounds have been identified in cold-pressed orange oil. A series of five homologous α,β-unsaturated aldehydes were isolated and partially characterized.

VAPOR ANALYSIS OF THE PRODUCTION BY BANANA FRUIT OF CERTAIN VOLATILE CONSTITUENTS. M.J. MYERS, P. ISSENBERG & E. L. WICK. *J. Food Sci.* **34,** 504–509 (1969)—Production of *iso*-amyl acetate by unripe slices was demonstrated based on experiments with metabolic inhibitors. The behavior of ripe and overripe slices was inconclusive since the vapor concentration of the acetate remained constant. The vapor concentration of *iso*-amyl alcohol was essentially unchanged at all ripeness stages. Investigations with L-leucine-U-^{14}C showed conclusively that leucine was a precursor and that both compounds were continually produced at all ripeness stages. The interpretation of direct vapor analyses over respiring fruit is considered.

PECTIN CHANGES IN THE RIPENING OF IRRADIATED AND STORED STRAWBERRIES. M. L. BELLI-DONINI & M. R. STORNAIUOLO. *J. Food Sci.* **34,** 509–514 (1969)—Strawberries at two successive stages of maturation and at different storage times were irradiated with a ^{60}Co source at 200 krads. The effects of irradiation on the pectic substances, pectin-methylesterase activity and anthocyanin content were investigated. In addition, changes in softening and extent of fungal growth were followed. A different response of strawberries irradiated at two stages of ripeness was found and the possible explanations are discussed.

EPIMYSIAL CONNECTIVE TISSUE SCORES AS RELATED TO BEEF TENDERNESS. R. A. FIELD, M. L. RILEY & YET-OY CHANG. *J. Food Sci.* **34,** 514–518 (1969)—Epimysial extension and thickness varied according to muscle location but at the same location it was not significantly different in steaks from tough versus tender steers. The LD epimysium was tougher in older bulls than in younger bulls. Shear values of epimysial tissue varied considerably within animals of the same age, especially when roasted to 140°F. Most connective tissue differences due to line of cattle were small. With few exceptions neither hydroxyproline, epimysial thickness nor connective tissue scores were significantly related to shear force of muscle.

COMPARISON OF SURVIVOR CURVES OF Bacillus subtilis SPORES SUBJECTED TO WET AND DRY HEAT. K. FOX & B. D. EDER. *J. Food Sci.* **34,** 518–521 (1969)—There were major differences in the shape of the wet vs. dry heat survivor curves. Wet heat resulted in convex curves at low temperatures, but straight lines at high temperatures. Dry heat resulted in concave curves at all temperatures. These results suggest that differences exist between wet and dry heat destruction of bacteria. Several possible explanations for the differences in the shape of the survivor curves were discussed.

TERPENE AND SESQUITERPENE HYDROCARBONS IN THE ESSENTIAL OIL FROM FRESH CELERY. C. W. WILSON III. *J. Food Sci.* **34,** 521–523 (1969)—The essential oils were prepared from celery puree by a batch distillation procedure, and by vaporization in a Votator Turba-Film evaporator and rectification of the vapors in a packed distillation column. The terpenes α-pinene, β-pinene, myrcene, limonene, γ-terpinene and cymene; and the sesquiterpenes β-elemene, β-caryophylene, α- and β-humulene and β-selinene were identified as constituents of the essential oils. The qualitative and quantitative relationships of the hydrocarbons between the two different oils were established and discussed.

THE RELATION OF FORCE TO SAMPLE DIMENSIONS IN OBJECTIVE MEASUREMENT OF TENDERNESS OF POULTRY MEAT. M. F. POOL & A. A. KLOSE. *J. Food Sci.* **34,** 524–526 (1969)—The resistance to shear was proportional to the equivalent diameter raised to a power of about 1.2, over a wide range of sizes, shapes and intrinsic toughness. Degree of compaction before shearing was significantly correlated ($r = 0.65$, significant at 0.1% level) with the force at first failure per unit of equivalent diameter, and the compaction naturally increased with increasing intrinsic toughness. These data will permit comparison of shear force measurements from meat samples of different sizes and shapes.

POLYGALACTURONASE CONTENT OF DATES AND ITS RELATION TO MATURITY AND SOFTNESS. S. HASEGAWA, V. P MAIER, H. P. KASZYCKI & J. K. CRAWFORD. *J. Food Sci.* **34** 527–529 (1969)—PG activity, virtually absent at the green stage, began to develop as maturity progressed. The greatest part of the development occurred at the late red stage and activity reached its maximum when the fruits commenced softening. Among the four grades o dates used in this study the activity was highest with natural, followec by waxy, number 1 dry and number 2 dry grades. The dates containec 0.9–1.3 units of PG per g of fresh tissue. The results of chemical anc physical analyses related to the texture are also reported.

SALT AND AGING TIME EFFECTS ON THE VIABILITY OF Trichinella spiralis IN HEAVY DRY-CURED HAMS AND SHOULDERS. J. D. CROUSE & J. D. KEMP. *J. Food Sci.* **34,** 530–53 (1969)—Salt and trichina larvae viability determinations were mad periodically by taking cores of lean samples after salt equalization anc weekly intervals during aging. Trichinae counts were made micro scopically after digesting samples in a H Cl-pepsin solution. Viabl larvae persisted throughout curing and salt equalization. However, the number of viable larvae was reduced greatly after 1 week of aging and no viable larvae could be found after 3 weeks aging. The averag salt concentrations for the hams and shoulders at this time were 3. and 4.5% respectively. Laboratory animals force fed lean sample aged 3 weeks or longer were found to be free from trichinae.

RELATIONSHIP BETWEEN MEAT SWELLING, VISCOSITY, EXTRACT-RELEASE VOLUME, AND WATER-HOLDING CAPACITY in EVALUATING BEEF MICROBIAL QUALITY. L. A SHELEF & J. M. JAY. *J. Food Sci.* **34,** 532–535 (1969)—With the microbially spoiled meats, the highest degree of correlation occurrec between SW and ERV, followed by ERV & WHC, SW & η, η & ERV and SW & WHC. These findings indicate that ERV and SW are quite similar and equally reliable in determining meat microbial quality while the relationships between η and ERV, and η & WHC were of lowe orders of significance. When fresh beef was homogenized with urea at levels between 0.5–5*M*, η correlated best with SW followed by ERV & η, and ERV & SW. Over the pH range 3–11, SW and ERV correlatec best followed rather closely by SW and η, and ERV & η.

IDENTIFICATION AND QUANTITATIVE ESTIMATION OF ALCOHOLS IN CELERY ESSENTIAL OIL. C. W. WILSON, III. *J Food Sci.* **34,** 535–537 (1969)—The alcohols in celery essential oil obtained from fresh celery by two essence recovery methods were separated by gas chromatography and identified by spectroscopic methods. Thirteen alcohols were reported. Twelve of the 13 alcohols have not been previously reported as constituents of celery essential oil. A semi-qualitative estimation of each constituent was made.

A COMPARISON OF FOUR METHODS FOR PASTRY TENDERNESS EVALUATION. C. G. STINSON & M. B. HUCK. *J. Food Sci.* **34,** 537–539 (1969)—Highly significant correlations were found between the subjective and objective methods of evaluation examined; the best agreement existed between the results of the organoleptic panel and those of the shear press. The panel and the shear press rated all four pastry types in the same order, the panel and shortometer disagreed on the tenderness of one type, and the tenderpen and panel ranked two of the four types similarly. The shear press was the most precise and the shortometer the least precise of the four methods investigated.

AUTOXIDATION OF METHYL LINOLEATE IN FREEZE-DRIED MODEL SYSTEMS. 4. Effects of Metals and of Histidine in the Absence of Water. K. H. TJHIO & M. KAREL. *J. Food Sci.* **34,** 540–543 (1969)—Histidine was antioxidant in the early stages of oxidation; later it became prooxidant. Cobalt chloride and manganese sulfate had strong catalytic activity at high (8.0 and 9.0) but not at low (4.0) pH. Histidine increased the catalytic activity of manganese but eliminated that of cobalt. Thin-layer chromatography showed that conditions under which histidine increased the prooxidant effect of manganese also resulted in the appearance of some decomposition products of histidine. These decomposition products may have catalytic activity.

ABSTRACTS:
IN THIS ISSUE

OMPOSITION OF MONTMORENCY CHERRY ESSENCE. 2. High-oiling Components. E. E. STINSON, C. J. DOOLEY, V. J. FILIPIC, C. H. HILLS *J. Food Sci.* **34,** 544–546 (1969)—The high-boiling eutral components of Montmorency cherry essence were concentrated y solvent extraction and separated by gas chromatography. The most bundant compounds were low molecular weight alcohols and benzalde-yde. Several terpenes and aromatic high molecular weight esters were ientified. The estimated concentrations of these components in the riginal essence are given.

VATER EXTRACTABLE PROTEINS OF PORCINE MUSCLE. T. ŻOLCZAK & M. WEBER. *J. Food Sci.* **34,** 547–552 (1969)—The water olubility of muscle proteins depend on the quality of the muscle, and s lowest in extremely PSE muscle. It was demonstrated with the aid of lectrophoresis on agar and acrylamide gels that some sarcoplasmic roteins which undergo precipitation 30 min after adding a citrate-hosphate buffer (pH 4.6) at temperature of 20°C, are extracted in maller amounts from PSE muscle than from normal muscle. One of nese fractions was benzidine positive.

NFLUENCE OF ORGANOPHOSPHATE INSECTICIDES ON AS-ORBIC ACID OXIDATION IN AQUEOUS SYSTEMS. B. M. REHFELD & D. E. PRATT. *J. Food Sci.* **34,** 551–553 (1969)—Or-anophosphate insecticides tested exhibited antioxidant activity. The ntioxidant activity of malathion in the presence of copper ions was ost when EDTA was added. Oxygenated, oxidized and hydrolyzed amples of organophosphates still had antioxidant activity. Experimental vidence indicates that the phosphate moiety of malathion is responsible or this antioxidant activity. It is postulated that the antioxidant activity night be due to the phosphate's ability to chelate metal ions.

EXTERNAL FAT COVER INFLUENCE ON RAW AND COOKED BEEF. 1. Fat and Moisture Content. A. P. WOOLSEY & P. C. PAUL. *Food Sci.* **34,** 554–557 (1969)—The moisture content of the lean amples was not affected by any of the variables. The total samples, which included the external fat cover, had moisture contents that re-lected the amount of crude fat present. The yield of crude fat was not ignificantly altered by the solvent used. Both solvents extracted signi-icantly more fat from the cooked lean samples than from the compar-ible raw ones. The lean samples from meat roasted at 163°C contained ignificantly more extractable fat than the samples roasted at 218°C.

STUDIES ON SOME PHYSICAL PROPERTIES OF BOVINE SKELETAL MUSCLE. R. C. KLINE, J. D. SINK, K. E. BEERY, F. H. ZIEGLER & L. L. WILSON. *J. Food Sci.* **34,** 557–560 (1969)—Generally, the steers had longer, heavier and lighter-colored muscles than the heifers. A negative relationship was noted between specific gravity and reflectance values for the crossbred beef muscles. The semimembranosus muscle was heaviest and accounted for the highest percent of the rough round in certain beef and dairy sire groups. In the dairy cattle, the weight of the b. femoris muscle was also affected by sire. Heritability estimates indicate that the weight of the semimem-branosus was highly heritable and its percent of rough round mod-erately heritable, in both beef and dairy cattle.

THERMOCHEMICAL FACTORS INFLUENCING THE DEATH KINETICS OF SPORES OF Clostridium botulinum 62A. L. N. KUZMINSKI, G. L. HOWARD & C. R. STUMBO. *J. Food Sci.* **34,** 561–567 (1969)—This investigation was undertaken to determine the death kinetics of spores of *Clostridium botulinum* on exposure to a dichlorodifluoromethane-ethylene oxide mixture. From the data ob-tained, survivor and thermochemical destruction curves for exposed spores were constructed, and from these, the resistance parameters, D and z, were determined. Variations in environmental moisture levels were reflected in changes in spore resistance and the increase in extent of spore death appeared as an exponential function of temperature. Time-temperature-moisture sterilization relationships were calculated to maintain the 12 D concept.

EXTERNAL FAT COVER INFLUENCE ON RAW AND COOKED BEEF. 2. COOKING TIME, LOSSES, PRESS FLUID and SHEAR FORCE VALUES. A. P. WOOLSEY & P. C. PAUL. *J. Food Sci.* **34,** 568–569 (1969)—Semitendinosus roasts were cooked either with or without the external fat cover to an internal temperature of 58°C at oven temperatures of 163° or 218°C. Roasts cooked at 218°C required a shorter total time in the oven. Presence or absence of the external fat cover had little effect on the min per lb. The higher oven temperature increased total cooking losses, as did presence of the fat cover. A greater amount of press fluid was obtained from roasts without the fat cover. The posterior cuts were more tender (Warner Bratzler shear) than the anterior ones. The lower oven temperature also reduced shear values.

COOKING METHODS AND HEATING EFFECTS ON DDT IN CHICKEN TISSUES. S. J. RITCHEY, R. W. YOUNG & E. O. ESSARY, *J. Food Sci.* **34,** 569–571 (1969)—DDT incorporated into chicken tissues during the growing period was reduced in concentration during cooking by either baking, frying, or steaming and during heating of tissues in closed containers for varying lengths of time. DDT was converted to DDD in each of the treatments, but the concentration of DDE was not altered significantly. Total losses of residue were greater when tissues were fried or steamed than when the samples were either baked or heated in closed containers. Losses of residue from chicken tis-sue occurred primarily through leaching of fat during the cooking process.

STUDIES ON BOVINE NATURAL ACTOMYOSIN. 2. Physico-chemical Properties and Tenderness of Muscle. H. K. HERRING, R. G. CASSENS, T. FUKAZAWA & E. J. BRISKEY. *J. Food Sci.* **34,** 571–576 (1969)—Reduced viscosity, ATP sensitivity and "actin" content (polyethylene sulfonate treatment) were higher for natural actomyosin prepared from muscle at 12–24 hr post-mortem than from pre-rigor muscle. Investigation of reduced viscosity of actomyosin from tough and tender muscle indicated a higher gel character in the actomyosin from tough muscle. No consistent difference was found in actomyosin from tough and tender muscle for ATP sensitivity, myosin content or actin content.

RELATION OF CHEMICAL STRUCTURE TO PLANT GROWTH-REGULATOR ACTIVITY IN THE PINEAPPLE PLANT: RETARDING SENESCENCE OF PINEAPPLE FRUIT WITH APPLICATIONS OF 2,4,5-TRICHLOROPHENOXYACETIC ACID AND 1-NAPHTHALENE ACETIC ACID. W. A. GORTNER. *J. Food Sci.* **34,** 577–580 (1969)—1-naphthaleneacetic acid and 2,4,5-trichlorophe-noxyacetic acid markedly retard further ripening of pineapple fruit and extend its marketable life as a fresh fruit. The effects are evident at dif-ferent stages of maturity and ripeness, different fruit densities, and from both pre-harvest and post-harvest applications. Even one ppm of 2.4.5-T has noticeable effect; 100 ppm appears optimum for senescence delay. For SNA, 500 ppm is an optimum level for dipping fruit. Refrigeration can supplement the effect of the chemical in retarding senescence.

STUDIES ON COMPOUNDS AND INDIVIDUAL LIPIDS OF WHEAT GERM. G. MORUZZI, R. VIVIANI, A. M. SECHI & G. LENAZ. *J. Food Sci.* **34,** 581–584 (1969)—A combination of chemical and chromatographic procedures demonstrated that 97% of total wheat germ lipids are nonpolar, while 3% are polar (glycolipids, phospholipids and proteolipids). The fatty acid composition in polar and nonpolar lipids was determined; the former contain a higher percentage of octadecatrienoic acid than the latter. Non polar lipids are tri-, mono- and diglycerides and sterol esters.

THE STEROLS AND TRITERPENES OF BANANA PULP. F. F. KNAPP & H. J. NICHOLAS. *J. Food Sci.* **34,** 584–586 (1969)—Using thin-layer chromatography and gas liquid chromatography the following substances were identified in banana pulp (*Musa sapientum L.*): Cycloartenol, cycloeucalenol, 24-methylene cycloartanol campesterol, β-sitosterol and stigmasterol. The major triterpene was 24-methylene cycloartanol while β-sitosterol accounted for greater than 72% of the sterol fraction.

WHISKEY COMPOSITION: IDENTIFICATION OF ADDITIONAL COMPONENTS BY GAS CHROMATOGRAPHY-MASS SPECTROMETRY. J. H. KAHN, P. A. SHIPLEY, E. G. LaROE & H. A. CONNER. *J. Food Sci.* **34,** 587–591 (1969)—Gas-liquid chromatographic methods are described for the identification of 87 components in distilled alcoholic liquors. Water-free concentrates of etherpentane or Freon extracts of the distillates were injected into different column systems and the column effluent transported to a mass spectrometer. To our knowledge, 34 of the identified compounds have not been reported previously in whiskey.

CALCIUM CHELATORS INFLUENCE ON SOME PHYSICAL AND CHEMICAL PROPERTIES OF RABBIT AND PIG MUSCLE. P. D. WEINER & A. M. PEARSON. *J. Food Sci.* **34,** 592–596 (1969)—Intravenous antemortem injections of EDTA, EGTA and CDTA all significantly inhibited muscle shortening during development of rigor mortis. Micro-injections of $CaCl_2$ increased shortening, but $MgCl_2$ had no measurable effect. Although average shear values for EDTA-treated rabbits were lower than those of untreated controls, the differences were not statistically significant. However, muscle from EDTA-treated pigs was signficantly improved in tenderness. A highly significant negative relationship existed between cooking losses and both initial pH and initial ATP values for both rabbit and pig muscle.

FRACTIONATION OF MUSCLE PROTEINS OF FRESH WATER FISH AND CHANGES DURING ICED STORAGE. B. R. BALIGA, M. N. MOORJANI & N. L. LAHIRY. *J. Food Sci.* **34,** 597–599 (1969)—Precipitation of actomyosin was complete at an ionic strength of 0.175 instead of at 0.225 as reported for chicken and mammalian muscle. But precipitation of fibrillar proteins was complete at an ionic strength of 0.05 as in chicken and mammalian muscle. The solubility of muscle-proteins in the buffer extract at an ionic strength of 0.55 showed a fall which was correlated with the maximum level of actomyosin in solution. Non-protein nitrogen and sarcoplasmic proteins did not change during iced storage. Viscosity of the buffer extracts was high or low depending on the state of rigor of the fish.

ENVIRONMENTAL INFLUENCE ON HIGH ENERGY PHOSPHATE METABOLITES IN PORCINE MUSCLE. E. D. ABERLE, N. W. THOMAS, J. M. HOWE & P. T. ARROYO. *J. Food Sci.* **34,** 600–603 (1969)—Longissimus dorsi muscle from pigs reared in 32°C environments had higher initial levels at ATP and CP and lower initial levels of G-6-P compared to muscle from animals reared at 21°C. Muscle from the latter group of animals exhibited more rapid postmortem glycolysis and increase in light reflectance. Results indicate that growing environment may affect the ability of porcine muscle to store and maintain high levels of high energy phosphate compounds.

PROVIDING UNIFORM MEAT CORES FOR MECHANIC SHEAR FORCE MEASUREMENT. C. L. KASTNER & R. L. HEN RICKSON. *J. Food Sci.* **34,** 603–605 (1969)—A significant ($P < 0.0$ diameter difference was observed between the hand and machine cores when the 1.90 cm and 1.27 cm bores were used. At these core ameters, there was less variation between machine bored cores and th removed by hand. There was no significant difference between c diameters of the two boring methods when the 2.54 cm bore was us However, in all cases the machine bored cores were larger in diame and produced a greater shear value than cores taken by hand.

TREATMENT AND POST-MORTEM AGING EFECTS ON T Z-LINE OF MYOFIBRILS FROM CHICKEN PECTORAL MU CLE. T. FUKAZAWA, E. J. BRISKEY, F. TAKAHASHI & T. YAS *J. Food Sci.* **34,** 606–610 (1969)—When the 24-hr stored samples w blendorized, electron micrographs showed two types of destruction the Z-lines of sarcomeres and myofibrillar fragments. (1) The degra tion and/or disappearance of Z-lines. (2) The breakdown of the ju tion of Z-line and I-filaments. A change in the state of the Z-line a the junction of the Z-line and I-filaments appeared to be indispensa for the fragmentation of the myofibrils. It was also shown throu phase contrast microscopic observations that sarcoplasmic protei participating in the glycolytic cycle, may play a role in the fragmen tion of the myofibrils.

COMPOSITION OF ORANGE ESSENCE OIL. R. L. COLEMA E. D. LUND & M. G. MOSHONAS. *J. Food Sci.* **34,** 610–611 (1969) The composition of midseason and Valencia orange essence oils h been determined. The 27 major components identified consist of monoterpenes, four sesquiterpenes, seven oxygenated compounds a two saturated cyclic hydrocarbons. A relatively large percentage valencene was found as well as a notable lack of long-chain aldehydes

CONSIDERATIONS FOR BEEF TENDERNESS EVALUATION G. C. SMITH, Z. L. CARPENTER & G. T. KING. *J. Food Sci.* 612–618 (1969)—Data were collected from 690 beef rib steaks evaluate the methodology of beef tenderness measurements. Resu indicate that cookery of frozen steaks with or without thawing has lit effect on tenderness or cooking loss. Evidence indicates that a tende ness gradient exists over the cross-section of the l. dorsi and sugge that core samples should be taken from as many and as varied positio as is feasible for existing research conditions.

ISOLATION, IDENTIFICATION AND COMPARISON OF TI VOLATILES OF PEACH FRUIT AS RELATED TO HARVE MATURITY AND ARTIFICIAL RIPENING. J. Y. DO, D. SALUNKHE & L. E. OLSON. *J. Food Sci.* **34,** 618–621 (1969) Volatiles of peach (*Prunus persica* L., Cultivar, Gleason Early H berta) fruit were studied by gas-liquid chromatography, thin-lay chromatography and infrared spectrometry. Hard-mature, firm-matu soft-mature, tree-ripe and artificially ripened hard-mature fruit we used. A total of 86 peaks were observed in the chromatogram of t tree-ripe peach volatiles. Major components of the identified volatil were gamma and delta lactones, esters, two aldehydes, one alcohol a one terpene. In general, concentrations of volatile components i creased with fruit maturity. Artificially ripened fruit, however, did n produce the same concentrations as did tree-ripe fruit.

GLYCOLYTIC ACTIVITY OF CHICKEN BREAST MUSCL MITOCHONDRIA. J. H. SOUTHARD & H. O. HULTIN. *J. Foo Sci.* **34,** 622–624 (1969)—A procedure is described for the preparati of chicken breast muscle mitochrondia which are capable of aerob respiration or anaerobic lactate production utilizing any of sever glycolytic intermediates including glucose. This indicates that the cor plete complement of glycolytic enzymes is associated with the prepar tion. The hexokinase-catalyzed phosphorylation of glucose is the rat limiting step both aerobically and anaerobically. The aerobic oxidatic of glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphc phate is equal to or greater than that with succinate. This demonstr tion of the association of the glycolytic complex with the mitochrond of muscle is the first other than with neural tissue and may have ir portant implications in post-mortem carbohydrate catabolism.

ABSTRACTS:
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'OLATILE COMPONENTS OF RAW PEANUTS: ANALYSIS BY GAS-LIQUID CHROMATOGRAPHY AND MASS SPECTROMETRY. H. E. PATTEE, J. A. SINGLETON & W. Y. COBB. *J. Food 'ci.* **34,** 625–627 (1969)—Ten volatile components have been isolated rom raw peanuts by means of low-temperature vacuum distillation and ifferential cryogenic trapping. Through the use of gas chromatographic nd mass spectrometric analyses, the isolated compounds have been dentified as: pentane, octane, methyl formate, acetaldehyde, acetone, nethanol, ethanol, 2-butanone, pentanal and hexanal. The "backbone" ompound of the raw peanut aroma appears to be hexanal, with other omponents adding the proper character.

AN AUTOMATIC SAMPLING SYSTEM FOR RESPIRATORY GASES AND RESPIRATORY RESPONSE OF IRRADIATED CITRUS FRUITS. G. F. GREEN, E. M. AHMED & R. A. DENNISON. . *Food Sci.* **34,** 627–629 (1969)—Continuous airflow at constant pressure and temperature was pumped through 24 chambers. The airflow was regulated by needle valves connected to 3-way solenoids which sequentially switched samples to a CO_2 infrared analyzer and recorder. A iming cycle was selected so that each sample was analyzed every 3 hr. The recorder was calibrated to read directly in percent CO_2. Results were expressed as ml CO_2/kg product/hr. Irradiated fruits exhibited narked increases in CO_2 production reaching a maximum 1–2 days fter treatment then gradually declining. Waxing limited the production of CO_2, especially with irradiated fruits.

CONCENTRATION EFFECT ON ODOR ADDITION OR SYNERGISM IN MIXTURES OF METHYL SULFIDE AND TOMATO UICE. D. G. GUADAGNI, J. C. MIERS & D. W. VENSTROM. . *Food Sci.* **34,** 630–632 (1969)—When methyl sulfide and tomato uice were diluted in distilled water to less than 10 ppb and 1,000 ppm espectively and then mixed, no significant interaction between juice nd Me_2S was observed in the mixture. The odor intensities of the two omponents (as measured by threshold values in distilled water) were dditive at subthreshold concentrations. As the concentrations of juice nd Me_2S were increased before mixing, an increasing degree of interction or synergism was observed in the mixture. At concentrations of 0^5 ppm juice and 1 ppm Me_2S, odor intensity of the mixture was pproximately double that which would be expected on an additive asis.

THE EFFECT OF pH TEMPERATURE TREATMENTS ON THE CALCIUM-ACCUMULATING ABILITY OF PURIFIED SARCOPLASMIC RETICULUM. M. L. GREASER, R. G. CASSENS, W. G. HOEKSTRA & E. J. BRISKEY. *J. Food Sci.* **34,** 633–637 (1969)—The calcium uptake activity of purified preparations from 0-hr old muscle was more than 20-fold higher than that from 24-hr old muscle. The ultrastructure of membrane fragments from 0- and 24-hr old muscle was nearly identical as revealed by electron microscopy of negative stained preparations. Incubation of sarcoplasmic reticulum fragments at pH 5.6 and 37°C for 1 hr caused a loss of calcium accumulating ability, which suggested that muscle pH and temperature may be responsible for the post-mortem inactivation observed in situ.

PACIFIC COD MUSCLE 5′-NUCLEOTIDASE. H. L. A. TARR, L. J. GARDNER & P. INGRAM. *J. Food Sci.* **34,** 637–640 (1969)—A 5′ nucleotidase purified about 20-fold from Pacific cod (*Gadus macrocephalus*) muscle was unstable, losing 85% of its activity in 1 hr at 37°C and 40% in 1 hr at 30°C. It was stable for 6 days at 0°C, but was destroyed rapidly at pH 4–5. The enzyme was strongly inhibited by EDTA, pyrophosphate, KF, $ZnCl_2$ (1–10 m*M*) and less markedly inhibited by GSH, 2-mercaptoethanol, carbonate and $CaCl_2$ (10–100 m*M*). The optimum pH was 7.6, the Km was 5×10^{-4} *M* with UMP and 8×10^{-4} *M* with IMP. The following nucleotides were hydrolyzed in order of effectiveness, UMP, IMP, CMP, d-AMP, GMP, d-IMP, d-GMP, d-UM and AMP.

THE MAILLARD REACTION AND ITS INHIBITION BY SULFITE. D. J. McWEENY, D. O. BILTCLIFFE, R. C. T. POWELL & A. A. SPARK. *J. Food Sci.* **34,** 641–643 (1969)—Studies on a sulfited glucose/glycine system showed that rapid color development, due to the Maillard reaction, did not begin until "free" SO_2 (i.e., sulfite/bisulfite ion) had almost disappeared from the system. At this time "free" SO_2 was less than 5% of that added and 28% was present in reversible combination with, e.g., carbonyl compounds. These results are considered in relation to recently published hypotheses on the intermediates involved in the Maillard reaction and on its inhibition by sulfites.

• NOTICES •

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Variations in Proximate Composition of North Carolina Scallop Meats

SUMMARY—*Bay scallops* (Aequipecten irradians), *calico scallops* (Aequipecten gibbus) *and sea scallops* (Placopecten magellanicus) *were sampled from known locations and months of harvest for determination of proximate composition. A scallop sample* (Chlamys hericius) *was obtained from near Whidbey Island, Puget Sound, for comparative purposes. The adductor muscles were analyzed for total moisture, protein, fat, glycogen and ash content. The proximate composition varied widely among locations and months of harvesting. In general, coefficients of variation were relatively low for moisture and protein but high for fat, glycogen and ash. The range for each component was fairly similar among species studied. The percent ranges for bay and calico scallop meats, respectively, were: moisture 74.15–83.66, 76.12–81.86; protein 13.44–21.57, 13.28–17.53; fat 0.23–0.91, 0.23–1.13; glycogen 0.13–3.86, 0.35–3.71 and ash 1.09–2.24, 1.17–1.91. Sea scallop percent ranges were: moisture 74.63–80.97; protein 13.87–18.11; fat 0.31–0.96; glycogen 0.27–8.74 and ash 1.28–1.81.*

INTRODUCTION

THERE IS A dearth of scientific information available on adductor meat composition. Igawa et al. (1962) reported that the ranges in proximate analyses of adductor muscle of sea scallops were 74.3–78.0% moisture, 0.31–0.37% fat, 16.87–18.75% protein and 1.7–6.8% glycogen. These scallops were taken off the coast of Japan but no specific reference to species was available. Glycogen was found to decrease gradually from June to September. Power et al. (1964) found glycogen level of 0.5–0.8% in sea scallops (*Placopecten magellanicus*). The authors noted that these values were low as compared to levels of 2–5% previously found in their laboratory. They presumed the variations were caused by sexual maturity, spawning season and feeding intensity. However, no consistent trend or data was established to support their hypothesis concerning these factors.

In other studies on sea scallop (*Placopecten magellanicus*) composition, Matsumoto et al. (1967) reported a proximate analysis of 80.0% water, 12.0% protein, 0.5% lipids and 1.0% ash. Idler et al. (1964) found a range of 1.04–1.09% fat in May and early June with a decrease to approximately 0.80% by September to December. Stansby (1962) reported that scallop meats had less than 5% fat and a range of 15–20% protein.

This study includes the determination of proximate composition variations as to location and date of harvest. Subsequently a detailed treatment of the data will be made to establish any relationships of proximate composition to environmental and physiological conditions.

A knowledge of proximate composition is of value in the study of product quality and processing methods. This investigation was designed to determine proximate composition of scallop adductor muscle.

EXPERIMENTAL

Sample collection

Bay scallops (*A. irradians*) and calico scallops (*A. gibbus*) were sampled in duplicate each month using commercial type scallop dredges. Scallops were loaded on the boat deck and transported to local docks. Transfer was made to shucking houses where the meats were hand shucked at room temperature, rinsed in tap water, packed in the sample containers and frozen. Hand shucking was accomplished by opening the shell, removing the vicera and severing the adductor muscle at the mantle. The delay in time from harvesting to shucking was 4–12 hr for calico and 4–8 hr for bay scallops.

Data were obtained on bay scallop composition at monthly intervals during 1965–66 from randomly selected sites in Bogue and Core Sounds, North Carolina. In the 1966–67 period Bogue Sound was sampled every other month, from September, 1966 through July, 1967, near Drum Shoals (34°43'N, 76°45'W) and Dog Island (34°42'N, 76°54'W). Core Sound was sampled every other month, from October, 1966, through August, 1967, near Whitehurst Island (34°39'N, 76°31'W) and Horse Island (34°47'N, 76°24'W). Calico scallops were taken monthly during 1965–67 from the Atlantic Ocean, 10–20 miles south-southeast of Beaufort Inlet Channel, North Carolina (vicinity 34°21'N, 76°42'W).

Sea scallop (*P. magellanicus*) samples were obtained from an area off the North Carolina-Virginia coast from September through December, 1966, and in May and July, 1967. Also, samples (*P. magellanicus*) and (*Chlamys hericius*) were obtained in November, 1966, from Georges Bank and Whidbey Island, Puget Sound. Sea scallop samples were obtained from commercial sources, frozen when removed from the vessel and shipped immediately in dry ice to the laboratory. The Puget Sound samples were collected by personnel of the Technological Laboratory, U.S. Bureau of Commercial Fisheries, Seattle, Washington, frozen and shipped in dry ice to the laboratory.

During the bay and calico scallop harvesting and shucking operations, data were recorded for harvest date, location, air temperature, water temperature, water salinity, initial and chilled meat temperature, individual meat weight and time from harvest to shucking. Samples were obtained from commercial lots and techniques of shucking and rinsing the samples were closely controlled, but similar to commercial practice.

Immediately following rinsing, the meats were placed in polyethylene lined, fiberboard, pint containers; tightly sealed; frozen with liquid nitrogen; packed in dry ice and transported to the laboratory. Upon arrival at the laboratory the frozen meats from all scallop samples were transferred to a −27 ± 2°C freezer and stored for approximately 30 days prior to completing chemical analyses. The adductor muscle was the only component of the scallop analyzed.

Apparatus and analytical procedures

Samples (about 30 scallops) were thawed at 3 ± 1°C for 24 hr and homogenized. The blended sample was subdivided into appropriate size units and analyzed for total moisture, protein, fat, glycogen and ash. Total nitrogen, ash and moisture were determined by the procedure of A.O.A.C. (1965), except that drying for total moisture was accomplished in a vacuum oven at 65°C for 16 hr. Total fat content was accomplished by the procedure of Dambergs (1956). Frazer's (1961) method was used to determine glycogen by a modification using a Whatman #2 filter paper. Polarimeter readings were standardized for scallop glycogen.

Data were analyzed statistically according to methods presented by Snedecor et al. (1957).

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Table 1—Proximate composition of bay scallop meats harvested in 1965–66 from randomly selected locations in Bogue and Core Sounds, North Carolina[1]

Month	Composition (%)				
	Moisture	Protein	Fat	Glycogen	Ash
Sept.	74.15	21.57	0.54	1.92	2.24
Oct.	81.59	16.30	0.46	0.19	1.55
Nov.	80.98	15.86	0.43	0.85	1.45
Dec.	80.09	15.48	0.42	1.87	1.36
Jan.	78.66	15.46	0.47	3.29	1.38
Feb.	81.16	14.75	0.49	1.82	1.23
Mar.	82.15	16.44	0.68	1.62	1.10
Apr.	80.63	16.24	0.61	1.90	1.27
May	78.55	17.02	0.54	1.48	1.52
June	80.09	15.12	0.48	2.45	1.32
July	81.77	13.55	0.50	1.26	1.22
Aug.	80.04	15.80	0.57	2.30	1.36
Mean, weighted[2]	79.90	15.75	0.51	2.04	1.39

[1] Each value was obtained by two or more determinations on each of two samplings.
[2] Obtained by calculating the mean in proportion to the number of samples for each period.

RESULTS

Bay scallop composition

The proximate composition of adductor muscle meats from bay scallop is presented in Tables 1 (1965–66) and 2 (1966–67).

The moisture content of bay scallop meats was significantly different at the 5% level between harvest years, sounds, locations within the sounds and among months within location. The coefficient of variation was 1.5% for the sample error on a per determination basis. The significant difference in variables tested and the low coefficient of variation showed that a moisture content standard cannot be established without specifying location and date of harvest.

The protein content of bay scallop meats harvested in 1965–66 was significantly higher than that of meats sampled in 1966–67 from Core Sound, but they were not significantly different from meats sampled from Bogue Sound. The 1966–67 samples from Bogue Sound were significantly higher in protein content than Core Sound samples. There was a significant difference in protein content among months sampled in 1965–66, but no significant difference was found in 1966–67. The coefficient of variation was 7.9% for error on a per determination basis; the protein content being fairly consistent for scallops at a specific location and month of harvest.

The fat content of bay scallop meats taken from 1966–67 Core Sound samples was significantly higher than 1966–67 Bogue Sound and 1965–66 random samples. The fat content was significantly higher in scallop meats taken from Dog Island than in samples from Drum Shoals in Bogue Sound. Also, samples from Horse Island were significantly higher in fat content than those from Whitehurst Island in Core Sound. However, only Core Sound samples were significantly different in fat content among months within location. The coefficient of variation was 25.2% for error on a per determination basis. Thus, the fat content of bay scallop meats was extremely variable within sampling areas and dates as well as between locations and among the months harvested. Fat content did not exceed 1.0% in any of the samples.

Glycogen content of bay scallop meats harvested from the 1965–66 random locations was significantly higher than that of the 1966–67 samplings. There was no significant difference in glycogen content between sounds. Glycogen content of meats taken from Drum Shoals was significantly higher than those from Dog Island in Bogue Sound. No significant difference was found between locations within Core Sound. Highly significant (1% level) differences were found in glycogen content among months for all locations. A high coefficient of variation (40.8%) was obtained for error on a per determination basis.

The ash content of the scallop meats was significantly higher in the 1966–67 Bogue Sound samples than either the 1966–67 Core Sound or 1965–66 random location samples. There was no significant difference in ash content between 1966–67 Core Sound and 1965–66 random location samples. A significant difference was found among months within both locations of Core Sound and within the 1965–66 random locations. No difference was found among months for Bogue Sound.

Coefficients of correlation between per-

Table 2—Proximate composition of bay scallop meats harvested in 1966–67 from two locations, each in Bogue and Core Sounds, North Carolina[1]

Month	Sound[2]	Composition (%)									
		Moisture location		Protein location		Fat location		Glycogen location		Ash location	
		A	B	A	B	A	B	A	B	A	B
Sept.	Bogue	81.02	80.62	16.36	15.77	0.28	0.27	0.71	1.28	1.46	1.47
Oct.	Core	83.39	83.66	15.56	15.50	0.28	0.37	0.13	0.22	1.51	1.38
Nov.	Bogue	83.05	81.44	13.64	15.14	0.57	0.42	0.36	0.22	1.39	1.73
Dec.	Core	81.19	83.06	15.46	13.44	0.58	0.68	0.98	1.09	1.55	1.25
Jan.	Bogue	78.14	82.17	15.89	15.39	0.47	0.53	2.77	0.31	1.50	1.42
Feb.	Core	82.69	79.30	13.46	14.12	0.91	0.64	1.41	2.51	1.12	1.68
Mar.	Bogue	77.40	81.91	17.00	15.74	0.36	0.60	3.86	0.99	1.40	1.30
Apr.	Core	80.82	83.38	14.82	13.44	0.67	0.66	[3]	[3]	1.26	1.09
May	Bogue	79.65	80.59	15.52	16.20	0.39	0.54	1.01	0.42	1.60	1.43
June	Core	[4]	[4]	[4]	[4]	[4]	[4]	[4]	[4]	[4]	[4]
July	Bogue	[4]	79.42	[4]	16.52	[4]	0.71	[4]	0.92	[4]	1.42
Aug.	Core	74.63	[4]	16.60	[4]	0.57	[4]	2.19	[4]	1.73	[4]
Bogue	Mean, weighted[5]	79.85	81.02	15.68	15.72	0.41	0.51	1.74	0.69	1.44	1.50
Core	Mean, weighted	80.54	82.35	15.04	14.12	0.64	0.76	1.18	1.41	1.41	1.36

[1] Each value was obtained by two or more determinations on each of two samples.
[2] Locations within sounds: Bogue A = Drum Shoals; Bogue B = Dog Island; Core A = Whitehurst Island; Core B = Horse Island.
[3] Samples were lost due to freezer failure prior to glycogen analysis.
[4] Scallops were unavailable at the times and locations indicated by lack of data. Statistical calculations were used to compensate for all missing data.
[5] Obtained by calculating the mean in proportion to the number of samples for each period.

Table 3—Proximate composition of Calico scallop meats harvested in different years from the North Carolina Atlantic coast[1]

Month	Composition (%)									
	Moisture		Protein		Fat		Glycogen		Ash	
	1965–66	1966–67	1965–66	1966–67	1965–66	1966–67	1965–66	1966–67	1965–66	1966–67
Sept.	79.30	78.68	15.88	16.40	0.70	0.48	2.37	1.74	1.27	1.43
Oct.	77.69	79.80	16.76	17.10	0.66	0.48	2.32	1.87	1.40	1.43
Nov.	78.91	79.45	16.36	15.35	0.43	0.27	2.10	1.18	1.37	1.82
Dec.	81.86	80.39	14.92	14.33	0.41	0.57	0.98	1.10	1.29	1.59
Jan.	[2]	79.31	[2]	16.14	[2]	0.23	[2]	0.57	[2]	1.91
Feb.	81.74	79.72	15.33	16.24	0.69	0.49	0.35	0.79	1.17	1.75
Mar.	80.94	80.33	17.40	16.94	0.66	0.44	0.58	[3]	1.29	1.40
Apr.	80.66	80.31	16.62	15.70	0.67	0.47	1.33	[3]	1.20	1.37
May	80.38	80.23	17.53	15.09	0.68	0.70	0.36	1.00	1.45	1.47
June	80.00	79.91	13.28	15.57	0.51	0.87	2.92	1.41	1.28	1.53
July	76.12	81.36	15.21	15.12	0.47	0.83	3.12	0.84	1.74	1.46
Aug.	78.27	78.95	15.81	14.93	0.56	1.13	3.71	1.21	1.50	1.55
Mean, weighted[4]	79.54	79.87	15.92	15.75	0.59	0.57	1.80	1.17	1.36	1.56

[1] Each value was obtained by two or more determinations on each of two samplings.
[2] Scallops were not obtained due to inclement weather during January, 1966.
[3] Samples were unavailable due to freezer failure prior to glycogen analysis. Statistical calculations were used to compensate for all missing data.
[4] Obtained by calculating the mean in proportion to the number of samples for each period.

cent chemical components of bay scallop meats are presented in Table 4. Significant correlations, although low, were found between total solids and glycogen and between ash and fat level. None of the other components had significant correlations.

Calico scallop composition

Proximate analyses for adductor muscle meats from calico scallops are presented in Table 3. Data were obtained from scallops harvested at regular monthly intervals from September, 1965 to August, 1967. Data were treated as two annual experiments to determine possible differences between years.

A statistical analysis of composition data showed that there were no significant differences in moisture, protein or fat levels between years of harvest or among months within years. The coefficients of variation for error on a per determination basis were moisture 1.9%, protein 11.8%, fat 33.1%. As found for bay scallop meats, fat content was low, being less than 1.13% for all samples, but highly variable within sampling units.

There was a highly significant difference in glycogen content between years and among months within years of harvest. The coefficient of variation was 37.7% for error on a per determination basis. The means and variabilities were comparable to those found in bay scallops in 1966–67.

Data for ash content of calico scallop meats indicated a highly significant difference between years of harvest, but differences among months within years were not significant. The coefficient of variation was 10.0% for error on a per determination basis, and was lower than that of bay scallops. Ash level was between 1.0–2.0% for all samples.

Coefficients of correlation between chemical components of calico scallop meats are presented in Table 4. A significant but low correlation was obtained between percent total solids and percent glycogen as was found with bay scallops. None of the other components showed a significant relationship.

Sea scallop composition

The proximate analyses of sea scallop and Puget Sound samples collected in 1966–67 are presented in Table 5.

The mean moisture content of the meats was similar for scallop species samples from the three locations. This level was approximately 2.0% lower than the moisture content of bay and calico scallop meats. Also, monthly variation in moisture content of meats harvested from the North Carolina-Virginia coastal area had a variation approximately equal to that of bay and calico scallop meats.

DISCUSSION

THE COEFFICIENTS of variation for moisture and protein content of bay and calico scallop meats indicated that, when

Table 4—Coefficients of correlation between chemical components of bay and calico scallop meats.[1]

Percentage	Bay scallops				Calico scallops			
	Chemical component (%)				Chemical component (%)			
	Ash	Fat	Protein	Glycogen	Ash	Fat	Protein	Glycogen
Total solids	0.32	−0.09	0.43	0.68	0.37	−0.06	0.13	0.69
Ash		−0.50	0.25	−0.22		−0.32	−0.08	−0.07
Fat			−0.28	0.19			−0.09	−0.05
Protein				0.02				−0.18

[1] Coefficients must exceed 0.49 to be significant at the 0.01 probability level; $n = 25$.

Table 5—Proximate composition of sea and Puget Sound scallops harvested in 1966–67 from randomly selected locations.

Location	Month	Composition (%)				
		Moisture	Protein	Fat	Glycogen	Ash
Whidbey Island, Puget Sound[1]	Oct. '66	77.09	20.11	0.47	0.60	1.79
Georges Bank	Oct. '66	77.97	19.27	0.25	0.78	1.62
North Carolina-Virginia Coast[2]	Sept. '66	74.63	15.20	0.47	8.74	1.28
	Oct. '66	78.75	17.27	0.42	1.04	1.81
	Nov. '66	74.76	13.87	0.31	7.34	1.58
	Dec. '66	80.97	18.11	0.55	0.27	1.66
	May '67	79.43	15.00	0.96	2.77	1.36
	July '67	76.75	15.64	0.88	5.38	1.47
	Mean	77.55	15.84	0.59	4.25	1.52

[1] *Chlamys hericius.*
[2] *Placopecten magellanicus.*

harvest location and month are specified, fairly consistent values are obtained (Tables 1 and 2). Significant differences between locations and among months of harvest showed that environmental factors are responsible for large variations in these components. Monthly samplings indicated no particular trend for moisture content. There was a slight trend for protein to increase in the fall and spring for both species. A direct relationship has been established between the protein and moisture content of skeletal muscle of domestic animals, but this was not obtained for scallop meats (USDA, 1965).

The fat and glycogen content of bay and calico meats indicated significant differences among sampling variables, even with high coefficients of variation. No consistent trends for harvest locations or months could be established. Since these components varied widely within a location and monthly sampling unit, the data suggests that both inherent and environmental factors influenced fat and glycogen deposition. Further investigations are underway to establish causes for large variations of these components within sampling units.

Variation of the ash content of scallop meats was low within a location and monthly sampling unit. The differences that were significant suggest that location of harvest was the major cause of variation.

Total combined levels of fat, glycogen and ash were relatively low when compared with moisture and protein levels.

Coefficients of correlation between total solids and glycogen suggest that glycogen deposition is inversely related to moisture retention (Table 4). Since the coefficient of variation was high for glycogen, this relationship may be of importance in subsequent investigations for evaluating quality.

The proximate analyses of calico scallop meats were similar in magnitude and degree of variation for error to those for bay scallop meats. However, variations in composition were not as large for calico as for bay scallops. The calico harvests were from an area having relatively uniform environmental factors such as water temperature, salinity and water depth. This suggests that environmental factors have a profound influence upon the proximate composition.

Results obtained in this study support the hypothesis that environmental factors contribute substantially to differences in proximate composition. Further treatment of these data is underway to determine if environmental factors and physiological conditions were directly related to proximate composition.

The data presented in Table 5 were obtained to compare proximate compositions with other species and locations during the term of this investigation. These results indicated that variations in sea scallop species sampled are similar in composition to that of calico scallops. Definite statements as to the proximate composition of scallop meats cannot be made unless location and date of harvest are specified for a particular species.

On the basis of these studies, the mean level of a particular component is of little value in defining the composition of scallop meats. However, when all variables are considered, the ranges in proximate composition were relatively similar among the species, but the variation for any selected component was large. As a result, variations in proximate composition were large enough to prohibit the establishment of standards for the industry.

The large variations occurring among scallop meats indicate that additional research is needed to determine the factors responsible for differences in proximate composition. Even with existing variations in components, these findings can be of value to investigators desiring to improve processing techniques or to develop methods of culturing scallops for commercial production.

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Hedonic Differences as a Function of Number of Samples Evaluated

SUMMARY—This study investigated the limit of the number of samples that can be reliably evaluated for preference in a single laboratory test session. Multiple samples were presented to 200 subjects randomly assigned to five conditions. Two critical samples were included under each condition and the number of other samples varied from zero to four. Two replications of each set were evaluated by each subject in a single session without his knowledge that samples were repeated. The total number of samples for the five experiments were 4, 6, 8, 10 and 12. The experiment was run separately with milk, soup and gravy base and maple syrup. There was no evidence that serving up to 12 samples in a single session adversely affected preference discrimination.

INTRODUCTION

IN THE TYPICAL laboratory situation, the time a subject actually spends in the taste-test booth is only a part of the total time he devotes to testing. Indirect time costs—breaking his job routine, walking to and from the laboratory, waiting to test or partaking of refreshments—taken together are probably greater than the direct time costs. Thus, it may be assumed that the more samples an individual can evaluate in one session, the more work the laboratory can produce with a given expenditure of subject time. Also, laboratory personnel can be more efficient because of economies in preparation of samples and administration. In central location testing, where paid subjects are recruited from outside the organization, costs can be reduced substantially by getting more information from each person (Girardot, et al., 1968).

Perhaps more important is that often, when each person has tested all samples, more straightforward experimental designs can be used instead of complicated ones such as balanced incomplete blocks. More precise estimates of inter-judge variability and judge-sample interaction can be used in the analyses of variance and the tedium and assumptions inherent in block adjustments can be avoided. Intuitively, there is a limit on the number of samples that can be presented. Most people can be induced to cooperate, but does acquiescence bring with it a loss in performance? Deterioration would not necessarily be due to sensory adaptation and loss of acuity, but might be a function of motivation as reflected in reduced attention, carelessness and general confusion.

Bradley, et al., (1954) reviewed much of the earlier literature on this topic. From two to eight samples per session have been advocated by various researchers, but usually there was no evidence that the recommended number was actually the optimum. These experiments as a group were inconsistent in the ranges of session length investigated. Some started with the assumption that the permissible maximum is two samples, but dared to go as high as three or four. Others went far beyond this limit.

Many experiments have dealt with sensory discrimination rather than with preference. Laue, et al. (1954) reported a loss of discrimination in the second of two successive triangle tests when maple syrup was the test material; however, this was not true for coffee. Mitchell (1956) found no loss of discrimination in duo-trio testing of beer even when the session was extended to four sets. Brandt, et al. (1956) went even further. Using various alcoholic beverages as test media, he tried as many as six duo-trios at one session, and reported that there was no loss of sensitivity. Pfaffman, et al. (1954) had subjects test repeatedly during 40 min sessions, employing the duo-trio and triangle tests with various food types, and found no significant loss of discrimination even after as many as 75 samples.

Preference testing may present a different kind of problem. Even though a subject's acuity might not be affected by continued exposure to food stimuli, his feelings may be altered. Bradley, et al. (1954) found no effect on levels of rating for various foods when they were served early vs. late in an eight-sample series. Sather, et al. (1960) had untrained subjects rate a series of 20 samples in four sets of five samples each. No loss of discrimination was found. Girardot, et al. (1968) reported that consumers' preference discrimination was better with the second pair than with the first pair of coffees.

In most preference testing the absolute levels of rating are of secondary importance. The main concern is with the direction and amount of the differences among competing samples. The present study took this approach in investigating the effect of extended testing. The basic question was: do differences in preference among food samples of the same subclass remain constant as the number of samples is increased?

EXPERIMENTAL

THERE WERE three replications of the basic experimental design which differed only in the products tested—milk, syrup and a broth made from soup and gravy base. The testing was done in the Food Acceptance Laboratory, U.S. Army Natick Laboratories, according to their normal practices. Samples were evaluated by ratings on a 9-point hedonic scale.

Subjects

Test subjects were selected randomly from the Natick Laboratory taste-test pool of about 700 persons. For each replication, five groups of 40, one for each experimental condition, were selected without replacement. Thus, no subject tasted a given food type more than once. Selection of the samples of subjects for each of the three replications was independent. Thus, it is possible that some people participated two or even three times in all.

Conditions

There were five conditions which varied only in the number of samples presented. Each condition was run in a separate session. In each session a subject evaluated all samples twice. Each time, subjects were told beforehand the number of samples but not of the duplications. The samples included in the various conditions were: first—A and B: second—A, B and C; third—A, B, C and D; fourth—A, B, C, D and E; and fifth— A, B, C, D, E and F. After rating all samples once, a subject immediately rated them again. The serving orders in each half of a condition were balanced insofar as possible, and the orders in the two halves were independent.

Selection of samples

Since the analysis focused on the difference between A and B, it was important that these two samples be neither too far apart nor too close together in preference. If the difference were very large, then the

maximum possible difference in rating might be obtained even in the least suitable condition. If the difference were too small, then even the most sensitive test method might not be able to demonstrate an effect and the negative results would be meaningless. Hence, the strategy was adopted of pilot testing the series of six samples to be used in the final test and designating the one with the second highest rating as Sample A and the one with the second lowest rating as Sample B.

This plan was carried out for soup and gravy base and milk, but revision was required for syrup. One of the original six samples was sorghum, which had a very low pretest rating (3.77). To have included this item might have induced contrast effects that would reduce discrimination among the other samples. Since it would have been tested in the 6-sample condition, the main effects of numbers might have been confounded with contrast effects in some complex manner. Hence, it was eliminated and the third-ranked sample was duplicated as both D and F. Another change was to designate the best syrup sample as A and the next best as B, since this contrast appeared to suit the purposes of the study. Table 1 describes the samples used in each replication, and gives certain details about the testing procedures.

RESULTS

THE RESULTS are reported separately by replication. Tables 2–4 show, for the three products, respectively, the mean rating of each sample in each condition by experimental half, then the totals. Table 5 demonstrates the effect of the experimental conditions on the ratings of the critical samples, A and B, for all three products. It gives the averages for the first and second halves and the differences between these averages. It also shows the differences between samples (A-B) for each experimental half, and, finally, the differences of the differences (second half minus first half). Note that a positive value in the last column indicates that the second half was more discriminating, while a negative value shows better discrimination in the first half.

Table 6 presents the analyses of variance for the critical samples for all products. Table 7 summarizes the most important information obtained from the analysis of variance made for each condition separately. Only the main effects of sample and experimental half, and the sample-half interaction, are given.

Table 1—Test samples and serving conditions.

Product	Sample description	Pretest rank order	Serving conditions
Soup and gravy base	A (All special formulations)	2	Temperature: 150–155° F
	B	5	
	C	3	Sample: 2 ounces
	D	4	
	E	1	Interval: 30 seconds
	F	6	
Milk	A 92% fresh, 8% recon.[1]	2	Temperature: room
	B 68% fresh, 32% recon.	5	
	C 84% fresh, 16% recon	3	Sample: 1 oz
	D 76% fresh, 24% recon.	4	
	E 100% fresh, 0% recon.	1	Interval: 30 sec
	F 60% fresh, 40% recon.	6	
Syrup	A Commercial	1	Temperature: 115–120°F
	B Commercial	2	
	C Pure maple	4	Sample: 1/2 oz
	D Govt. standard[2]	3	
	E Commercial	5	Inveral: 60 sec
	F Govt. standard[2]	3	

[1] Reconstituted—33% condensed milk and 67% water.
[2] Samples D and F were the same.

Table 2—Soup and Gravy Base. Mean ratings of samples in each experimental condition (N = 36 in 6-sample, 40 in all others).

	Experimental condition				
	2 Sample	3 Sample	4 Sample	5 Sample	6 Sample
Sample A					
1st half	7.13	6.93	6.68	6.83	6.58
2nd half	6.70	6.98	6.45	6.33	5.81
Total	6.91	6.95	6.56	6.58	6.19
Sample B					
1st half	6.85	6.38	6.28	6.13	5.67
2nd half	6.48	5.58	5.88	5.48	5.14
Total	6.66	5.98	6.08	5.80	5.40
Sample C					
1st half	—	6.85	6.90	6.93	6.28
2nd half	—	6.05	6.23	6.63	6.25
Total	—	6.45	6.56	6.78	6.26
Sample D					
1st half	—	—	6.80	7.33	6.86
2nd half	—	—	6.80	6.68	7.53
Total	—	—	6.80	7.00	6.69
Sample E					
1st half	—	—	—	6.88	6.83
2nd half	—	—	—	6.63	6.06
Total	—	—	—	6.75	6.44
Sample F					
1st half	—	—	—	—	4.56
2nd half	—	—	—	—	3.81
Total	—	—	—	—	4.18
Total					
1st half	6.99	6.72	6.66	6.82	6.23
2nd half	6.59	6.20	6.34	6.35	5.60
Total	6.79	6.46	6.50	6.58	5.86

Soup and gravy base

Ratings were obtained from only 36 subjects for the 6-sample condition. Thus, for purposes of the analyses presented in Tables 5 and 6 the ratings given by four randomly selected subjects were eliminated from each of the other conditions to equalize the *N*.

Across the entire experiment Samples A and B differed by 0.61 scale points (Table 5), a difference significant at the 0.1% level (Table 6). Samples were rated consistently higher in the first half than in the second half of the sessions by an average of 0.44 scale points. The main effect of condition was significant at the 1% level. Most of this effect was probably attributable to the 6-sample condition, where the samples were rated particularly low, since the effect was not significant in a separate analysis which excluded the 6-sample part. Note that the average ratings tended to decrease as the number of samples increased (Table 2).

No interaction involving sample, half or experimental condition was statistically significant (Table 6). We would conclude that, although ratings tended to be lower in the second half than in the first, there was no evidence that the differences between the samples were affected either by the number of samples or by whether they were presented in the first half or in the second half of the session.

The summary of the separate analyses by condition (Table 7) shows that Samples A and B always differed significantly (0.1% level) with the exception of the 2-sample condition. Also, the main effect of experimental half was always significant. The interaction of sample and half was significant only for the 3-sample condition (5% level). For this condition, the

difference between the samples was much greater in the second than in the first half (1.40 vs. 0.55); however, this increased differentiation failed to appear in the other four conditions. Thus, again, there was no evidence that either the total number of samples or position in the serving order had any consistent effect on the difference between the critical samples.

Milk

The difference of 1.08 scale points between Samples A and B (Table 5) was larger than was anticipated on the basis of the pilot test results. Again, ratings in the first half were significantly (5% level) higher than in the second half (Table 6). The absence of a significant interaction between sample and half implies that the difference between Samples A and B in the first half (0.97) was not significantly smaller than the difference in the second half (1.21).

The main effect of condition was not significant, so there is no evidence that the total number of samples affected the level of rating. Since none of the interactions was significant, we cannot conclude that the total number of samples or position in the serving order affected the difference between Samples A and B. The figures in Table 5 would seem to indicate otherwise. The difference between halves was 0.60 for the 2-sample condition and dropped successively to −0.08 for the 6-sample condition, but the sample-half-condition interaction was not significant; hence, this apparent trend has no statistical support.

The results of the separate analyses for each condition (Table 7) maintain a consistent pattern. The main effect of sample was highly significant (0.1% level) in each case, but none of the sample-half interactions was significant. The only other significant source of variation in any analysis was the main effect of half in the 6-sample condition (1% level).

Syrup

Samples A and B were significantly different (0.1% level) in preference (Table 6); however, one finding was totally unexpected. In the pilot test, Sample A had rated 0.57 scale points higher than Sample B. In the main experiment Sample B was rated an average of 0.34 scale points higher than Sample A and there were only two instances out of ten where Sample A was higher in any half in any condition (Table 5). There was some evidence that the actual formulation of Sample B, a commercial product, had been changed between the pilot test and the main experiment.

As in the other replications, the effect of first vs. second half was significant (5% level), with the second half rated lower. Here the effect of experimental condition was also significant (5% level). The average of A and B dropped from 6.90 in the 2-sample condition to 6.00 in the 6-sample condition (Table 4), but the decrease was not monotonic. The only other significant effect (5% level) was the interaction between half and condition. The right-hand portion of Table 5 illustrates this, but there is no clear reason why it occurred. In the first half, the difference between the samples was highest for the 3-sample and 5-sample conditions; in the second half, it was highest for the 2-sample and 5-sample conditions and almost zero for the 4-sample and 6-sample conditions. There was no linear

Table 3—Milk. Mean ratings of samples in each experimental condition (N = 40 each condition).

	Experimental condition				
	2 Sample	3 Sample	4 Sample	5 Sample	6 Sample
Sample A					
1st half	6.78	6.65	6.75	6.90	6.58
2nd half	6.73	6.75	6.75	6.68	6.38
Total	6.75	6.70	6.75	6.79	6.48
Sample B					
1st half	5.95	5.67	6.08	5.75	5.35
2nd half	5.30	5.28	6.00	5.45	5.23
Total	5.63	5.48	6.04	5.60	5.29
Sample C					
1st half	—	6.23	6.63	6.30	6.08
2nd half	—	6.45	6.33	6.15	5.82
Total	—	6.34	6.48	6.23	5.95
Sample D					
1st half	—	—	6.45	5.90	5.85
2nd half	—	—	6.13	5.60	5.55
Total	—	—	6.29	5.75	5.70
Sample E					
1st half	—	—	—	6.90	6.75
2nd half	—	—	—	6.58	6.35
Total	—	—	—	6.74	6.55
Sample F					
1st half	—	—	—	—	5.45
2nd half	—	—	—	—	4.63
Total	—	—	—	—	5.04
Total					
1st half	6.36	6.18	6.48	6.35	6.01
2nd half	6.01	6.16	6.30	6.09	5.66
Total	6.19	6.17	6.39	6.22	5.83

Table 4—Syrup. Mean ratings of samples in each experimental condition (N = 40, each condition).

	Experimental condition				
	2 Sample	3 Sample	4 Sample	5 Sample	6 Sample
Sample A					
1st half	7.03	6.75	6.60	6.28	5.90
2nd half	6.25	6.38	6.33	6.23	5.90
Total	6.64	6.56	6.46	6.25	5.90
Sample B					
1st half	7.38	7.38	6.43	6.95	6.33
2nd half	6.93	6.60	6.35	6.85	5.88
Total	7.15	6.99	6.39	6.90	6.10
Sample C					
1st half	—	5.45	5.30	4.75	3.55
2nd half	—	4.70	3.98	3.48	2.88
Total	—	5.08	4.64	4.11	3.21
Sample D					
1st half	—	—	5.93	5.70	5.55
2nd half	—	—	4.73	4.33	4.90
Total	—	—	5.33	5.01	5.23
Sample E					
1st half	—	—	—	4.88	5.23
2nd half	—	—	—	4.35	4.80
Total	—	—	—	4.61	5.01
Sample F					
1st half	—	—	—	—	5.47
2nd half	—	—	—	—	4.80
Total	—	—	—	—	5.14
Total					
1st half	7.20	6.53	6.06	5.71	5.34
2nd half	6.59	5.89	5.34	5.05	4.86
Total	6.90	6.21	5.70	5.38	5.10

trend for the difference to vary according to the number of samples tested, whether one considers each half separately or both combined.

The absence of an interaction between samples and conditions, or between samples and half, is consistent with the results from the other two replications. Thus, level of ratings might be affected by whether samples appeared in the first or second half; but, on an overall basis, the conclusion that Sample B is preferred to Sample A does not depend upon the half in which they were tested or upon the number of samples with which they were evaluated or upon the interaction of the two variables.

The separate analyses by experimental condition (Table 7) again clearly show differences between the critical samples and between halves. In each case Sample B rated higher and level of rating was higher in the first half of the sessions. In the 4-sample and 5-sample conditions, interactions between sample and half were significant.

In the 4-sample condition, Sample C dropped by 1.32 scale points from the first to the second half, and Sample D dropped by 1.20 scale points (Table 4). However, the mean rating of Sample A was only 0.27 scale points lower in the second half, and the mean rating of Sample B was only 0.08 scale points lower. Similarly, in the 5-sample condition, Samples C and D dropped 1.27 and 1.37 scale points, respectively, in the second half; but Samples A and B dropped only 0.05 and 0.10 scale points. Thus, the nature of the interaction between sample and half was nearly the same in each of the two conditions.

The four samples which appeared in the 4-sample and 5-sample conditions also were present in the 6-sample condition; yet the sample-half interaction was not significant. Why not? Note (Table 4) that mean ratings for Sample C and D were substantially lower in the first half of the 6-sample condition than in the 4-sample condition and to a lesser extent than in the 5-sample condition. The two samples did drop in the second half of the 6-sample condition by 0.67 and 0.65 scale points, but for some reason Sample B dropped by an unusually large amount, 0.45 scale points, relative to the preceding two conditions. Thus, the absence of a sample-by-half interaction in the 6-sample condition may have been due to abnormally low first-half ratings for Samples C and D or to the abnormally large decrease for Sample B. Either or both of these effects would work in the direction of supporting the null hypothesis for the sample-by-half interaction.

Even in the two conditions where the sample-by-half interaction was significant, the rank order of preference within half remained the same; Sample B always had the highest mean rating, Sample A the second highest. Sample D next and Sample C the lowest. The range of ratings was somewhat higher in the second half than in the first half: 2.37 vs 1.30 for the 4-sample condition, and 3.37 vs 2.20 for the 5-sample condition. There is no evidence that the differences among samples are attenuated either in the second half or in the experimental conditions involving a larger number of samples.

DISCUSSION

ALL THREE replications (food items) yielded similar interpretations. First, the ratings in the second half were significantly lower than in the first half. This point is inconsequential when considering one major purpose of taste-tests, which is to determine differences among samples rather than to establish levels of rating. However, some users of the data might be pleased or dismayed at the lower ratings in the second half and might have to shift their frames of reference when using such ratings as absolutes.

Second, within each replication the samples were intended to differ sufficiently to allow the effects of other variables to come into play. An unanswered question is whether the differences were, in fact, too great so that they obscured the effects of these other variables. This may have occurred for milk, where the

Table 5—Effect of experimental condition on ratings of the critical samples A and B.

Conditions	Average (A + B)/2) 1st half	2nd half	Difference 1st–2nd	Difference (A − B) 1st half	2nd half	Difference 1st–2nd
Soup & gravy base (N = 36)						
2-Sample	7.02	6.56	0.46	0.19	0.23	0.04
3-Sample	6.72	6.34	0.38	0.55	1.40	0.85
4-Sample	6.44	6.24	0.20	0.50	0.47	−0.03
5-Sample	6.42	5.90	0.52	0.67	0.69	0.02
6-Sample	6.12	5.48	0.64	0.91	0.67	−0.24
Total	6.54	6.10	0.44	0.55	0.66	0.11
Average of halves	6.32			0.61		
Milk (N = 40)						
2-Sample	6.36	6.01	0.35	0.83	1.43	0.60
3-Sample	6.16	6.02	0.14	0.98	1.47	0.49
4-Sample	6.42	6.38	0.04	0.67	0.75	0.08
5-Sample	6.32	6.06	0.26	1.15	1.23	0.08
6-Sample	5.96	5.80	0.16	1.23	1.15	−0.08
Total	6.24	6.05	0.19	0.97	1.21	0.24
Average of halves	6.14			1.08		
Syrup (N = 40)						
2-Sample	7.20	6.59	0.61	0.35	0.68	0.33
3-Sample	7.06	6.49	0.57	0.63	0.22	−0.41
4-Sample	6.52	6.34	0.18	−0.17	0.02	0.19
5-Sample	6.62	6.54	0.08	0.67	0.62	−0.05
6-Sample	6.12	5.89	0.23	0.43	−0.02	−0.45
Total	6.70	6.37	0.33	0.38	0.30	−0.08
Average of halves	6.54			0.34		

Table 6—Analysis of variance for critical samples (A and B) across experimental conditions.

Source of variation	df	ms	F
Soup & gravy base (N = 36)			
A—Sample	1	66.61	35.44[1]
B—Half	1	35.11	30.27[1]
C—Condition	4	20.16	3.65[2]
A × B	1	0.51	[3]
A × C	4	2.57	1.37
B × C	4	0.98	[3]
A × B × C	4	1.28	1.58
D—Subject (within C)	175	5.53	
A × D	175	1.88	
B × D	175	1.16	
A × B × D	175	0.81	
Milk (N = 40)			
A—Sample	1	236.53	123.19[1]
B—Half	1	7.41	6.18[4]
C—Condition	4	5.56	[3]
A × B	1	2.77	2.72
A × C	4	1.81	[3]
B × C	4	0.56	[3]
A × B × C	4	0.88	[3]
D—Subject (within C)	195	6.82	—
A × D	195	1.92	—
B × D	195	1.20	—
A × B × D	195	1.02	—
Syrup (N = 40)			
A—Sample	1	23.46	13.72[1]
B—Half	1	11.76	6.53[4]
C—Condition	4	19.45	3.34[4]
A × B	1	0.56	[3]
A × C	4	3.25	1.90
B × C	4	4.99	2.77[4]
A × B × C	4	1.14	1.24
D—Subject (within C)	195	5.83	—
A × D	195	1.71	—
B × D	195	1.80	—
A × B × D	195	0.92	—

[1] Significant at the .1% level.
[2] Significant at the 1% level.
[3] F-ratio less than 1.00.
[4] Significant at the 5% level.
Testing of effects:
A tested against A × D.
A × C tested against A × D.
B tested against B × D.
B × C tested against B × D.
C tested against D.
A × B × C tested against A × B × D.
A × B tested against A × B × D.

overall mean difference between Sample A and Sample B was 1.08 scale points (Table 5); but the differences of 0.61 for soup and gravy base and of 0.34 for syrup approach the specifications for this experiment. Because the results correspond so well among the three replications, we do not believe that the large difference with milk constitutes a serious problem. The fact that the level of rating varied among experimental conditions in the first and third replications does not in itself mean very much. This could be caused by the effects of the other samples evaluated with A and B or by the psychological effects on the judges of having to rate varying numbers of samples.

The crucial source of variation is the interaction between experimental condition and specific samples. In none of the three analyses of variance involving only Samples A and B was this source significant. In the analyses of variance of the individual conditions, in only one condition of the soup and gravy base replication and in two conditions of the syrup replication was the interaction between sample and half significant. In each of these cases, the rank orders of preference in the two halves were identical. If anything, the ratings were spread out more in the second half than in the first half.

Thus, there is no evidence that increasing the number of samples up to 12 would lessen the relative differences in ratings among food samples. If there is some upper limit, we have not attained it. These negative results are meaningful and important since they give one confidence in conducting taste tests involving a greater number of samples than the typical three, four or five. However, we do not know the effect of lengthened tests upon the panel population.

Perhaps most participants would not object to an occasional ten or twelve sample test, but if such tests were to become common, then some might be induced to withdraw. Perhaps some people especially welcome frequent or short tests as breaks from their everyday activities, and might dislike less frequent and longer ones. Even so, an occasional longer test might productively be used. There is no contrary experimental evidence.

The data suggest several other problems worthy of study. A few significant interactions between sample and half were noted. It would seem that some samples are affected by certain others with which they are served, such that in the second half they drop disproportionately.

Table 7—Summary of analysis of variance for each experimental condition (N = 40 each condition).

Condition	Source of variation: Sample df	Sample Signif.	Experimental half df	Experimental half Signif.	Sample-half interaction df	Sample-half interaction Signif.
Soup & gravy base[1]						
2-Sample	1	ns	1	5%	1	ns
3-Sample	2	0.1%	1	0.1%	2	5%
4-Sample	3	0.1%	1	0.1%	3	ns
5-Sample	4	0.1%	1	0.1%	4	ns
6-Sample	5	0.1%	1	0.1%	5	ns
Milk						
2-Sample	1	0.1%	1	ns	1	ns
3-Sample	2	0.1%	1	ns	2	ns
4-Sample	3	0.1%	1	ns	3	ns
5-Sample	4	0.1%	1	ns	4	ns
6-Sample	5	0.1%	1	1%	5	ns
Syrup						
2-Sample	1	5%	1	5%	1	ns
3-Sample	2	0.1%	1	1%	2	ns
4-Sample	3	0.1%	1	1%	3	0.1%
5-Sample	4	0.1%	1	0.1%	4	1%
6-Sample	5	0.1%	1	1%	5	ns

[1] $N = 36$ in 6-Sample condition.
Testing of effects:
Sample against sample-subject interaction.
Half against half-subject interaction.
Sample-half against sample-half-subject interaction.

This phenomenon, which did not always appear, might be a manifestation of contrast and convergence effects (Kamenetzky, 1959). Certain samples of a product might achieve a fairly high average rating the first time, but drop when tested again, because some judges do not become aware of these deficiencies until they have had intervening experience with good quality products.

If some samples are disproportionately affected when repeatedly evaluated by the same person, then one would hypothesize that they would be more subject to monotony effects if they became standard items of issue than samples which showed only the normal loss in preference in the extended test situation. The reason is that the more often a susceptible food is served, the greater the opportunity for deficiencies to be noticed. For example, if firmer evidence were available that preferences for syrup Samples C and E are nearly equal (see Table 4, 5-sample condition), one might hypothesize that preference for the former would decline more sharply than preference for the latter, assuming an equal rate of use.

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A Simplified and Rapid Method to Uniformly Size Rib Steak Pieces for Taste Panel Evaluation

SUMMARY—Uniformity of size and preparation of steak samples are important prerequisites for accurate taste panel analysis. A system is described to utilize a Broiling and Sectioning Apparatus (BSA) to obtain this uniformity. Frozen beef rib steaks are uniformly sized before broiling and then sectioned into bite-size pieces for presentation to a taste panel.

INTRODUCTION

ORGANOLEPTIC evaluation of beef rib steaks can provide an important subjective index to their tenderness, flavor, juiciness and total acceptability. However, reliability of results can only be ascertained by providing uniformity in preparation of individual rib steaks, steak samples and steak pieces.

A simple and rapid method for preparing beef rib steaks for taste panel analysis was needed to provide physical uniformity of samples and to insure uniformity of broiling. Reported herein is a description of the procedure developed to remove uniform steak samples from frozen beef rib steaks, broil them simultaneously on both sides until a standardized internal temperature has been reached and uniformly section them into bite-size steak pieces using a slotted cutting box system.

Previous work by Alsmeyer (1965, private communication) suggested eight samples as the maximum number to be evaluated at one panel sitting as members begin to fatigue after that number of samples. The panel number at The Pennsylvania State University was standardized at eight. Cover et al. (1960) found a 1.0 cm thick by 2.0 cm^2 steak piece acceptable for panel analysis.

EXPERIMENTAL

Apparatus

An eight steak sample, stainless steel, Broiling and Sectioning Apparatus (BSA) for beef rib steak taste panel analysis (Fig. 1) consisted of eight individual steak sample holders held in line with 1.9 cm wide angles 66.0 cm long. The holders were spot welded to the angles 5.2 cm from each end and 2.0 cm between holders.

Holders were fabricated from 20 gauge stainless steel sheet in a rectangular shape 5.0 cm wide, 11.0 cm long and 4.5 cm high. Nine cutting slots were placed on each side of the holders. These side slots were aligned with the slots on the opposite side of the holder as well as the slots of the other holders. Each holder also had three aligned slots on each end. Both end and side cutting slots were each 3.5 cm deep and 0.16 cm wide. Each of the slots were 1.0 cm apart with 1.5 cm internal measurement between the center of the last slots and the ends or sides.

Four 0.17 cm grating pin holes were placed on each end of all holders 0.75 cm from the bottom of the holder and 1.0 cm apart with 1.0 cm between the last pin holes and the ends as measured internally. The stainless steel 0.15 cm diameter grating pins were each 13.32 cm total length with a ring bent on one end for ease in their extraction.

As a means to raise or lower the BSA and to locate the steak sample equidistant from the heating surfaces of the oven, the angles have been tapped vertically 2.0 cm from the ends with (10 × 32 NF-2) tapped holes and fitted with four (10 × 32 NF-2) by 5.1 cm long pan head screws each locked with a matching (10 × 32 NF-2) hexagonal nut.

To facilitate handling the BSA, 0.68 cm holes were drilled horizontally through the angles 0.7 cm from the ends of each angle and fitted with 0.64 cm diameter rods each 13.5 cm long to serve as handles. The rods passed through both angles and were secured with a (1/4 × 20 NC-2) plain hexagonal nut and a 0.64 cm spring lock washer on each end. As a means of identifying steak samples, the sample holder was lettered from

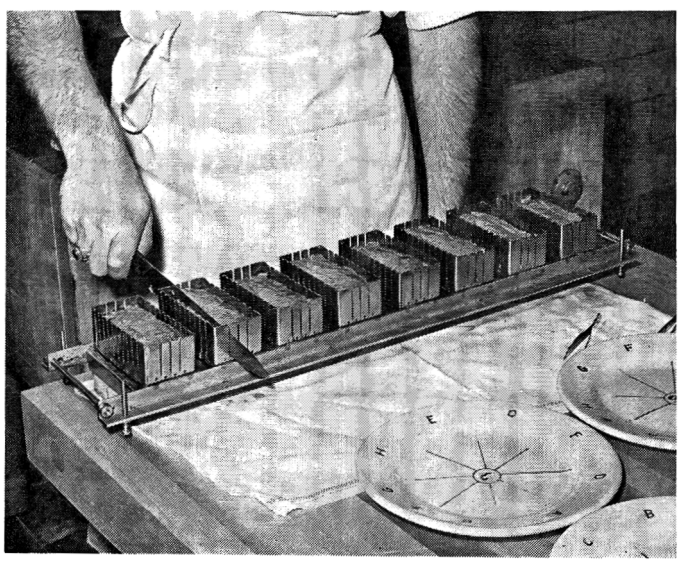

Fig. 1—Broiled steaks being sectioned in the BSA.

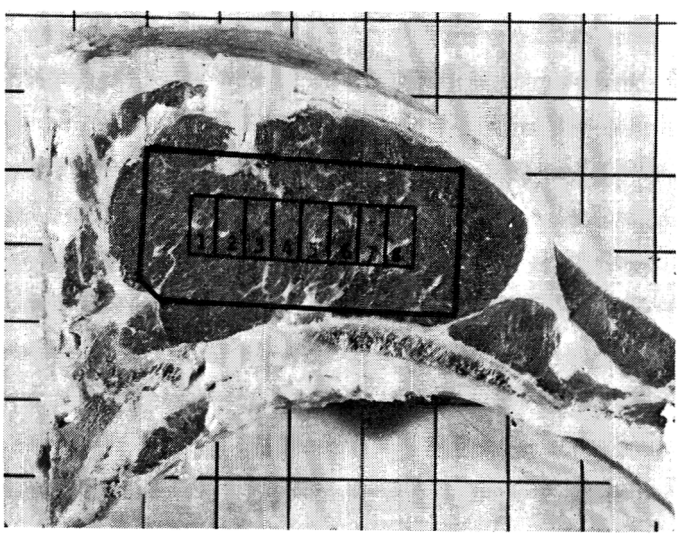

Fig. 2—Twelfth right beef rib steak showing size and location of steak sample and eight steak pieces.

A to H on the angle immediately in front of it.

To complete the system, a cutting base was designed to exactly mesh with the eight holders of the BSA. This unit consisted of eight graphite infused nylon cutting blocks each 1.3 cm high, 10.7 cm long and 4.7 cm wide mounted on a stainless steel support using two (6 × 32 NC-2) by 0.64 cm long pan head screws to secure each block. The 20 gauge support was 10.9 cm wide and 57.5 cm long with the four edges turned down 4.0 cm.

A thermostatically controlled isothermal research oven was used for broiling steak samples. The oven consisted of an enclosed heating chamber, 98.0 cm long, 36.0 cm wide and 9.3 cm high. The oven included two separate heating units, a stationary lower unit and a movable top unit of identical heating capacity but which could be raised 31.0 cm to completely expose the lower unit.

Procedure

A uniformly sized steak sample (Fig. 2) consisted primarily of Longissimus dorsi muscle removed with a band saw from the frozen right twelfth rib steak. Size of the frozen steak sample was 11.7 cm long, 5.5 cm wide and 2.5 cm thick. Thickness can vary depending on product and conditions from a minimum of less than a centimeter to a maximum of 3.3 cm, slightly less than the thickness of a rib steak.

The BSA was prepared by inserting the grating pins and joining these pins together by two 0.30 cm diameter by 30.0 cm long extracting rods each inserted through the rings of 16 pins to enable simultaneous extraction following broiling.

After thawing 12 hr at 2°C, coded steak samples were placed in their individual holders. As an eight steak sample broiling unit, the BSA was placed in the isothermal oven. An important feature of the BSA was the ease and speed with which the eight steak samples can be handled during broiling and sectioning operations.

After a standardized internal temperature was reached, as indicated by an iron thermocouple potentiometer, the BSA was removed from the oven and placed upon the specially prepared cutting base. Grating pins were removed and steaks automatically lowered onto the nylon cutting blocks.

Steak samples were sectioned by first making two parallel longitudinal cuts with a thin bladed knife (e.g. Dexter No. 44910) using the two outer end sectioning slots of each holder as guides.

Sectioning was completed by drawing the knife across the steak samples using the nine side sectioning slots as guides. The eight internal steak pieces (each 1.0 cm thick and 2.0 cm wide with variable length, normally 2.0 cm) were removed from the BSA with tweezers and placed according to code on the panelists' plates. In addition, 16, 1.0 cm × 1.0 cm steak pieces, again with variable length, can be sectioned if desired. By this method of sampling and sectioning the evaluated steak pieces were cut longitudinally to the muscle fibers rather than across to more closely reflect fiber tenderness.

RESULTS & DISCUSSION

IN USE with 164 beef rib steak samples, the BSA system was found to be a convenient and rapid method. The size of the individual steak sample holder is adequate to sample the Longissimus dorsi muscle from the twelfth rib of 226.8 kg and larger beef carcasses. The speed with which the eight samples can be prepared, broiled, sectioned and served greatly increased the number of samples that can be efficiently handled. Because steak samples can be processed simultaneously, steak pieces stay much warmer during panel analysis.

Steak samples after broiling had moist surfaces and showed uniform heat penetration throughout as evidenced by their uniform color. Conduction of heat by the stainless steel steak sample holders or grating pins to the steaks was not noticed in excessive surface overheating. The nylon block-stainless steel cutting base provided a uniformly resistant cutting surface that withstood repeated use without excessive block erosion or knife dulling. The entire BSA and accessories were conveniently cleaned with a cleaning agent in hot water and rinsed with distilled water for future use.

The authors are confident that this system could readily be adapted for use with any meat sample either hot or cold of sufficient size or any other food product where uniformly sized pieces are necessary for evaluation. Use of the BSA also permits easy identification of sections to be evaluated within the total sample. In addition, the BSA could be conveniently adapted for use with conventional style ovens by attaching a pivotal arrangement and a top sample retention grate.

Thickness of steak samples could be changed in order to vary the size of the individual steak pieces presented to the panel. However, at The Pennsylvania State University Meat Laboratory with trained adult female panelists, the size described appeared most satisfactory.

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Lipid in Ripening Banana Fruit

SUMMARY—Lipid extracts were isolated from unripe and ripe banana pulp and peel and the fatty acid composition of the extracts was determined. Unsaturated acids, particularly linoleic and palmitoleic, decreased about 3-fold in the pulp while more than a 2-fold increase in stearic acid occurred. In general, unsaturated fatty acids decreased in both the pulp and peel during ripening. The peel contained almost 4 times more lipid than the pulp.

INTRODUCTION

VOLATILE constituents of banana fruit were isolated and identified (Wick et al., 1969) and L-leucine was investigated as a precursor to important aroma constituents (Myers et al., 1970). It was postulated that fatty acids and sugars might also be precursors to the volatiles produced during ripening. The major conversion of starch to soluble sugars during ripening is well known (von Loesecke, 1950; Simmonds, 1959) but little is known of lipids in banana fruit. They, in general, constitute 0.2 to 0.5% of the fresh weight of the pulp at any stage of ripeness (von Loesecke, 1950). Grosbois et al. (1964) reported that the major fatty acids in the lipid of both pulp and peel were palmitic, oleic and linoleic acids. They observed that during ripening the relative amounts of unsaturated fatty acids in the pulp lipid, especially palmitoleic, decreased. The magnitude of this decrease was not reported. An increase in the relative amount of fatty acids in the peel lipid during ripening was also observed but the magnitude of this increase was not reported.

This investigation was undertaken to determine the lipid composition of unripe and ripe banana pulp and peel.

EXPERIMENTAL

UNRIPE bananas, variety Valery, were obtained from the United Fruit Co., New York, N.Y., and divided into two groups of 51 fingers each. Lipids were extracted from the first group at an unripe stage when the peel color was bright green to yellow. The pulp was hard, astringent and lacking in characteristic banana aroma and flavor. The second group was re-wrapped in polyethylene film and left for five days at ambient temperature (21°C). Peel color was yellow-brown and the pulp was nonastringent, sweet and had the characteristic flavor and aroma of ripe bananas. Lipid extraction was carried out at this post-climacteric stage.

Lipid extraction

The extraction procedure was adapted from Goldstein et al. (1963), Nichols (1964) and Nichols et al. (1964).

Bananas (3 per batch) at the given ripeness stage were cut in half and a ¼-in. slice removed from each. The slices were peeled rapidly with forceps, weighed (combined weight was about 25 g), and dropped into 500 ml of analytical grade *iso*-propanol in a Waring Blendor. These operations were performed rapidly (in less than 2 min) to minimize enzymatic change in complex lipids. The pulp: *iso*-propanol mixture [1:20 (w/v)] was blended 3 min at a blendor setting of 60 volts. The resulting suspension was filtered by suction through a course, sintered-glass funnel. The solid residue was again extracted with *iso*-propanol [1:20 (w/v)] in the same manner. The insoluble residue was treated with chloroform : *iso*-propanol [2:1 (v/v)] in 1:20 (w/v) proportions and blended 5 min. After filtration all *iso*-propanol and chloroform : *iso*-propanol filtrates were combined (total volume 1.65 l). A total of about 300 g each of unripe and ripe pulp was extracted by an overall volume of about 20 L of solvent. The extracts were stored at −22°C under nitrogen.

Batches (about 25 g) of peel taken from a 2 in. wide middle strip from two bananas were cut rapidly into 1 cm squares and extracted. The procedure was the same as that described above except that the blendor was set at 100 volts to aid in maceration of the fibrous tissue, and two extractions with chloroform : *iso*-propanol [2:1 (v/v)] were performed. A total of approximately 75 g of peel were extracted by a total solvent volume of 6.4 l. The extracts were combined and stored at −22°C under nitrogen.

Concentration and purification of lipid extracts

Each extract was treated in the following manner. Concentration at 35°C under reduced pressure in a rotary flash evaporator to a final volume of about 30 ml was carried out. Chloroform : methanol [2:1 (v/v)] was added to this residue until the total volume was 250 ml. The resulting mixture was filtered through a course sintered-glass funnel to remove insoluble sugars and gums. The solid was washed thoroughly with chloroform, the filtrates were combined and concentrated to dryness under vacuum at 0°C. The lipid concentrate was dissolved with 10 successive 2 ml-aliquots of chloroform: methanol [2:1 (v/v)] which were, in turn, transferred to a 40 ml graduated centrifuge by means of a Pasteur pipette.

The solution was brought to a final volume of 20 ml and treated with 5 ml of 0.7% (w/v) aqueous sodium chloride. After thorough mixing the solution was centrifuged for 30 minutes at 1800 rpm and at 3°C in a refrigerated centrifuge. The layers were separated. The aqueous layer was washed with 15 ml and then 10 ml of a mixture of chloroform : methanol : water [86:14:1 (v/v)]. After each washing the layers were centrifuged and separated.

The combined chloroform phase was evaporated to dryness under vacuum at 0°C. The residue was dried overnight in a vacuum desiccator which contained potassium hydroxide. It was then weighed, dissolved in chloroform and brought to a final volume of 5 ml. All chloroform solutions were stored in the dark at −22°C under nitrogen.

The yields of lipid obtained from unripe and ripe banana pulp and peel are summarized in Table I.

Transesterification of lipids

Transesterification of the total lipid extracts from the pulp and peel of unripe and ripe bananas was carried out to allow determination of their fatty acid composition. The method and apparatus of Stoffel *et al.* (1959) was employed. Lipid (about 5 mg) was refluxed in dry 5% methanolic hydrochloric acid for 2 hr at 85–90°C. The methyl esters were extracted with petroleum ether, sublimed at 60°C and 20 microns pressure onto a cold finger, and washed into a 1.0-ml graduated flask with analytical grade hexane.

Determination of fatty acid composition

The fatty acid composition of the total lipid extracts from pulp and peel of unripe and ripe bananas was determined by gas chromatographic analysis of the products of

Table 1—Lipid extracted from unripe and ripe bananas (variety Valery).

State of ripeness	Tissue extracted	Moisture (%)[1]	Lipid (% dry wt)[1]
Unripe	Pulp	28.0	0.92
	Peel	11.2	6.12
Ripe	Pulp	23.2	1.10
	Peel	16.5	6.98

[1] Average of three determinations.

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Table 2—Comparison of retention times (relative to methyl palmitate[1]) of banana methyl esters with those of known methyl esters.

Known methyl esters		Esters from pulp lipid		Esters from peel lipid	
	Rel_{tr}	Unripe	Ripe	Unripe	Ripe
14:0	0.52	0.54	0.54	0.53	0.53
15:0	—	0.74	0.74	0.73	0.73
16:0	1.00	1.00	1.00	1.00	1.00
16:1	—	1.22	1.22	1.18	1.20
17:0	—	1.37	1.37	1.38	1.37
16:2	—	1.65	1.65	1.65	1.69
18:0	1.92	1.86	1.85	1.91	1.91
18:1	2.22	2.20	2.19	2.22	2.23
18:2	2.87	2.83	2.82	2.87	2.86
20:0	3.70	3.49	3.45	3.71	3.67
18:3	3.95	3.92	3.85	3.96	3.94

[1] The retention time of methyl palmitate (16:0) is about 6 min.

Table 3—Fatty acid composition of lipid extracts from unripe and ripe banana pulp.

Banana methyl ester	Unripe pulp[1] (%)	Ripe pulp[1] (%)
14:0	Trace	Trace
15:0	Trace	Trace
16:0	28.80	41.90
16:1	5.75	2.10
17:0	Trace	Trace
16:2	2.91	Trace
18:0	1.59	3.83
18:1	11.35	14.40
18:2	33.88	16.10
20:0	Trace	Trace
18:3	15.80	21.70

[1] Average of two determinations.

transesterification. Separations were carried out in a chromatograph fitted with a flame ionization detector. A 5 ft × ⅛ in. stainless steel column maintained at 173 ± 1°C diethylene glycol succinate (DEGS) on 100/110 Anak ABS (Analabs) was employed. Carrier gas (nitrogen) flow was 18 ml/min. Assignment of identity was based on comparison of retention times (relative to methyl palmitate) of banana esters with those of authentic reference samples. The results are summarized in Table 2 and Figure 1.

Preliminary analyses of known reference compounds showed that transesterification of trilinolein samples which ranged from 0.5, 1.0, 2.0, 4.0 to 8.0 mg gave essentially 100% yields of methyl linoleate. Using gas chromatographic separation and internal normalization of peak areas, the percentage composition of a known mixture (Applied Science K-108) of methyl esters of 16:0, 18:0, 18:1, 18:2 and 18:3 acids was confirmed with better than ±1% precision and accuracy. The total amounts of esters injected ranged from 1.25 to 10.0 μg while the amounts of individual esters detected varied from 0.25 to 2.0 μg. Gas chromatographic detector response was linear over the range studied. Methyl ester concentrations of 1 mg/ml produced an average peak area of 349 cm^2.

On the basis of the results with trilinolein, conversion of banana lipid extracts to methyl esters was assumed to be complete. The percentage compositions of methyl esters in unripe banana pulp were determined by internal normalization of peak areas. They are

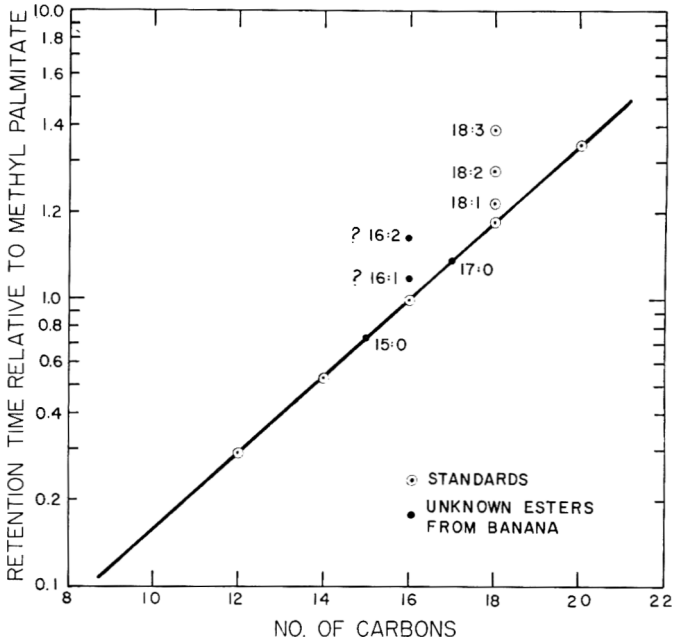

Fig. 1—Log-plot of retention times relative to methyl palmitate of known methyl esters and of unknown esters from banana lipid.

summarized in Table 3. Division of each peak area by 349 gave the amount (mg) of each ester in 1 ml of solution. Since the weight of each lipid extract taken for transesterification was known, the weight of each fatty acid per 10 g dry weight of banana tissue was calculated. The results are given in Table 4.

CONCLUSIONS

THE TOTAL weight of lipid extracted from banana pulp and peel did not change substantially during ripening (Table 1). This was in agreement with von Loesecke (1950). On a dry weight basis pulp from unripe fruit contained 0.92% lipid while pulp from ripe fruit contained 1.10% lipid. On the same basis unripe and ripe peel contained 6.12 and 6.98% of lipid respectively.

Transesterification of the lipid extracts and gas chromatographic analysis of the resulting methyl esters indicated that the compounds given in Table 2 were present. Retention times relative to methyl palmitate (16:0) of reference compounds and of banana methyl esters provided evidence of identity. The possible presence of esters of 15:0, 16:1, 17:0 and 16:2 acids was indicated based on the log plot shown in Figure 1 and on the known behavior of unsaturated methyl esters on DEGS columns (Stoffel et al., 1959).

The percentage composition of major fatty acids in lipid extracts from unripe and ripe banana pulp is given in Table 3. Similar information but in terms of mg of methyl ester/10 g of banana tissue (dry weight), is summarized in Table 4. A three-fold decrease in methyl linoleate, from 12.58 to 4.88 mg/10 g dry weight, was observed as the pulp ripened. A smaller decrease in palmitoleate (16:1, tentative identity) from 2.21 to 0.84 mg/-

Table 4—Major fatty acids of banana (pulp and peel) during ripening (results in terms of mg fatty acid: 10 g dry weight).

Tissue	State of ripeness	Components present[1,2]									Total acids	
		14:0	15:0	16:0	16:1	16:2	18:0	18:1	18:2	18:3	Sat.	Unsat.
Pulp	Unripe	—	0.33	10.89	2.21	1.16	0.63	4.44	12.58	6.08	11.85	26.47
	Ripe	—	Trace	11.92	0.84	Trace	1.68	4.08	4.88	6.84	13.60	16.64
	Ripe / Unripe	—	—	1.10	0.38	—	2.66	0.92	0.38	1.12	1.08	0.66
Peel	Unripe	1.35	—	56.30	—	—	7.32	8.70	38.00	19.80	64.97	66.50
	Ripe	1.43	—	62.80	—	—	6.46	9.50	26.70	18.40	70.69	54.60
	Ripe / Unripe	1.06	—	1.12	—	—	0.88	1.08	0.70	0.93	1.09	0.82

[1] Identifications based on retention data.

[2] Results based on the average of two gas chromatographic analyses of each of four independent transesterifications of banana lipid extracts.

10 g dry weight also occurred. At the same time, however, stearate increased from 0.63 to 1.68 mg/10 g.

Overall trends are indicated by the ratios shown in Table 4 of the amount of fatty ester in ripe pulp or peel compared to the amount present in unripe pulp or peel. In general, the unsaturated acids decreased in both pulp and peel during ripening. The peel contained almost four times more lipid than did the pulp.

A marked change in the fatty acid composition of pulp lipid was observed with ripening. It cannot be said whether the change is related in any way to production of volatile constituents and flavor development. Continued research on the nature of the complex lipid should provide additional information.

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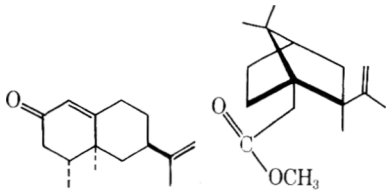
Photoinduced Reduction of Nootkatone

SUMMARY—Photolysis of the principal flavoring constituent of grapefruit, nootkatone, in methanol gave rise to a number of compounds. One of them has been isolated and shown to be 1,10-dihydronootkatone.

INTRODUCTION

LITTLE ATTENTION has been given in the past to the mode of decomposition of flavoring constituents when exposed to ultraviolet light. In a continuing effort to understand and prevent loss of desirable flavors and formation of undesirable characteristics, we have isolated and characterized another photoproduct from irradiated nootkatone (I), the principal flavoring constituent of grapefruit (MacLeod et al., 1964). The main product of the photolysis, photonootkatone (II), has been reported in an earlier publication (Stevens et al., 1968).



I II

EXPERIMENTAL

Apparatus

All NMR spectra were run in dilute carbon tetrachloride solutions with tetramethylsilane as an internal standard. A Varian HR 100 spectrometer was used. A Perkin-Elmer model 237 infrared spectrometer was used and the samples were run neat. An EAI model 300 quadrupole mass spectrometer was used to determine mass spectra. A Cary model 60 ORD spectrometer with a Cary 6001 CD attachment was used to determine ORD and CD spectra respectively. Spectra were determined in cyclohexane.

Methods

Isolation of nootkatone. Described in previous publication (MacLeod et al., 1964).

Photolysis of nootkatone. Described in previous publication (Stevens et al., 1968).

Isolation of photoproduct (III). The reaction mixture, after photolysis, was analyzed on a 75 ft × 0.01 in. Igepal column (Fig. 1) and the desired compound, III, was isolated by a combination of chromatography on silica gel (100–200 mesh, Silic AR-CC-7, eluted with 70:30 hexane/ether) and gas chromatography on a $^1/_2$ in. × 12 ft Carbowax 20M column (1% on 70–80 mesh Chromasorb G) at 175°C and 20 psi pressure. Analysis performed on the 75 ft × 0.01 in. analytical column showed the material to be pure.

Hydrogenation of photoproduct III. The photoproduct (III), 30 mg, was dissolved in 5 ml of 95% ethanol and 50 mg of 5% palladium on charcoal (50% wet) were added. Hydrogenation was carried out at room temperature and atmospheric pressure and was completed in 5 min. Filtration and evaporation of the ethanol left an oil which was homogeneous by gas chromatography.

Tetrahydronootkatone. Nootkatone (I), 200 mg, was dissolved in 10 ml of 95% ethanol, and 200 mg of 5% palladium on charcoal (50% wet) were added. The material was hydrogenated at room temperature and atmospheric pressure. Hydrogen uptake ceased in 25 min after which the mixture was filtered, and the ethanol evaporated. The remaining oil was pure, determined by gas chromatography.

RESULTS & DISCUSSION

THE NEWLY isolated photoproduct showed an infrared spectrum having relevant adsorption peaks at 1715 cm^{-1} (nonconjugated carbonyl), 1644 cm^{-1} (carbon-carbon double bond stretch), and 886 cm^{-1} (terminal methylene, out-of-plane deformation). The mass spectrum showed a parent peak at m/e 220, two mass units higher than nootkatone.

The NMR spectrum (Fig. 2) shows peaks at δ 4.62 (S, 2H), which can be ascribed to two terminal methylene protons, and at δ 1.68 (S, 3H), which can be assigned to a vinyl methyl group. In addition, a singlet occurs at δ 0.93 and a doublet at δ 0.89, part of which is superimposed on the singlet. The integrated area of these peaks represents six protons and may be ascribed to the tertiary and secondary methyl groups.

These data strongly suggest that the photoproduct (III) is 1,10-dihydronootkatone. This structure was confirmed by comparing the hydrogenation product of III with tetrahydronootkatone. The two compounds were identical with respect to retention time, NMR, IR, and MS.

The unsensitized reduction of conjugated double bonds upon photolysis is

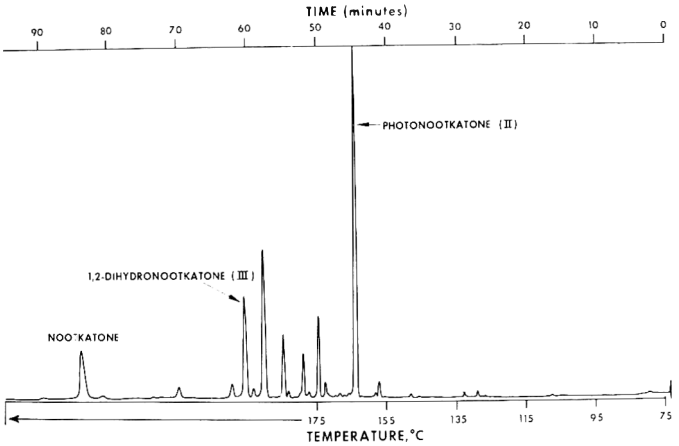


Fig. 1—Gas chromatogram of reaction products from irradiated nootkatone. Analysis was performed on a 75 ft × 0.01 in. Igepal column.

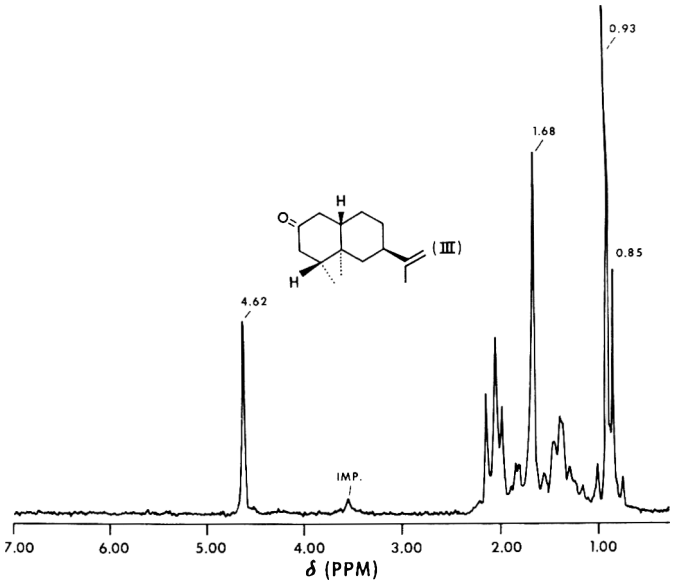
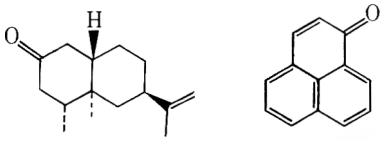


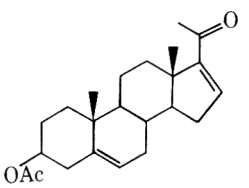
Fig. 2—NMR spectrum of 1,2-dihydronootkatone (III) at 100 MHz using tetramethylsilane as an internal standard and carbon tetrachloride as a solvent.

not without precedence. Köller et al. (1964) reported the reduction of phenalen-1-one (IV) in alcohols to give 2,3-dihydrophenalen-1-one. Williams et al. (1964) reported that when 3β-acetoxypregna-5,16-dien-20-one (V) was subjected to ultraviolet light in alcoholic solvents, one of the components was 3β-acetoxypregna-5-en-20-one.



III

IV



V

Comparison of the ORD and CD curves of III with tetrahydronootkatone showed them to be practically superimposable. Catalytic reduction of nootkatone gives tetrahydronootkatone with a *trans* ring junction. Consequently, the photoproduct must have a *trans* ring junction, since a *cis* configuration would result in the ORD and CD curves being significantly different.

Selective catalytic hydrogenation of double bonds relies upon the fact that a difference in rate may be realized. For instance, one may selectively hydrogenate the isolated double bond of nootkatone by using appropriate conditions, whereas it becomes extremely difficult to selectively hydrogenate the conjugated double bond. The photoinduced reduction of nootkatone, leading to the 1,10-dihydro product, thus becomes a convenient method of carrying out this reaction and might have some utility in other systems as well.

Interestingly, the photoproduct (III) does not possess the characteristic aroma of nootkatone but has a rather bland, nondescript odor. Also, the entire photoproduct mixture has little, if any, of the characteristic grapefruit aroma. The other photoproducts shown in the chromatogram (Fig. 1) are currently being investigated and will be the subject of a future publication.

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Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

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Palatability and Selected Related Characteristics of Three Types of Roasted Porcine Muscle

SUMMARY—A completely randomized design was followed to evaluate 48 roasts (posterior third of the loin) from 12 Duroc and 12 Poland China barrows. Antemortem treatment of pigs produced "normal," pale-soft-exudative (PSE) and dark-firm-dry (DFD) longissimus dorsi (LD) muscle. Meat was roasted at 350°F to an internal temperature of 167°F and evaluated by organoleptic and selected objective measurements. There was no significant organoleptic preference for one type ("normal," PSE, DFD) of LD. Also, differences among types of muscle were not significant for roasting time, volume of press fluid or total moisture (press method); whereas roasting losses, total moisture (Brabender) and pH of LD were affected significantly by type of muscle. In general, there were significant ($P < 0.05$) differences between PSE and DFD muscle, and between "normal" and DFD muscle. DFD muscle exhibited the smallest roasting loss and greatest total moisture, whereas PSE muscle had the greatest roasting loss and least total moisture. DFD muscle rated highest in pH and lowest in Warner-Bratzler shear value. LD from Durocs was more tender ($P < 0.05$), had a higher ($P < 0.05$) pH, and contained less ($P < 0.01$) total moisture (Brabender) than LD from Poland Chinas. Cooking losses were greater ($P < 0.05$) for roasts from Durocs than for roasts from Poland Chinas.

INTRODUCTION

SEVERAL new strains of hogs have been developed since the late 1930s to meet consumer preference for a lean type of pork. Concurrently, processors began to notice pale, soft and exudative (PSE) porcine muscle. PSE muscle has been attributed to such factors as breed, weight, and sex of the animal, ration fed and processing procedures.

Research on the physical and chemical properties of porcine muscle has been extensive. Three types of porcine muscle have been recognized; PSE, dark-firm-dry (DFD) and "normal." Although physical and chemical properties have received considerable attention, little has been done to ascertain the acceptability of those three types of muscle from the standpoint of eating quality.

Judge et al. (1960) reported that the tenderness of broiled chops increased as loins became less firm and as pH decreased. Conversely, Lewis et al. (1962) found that dark muscle of high pH was significantly more tender than pale, watery muscle. After determining what he referred to as loose water in porcine loins, Wismer-Pedersen (1959) submitted the loins to a taste panel, who found the meat acceptable over a wide range of loose water numbers. Scores for taste and texture of fried chops decreased slightly as the water holding capacity decreased.

Sayre et al. (1964) also found water-binding associated with tenderness. Muscles that had low cooking loss were tender, whereas muscles with high cooking loss lacked tenderness as determined by Warner-Bratzler shear values.

This study attempted to determine the palatability and selected related characteristics of PSE, DFD and "normal" longissimus dorsi (LD) muscle in roasted pork loin.

EXPERIMENTAL

48 PORK LOIN roasts (818–1381 g) were available from 12 Poland China and 12 Duroc barrows that had received ante- and post-mortem treatments designed to develop PSE, DFD and "normal" muscle (Bowers et al., 1968). The animals were divided into three lots of four from each breed. Lot I was fed a basal ration. Lot II was given the basal ration until seven days before slaughter, then a 50-50 ration, by weight, of sucrose and the basal ration. Lot III was given the basal ration until 48 hr before slaughter; immediately prior to slaughter exhaustive exercise was induced. Post-mortem treatment consisted of two chilling temperatures. Half of each carcass was assigned at random to coolers maintained at 30 and 42°F.

Roasts consisting of the section of the loin from posterior to the first lumbar vertebra to the anterior end of the hipbone were wrapped in 0.0015-gauge aluminum foil, blast frozen and stored at −4°F for 7–9 months. Roasts were removed from the freezer approximately 18–20 hr before cooking, allowed to thaw in their wrapping at room temperature (78.8 ± 3.6°F), unwrapped and placed with the ribs down on racks in individual shallow pans with a thermometer inserted into the center of the LD (Fig. 1.). The internal temperature of roasts after thawing averaged 68 ± 7.8°F.

A completely randomized design was followed to evaluate 48 roasts representing "normal," PSE and DFD musculature. At each evaluation period three roasts were cooked to an internal temperature of 167°F in a rotary hearth gas oven at 350°F (Pengilly et al., 1966).

The LD was separated from the bone, all exterior fat and brown surface removed, and the muscle divided 2½ in. from the anterior end into two pieces. Sampling is illustrated in Figure 2.

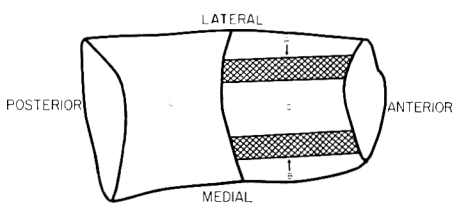

Fig. 2—Sampling plan for longissimus dorsi muscle. A. Lateral shear core (½ in.). B. Medial shear core (½ in.). C. Organoleptic samples (½ in. cubes). D. Press fluid, total moisture and pH.

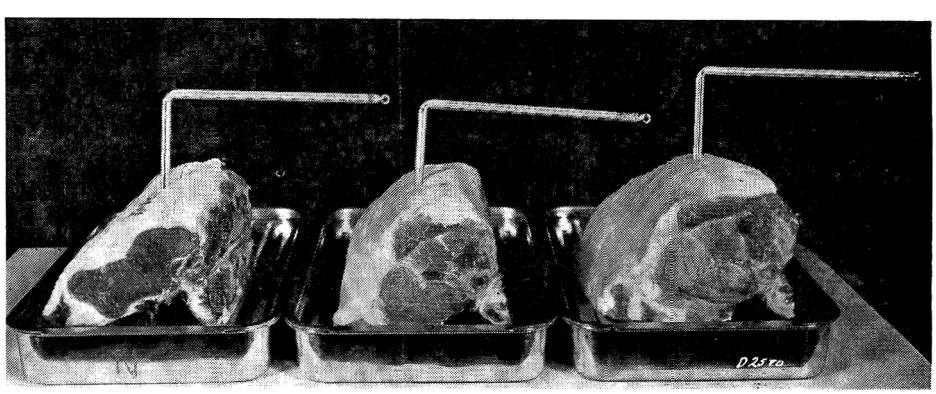

Fig. 1—Appearance of loin prior to roasting, from left to right. A. DFD loin, anterior surface, first lumbar region. B. "Normal" loin, posterior surface, anterior end of hip bone. C. PSE loin, posterior surface, anterior end of hip bone.

Evaluation of roasts

Total roasting time was noted, and roasting time on the basis of min/lb and percentage total, volatile and dripping roasting losses were calculated. Shear values were obtained for two cores ($^1/_2$ in.) from the LD, one lateral and one medial. Each core was sheared three times on a Warner-Bratzler shearing apparatus equipped with a 25-lb dynamometer.

An experienced 8-member panel evaluated $^1/_2$-in. cubes of the LD muscle for tenderness (based on chews), juiciness and flavor on a 7-point scale. Juiciness and tenderness were scored on an intensity scale, whereas flavor was scored on degree of desirability.

Percentage total moisture was determined by drying duplicate 10-g samples of ground, cooked muscle in a C.W. Brabender semi-automatic moisture tester for 1 hr at 121°F. Press fluid was measured for duplicate 25-g samples of ground, cooked muscle packed in a cheesecloth-lined (2 layers 14.5 cm in diameter) cylinder of a Carver Laboratory Press by alternating the sample, roughly divided into thirds, with 4 circles of Whatman No. 1 filter paper (5.5 cm). The sample was pressed following a standardized 15-min time-pressure schedule with a maximum pressure of 4,000 psig. The press fluid was poured into weighed centrifuge tubes graduated in 0.1 ml, capped with aluminum foil, and placed in a refrigerator until the next day, when the volume of total fluid, serum and fat was read.

To obtain percentage moisture in the press fluid, the centrifuge tubes were placed in a freezer 4–6 hr, the frozen fat was removed from the press fluid with a stainless steel laboratory spatula and the sides of the centrifuge tube above the frozen serum wiped with a tissue that had been wrapped around the laboratory spatula. The tubes were allowed to stand at room temperature with the aluminum caps in place for 1 hr before being weighed on an analytical balance. Percentage moisture in the press fluid was calculated as follows: [wt (g) of serum expressed/wt of ground meat sample (25 g)] × 100 = % moisture in press fluid.

Also, percentage moisture in the residue (press cake) from the ground meat sample was determined. The press cake was broken into six pieces, and the cheesecloth covering cut into pieces approximately $^3/_4 \times {}^1/_4$ in. The partially divided press cake and cheesecloth were mixed at high speed in a household blender for a total of 2 min with intermittent scraping down of the sides of the blender jar as necessary. A sample (10 g) of the finely divided press cake and cheesecloth was dried in a C.W. Brabender semi-automatic moisture tester as described for ground, cooked muscle.

Duplicate pH measurements were made on homogenates of ground, cooked muscle, using a Beckman pH meter, model 76. To prepare the homogenates, 5 g of muscle were blended with 50 ml of distilled water.

Statistical analyses

Data for each measurement made to evaluate the roasts were subjected to analysis of variance. When the F-test showed significant differences among three treatments, least significant differences ($P < 0.05$) were calculated. Correlation coefficients were computed for data within each type of muscle and with data for all three types of muscle pooled for each measurement with every other measurement. Shear values for cores from medial and lateral positions in the LD were analyzed by Student's *t*-test.

RESULTS & DISCUSSION

LOIN ROASTS were evaluated by palatability scores and values for selected objective measurements. None of the measurements was affected significantly by chilling temperature (post-mortem treatment of the carcass). Therefore, the data are reported according to the effect of type of musculature ("normal," PSE, DFD) and breed (Duroc and Poland China) irrespective of chilling temperature. For all characteristics measured, there was little variation among data within each type of muscle.

Palatability factors

There was no significant organoleptic preference for one type of musculature (Figs. 3, 4, 5). Tenderness, juiciness and flavor scores for LD were not affected by type of musculature, and only tenderness scores were affected ($P < 0.05$) by breed. Muscle from Durocs was more tender than muscle from Poland Chinas.

Objective measurements

There were significant ($P < 0.05$) differences in Warner-Bratzler shear values for LD from Poland Chinas attributable to type of musculature, whereas shear values for LD from Durocs were not affected by type of musculature. DFD muscle from Poland Chinas had significantly ($P < 0.05$) lower shear values (more tender) than "normal" or PSE muscle, but the difference between "normal" and PSE muscle was not significant. In general, shear values were lower (muscle more tender) for LD from Durocs than for LD from Poland Chinas, which agrees with tenderness scores (Fig. 3).

When shear values were analyzed by *t*-test, irrespective of type of musculature

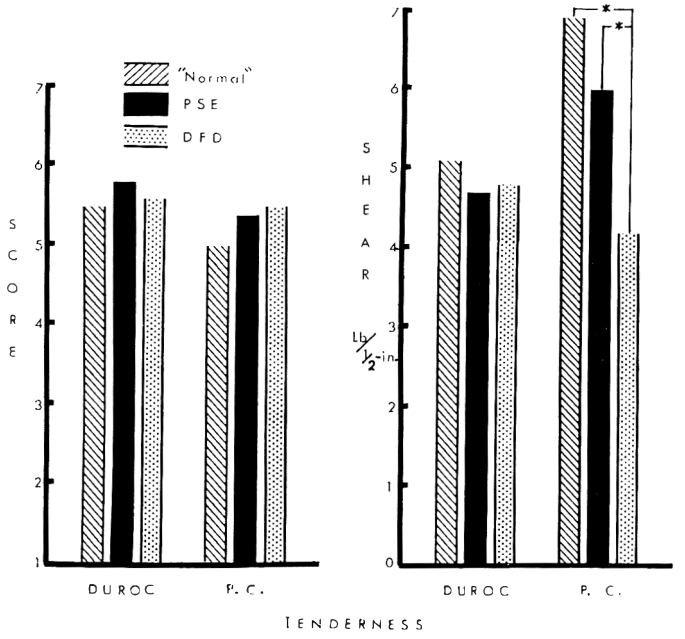

*Fig. 3—Mean tenderness scores and shear values for "normal," PSE, and DFD porcine longissimus dorsi. *, $P < 0.05$*

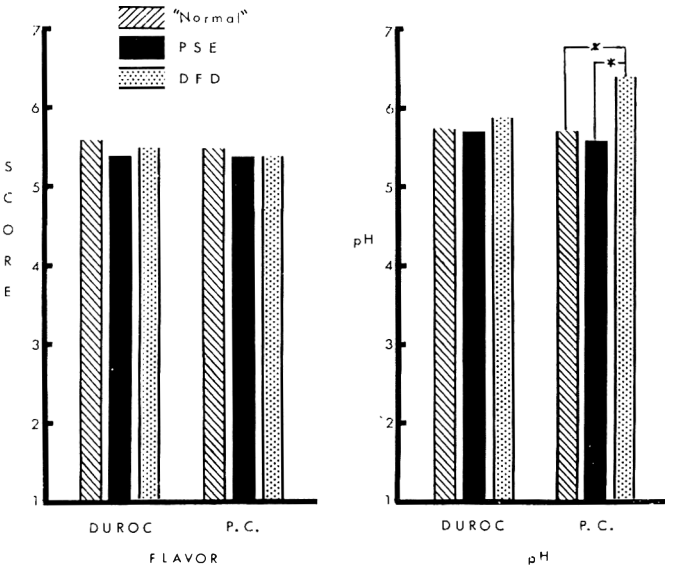

*Fig. 4—Mean flavor scores and pH values for "normal," PSE, and DFD porcine longissimus dorsi. *, $P < 0.05$*

or breed, values for the cores taken at the lateral position of the LD were significantly ($P < 0.001$) lower than those for cores from the medial position. That supports the findings of Urbin et al. (1962), but opposes those of Pengilly et al. (1966), Murphy et al. (1961) and Onate et al. (1963).

Differences in pH were significant ($P < 0.05$) between DFD and both "normal" and PSE muscle from Poland Chinas, but not for muscle from Durocs. In general, values for pH of muscle from Durocs were higher than those for muscle from Poland Chinas (Fig. 4).

Percentage total moisture, as measured by the Brabender moisture tester, was affected ($P < 0.001$) by both type of musculature and breed, and by interaction between those factors ($P < 0.05$). DFD muscle from Poland Chinas contained significantly ($P < 0.05$) more moisture than "normal" or PSE muscle, but the difference between "normal" and PSE muscle was not significant (Fig. 6). Total moisture as measured by the Carver Laboratory Press (percentage moisture in serum of press fluid and press cake) and total press fluid yield were not affected significantly by type of musculature or breed.

Correlation coefficients for total moisture as measured by the Brabender Moisture Tester vs the Carver Laboratory Press were high for DFD muscle ($r = 0.82P < 0.001$) and moderate for "normal" ($r = 0.58P < 0.05$), PSE ($r = 0.77P < 0.001$), and all types of musculature combined ($0.79P < 0.001$). A larger portion of the moisture, as measured by the Carver Laboratory Press, was retained by the press cake (37.5–40.7%) than was expressed in the press fluid serum (24.4–27.3%). However, the relationship between total moisture (Carver Press) vs moisture in the press cake ($r = 0.59P < 0.001$) was not as high as the relationship between total moisture (Carver Press) vs serum in the press fluid ($r = 0.84P < 0.001$).

Irrespective of type of musculature, correlation coefficients between panel scores for juiciness and total moisture as obtained both by the Brabender Moisture Tester ($r = 0.31$ $P < 0.05$) and the Carver Laboratory Press ($0.33P < 0.05$) were low. Also, the correlation coefficients for total moisture vs tenderness scores were low (Brabender, $r = 0.10$, not significant; Carver Press, $0.38P < 0.01$).

Differences in total roasting losses were attributable to type of musculature ($P < 0.001$) and to breed ($P < 0.05$). Losses within each type of musculature were greater for roasts from Durocs than for roasts from Poland Chinas. For both breeds, significant ($P < 0.05$) differences occurred between DFD and both "normal" and PSE roasts, but not between "normal" and PSE roasts. Also, for both breeds, roasting losses were greatest for PSE roasts, least for DFD roasts, intermediate for "normal" roasts (Fig. 6). Similarly Meyer et al. (1963) reported weight losses from cooked gluteus medius PSE muscle significantly ($P < 0.05$) greater than losses from DFD gluteus medius. Lewis et al. (1962) found that stress significantly ($P < 0.05$) decreased total roasting losses from the LD, but that sugar feeding had no effect on roasting losses.

Roasting time, both in total min and in min/lb, was not affected significantly by type of musculature or breed. It was suggested (Sayre et al., 1964) that cooking rate decreases with high evaporative loss because of the cooling effect of evaporation. In this study DFD roasts from both breeds had significantly ($P < 0.05$) lower volatile roasting losses (Duroc, 11%; Poland China, 9.5%) than PSE roasts (Duroc, 12.8%; Poland China, 11.5%), and required slightly less total roasting time (DFD—Duroc, 74.2 min; Poland China, 73.2 min; PSE—Duroc, 75.7; Poland China, 74.0). "Normal" roasts from Durocs had significantly ($P < 0.05$) lower volatile losses ("normal," 11.7%) than PSE roasts (12.8), but required slightly longer total roasting time ("normal," 77.9 min; PSE, 75.7 min).

There was no difference in volatile losses between Poland China "normal" (11.5%) and DFD (11.7%) roasts. However, PSE roasts from Poland Chinas required slightly longer to cook (74.0 min) than "normal" roasts (70.3 min).

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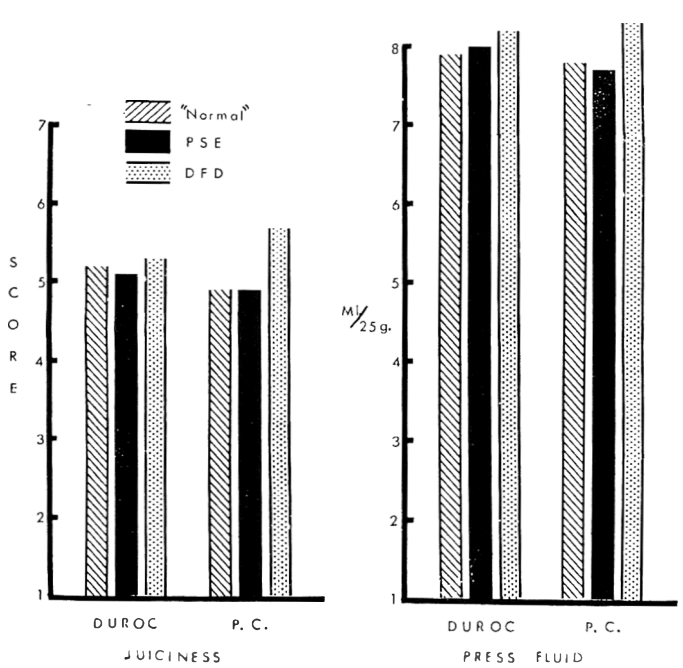

Fig. 5—Mean juiciness scores and press fluid yield for "normal," PSE, and DFD porcine longissimus dorsi.

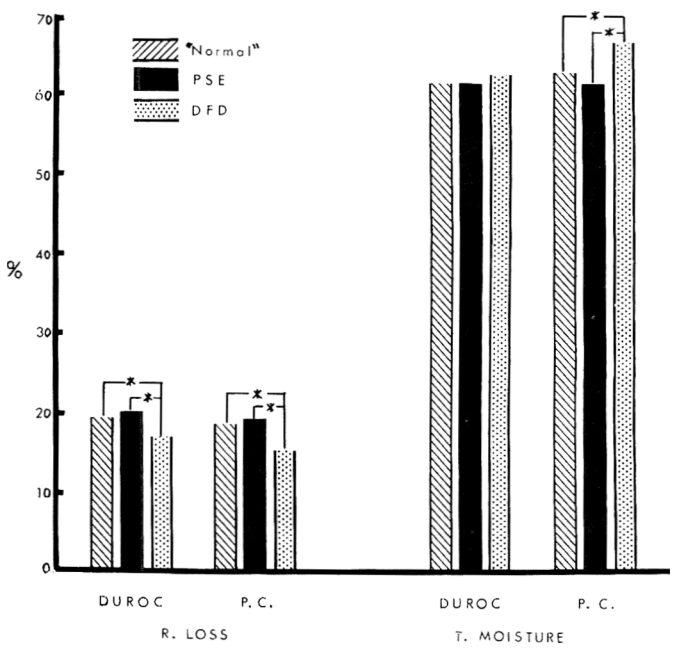

*Fig. 6—Mean percentage roasting losses and total moisture for "normal," PSE, and DFD porcine longissimus dorsi. *, $P < 0.05$*

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Relative Efficiency of Paired Comparisons and Rank Order in Preference Discrimination among Coffees

SUMMARY—Four samples of instant coffee, which varied only in strength, were preference tested by 1152 coffee drinkers by three different methods. Half tested by paired comparisons with two pairs served, one-third used 3-sample rank order and one-sixth used 4-sample rank order. The direction of preference was the same with all methods. The weaker coffees were preferred over the stronger ones. Within the paired comparison method, discrimination was slightly better with the second than with the first pair. Paired comparisons and 3-sample rank order discriminated equally well. Discrimination by 4-sample rank order was slightly poorer than with the other two methods when the between-sample differences were small.

INTRODUCTION

THE PAIRED comparisons method is considered the elite among psychometric methods. It is used more than any other method for evaluating feelings and opinions related to many kinds of stimuli, including foods. Simplicity, both logical and operational, is a primary advantage. Subjects understand it readily, results are easy to interpret and they have strong face validity. It is easy to understand why this method is the reference against which other methods are judged.

There is evidence that the method of paired comparisons should share its honored position. Experiments demonstrated that in certain situations variations of other methods discriminate just as effectively (Pilgrim et al., 1955). Seldom, if ever, has the general case been proven that other methods are superior from the standpoint of discrimination, but they have other advantages. The main one is efficiency.

Whenever there are more than two samples, both rank order and rating scale methods require less material, less experimenter time and less time and attention by subjects. For example, with four samples, rank order requires only the one-time presentation to a single subject to generate the complete set of intercomparisons, whereas paired comparisons require presentation of six pairs. As it is inadvisable, at least with foods, to present as many as 12 samples at a session, either multiple testing sessions or a series of judgments distributed among several subjects are required. The rank order method is not problem-free either. As the number of samples increases, greater demands, such as intensified physiological adaptation and complexity of judgment, are placed on the subject.

Although the work reported here was based on the theoretical background described briefly above, its objective was highly utilitarian. Consumer testing of various food products had been done by the method of paired comparisons, usually serving only one pair to each subject. Alternate procedures were considered that might increase efficiency by reducing time and cost without any important loss of discrimination. Three questions were inherent in the design of the study:

1. Does including a second pair in the paired comparison test have any effect on preference discrimination?
2. Does 3-sample rank order discriminate as effectively as paired comparisons?
3. Does including a fourth sample in the rank order test reduce capacity for preference discrimination?

METHODS

Test products

Test samples were instant coffees which differed only in strength. The between-sample relationships are shown below in terms of concentration of instant in the "usual" (5 ounce) cup of coffee.

Sample A—X g/150 ml
Sample B—X + 0.2 g/150 ml
Sample C—X + 0.4 g/150 ml
Sample D—X + 0.6 g/150 ml

Previous consumer testing showed that there should be preference differences with this series. Sample D was expected to be too strong; however, there was no prior expectation about the optimum concentration.

The proper quantities of instant for 60 ounces of finished coffee were pre-weighed in the laboratory. Tap water available at each test location was used in the preparation. Water was heated in thermostatically controlled electric percolators to 190°F, at which point the instant was added. The four samples were always prepared at the same time, and were maintained at 185° ± 3°F until drawn for serving. There was a maximum holding time of 30 min.

Test subjects

Test subjects were recruited in the Chicago area by working with various social groups who were paid for their services. All subjects were adults, with a minimum age of 17 and no maximum. The group was equally divided between men and women.

[a] The Coca-Cola Company, Atlanta, Georgia.
[b] Peryam & Kroll Research Corporation, Chicago, Illinois.

The only requirement was that all should normally drink coffee.

Test procedure

All subjects from a given organization were tested during the same session at some convenient location where adequate facilities were available. People reported in small groups, numbering up to about 12. They were seated, spaced apart at tables and each completed a short questionnaire about himself and his coffee drinking habits. The test instructions, also printed on the questionnaires, were given orally. Any questions that arose were answered. Then the group was enjoined to silence for the duration of the test.

Full cups of coffee were served in 5 oz china cups, identified by 3-digit code numbers, which also appeared on the questionnaires. One set of four code numbers was used for the rank order tests and for the first pair in the paired comparison test, and another set was used for the second pair. The assignment of codes to samples remained constant within a given experimental session. However, the assignment was rotated from one session to the next so that, over the entire test, each code number was used equally often for each sample. Cream (half & half) and small cubes of sugar were available for ad lib use. Subjects were cautioned to use the same amounts of sugar and cream in each cup.

The questionnaire for paired comparisons is shown in the appendix. Those for the rank order tests were similar. They showed the sample codes written in order of testing and the same instructions about the use of cream and sugar. Minor changes were necessary in the instructions about the procedure for tasting to adjust to three or four samples. Subjects recorded their preferences by writing the code numbers of the appropriate samples to complete a series of statements in the form: "The coffee I like best is_____," "The coffee I like next-best is __," etc . . . .

In the rank order tests, all samples were served at the same time. With the paired comparison method, the second pair was served a few minutes after the first judgment was made. Order of tasting the samples was controlled by the position of the cups, coupled with the instruction to subjects always to try them in order from left to right. If a subject failed to reach a decision on the ordering of the samples, which rarely happened, the questionnaire was discarded.

All subjects present in the room at one time were tested by the same method. However, methods were changed from one small group to the next insofar as possible. Thus, method was only partially confounded with location and social group.

Test design

With four samples, there were six possible inter-sample comparisons. The test was designed to equate the three methods on the basis of the number of times each comparison was made. Table 1 presents key features of the design.

Considering order of tasting, there are 12 possible ways of presenting the four samples in pairs, and 144 (12 × 12) ways of presenting two pairs. All possible combinations of pairs and of samples and orders within pairs were used, including arrangements where the first and second pairs were identical. This was the basic "block" of the design for paired comparisons. It was repeated twice for men and twice for women, requiring a total of 576 subjects. There was a total of 1152 judgments, and 192 for each pair, equally divided between first-pair and second-pair.

Table 1—Features of the test design.

	Paired comparisons	3-Sample rank order	4-Sample rank order
Subjects			
Men	288	192	96
Women	288	192	96
Total	576	384	192
Samples tested per subject	4	3	4
Comparisons per subject			
1st pair	1	—	—
2nd pair	1	—	—
Total	2	3	6
Total comparisons	1152	1152	1152
Comparisons per pair			
1st pair	96	—	—
2nd pair	96	—	—
Total	192	192	192

With three samples, there are four combinations and six permutations in each combination, again giving a total of 24; however, each ranking produces only three comparisons. Hence, it was necessary to repeat the "block" 16 times, eight times each for men and women, to get the 1152 comparisons. The total subject expenditure was 384.

With four samples, there are 24 permutations, and each ranking produces all six inter-sample comparisons. Thus, only eight "block" repetitions, four each for men and women, were required to get the same total number of comparisons from a total of 192 subjects.

Data analysis

Paired comparison results were analyzed in the usual way by obtaining the percentage of choice for each sample within each pair. The rank order data were analyzed as if they were paired comparisons in order to have them in the same form. Each ranking was converted to a set of comparisons between pairs of samples. For example, if a person ranked three samples in the order, A-B-C, this would produce three "choices": A over B, A over C and B over C. With the 4-sample rank order method, each ranking gave six comparisons, one for each pair of samples. Then these converted data were handled just as with paired comparisons.

Scale values (Table 2) for the paired comparison method were derived using L.L. Thurstone's scaling method, assuming Case V (Guilford, 1954). This method derives a measure, expressed in terms of a sigma distance, for each item based on the average of its proportion of choice against each other item.

The significance of percentages was tested by means of the z-distribution, which is an approximation to the binomial.

Table 2—Preference ranking for coffees of varying strength by different test methods.

	Paired comparisons[1]	3-Sample rank order (ave. rank)	4-Sample rank order (ave. rank)
Sample A—weakest	0.00	1.83	2.25
Sample B ↑	0.98	1.91	2.49
Sample C ↓	2.69	2.00	2.48
Sample D—strongest	4.29	2.26	2.77

[1] Thurstone scale values. The value 0.00 has been arbitrarily assigned to the best-liked sample, and the sigma distances have been multiplied by 10.

RESULTS

TABLE 2 gives the preference rankings for the four samples. Those for the rank order methods are average ranks, while that for paired comparisons was calculated by Thurstone's scaling method. The between-sample differences were significant at the 1% level, but there is no method of determining significance for the Thurstone values. Two points should be noted. The close correspondence of the three rankings means that the methods are in essential agreement, as one would expect. Thus, the only thing at issue is the relative precision of discrimination. Next, preference is inversely related to coffee strength. This means that the strength of the test coffee which would be optimum for this group of consumers is at or below the concentration of sample A.

This regular "weaker is better" relationship facilitated both the analysis and the presentation of results. Percentages could be combined across all pairs to provide an overall measure of tendency to prefer the weaker sample. To facilitate discussion, this tendency is referred to as "capacity to discriminate." Even though there could have been discrimination without a preference trend, the existence of the trend shows that discrimination occurred.

Table 3 presents the basic results in terms of percentage of choice of the weaker over the stronger coffee in each pair by each method. The totals across all six pairs are also shown.

The "total all methods" column reaffirms the generality suggested by the average ranking. In only two cases did preference for the weaker sample fail to reach significance at or beyond the 1% level. The fact that these occurred at the low-concentration end of the series, A vs B and B vs C, suggests that the weaker samples were near the preference optimum. Over the entire study, the weaker sample was preferred 57.5% of the time.

Let us first consider the matter of the utility of serving a second pair by the

Table 3—Preference for weaker over stronger coffee by different methods.

Comparisons: Weaker sample	Comparisons: Stronger sample	N =	Paired comparisons, %: 1st Pair 96	Paired comparisons, %: 2nd Pair 96	Paired comparisons, %: Total 192	Rank order, %: 3-Sample 192	Rank order, %: 4-Sample 192	Total all methods, % 576
A	B		46.9	54.2	50.5	56.2[1]	54.2	53.6[1]
B	C		49.0	62.5[2]	55.7	53.1	50.0	53.0
C	D		55.2	62.5[2]	58.9[2]	57.8[2]	56.3[1]	57.6[3]
A	C		58.3	64.6[3]	61.5[3]	55.2	54.7	57.1[3]
B	D		54.2	59.4[1]	56.8[1]	67.2[3]	55.2	59.7[3]
A	D		65.6[3]	57.3	61.5[3]	64.1[3]	65.6[3]	63.7[3]
Total all pairs			54.9[2]	60.1[3]	57.5[3]	58.9[3]	56.0[3]	57.5[3]
Total N			576	576	1152	1152	1152	3456

[1] Significant at the 10% level.
[2] Significant at the 5% level.
[3] Significant at the 1% level.

Significance of differences between percentages:
5% level: Pair B-D, 3-sample rank order over both 4-sample rank order and paired comparisons.
10% level: Pair B-C and total, second pair over first pair paired comparisons.

Table 4—Effect of subject characteristics on preference for weaker over stronger coffee (figures are percentages of choice for all pairs combined).

	N	%
Sex		
Men	1,728	55.7[1]
Women	1,728	59.2[1]
Daily coffee use		
1–4 cups/day	1,741	58.2
5+ cups/day	1,715	56.7
Age		
Under 30	335	61.8[2]
30–39	957	58.8
40–49	1,332	56.0
50–59	562	56.2
Over 59	270	57.0
Use of cream and/or sugar		
Cream & sugar	1,187	57.7
Black	1,117	55.9
Sugar only	167	68.9[3]
Cream only	985	57.1

[1] Difference between percentages significant at the 5% level.
[2] Significantly higher (10% level) than percentages for 40–49 and 50–59 groups.
[3] Significantly higher (1% level) than percentages for all other groups.

paired comparisons method. Averaged across all pairs, the first pair judgments discriminated, but only weakly, with 54.9% of choices for the weaker sample. Considering individual pairs, they established a significant difference only for A vs D, where the difference was greatest. In contrast, the second pair judgments discriminated significantly with four pairs and the total across all pairs was 60.1% of choices for the weaker sample. Differences between the two methods were significant for B vs C and for the total across all pairs, but only at the 10% level. While these results do not definitely prove the superiority of second-pair over first-pair testing, they leave little doubt that it is equally as good.

Combining all of the paired comparison results gives the same number of judgments per pair (192) for each of the three methods. Also, serving two pairs makes the methods about operationally equivalent in terms of number of samples considered by each subject. Totals across all pairs show that 3-sample rank order was most discriminating, with 58.9% of choices for the weaker sample, followed by paired comparisons with 57.5%, then 4-sample rank order with 56.0%. Differences between these percentages seem small and, in fact, none are significant.

If one considers individual pairs, there appear to be differences. There was significant discrimination by all methods with the A vs D pair which represented the greatest physical difference. With 4-sample rank order, there was significant discrimination for one other pair, and this only at the 10% level, whereas both paired comparisons and 3-sample rank order discriminated significantly on three other pairs. The differences between the proportions obtained by the various methods were significant only for the B vs D pair, where the 67.2% proportion achieved by 3-sample rank order was significantly higher (5% level) than the proportions for both of the other methods. Although the general case for the inferiority of 4-sample rank order remains unproved, the data suggest that it is less likely to detect small differences.

Table 4 presents some interesting auxiliary information, not necessarily related to the main purpose of the study. Data were classified according to various characteristics of the test subjects on which information was obtained. Results for all six pairs by all three methods were combined to give a general index of capacity to discriminate. All of the percentages shown in the table are significant at or beyond the 1% level, which means that, no matter what the classification, all groups preferred the weaker coffee.

Women were more discriminating than men (59.2% vs 55.7%) a difference significant at the 5% level. This supports the popular belief that housewives are the most discriminating judges of food products. Perhaps this finding should be qualified. It may show only that women are more likely than men to prefer weaker coffee.

Younger people (under 30) had 61.8% of choices for the weaker sample, while the percentages for the other age groups fell into a narrow range. The differences between 61.8% and the two lowest percentages reached the 10% level. Again, younger people are either sharper judges or have stronger preferences for the weaker coffee.

Amount of daily coffee usage had no effect on discrimination. Both the lower usage group (1–4 cups) and the higher usage group (5 or more cups) preferred weaker coffees, and to about the same extent.

The figures for manner of using cream and sugar seem to show something. "Sugar only" users, with an index of 68.9%, were significantly higher (1% level) than all of the other groups, whose proportions of choice for the weaker sample fell into a narrow range. However, this group was represented by only 58 persons who made a total of 167 comparisons, so the generality of the finding is questionable. These data were also analyzed to contrast people who use cream with those who do not, and people who use sugar with those who do not. Neither of the differences between percentages was significant.

DISCUSSION

THE SERIES of concentrations was designed to obtain a preference differential. Determination of an optimum concentration was an incidental objective. There was a consistent tendency to prefer the lower concentration coffees; however, the terms "Weak" and "Strong" have been used here only for convenience. Results should not be interpreted as proof that consumers in general like weak-tasting coffee, since there is no evidence that even the lowest concentration was perceived as "weak." This is the reason why the actual concentrations are not given. The results are probably valid for the particular instant coffee which was used. For example, they might serve as a guide for writing label instructions. However, they are not necessarily representative of all instant coffees.

To what degree can these results be generalized to other situations? Certainly the study was extensive compared to most laboratory or even field investigations; however, testing was limited to only one product, and one variable within that product. How important is this qualification? To establish that two methods are about equal in their capacity to discrimi-

nate contributes little that is new to the general fund of knowledge. This is the usual finding when direct comparisons are made among the three basic psychometric methods—paired comparisons, rank order and single stimuli. All of the methods are adequate, and differences will be marginal if proper test controls are exercised.

Pilgrim, et al. (1955) tested many types of foods and found that paired comparisons and hedonic scale ratings discriminated preferences equally well. Simone, et al. (1957) did a similar experiment with a highly-liked food—canned peaches. They reported that the two methods established the same preference order among samples, but that paired comparisons "appeared" to differentiate among samples somewhat better.

The other factor involved in this study was the number of samples considered by a subject at a single session. Present results show that extending the number within reasonable limits has little deleterious effect on capacity to discriminate. This finding is not uncommon, and has been made for both preference and difference testing. Some investigators have extended test sessions to "unreasonable" limits without losing discrimination. It is logical to suppose that the permissible number of samples should depend upon the physiological effects of the type of material evaluated; e.g., a subject's sensitivity should be maintained longer with a bland food like milk than with a heavy, cloying substance like maple syrup, although the evidence on this point is inconclusive.

The first-pair vs second-pair contrast in the present study suggests the facetious conclusion that, if one insists on single-pair paired comparison results, he should discard the first pair. Other work has shown that this is not always the case. Laue, et al. (1954) reported results from experiments where subjects took two triangle tests in immediate succession. With black coffee the proportion of successes was slightly higher in the second test, but, with maple syrup, there was a significant loss of discrimination. Extensive two-pair preference testing of candies has shown that first-pair and second-pair results are about equally discriminating (D. R. Peryam, personal communication).

Difference testing with college students extended the number of samples to what might be considered "unreasonable" (Pfaffmann, et al., 1954). Subjects were tested continually during 40-min sessions, using the triangle and duo-trio methods. The test foods were fruit juices and brown bread. They reported that loss of capacity to discriminate was negligible even after tasting 60–75 samples. This was difference testing; however, neither is preference discrimination always inhibited by "unreasonable" session length. Kamen, et al. (1968) had subjects rate up to 12 samples of milk, soup or maple syrup in one continuous session. Discrimination was not significantly poorer with 12 than with only four samples.

Efficiency has another aspect. Once the equivalence of methods in capacity to discriminate has been demonstrated, it is legitimate to examine the costs of testing. The superiority of the rank order methods on this criterion is abundantly clear. Let us assume that the per subject cost for recruiting and testing would be approximately the same for all methods. Then, if a single subject cost $10.00, the overall expense of evaluating the four coffees by the various methods would have been:

One-pair paired comparison,
1152 subjects—$11,520
Two-pair paired comparison,
576 subjects—$5,760
Three-sample rank order,
384 subjects—$3,840
Four-sample rank order,
192 subjects—$1,920

These differences are dramatic—the most expensive method is six times the least expensive. Of course, there is another factor to be considered, namely, the breadth of the sampling. A result based on 1152 consumers is certainly better than one based on only 192. However, this works both ways. Holding cost constant, by the four-sample rank order method, one could have results for each pair based on 1152 subjects, whereas the figure would be only 192 for one-pair paired comparisons.

APPENDIX

Coffee preference test

You will be served two pairs of coffees. The pairs will be served one at a time.

You will notice that there is a code number on each cup and that these numbers are shown on the questionnaire. Please leave the cups in the positions in which they are served.

When the coffees are served, first fix both cups with cream and sugar (or leave them black), just the way you usually fix your coffee. Pitchers of cream and small sugar cubes are on the table. Be careful to use exactly the same amounts of cream and sugar in both cups. Now you are ready to test.

First try the cup on your left, which is also the one whose number is written on the left side of the questionnaire. Drink enough so that you get the full flavor of the coffee. Wait about 30 seconds and take a drink of water. Then try the second cup in the same way. You may go back and try the coffees again if you wish.

First pair. Which of the two coffees do you like better? Circle its number below:

369 587

When you have finished with the first pair, signal the operator. She will then serve your second pair of samples. Be sure to take a drink of water while you are waiting. When the second pair is served, prepare the coffees and test them in just the same way as the first pair.

Second pair. Which of the two coffees do you like better? Circle its number below.

264 196

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Wheat Flavor Components

SUMMARY—The volatile flavor components of a lightly milled, whole-grain soft wheat, Moro variety, were isolated and studied by gas-liquid chromatography, mass spectrometry, paper chromatography and/or chemical tests. The following 11 compounds were identified in the wheat flavor essence or in headspace vapors: acetaldehyde, isobutyraldehyde, butyraldehyde, hexanal, heptanal, octanal, crotonaldehyde, 3-methyl-2-butanone, 2,2-dimethyl-3-pentanone, diacetyl and ethyl acetate. Tentative identifications were made of seven additional compounds: butanone, valeraldehyde, isovaleraldehyde, cyclopentanone, phenylacetaldehyde, amyl alcohol and isoamyl alcohol.

INTRODUCTION

COMPONENTS of the flavor fractions of many foods have been the subject of intensive research in recent years. Yet the realm of cereal flavors has been practically untouched despite the fact that cereal grains constitute the basic food supply for many peoples of the world. In anticipation of food shortages throughout the world, new food products are being developed from previously unexploited sources. One well-known example of such a food formulated by man is Incaparina.

Palatability as well as nutritional value is of prime importance in securing consumer acceptance of new foods. The use of well-accepted flavors such as those of cereal grains in new food products should contribute to their acceptance and utilization. When the significant components in the cereal volatiles are identified, synthesizing these important flavors will be possible.

In this study the volatile flavor components of whole grain wheat, Moro variety, were investigated. Eleven of the low-boiling compounds in the wheat volatiles were identified and seven were tentatively identified.

Two papers dealing directly with the flavor of cereal grains were found. In studies on rice flavor, Yasumatsu et al. (1966a) positively identified acetaldehyde and n-caproaldehyde, and tentatively identified methylethylketone, n-valeraldehyde, and either propionaldehyde or acetone from the vapor of cooked rice. The stale flavor of stored rice was found to be due, at least in part, to the presence of n-caproaldehyde (Yasumatsu et al., 1966b).

Although bread doughs, pre-ferments and baked bread are more complex than the cereal grain alone, some of the important flavor components will arise from the cereal portion of the mix. Ng et al. (1960) identified acetaldehyde, ethyl pyruvate and furfural as constituents of fresh white bread volatiles, and isobutyraldehyde and n-valeraldehyde as compounds in the oven vapors. Acetaldehyde, acetone, 2-ethyl hexanal, 2-hexanone, 3-heptanone, crotonaldehyde, diacetyl, pyruvaldehyde and furfuraldehyde were identified in fresh bread by Wiseblatt et al. (1960).

Johnson et al. (1966) summarized studies conducted to that date in which 22 carbonyl compounds had been identified in pre-ferments, doughs, oven vapors, and bread. Ten esters had been detected in pre-ferment and bread, 24 organic acids in pre-ferments, doughs and bread, and seven alcohols in pre-ferments, oven vapors and bread.

EXPERIMENTAL

ANALYSES were carried out on a wheat flavor concentrate and on headspace vapor. The concentrated essence of the wheat flavor volatile components was prepared using a modification of the procedure of Herz et al. (1966). A sample of 1500 g wheat was mixed with 6.5 L of distilled water saturated with sodium chloride. Steam was directed through this slurry and the condensate was collected for 4 hr under vacuum. At the end of the distillation period the condensate in the traps was combined and extracted with ether. The ether was evaporated to yield 0.15 ml of a viscous yellow essence which had an intense, wheaty aroma.

Headspace vapor was obtained fresh for each analysis by bubbling steam through the wheat sample (200 g wheat and 600 ml distilled water) which was heated in a simple distillation apparatus under atmospheric pressure. A removable thermometer in the side arm of the flask allowed ready access to the exiting vapors.

Identification of components of the volatile flavor fraction of wheat was accomplished by means of functional group analysis, melting point determinations and paper chromatography of 2,4-dinitrophenylhydrazone derivatives of the carbonyl compounds present, retention times during gas-liquid chromatography, and mass spectrometry.

For functional group analysis a 5-ml sample of the vapors was injected into the chromatograph. As the various peaks emerged, the effluent stream was diverted into five vials containing selected reagents (Walsh et al., 1960). The reagents, chosen to indicate the presence of alcohols, carbonyls, esters, amines and mercaptans, were removed when a given peak (or peaks) had passed, and were replaced with a complete set of fresh reagents.

Collection times were 50–66 sec, 67–78, 79–100, 105–130, 133–160, and 160–180 sec total elapsed time from injection to elution. Each collection time corresponded to the elution of one or more components. The 6 ft $\times$ $^1/_4$ in. I.D. column, packed with 6% diethylene glycol succinate supported on 90-100 mesh Anakrom ABS, was operated isothermally at 75°C under a nitrogen pressure of 5 psi.

Although a concentrate had been prepared by ether extraction of the vacuum condensate, enough of the volatile flavor components remained in the condensate to permit preparation of 2,4-dinitrophenylhydrazone derivatives (Shriner, et al., 1964). The crystals formed slowly, thus enabling partial separation of the various derivatives by filtration. Further separation was achieved by recrystallizing from ethyl alcohol, and by manual separation with the aid of a magnifying glass. Melting points of these derivatives were determined and compared with the melting points of known carbonyl derivatives prepared in the laboratory and also compared with values reported in the literature.

Column chromatography (Schwartz et al., 1962) and ascending paper chromatography, as outlined by Gaddis et al. (1959) and Ellis et al. (1958), were used to separate the aforementioned carbonyl derivatives into classes and to subsequently identify them as specific compounds.

Retention times of known compounds were determined by measuring the elapsed time from injection to the height of the peak, using a Barber-Colman gas-liquid chromatograph equipped with a flame ionization detector. Retention times were noted for three column packings, 6% Apiezon M, 6% diethylene glycol succinate and 3% Free Fatty Acid Phase; all supported on 90-100 mesh Anakrom ABS. Glass columns, 6 ft $\times$ $^1/_4$ in. I.D. were used.

The columns were operated isothermally at 75°C with a nitrogen carrier pressure of 5 psi. Samples of wheat flavor essence, 0.2 μl

in size, and of wheat headspace vapor, 5 ml in size, were analyzed on the same columns under the same conditions. Retention times of the peaks in the chromatograms of compounds arising from the wheat flavor essence and headspace vapor were compared with those of known compounds.

For mass spectrometry, a 1 μl sample of the wheat flavor essence was injected into a Perkin-Elmer gas chromatograph which housed a 300 ft $\times$ 0.01 in. I.D. stainless steel capillary column coated with butanediol succinate. Operation was isothermal at 60°C for 15 min, followed by programmed heating at the rate of 4°C per min to a final hold temperature of 182°C. The effluent from the gas chromatograph was introduced directly into the Atlas-MAT CH-4 mass spectrometer (9-in., 60° sector, single-focusing instrument) with a rapid magnetic scan from m/e 25 to 200 requiring 2 sec. Spectra of the eluting peaks were recorded by a Honeywell Model 1508 Visicorder.

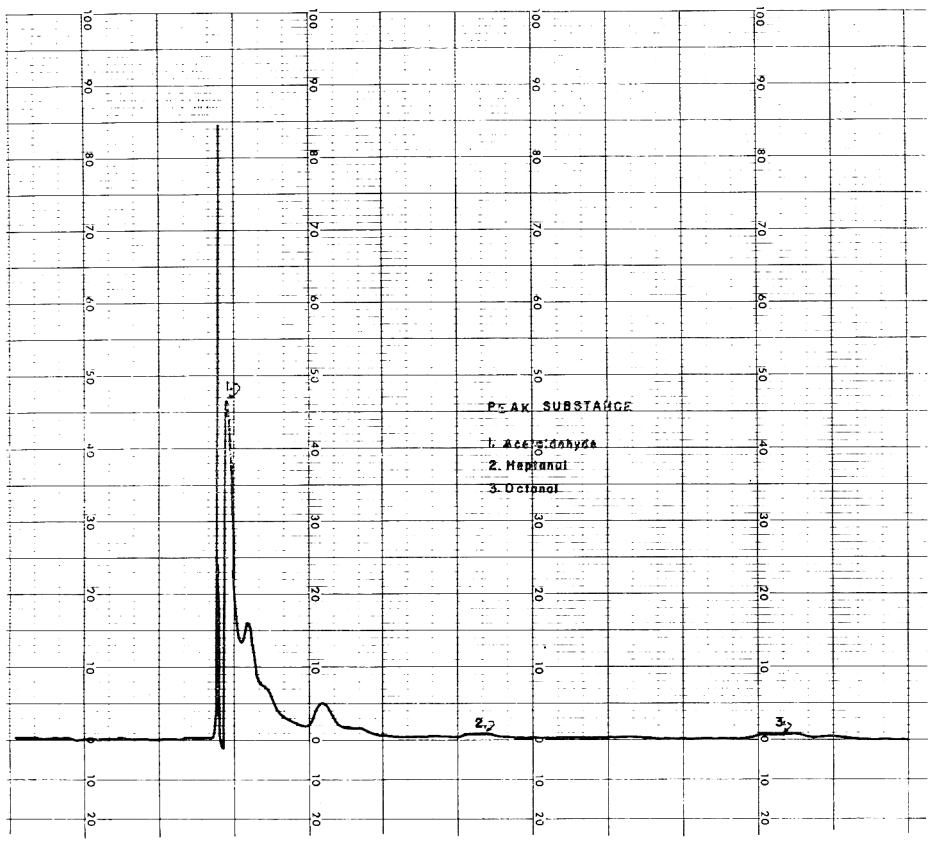

Fig. 1—Wheat headspace vapor, Apiezon M. 6%.

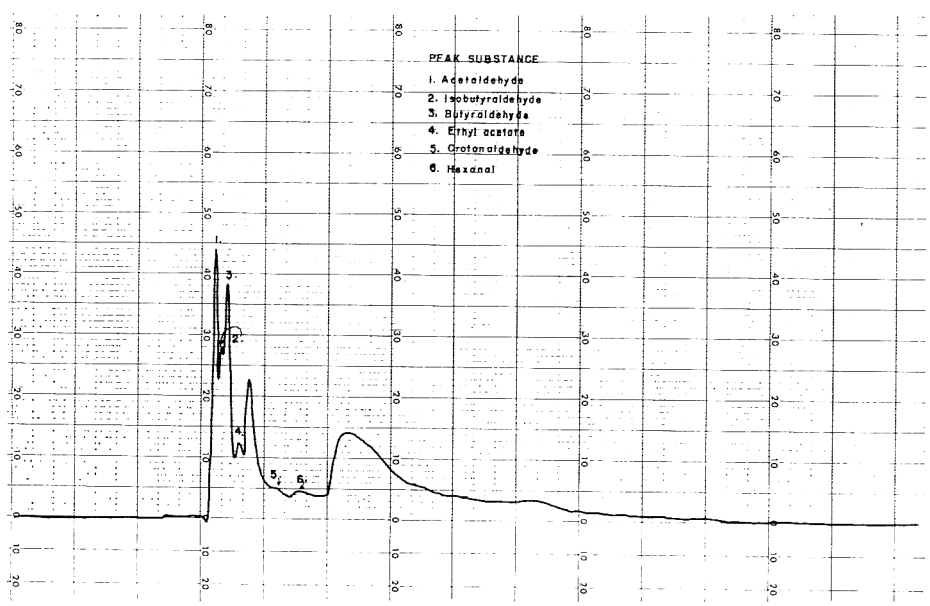

Fig. 2—Wheat headspace vapor, FFAP 3%.

RESULTS

CHROMATOGRAMS of headspace vapor and wheat flavor essence obtained by means of gas-liquid chromatography are shown in Figures 1 through 4. Retention times of wheat vapor components, Figures 1 through 3, indicated the presence of: 6% Apiezon column,—acetaldehyde, heptanal and octanal; 3% FFAP column, —acetaldehyde, isobutyraldehyde, butyraldehyde, 3-methyl-2-butanone, crotonaldehyde and hexanal; 6% DEGS column,—acetaldehyde, isobutyraldehyde butyraldehyde and crotonaldehyde.

The chromatogram of wheat flavor essence, Figure 4, indicates the presence of 3-methyl-2-butanone, crotonaldehyde, hexanal and heptanal. Tentative identification is indicated for cyclopentanone, isoamyl alcohol and amyl alcohol.

Functional group analysis of the effluent from the DEGS column during the periods mentioned under "Procedure" indicated the presence of carbonyl compounds through 180 sec. During each of the collection periods, precipitates formed in the vials containing 2,4-dinitrophenylhydrazine reagent, indicating the presence of the following compounds: 50–66 sec, acetaldehyde and/or isobutyraldehyde; 67–78 sec, butyraldehyde; 79–100 sec, 3-methyl-2-butanone, isovaleraldehyde and/or butanone; 105–130 sec, 2,2-dimethyl-3-pentanone, valeraldehyde and/or diacetyl; 133–160 sec, crotonaldehyde; 160–180 sec, hexanal.

Melting point determinations of the 2,4-dinitrophenylhydrazine derivatives of the unknowns in the flavor volatiles were compared with those obtained from known carbonyl derivatives prepared in the laboratory. Results indicated the presence of acetaldehyde, isobutyraldehyde, butyraldehyde, crotonaldehyde, 3-methyl-2-butanone, valeraldehyde, hexanal, 2,2-dimethyl-3-pentanone, heptanal, octanal, butanone and isovaleraldehyde. A comparison of observed melting points with values reported in the literature is presented in Table 1.

Additional confirmation of the presence of acetaldehyde, crotonaldehyde, and 3-methyl-2-butanone was secured by separating the 2,4-dinitrophenylhydrazone derivatives into classes by means of paper chromatography. The presence of 3-methyl-2-butanone was shown by eluting 2-alkanone region and subsequently chromatographing this sample with a derivative of the known compound. A similar procedure, using the alk-2-enal region, was used to identify acetaldehyde and crotonaldehyde. Ellis et al. (1958) had previously demonstrated that acetalde-

Table 1—Comparison of melting points of 2,4-dinitrophenylhydrazine derivatives of wheat volatiles with those of known compounds reported in the literature.

Carbonyl	Melting point of 2,4-dinitrophenylhydrazone, °C	
	Laboratory	Literature[1]
Acetaldehyde	168	168
Isobutyraldehyde	182	182
Butyraldehyde	125	122
Crotonaldehyde	190	190
3-Methyl-2-butanone	119	117
Valeraldehyde	110	107
Hexanal	104	104
2,2-Dimethyl-3-pentanone	178	175
Heptanal	110	108
Octanal	109	106
Butanone	116	114
Isovaleraldehyde	125	122

[1] Values as reported in Shriner et al. (1964) except those for valeraldehyde, 3-methyl-2-butanone, and 2,2-dimethyl-3-pentanone which were reported in Heilbron et al. (1953).

hyde was separated in the class separation step with the alk-2-enals rather than with the expected n-alkanals.

Mass spectral identifications for three compounds were made by comparing the spectra with those published in the ASTM tables (1963). The capillary column used in conjunction with the mass spectrometer provided good separation of volatile components of the wheat flavor essence.

In the first spectrum, the parent ion was at m/e 44 and the base peak was at m/e 29. This fragment suggests a cleavage between the first and second carbon atoms, and the relative abundance of the fragment ion at m/e 43 was consistent with the value reported in the ASTM tables for acetaldehyde. This spectrum was thus confirmed as being acetaldehyde.

Similarly, a second spectrum was identified as being ethyl acetate. The parent ion was at m/e 88 and the base peak was at m/e 43. The ions observed at me/43 and m/e 45 were the result of the cleavage of the ester linkage.

A third spectrum was analyzed, and the parent ion was at m/e 86. The base peak at m/e 43 was the result of a split in the middle of the symmetrical molecule, diacetyl. The intensities observed in both spectra 2 and 3 were consistent with the intensities for ethyl acetate and diacetyl, respectively, in the ASTM tables.

A fourth spectrum was examined and the parent ion was at m/e 120, and the base peak at m/e 91. Comparison of this spectrum with the values reported in the tables led to tentative identification of this spectrum as that of phenylacetaldehyde.

The identifications of components are summarized in Table 2.

DISCUSSION

ONE OF THE pitfalls of the flavor researcher is the possibility of creating or introducing experimental artifacts during the concentration process and ultimately endowing them with a natural existence. The wheat odor was carefully noted when the shipment arrived. The aroma of the heated sample for headspace vapor analysis was compared with the original aroma, as were the odors at the various stages of concentration during the processing of the wheat essence.

The aroma issuing from the headspace vapor apparatus when the thermometer was removed from the system was identified as strongly wheat-like. The odor of the wheat essence that resulted after extraction with anhydrous diethyl ether and distillation of the ether was intense and was easily recognized as that of wheat. The trapped effluent from the gas chro-

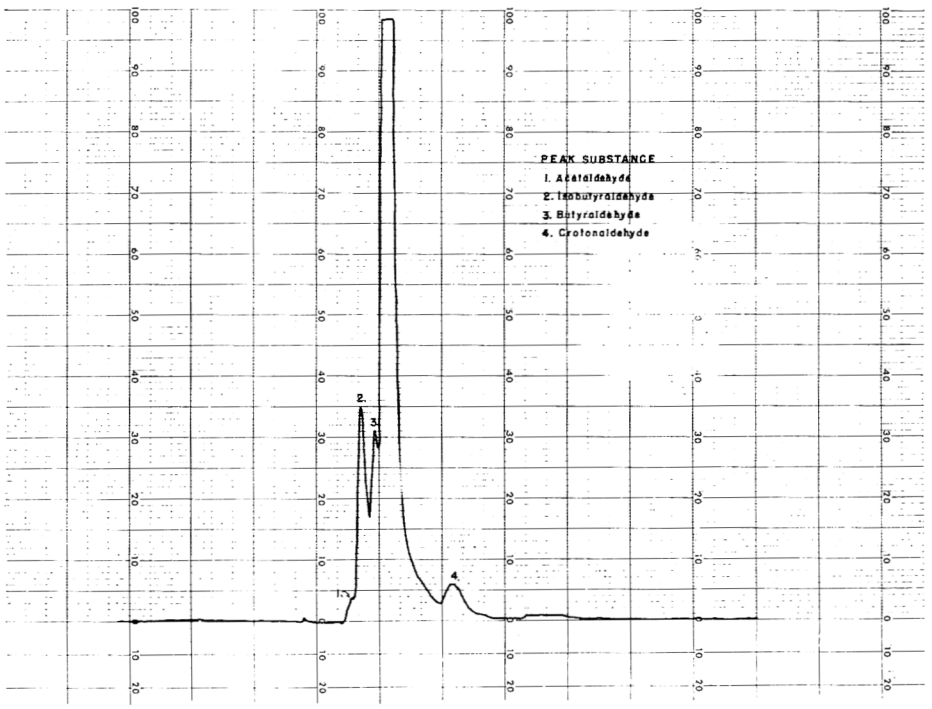

Fig. 3—Wheat headspace vapor, DEGS 6%.

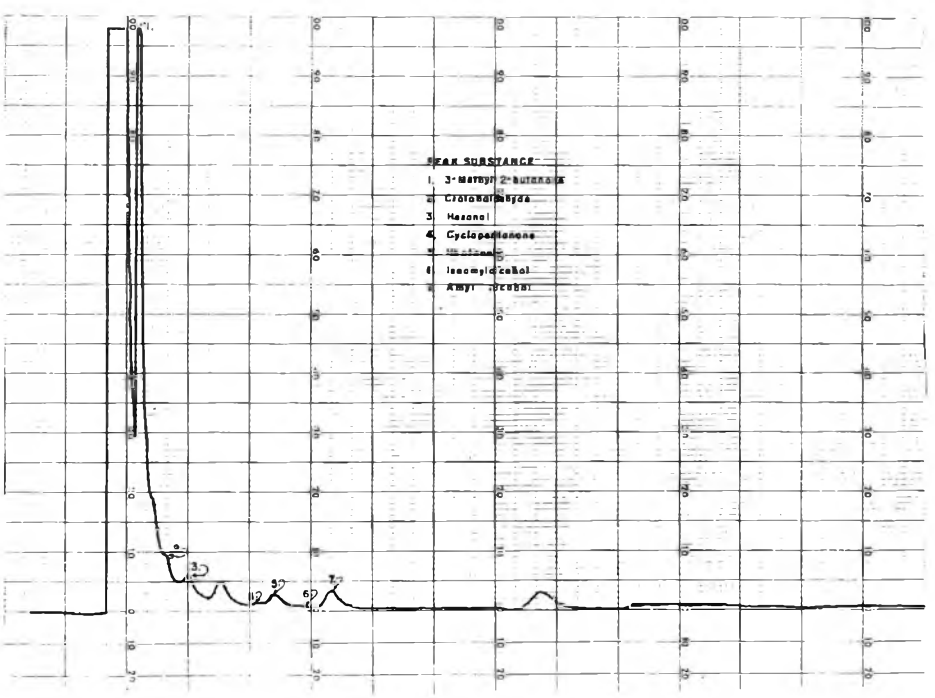

Fig. 4—Wheat flavor essence, FFAP 3%.

Table 2—Summary of identification of components in wheat headspace vapor or essence.

Compound	Effluent formed a precipitate in 2,4-DNPH reagent at retention times of known compound	Paper chromatography	Gas-liquid Chromatography						Mass Spectrometer	Melting point of 2,4-DNPH derivative
			Headspace Vapor			Essence				
			AM	FFAP	DEGS	AM	FFAP	DEGS		
Acetaldehyde	x	x	x	x	x				x	x
Isobutyraldehyde	x			x	x					x
Butyraldehyde	x			x	x					x
Hexanal	x			x			x			x
Heptanal			x	x			x			x
Octanal			x							x
Crotonaldehyde	x	x		x	x		x			x
3-Methyl-2-butanone	x	x		x			x			x
2,2-Dimethyl-3-pentanone	x									x
Diacetyl	x								x	
Ethyl acetate									x	
Isoamyl alcohol							x			
Butanone	x									x
Isovaleraldehyde	x									x
Valeraldehyde	x									x
Cyclopentanone							x			
Phenylacetaldehyde									x	
Amyl alcohol							x			

matographic column was also readily identified as having the odor of wheat.

Experimental evidence from chemical, gas chromatographic and mass spectrometric techniques was used in identifying wheat flavor components. All results were brought to bear in establishing identification of 11 compounds and tentative identification of seven others.

Of the 18 compounds noted in this paper, all but 3-methyl-2-butanone and 2,2-dimethyl-3-pentanone have been reported as components of pre-ferments, doughs, oven vapors or bread in the summary by Johnson et al. (1966). It appears that much of the flavor of bread may arise from components present in the wheat.

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Identification and Quantitative Analysis of Phospholipids in Cocoa Beans

SUMMARY—*The lipids extracted from several common cocoa bean varieties were separated into neutral lipid, glycolipid, and phospholipid fractions. The composition of the total lipid extract was 98% neutral lipid and 1 to 2% polar lipid of which approximately 70% was glycolipid and 30% was phospholipids. Two-dimensional thin-layer chromatography was used to separate all of the known major phospholipids. The relative distribution of the phospholipids was determined by quantitative phosphorus analyses of individual spots scraped from two-dimensional thin-layer plates. The major components were lysophosphatidyl choline, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol. Phosphatidyl choline was found to contribute 36 to 40% of the phospholipids of cocoa beans. The phospholipid composition of Accra, Arriba, and Bahia beans was shown to be quite similar although minor variations were observed.*

INTRODUCTION

SINCE PHOSPHOLIPIDS are surface-active compounds, they have a significant influence on the physical properties of chocolate products (Cook, 1963). Lecithin, commercially derived from soybeans, is commonly used in chocolate to reduce the interfacial tension between the cocoa butter and the non-fat particles. Less cocoa butter is needed and during conching viscosity is reduced much more rapidly when lecithin is incorporated into chocolate.

The demonstrated effectiveness of lecithin in controlling viscosity focuses attention on the natural phospholipids of cocoa beans. Very little is known about them, except that they are present in concentrations of 0.3 to 0.4% by weight (Knapp, 1937). Because of the great importance of phospholipids in the control of viscosity, identification of the compounds present in cocoa beans and the determination of qualitative and quantitative differences among beans from various sources is needed.

Methods developed for the separation of phospholipids from various animal tissues give unsatisfactory results when applied to the separation of plant lipids. This is attributed to the differing nature and greater complexity of plant lipids (Nichols, 1964). One problem in the separation of the individual classes of phospholipids is the inherent difficulty of isolating all the individual components by silicic acid column chromatography or one-dimensional thin-layer chromatography (TLC).

Hawke (1963) was not able to separate phosphatidyl ethanolamine and phosphatidyl serine. Phosphatidyl inositol and phosphatidyl serine were separated by Skipski et al. (1964), but cardiolipin and phosphatidic acid could not be differentiated. Morrison et al. (1965) used two solvent systems on two separate plates to resolve most of the polar lipids from bovine milk but phosphatidyl inositol was not separated in their study.

Two-dimensional TLC methods (Abramson et al., 1964, Feldman et al., 1966, Nichols, 1964, Rouser et al., 1965) have yielded improved separations but phosphatidic acid, phosphatidyl inositol, and phosphatidyl serine were not completely resolved.

Recently, a two-dimensional TLC system (Parsons et al., 1967) was developed which overcomes these difficulties. Using this and related procedures, an investigation was undertaken to determine the levels of lipid phosphorus and the identities and relative distribution of the phospholipids of cocoa beans.

EXPERIMENTAL

Cocoa beans

Raw beans were provided by several member companies of the Chocolate Manufacturer's Association of the USA. Several samples of common cocoa bean varieties, Accra, Arriba, and Bahia, plus single samples of Caracas, Costa Rica, Ghana, Ivory Coast, Lagos, Sanchez, Tabasco, and Trinidad beans were supplied. Each raw bean sample was a composite from several bags to insure a representative product. Samples were stored in an air-conditioned laboratory until analyzed.

Extraction and purification of lipids

Total lipid was extracted from 20 g of shelled raw beans by grinding in a "Virtis" model 23 homogenizer (Virtis Company, Inc., Gardner, New York) with chloroform-methanol (2:1 v/v) solvent as recommended by Folch et al. (1957). Suspended cocoa fiber was removed from the lipid extract by filtering through a layer of Celite 545 (Johns-Manville, Pittsburgh, Pennsylvania) and a disk of filter paper (Whatman No. 1) on a Buchner funnel under reduced water aspirator pressure.

Nonlipid contaminants were removed by the washing procedure of Folch et al. (1957). Since the moisture content of cocoa beans is approximately 6%, it was necessary to modify the washing procedure so that chloroform, methanol, and water would be present in the proportions 8:4:3 by volume.

The modified Folch procedure consisted of washing the lipid extract with 0.24 times its volume of a 0.04% $CaCl_2$ solution. The solvent was removed from the washed extract on a rotating evaporator under reduced pressure at 35°C and the total lipid sample was taken up in a small volume of diethyl ether.

Separation of the lipid extract into classes

The silicic acid column chromatographic method of Hirsch et al. (1958), slightly modified, was employed to separate the total lipid extract into neutral lipid, glycolipid, and phospholipid fractions. Thin-layer chromatograms showed no detectable levels of phosphorous in the neutral lipid and glycolipid fractions, based on the specific phospholipid spray of Dittmer et al. (1964).

Weight determinations were made on aliquots of each fraction to estimate the gross composition of the total lipid extract. The phospholipid fraction was evaporated and taken up in a small volume of chloroform-methanol (2 : 1 v/v).

Analysis of the phospholipids of cocoa beans

The two-dimensional TLC procedure of Parsons et al. (1967) was used to separate the individual phospholipids of cocoa beans. The solvent systems are modifications of those reported by Rouser et al. (1965). For the analysis of phospholipid phosphorus the spots were made visible by exposure to iodine vapor and immediately outlined with the point of a needle.

Other detection methods included spraying with one of the following reagents: ninhydrin reagent (0.2% in ethanol) for phospholipids containing free amino groups, the specific phospholipid spray of Dittmer et al. (1964) and the sulfuric acid potassium dichromate

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reagent (Privett et al., 1965) to reveal the presence of all lipid material. Individual phospholipids were identified by means of these selective spray reagents and by comparing R_f values with those of purified reference compounds.

The method of Saliman (1964) was used for the determination of phosphorus in total lipids extracts. Analysis of phospholipid phosphorus from two-dimensional thin-layer chromatograms was determined by the method of Rouser et al. (1966) with minor modifications.

RESULTS & DISCUSSION

Polar lipid composition of cocoa beans

In order to obtain quantitative data on the phospholipid composition of cocoa beans, it was necessary to separate the glycolipids from the phospholipids. This was achieved by a modification of the silicic acid column chromatographic method in which acetone was used to elute the glycolipid fraction followed by methanol to recover the phospholipid fraction.

Weight determinations of the column fractions provided evidence that cocoa beans contain 1 to 2% polar lipid of which approximately 70% was glycolipid and 30% was phospholipid. The phospholipid levels of Accra, Arriba, and Bahia beans were found to be quite similar. However, Arriba beans contained higher levels of glycolipid than either Accra or Bahia beans. This accounted for the slightly higher concentration of polar lipids found in Arriba beans.

Evidence available when the study was initiated suggested that cocoa beans contain 0.3 to 0.4% phospholipid (Knapp, 1937) of which phosphatidyl choline was believed to be the major component. However, one of the most important findings revealed in this study is the dominance of the glycolipids in the polar lipid fraction. The surface-active properties of the phospholipids are well known. Although the chemical and physical properties of glycolipids and phospholipids are similar, the significance of the surfactant properties of glycolipids in chocolate products is presently unknown.

It can be seen in Figure 1 that the patterns of phospholipids from the various beans (Accra, Arriba, and Bahia) are virtually identical. Figure 2 shows a representative two-dimensional chromatogram of the phospholipids of raw Accra beans. The separation of the components into the known major phospholipids is as complete as possible.

Unknown phospholipids represented by X_1, X_2, X_3 and X_4 are all positive to the Dittmer et al. (1964) spray reagent, but have not yet been identified. Since spot X_1 is also ninhydrin-positive, it may be lyso-phosphatidyl ethanolamine, and it is not inconceivable that X_2, X_3 and X_4 are phosphatidyl glycerols. Compound X_2 co-chromatographed with a phosphatidyl-glycerol sample and the behavior of X_3 was suggestive of diphosphatidyl glycerol.

Table 1—Percent phospholipid of several varieties of raw cocoa beans calculated from lipid phosphorus levels.

Sample	mg lipid phosphorus/ 100g raw beans	Percent phospho-lipid[1]
Accra	11.4	0.28
Arriba	16.6	0.41
Bahia	16.2	0.40
Caracas	12.9	0.32
Costa Rica	16.5	0.41
Ghana	15.0	0.37
Ivory Coast	15.0	0.37
Lagos	13.0	0.32
Sanchez	16.2	0.40
Tabasco	18.0	0.45
Trinidad	15.5	0.39

[1] Calculated as g lipid phosphorus per 100 g raw beans × 25.

Comparison of lipid phosphorus levels of several varieties

Quantitative lipid phosphorus analyses of the chloroform methanol (2:1 v/v) extracts of eleven common varieties of cocoa beans are presented in Table 1. Five lots of Accra, Arriba and Bahia beans, but only single samples of other bean types were available for analysis. When expressed as percent by weight phospholipid, the values are in agreement with those reported by Knapp (1937)

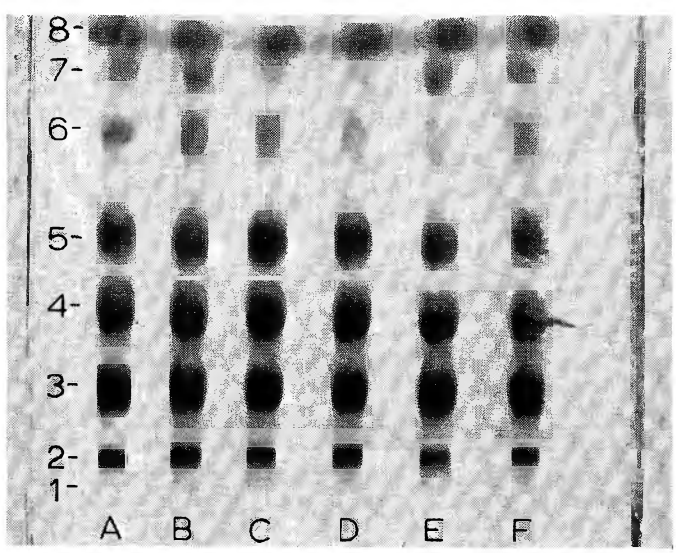

Fig. 1—Thin-layer Chromatographic Separation of Phospholipids from Cocoa Beans. A, B—Accra; C, D—Bahia, E, F—Arriba. The chromatogram was prepared from Silica Gel H-HR and developed with chloroform-methanol-water-28% aqueous ammonia (130:70:8:0.5). Lipids were detected by sulfuric acid-potassium dichromate spray-reagent (Privett et al., 1965). 1, origin; 2, lyso-phosphatidyl choline; 3, phosphatidyl choline; 4, phosphatidyl inositol; 5, phosphatidyl ethanolamine; 6, unknown; 7, unknown; 8, neutral lipids at the solvent front.

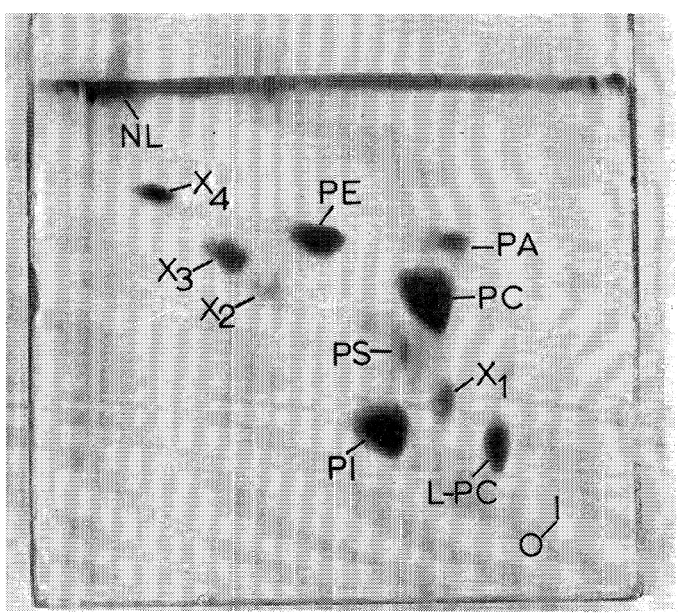

Fig. 2—Two-Dimensional TLC of Phospholipids from Raw Accra Beans. The chromatogram was prepared from Silica Gel H-HR and developed in the horizontal direction from right to left with chloroform-methanol-water-28% aqueous ammonia (130 : 70 : 8 : 0.5) and in the vertical direction with chloroform-acetone-methanol-acetic acid-water (100 : 40 : 20 : 20 : 10). Lipids were detected by sulfuric acid-potassium dichromate spray reagent (Privett et al., 1965). O, origin; L-PC, lyso-phosphatidyl choline; PC, phosphatidyl choline; PA, phosphatidic acid; PS, phosphatidyl serine; PI, phosphatidyl inositol; PE, phosphatidyl ethanolamine; NL, neutral lipid; and unknown substances listed as X_1, X_2, X_3 and X_4.

who found 0.3 to 0.4% phospholipid in cocoa beans.

While there was a tendency for African beans, namely Accra, Ivory Coast, Ghana and Lagos, to have lower concentration of phospholipids than beans of American origin, the number of samples examined was entirely too small to allow any conclusions to be made in this regard.

Data for the several samples of Accra, Arriba and Bahia beans indicate that the phospholipid concentration may vary considerably among different samples of the same type of bean. For instance, the range of values found for the five Accra samples was 7.8 to 16 mg of lipid phosphorus per 100 g of beans.

Table 2—Phospholipid composition of raw Accra, Arriba, and Bahia beans.

Phospholipid	Percent phospholipid by TLC[1,2]		
	Accra	Arriba	Bahia
Phosphatidic acid	1.7 ± 0.6	3.7 ± 0.5	0.6 ± 0.3
Lyso-phosphatidyl choline	9.0 ± 1.1	6.5 ± 0.3	4.5 ± 0.5
Phosphatidyl choline	36.2 ± 0.4	40.4 ± 1.0	36.1 ± 1.3
Phosphatidyl serine	2.9 ± 0.6	2.3 ± 0.6	3.3 ± 1.0
Phosphatidyl inositol	25.5 ± 1.4	27.5 ± 1.2	29.0 ± 1.4
Phosphatidyl ethanolamine	13.5 ± 0.3	11.3 ± 0.7	18.6 ± 1.3
Unknown X_1	4.0 ± 0.8	2.4 ± 0.7	2.7 ± 0.7
Unknown X_2	1.8 ± 0.8	1.7 ± 0.8	0.7 ± 0.3
Unknown X_3	3.8 ± 0.6	3.4 ± 0.6	3.9 ± 0.5
Unknown X_4	1.7 ± 0.5	1.0 ± 0.8	0.8 ± 0.1

[1] Average recovery 90 to 100%.
[2] Mean ± SD, n = 6.

Comparison of the phospholipid composition of three common varieties

The phospholipid compositions of Accra, Arriba and Bahia beans are presented in Table 2. Quantitative analyses involved the separation of the components by two-dimensional TLC followed by phosphorus analyses of the spots scraped from the thin-layer plate. Usually spots from two chromatograms were pooled for minor components. Average values were thus obtained from six complete determinations on a total of twelve two-dimensional thin-layer plates.

Recovery of lipid phosphorus was 90 to 100%. Some of the loss may be attributed to minor components not determined and to the fact that only the spots plus corresponding blanks were scraped from the TLC plate.

The presence of at least 10 classes of phospholipids in cocoa beans has been established. The two-dimensional thin-layer chromatogram shown in Figure 2 is representative of the phospholipid composition of the three varieties of cocoa beans. The major components were lyso-phosphatidyl choline, phosphatidyl choline, phosphatidyl ethanolamine, and phosphatidyl inositol. The remaining phospholipids were present in amounts varying from 0.7 to 5.0%.

Phosphatidyl choline contributes 36 to 40% of the phospholipids of cocoa beans. Previously it was assumed to be the major phospholipid and present at levels much greater than 50%.

Lyso-phosphatidyl choline was present in all samples studied. The relatively high concentration of lyso-phosphatidyl choline may be attributed in part to the action of enzymes during the fermentation process. Phosphatidic acid may be formed by a similar process, but this seems unlikely because Arriba beans are usually only slightly fermented and have the highest level of phosphatidic acid, 3.7%.

The phospholipids of cocoa beans are important as surface-active compounds influencing the physical properties of chocolate products (Cook, 1963). It is now evident that the phospholipid level in the common varieties of cocoa beans is relatively constant at 0.3 to 0.5%. The phospholipid composition of raw Accra, Arriba and Bahia beans has been shown to be quite similar although minor variations were observed.

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Bisulfite Effect on the Chemistry and Activity of Horseradish Peroxidase

SUMMARY—Bisulfite retarded the inactivaton of horseradish peroxidase by weak acids. Spectral analysis indicated that the retardation was accomplished by the stabilization of the linkage between the iron containing prosthetic group and the protein. Cyanide, azide and fluoride, which form reversible complexes with peroxidase iron, exerted a similar effect; thus, it is inferred that bisulfite also forms a complex with peroxidase iron. A kinetic method was used to calculate a dissociation constant of 0.2M for the bisulfite peroxidase complex.

INTRODUCTION

THE EFFECT of sulfurous acid and its salts on peroxidase has not been extensively studied. Klebanoff (1961) reported that sulfite activated the nonperoxidative oxidation of NADH and NADPH as catalyzed by peroxidase. Chmielnicka (1963, 1964) and Monikowski et al. (1964) found that SO_2 destroyed the H_2O_2 in a system containing this peroxide and tomato or horseradish peroxidase and stopped the activity of the enzyme. Maehly (1952) noted that acids in general split the prosthetic group of horseradish peroxidase from its protein, thus inactivating the enzyme.

The purpose of this study was to observe and explain some of the effects of bisulfite on the chemistry and enzymatic activity of horseradish peroxidase (HRPR).

MATERIALS & METHODS

THE ENZYME used in this work was "type II" horseradish peroxidase from the Sigma Chemical Company. Its activity was 110 purpurogallin units per mg.

Enzymatic activity was determined by adding 0.1 ml of the peroxidase solution, properly diluted (usually 1:40), to a mixture of 1 ml 0.5*M* phosphate buffer pH 6.5, 1 ml 20 m*M* gualacol, 0.1 ml 10 m*M* H_2O_2 and 0.8 ml H_2O. The reaction mixture was in a 1-cm-path Beckman cuvette and the enzyme solution was added with a square Teflon plunger provided with a groove and three orifices and connected to a stainless steel handle. Color formation in the solution was measured with a Beckman DU spectrophotometer connected to a Ledland log-converter and a Sargent SR recorder. The rate of reaction was measured by the tangent at the origin of the curve obtained with the recorder.

Spectra were obtained with a Bausch & Lomb Spectronic 505 recording spectrophotometer.

Table 1—The effect of bisulfite on the activity of HRPR solutions.

pH	Without bisulfite	Containing 0.067*M* HSO_3^-
4	37	87
5	80	92
6	100	96
7	100	98

Each solution contained 0.5 mg HRPR and 0.66*M* citrate-phosphate buffer in a total volume of 3 ml. The figures are the percent enzymatic activity remaining after 30 hr incubation under anaerobic conditions.

RESULTS & DISCUSSION

WHEN HRPR solutions were held for thirty hours at the pH range 4.0–7.0, there was a loss of enzymatic activity at the acid end of the range (Table 1), presumably because of loss of the prosthetic group as described by Maehly (1952). With bisulfite present in the solutions, however, there was a considerable retention of activity at the lower pH levels. The opposite effect would be expected as the increase in ionic strength, caused by the addition of bisulfite, should accelerate the detachment of the prosthetic group by acid. This is illustrated in Table 2, where it can be seen that the addition of sodium sulfate hastens the loss of enzymatic activity at pH 4.0, in contrast to the addition of bisulfite.

Table 2—The effect of sulfate and bisulfite on the activity of HRPR solutions at pH 4.0.

Time, hr	No $SO_4^=$ or HSO_3^-	With 0.05*M* $SO_4^=$	With 0.05*M* HSO_3^-
0	100	100	100
1.5	91	69	94
3.0	80	45	86
4.5	72	29	82
6.5	59	17	78
8.5	43	8	72
10.5	34	0	71

Each solution contained 0.03 mg HRPR in 6 ml 0.05*M* citrate buffer, pH 4.0. The figures are the percent activity remaining after varying periods of incubation.

The protective effect of bisulfite was also observed at pH levels below 4.0 (3.5 and 3.0) where the inactivation of peroxidase is much more rapid, taking place in only a few hours. A similar protective effect on the Soret adsorption band was observed when bisulfite was present in the HRPR solutions of pH 4.0. Line 1 of Figure 1(A) represents the normal absorbance spectrum of HRPR in the Soret region, with a peak at 403 nm. Line 2 represents the spectrum of the same material 6 hr later; the absorbance has fallen off because a portion of the enzyme has lost its protohematin prosthetic group. Line 3 represents the spectrum after 12 hr and line 4 after 24 hr of incubation. By

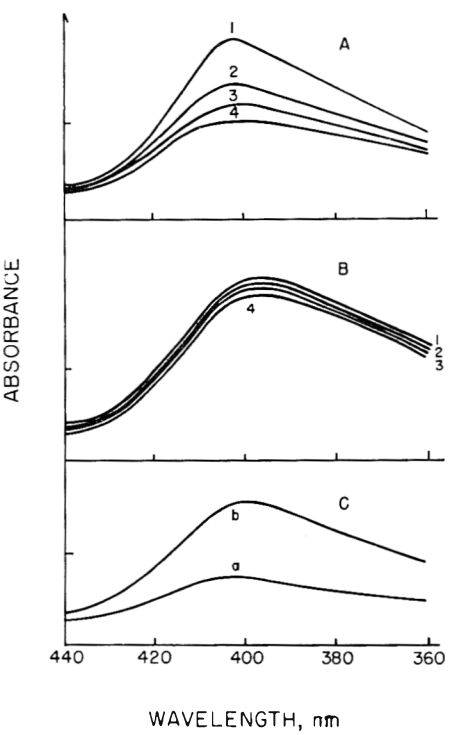

Fig. 1—Effect of bisulfite on the spectrum of HRPR. (A) Spectra of a solution containing 1 mg HRPR in 5 ml of 0.04M citrate buffer, pH 4.0. Lines 1, 2, 3 and 4 correspond to the spectra at 0, 6, 12 and 24 hr of incubation, respectively. (B) Spectra of same solution containing 0.1M bisulfite. (C) Line a is the spectrum of solution A, and b of B, both after 24 hr incubation followed by 24 hr dialysis.

that time, the enzyme had become nearly colorless and inactive.

Figure 1(B) illustrates the spectral curve of a similar solution of peroxidase at pH 4.0, with bisulfite added. It can be seen that the curve did not fall with time and after 24 hr the only change was a shift of the peak from 403 nm to about 395 nm.

Both solutions were dialyzed for 24 hr at 5°C against demineralized water and their Soret spectra then compared [Fig. 1(C)]. The peroxidase which had been treated with bisulfite had an almost normal spectrum, with its peak shifted back to 402 nm, whereas the untreated peroxidase had a flattened spectrum.

The enzymatic activities of these solutions at the times their spectra were determined are given in Table 3.

Table 3—Loss of enzymatic activity of solutions A and B with time (See Fig. 1).

Incub. time	Solu. A	Solu. B
0	100	100
6	45	96
12	25	80
24	15	75

Figures are percent activity remaining after incubation.

These findings indicate that bisulfite retards the loss of the prosthetic group by acid. It may do this by complexing with the sixth coordinate position of the iron atom in the protohematin group and stabilizing its linkage with the protein.

To test this hypothesis, four peroxidase solutions with the same HRPR and buffer compositions as those in Table 1 were held for 24 hr at pH 4.0. Solutions 1, 2 and 3 also contained 0.033*M* KCN, 0.033*M* NaN_3, and 0.033*M* NH_4F, respectively; the fourth solution was a control. Cyanide, azide and fluoride all form spectroscopically distinctive complexes with peroxidase iron (Keilin et al., 1951).

After the 24-hr incubation, the four solutions were dialyzed for 36 hr against demineralized water at 5°C. With the removal of the complexing agents the normal Soret spectrum of each peroxidase solution was restored. The spectrum of the control solution was very flat in comparison. When the enzymatic activities of the dialyzed solutions were determined, it was found that solution 1 retained 81% of its original activity; solution 2, 72%; and solution 3, 84%; the control retained only 14% of its original activity.

It appears, therefore, that complexing agents in general stabilize the enzyme against attack by weak acids. It may be inferred that bisulfite, since it exerts a similar stabilizing effect, is also a complexing agent for peroxidase. The fact that the bisulfite-peroxidase complex is active, whereas the other complexes are not, may be due to the rapid oxidation of the bisulfite upon addition of H_2O_2 for the assay.

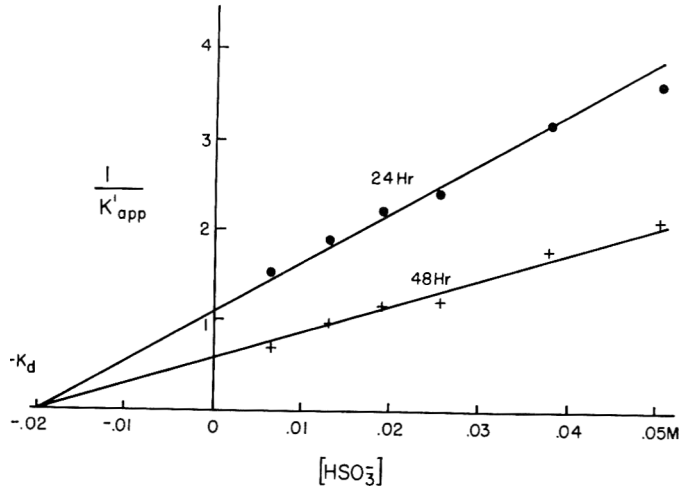

Fig. 2—Mildvan-Leigh Plot of 1/k'app. vs [HSO_3^-]. Each reaction mixture contained 0.5 mg of HRPR, 0.05M citrate buffer, pH 4.0 and varying quantities of bisulfite, in a total volume of 4 ml. The enzymatic activity was measured at 24 and 48 hr of incubation.

Assuming the bisulfite-peroxidase complex is exactly as active as free peroxidase, its dissociation constant was determined by the kinetic method of Mildvan et al. (1964). This method is applicable in cases where an enzyme is gradually inactivated by some inhibitory agent and a cofactor or other substance combines with the enzyme and retards the inactivation. The key equation is as follows:

$$\frac{1}{k_{app}} = \frac{1}{k} + \frac{[lig.]}{kK_d}$$

where K_d is the dissociation constant of the ligand-enzyme complex and [lig.] is the molar concentration of the complexing agent. k and k_{app} are, respectively, the actual and apparent rate constants for the inactivation of the enzyme. k_{app} is defined in the equation:

$$\log \frac{[E_0]}{[E]} = \frac{k_{app} \cdot t \cdot [I]}{2.3}$$

where $[E_0]$ is the total enzyme concentration, [E] is the active enzyme concentration after time t of incubation with inhibitor present in concentration [I].

K_d is determined by plotting $1/k_{app}$ against [lig.], and the intercept on the X axis has the value of $-K_d$.

In our study, four HRPR solutions containing varying amounts of bisulfite were deoxygenated by passing nitrogen through them and incubated for 24 hr at pH 4.0 at room temperature. The activities of the solutions were determined both at zero time and after incubation. The solutions were then held for an additional 24 hr and their activities again determined. These data were used to determine a simplified k_{app}, k'_{app}, in the following manner: the value E_0/E was taken as the ratio of the activity at zero time to that after a given incubation time, and log E_0/E was considered equal to k'_{app}; this simplification is based on the fact that incubation time, t, and acid concentration, [I], did not enter into the final value of K_d.

Figure 2 gives the plot of $1/k'_{app}$ vs. (HSO_3^-) for both the 24 hr and 48 hr incubation periods. The intercept on the X axis indicates a value of about 0.02*M* as the dissociation constant of the bisulfite-HRPR complex.

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Aldehydes, Ketones and Esters in Valencia Orange Peel Oil

SUMMARY—The aldehydes, ketones and esters in the volatile portion of Valencia orange oil have been isolated and identified. Seventeen aldehydes and ketones and three esters are included, 10 of which have never been identified as constituents of Valencia oil. Cis- and trans-limonene oxide have also been found to occur in the same fraction. This is the first time that these compounds have been identified in cold-pressed orange oil. A series of five homologous α,β-unsaturated aldehydes were isolated and partially characterized.

INTRODUCTION

AS PART of a continuing effort by this laboratory to isolate and identify citrus oil components which may influence flavor, aroma and color, a fraction of cold-pressed Florida Valencia orange oil has been isolated and its components, consisting of aldehydes, ketones and esters identified.

The literature contains numerous references to the identification of citral in orange peel oil (Nelson et al., 1934; Guenther et al., 1938; Foote et al., 1943; Naves, 1947; Schweisheimer, 1956). Straight chain, saturated aldehydes, principally decanal and octanal, have been reported as constituents of California Valencia orange oil and a quantitative study of their relative concentrations was made (Stanley et al., 1961).

Recently, a number of other aldehydes, ketones and esters have been isolated and identified as constituents of California Valencia orange oil. These include carvone (Stanley et al., 1961; Kesterson et al., 1962; Teranishi et al., 1966), piperitenone and methyl heptenone (Moshonas, 1967), n-octyl acetate (Ohta et al., 1966), and α- and β-sinensal (Stevens et al., 1965; Flath et al., 1966).

Others identified are nootkatone (MacLeod et al., 1964), perillaldehyde, perillyl acetate and 1,8-p-menthadiene-9-yl acetate (Ohta et al., 1966), hexanal (Teranishi et al., 1966), heptanal, octanal, nonanal and decanal (Stanley et al., 1961; Teranishi et al., 1966), undecanal and dodecanal (Stanley et al., 1961).

This paper reports the isolation and identification of all of the above compounds as well as 10 compounds never before identified as constituents of orange oil. A separation technique was used which had been developed and used previously in this laboratory (Hunter et al., 1965a; Hunter et al., 1965b). It involved distillation of the oil into several cuts followed by fractionation of each cut on a neutral alumina column and finally gas-liquid chromatographic analysis of the fractions from the column.

Table 1—Compounds identified in Valencia orange oil[1]

1. *n*-hexanal
2. *n*-heptanal
3. *n*-octanal
4. 6-methyl-5-hepten-2-one
5. *n*-nonanal
6. trans-limonene oxide[2,4]
7. cis-limonene oxide[2,4]
8. octyl acetate[4]
9. citronellal[4]
10. *n*-decanal
11. *n*-undecanal
12. neral[4]
13. geranial[4]
14. *n*-dodecanal
15. carvone
16. perillyl acetate[4]
17. perillaldehyde[4]
18. 1,8-*p*-menthadiene-9-yl acetate[4]
19. piperitenone
20. aldehyde A
21. nootkatone[4]
22. β-sinensal[3]
23. aldehyde B
24. α-sinensal[3]
25. aldehyde C
26. aldehyde D
27. aldehyde E

[1] Listed in order of increasing retention time.
[2] Authentic samples were obtained by GLC fractionation of a mixture of the cis- and trans-epoxides obtained from Dr. W. F. Newhall of the Citrus Experiment Station of the University of Florida, Lake Alfred, Florida.
[3] See Buchi et al., 1967 for nomenclature. Samples for identification obtained from the Western Regional Research Laboratory, ARS, USDA, Albany, California.
[4] Isolated and identified as a component of Valencia orange peel oil for the first time.

EXPERIMENTAL

Spectroscopic measurements

Mass spectra were obtained with a Bendix Model 3012 (TOF) mass spectrometer, infrared spectra on a Perkin-Elmer Infracord Model 137-A, NMR spectra on a Varian Model A60, and UV data on a Cary Model 14 spectrophotometer.

Separation procedure

Cold-pressed Valencia orange oil (4750 ml) was distilled at 36°C and 2 mm Hg in a rotary evaporator until most of the volatile portion came over. The residue (197 ml) was distilled on a Nester/Faust 10 mm × 36 in. spinning band column and a fraction weighing 35 g collected at a head temperature of 35–115°C at 1–0.7 mm Hg.

Ten g of the distillate from the spinning band column were separated into three fractions on a 2.5 × 45 cm column of Fisher Activity II neutral alumina. The fractions were eluted by washing the column successively with 300 ml portions of hexane, ethyl ether and ethanol. The weight of material in grams in each of the fractions was as follows: hexane, 6.2; ether, 1.5; ethanol 2.3. Most of the carbonyl containing compounds were in the ether fraction based on the relative intensity of the carbonyl stretching band in the infrared spectrum. However, since infrared analysis also indicated weak carbonyl bands in the hexane and alcohol fractions, each of the three fractions were analyzed for carbonyl containing compounds.

Analysis of the fractions was carried out on an F&M Model 810 gas chromatograph equipped with a thermal conductivity detector using a ¼ in. by 20 ft column packed with 20% Carbowax 20M on 60/80 mesh Chromosorb P. The helium flow rate was 60 ml/min and the column was programmed from 135–225°C at 1°/min. Before the final GLC analysis, the hexane fraction was subjected to a preliminary fractionation on a preparative gas chromatograph, using a Nester/Faust model 850 equipped with a ¾ in. by 6 ft "Bi-Wall" annular column packed with 25% SE-30 on chromosorb.

RESULTS & DISCUSSION

THE COMPOUNDS isolated are shown in Table 1. Identification was made by the comparison of infrared and mass spectra of known and unknown compounds.

A quantitative evaluation of the relative amount of the compounds in Table 1 could not be obtained because of the significant losses of material which occurred during the separation procedure.

An additional difficulty in obtaining quantitative data results from the fact that a number of the peaks from the gas-liquid chromatogram were mixtures of two or more compounds and the compounds indicated were obtained from shoulders of peaks. The most abundant constituents are *n*-octanal, *n*-nonanal, citronellal, *n*-decanal, neral, geranial, carvone, nootkatone, β-sinensal and α-sinensal as estimated from the relative size of the peaks in the gas-liquid chromatogram.

All of these abundant constituents have potent and distinctive odors which probably contribute significantly to the characteristic orange aroma. The saturated aliphatic aldehydes contribute a sweet pungent fatty odor. Neral, geranial and citronellal have a pungent citrus-like odor, carvone a minty odor, nootkatone the odor of grapefruit and the sinensals a

sweet pungent penetrating smell.

Trans- and cis-limonene oxide have never been reported as constituents of cold-pressed orange oil. Although not carbonyl containing compounds, they were included because they were found in one of the fractions along with the carbonyl compounds. A number of the alcohols in orange oil (Hunter et al., 1965b) are related to limonene oxide and are possibly formed by acid-catalyzed ring opening of the oxides.

Esters 16 and 18 were identified by hydrolysis with NaOH in aqueous ethanol, isolation of the alcohol and comparison of the infrared and mass spectrum of the alcohol with those of known compounds. Compound 16, perillyl acetate, yielded an alcohol identical with a sample of perillyl alcohol obtained from Fritzsche Brothers. Esters 18 gave 1,8-p-menthadiene-9-ol (Hunter et al., 1965a). The presence of an acetate unit in the original ester was inferred from the difference in molecular weight between the alcohol and the ester as shown by the respective mass spectra, combined with infrared bands characteristic of acetate esters at 5.7 and 8.1 μ.

The perillyl aldehyde was usually isolated as a mixture containing minor amounts of another compound. The contaminant could not be identified because it had the same GLC retention time as perillyl aldehyde on both polar and nonpolar columns. However, a sample of the pure perillyl aldehyde was obtained from one of the orange oil samples. It could be identified by comparison of the infrared spectrum with that of an authentic sample prepared either by pyrolysis of myrtenal in a sealed tube under nitrogen at 400°C for 5 min (Tsuruta et al., 1951) or by manganese dioxide oxidation of perillyl alcohol. The identification was also confirmed by reduction of the unknown with PtO_2 and hydrogen at 70 psi to a mixture of cis- and trans-*p*-menthane-7-ol, identified by comparison of the infrared spectra with those of known samples.

Aldehydes A-E represent a series of compounds with similar infrared spectra characterized by absorption bands at 3.7 μ (C—H on aldehyde carbonyl); 5.9 μ (conjugated $\overset{\overset{O}{\|}}{C}$) and 6.2 μ (—C=C—). The mass spectra indicated molecular weights of 238, 252, 266, 280, and 280, respectively for aldehydes A, B, C, D and E. The UV spectra of A-E in ethanol indicated a high intensity absorption maximum at 230 mμ in each case which could be assigned to a disubstituted α,β-unsaturated aldehyde chromophore.

An NMR spectrum was obtained of aldehyde C in CCl_4 with multiplet signals at 0.9, 1.3, 1.7 and 2.2 and a singlet at 9.3 (δ values in ppm relative to TMS). The unsplit aldehyde C—H signal at 9.3 eliminated the possibility of a proton on the alpha carbon.

At this point one could assign the general formula

$$R—CH=\underset{\displaystyle R'}{\overset{\displaystyle |}{C}}—CHO$$

to aldehyde C where R and R′ are acyclic saturated hydrocarbon radicals. Assuming that the other members of the series likewise lack a proton at the alpha carbon, they would also possess the above general formula and would differ from one another by the number of —CH_2— units in R and R′. The nature of the structure of R and R′ is presently under investigation.

The odor of these unknown aldehydes was found to have a faint, burnt, fatty quality. In flavor, they could be described as cardboard-like.

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References to specific products of commercial manufacture are for illustration and do not constitute endorsement by the U.S. Department of Agriculture.

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Vapor Analysis of the Production by Banana Fruit of Certain Volatile Constituents

SUMMARY—*Direct gas chromatographic vapor analyses were utilized to determine whether banana slices at different stages of ripeness in an in vitro system produced* iso-*amyl acetate and* iso-*amyl alcohol, known banana aroma constituents; and whether a precursor-product relationship could be observed between these compounds and L-leucine. Production of* iso-*amyl acetate by unripe slices was demonstrated based on experiments with metabolic inhibitors. The behavior of ripe and overripe slices was inconclusive since the vapor concentration of the acetate remained constant. The vapor concentration of* iso-*amyl alcohol was essentially unchanged at all ripeness stages. Investigations with L-leucine-U-*^{14}*C showed conclusively that leucine was a precursor and that both compounds were continually produced at all ripeness stages. The interpretation of direct vapor analyses over respiring fruit is considered.*

INTRODUCTION

ONE OF THE MOST prominent but least understood of the many changes that occur in ripening fruit is the development of flavor and aroma. Since the compounds which give a food its characteristic aroma are usually volatile, direct gas chromatographic analysis of the vapors over them has proved (Bailey et al., 1962; Buttery et al., 1963; McCarthy et al., 1963; Lim et al., 1964; Romani et al., 1966) to be a most useful method for monitoring changes in the vapor composition brought about by ripening, processing, or storage.

A particularly successful application of direct vapor analysis was the correlation of gas chromatographic data with flavor profiles and chemical identity (Issenberg et al., 1963) of certain volatile constituents of ripening banana fruit (McCarthy et al., 1963; McCarthy et al., 1964). As ripening proceeded the amount and number of volatiles increased and flavor and aroma developed.

In the course of continuing research in this laboratory on the isolation and identification of volatile banana constituents, and on the relationships between known aroma components and potential precursors (Wick et al., 1966; Myers et al., 1970; Wick et al., 1969), headspace analyses were employed to follow production of volatiles by banana slices in an in vitro system.

Since banana aroma concentrates contain about 200 components, the investigation was limited to constituents that (1) had already been identified, (2) were relatively easily separated and isolated in quantities large enough to work with, (3) are important contributors to banana flavor, and (4) whose precursors can be predicted based on past evidence. The relationship between *iso*-amyl acetate and *iso*-amyl alcohol (odor constituents) and L-leucine (the precursor; Ingraham et al., 1960; Ingraham et al., 1961) met these requirements. A similar approach was taken by Weurman (1961) in a study of volatile production by raspberries, and by Meigh et al., (1966) who followed the production of carbonyl compounds by tomato fruit tissue in the presence of aliphatic alcohols.

The purpose of this investigation was to utilize direct gas chromatographic vapor analyses to determine whether banana slices at different stages of ripeness in an in vitro system produced *iso*-amyl acetate and *iso*-amyl alcohol, and whether a precursor-product relationship could be observed between these compounds and L-leucine. Comparison of the results obtained with those of an investigation described elsewhere (Myers et al., 1970) with L-leucine-U-^{14}C of this relationship provided insight in the interpretation and meaning of direct vapor analyses over respiring fruit.

EXPERIMENTAL

HANDS OF GREEN bananas (*Musa cavendishii*, variety Valery) were obtained from the Tropical Banana Company, Cambridge, Mass. and ripened at 20°C and high relative humidity. The stage of ripeness was estimated by peel color. Less than ripe fruit was defined as that which ranged in color from green-yellow to yellow with green tips (Ripeness index [R.I.] 3 to 4); ripe fruit was full yellow (R.I. 5–6) and overripe fruit was yellow with brown flecks (R.I. > 6)

As judged by peel color, less than ripe fruit was at or near the climacteric while ripe and overripe fruit were in the post-climacteric stage (Looney et al., 1967; McCarthy et al., 1964; Wyman et al., 1964). In Valery banana fruit flavor development begins at about R.I. 3 to 4 when peel color begins to become yellow.

Incubation of slices

Slices (3 × 20 mm) were prepared from banana pulp tissue with a hand microtome and a No. 14 cork borer. They were randomized and soaked in tap water for up to 1 hr. In each experiment fingers from the same hand of fruit were used. Sets of 10 slices (about 10 g) were placed in 250 ml wide-mouthed Erlenmeyer flasks containing 10 ml of the appropriate incubation solution. The flasks were covered by aluminum foil (Buttery et al., 1963) and then by a 1$^{3}/_{4}$" Davol "Sani-Tab" serum cap. This closure provided an air-tight seal and minimized adsorption of volatiles on the serum cap.

The reaction flasks were equilibrated for 1 hr at room temperature with mild agitation provided by a wrist-action shaker. Vapor samples were then taken. The seals were removed and replaced every hour whether or not a sample had been taken, in order to insure aerobic conditions. Between samples the gas-tight syringe (Hamilton Co.) was dismantled, the plunger rinsed in distilled water and blotted dry. The needle and barrel were rinsed with distilled water and heated in an oven at 80°C for at least 15 min. Preliminary studies had shown that this cleaning procedure removed all detectable volatile components from the syringe.

Pasteurized, dehydrated, pure banana flakes (11.0 g, "Trop-Bana," Beatrice Foods Co.) in 20 ml of distilled water, or in 0.05 *M* phosphate buffer (pH 6.0), provided biologically inactive samples (with respect to volatile production) for use in interpretation of vapor analyses over banana slices. Headspace samples were taken in the manner described above.

The following aqueous solutions were used as incubation media.

1. 10 ml distilled water.
2. 10 ml 0.05 *M* phosphate buffer, pH 6.0 (control).
3. 1 ml 0.1 *M* sodium arsenate and 9 ml phosphate buffer, pH 6.0.
4. 0.1 ml 0.01 *M* 2,4-dinitrophenol (DNP) and 9.9 ml phosphate buffer.
5. 1 ml 0.1 *M* potassium cyanide and 9 ml phosphate buffer, pH 6.0.
6. 10 ml 0.01 *M* D,L-leucine in phosphate buffer, pH 6.0.

[a] Present Address: The Coca Cola Company, Atlanta, Georgia 30301.

7. 1 ml 0.01 *M* D,L-leucine and 9 ml phosphate buffer, pH 6.0.
8. 0.1 ml 0.10 *M* D,L-leucine and 9.9 ml phosphate buffer, pH 6.0.

Determining production of *iso*-amyl acetate and *iso*-amyl alcohol

Vapor samples—10 ml for less than ripe (R.I. 3 to 4) and 5 ml for ripe (R.I. 5–6) and overripe slices (R.I. > 6) were taken at 1 or 2 hr intervals by means of a Hamilton Gas-Tight syringe fitted with a Chaney adapter. They were injected on a 6′ × 1/4″ O.D. stainless steel column containing 2% Ucon 50HB 2000 on Anakrom ABS (Analabs, Inc.) 60/70 and maintained isothermally between 45 to 50°C. Carrier gas (nitrogen) flow was 30 ml/min. Hydrogen flow was 30 ml/min and the injection port temperature was 205°C. The chromatograph was an Aerograph 600-C (Varian Aerograph, Walnut Creek, Calif.) fitted with a flame ionization detector and an Aerograph 326 linear temperature programmer.

Approximately 30 min were required for each chromotogram to be recorded. No peaks were detected after that time. When duplicate sets of slices were studied batches of slices were prepared at 30 min intervals. Excellent separation of *iso*-amyl acetate and *iso*-amyl alcohol from each other and from other volatile constituents was achieved (see Fig. 1). Peak areas of *iso*-amyl alcohol and *iso*-amyl acetate were calculated by the triangulation method. The change in the relative amount of each compound in terms of peak area over a period of time was determined from the ratio of peak area at selected time intervals to the initial peak area after 1 hr incubation.

The precision of the vapor analyses was determined by analyzing the headspace over rehydrated banana flakes. The headspace over freshly prepared samples was studied over periods of 5 hr in 7 days throughout a 3 week period. The concentration of *iso*-amyl acetate remained essentially constant. The coefficient of variation was 1.5%.

RESULTS & DISCUSSION

THE TYPICAL distribution of components in the vapor over slices from overripe (R.I. 8–9) banana pulp incubated 3 hr in phosphate buffer is shown in Figure 1. Review of the peak attenuations shows that 3 to 4 times more *iso*-amyl acetate was present than *iso*-amyl alcohol. When unripe slices (R.I. 3–4) were incubated *iso*-amyl alcohol was usually not detectable in the vapor.

Chromatograms obtained at all stages of ripeness were very similar to those published by McCarthy et al. (1963). In all cases the concentration of *iso*-amyl acetate was 2 to 4 times greater than that of *iso*-amyl alcohol in the headspace. This was in direct contrast to the relative amounts of the same compounds found in odor concentrates isolated by vacuum distillation (Myers et al., 1970) from the same slices over which vapor analyses had been taken. In the odor concentrates *iso*-amyl alcohol at all stages of ripeness was present in at least twice the amount of *iso*-amyl acetate. This is illustrated in Figure 2 by the separation of an odor concentrate from incubated ripe (R.I. 5.5 to 6) banana slices.

Comparison of Figures 1 and 2 emphasizes the quantitative differences that result in the composition of volatile isolates obtained by different isolation procedures. Apparently the polarity and interactions of *iso*-amyl alcohol with banana slices in the incubation medium decreased its concentration in the vapor very significantly relative to *iso*-amyl acetate. This occurred even though the alcohol's boiling point (130°C) is lower than that of acetate (b.p. 142°C).

The time-course of change in *iso*-amyl acetate concentration in the vapor over slices at three stages of ripeness is shown in Figure 3. Duplicate vapor samples were taken at each time interval. The 95% confidence intervals of the mean increase (or decrease) at each point are presented in Table 1. The concentration of *iso*-amyl acetate increased approximately 9-fold during a 7-hr incubation of less than ripe (R.I. 3.5) slices. The relative increase was only 2-fold for slices at R.I. 5 and no increase was observed in the vapor of

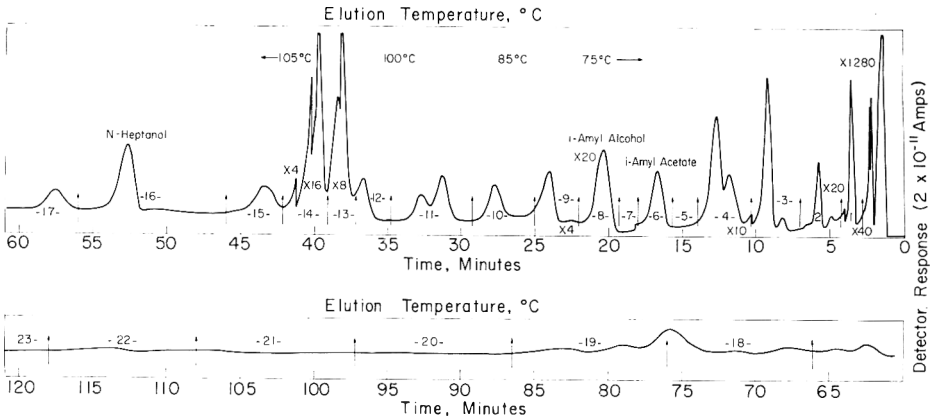

Fig. 2—Chromatogram of an odor concentrate isolated by vacuum distillation from ripe (R.I. 5.5–6) banana slices incubated in phosphate buffer in the presence of L-leucine-U-^{14}C. Peaks were trapped at the points indicated.

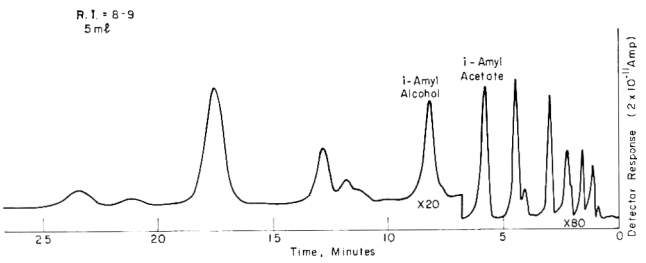

Fig. 1—Aromagram from overripe (R.I. 8–9) banana slices incubated 3 hr in phosphate buffer, pH 6.0.

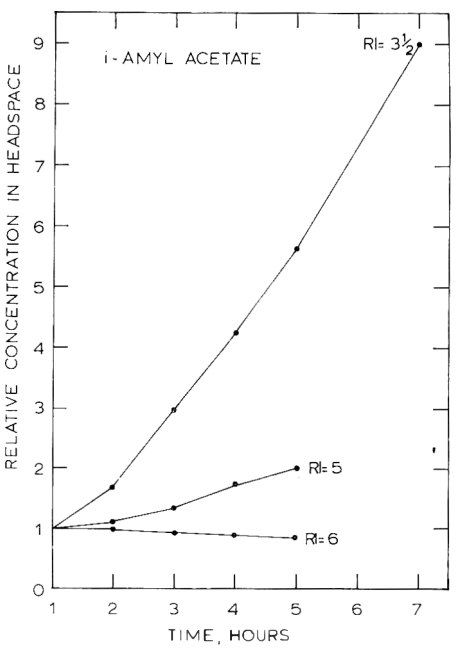

Fig. 3—Relation between stage of ripeness and changes in the relative iso-amyl acetate concentration in vapor over banana slices incubated in distilled water.

Table 1—Ninety-five percent confidence intervals of mean values for the increase in amount of iso-amyl acetate in the headspace.

Ripeness index	Time (hr)	Relative amount[1]	Average	95% interval
3½	1	1.00 —	—	—
	2	1.83; 1.67	1.75	0.61–2.89
	3	3.00; 3.00	3.00	3.00
	4	4.50; 4.00	4.25	1.07–7.43
	5	6.33; 6.00	6.16	4.13–8.19
5	1	1.00 —	—	—
	2	1.06; 1.06	1.06	1.06
	3	1.39; 1.36	1.38	1.13–1.63
	4	1.73; 1.71	1.72	1.60–1.84
	5	2.18; 1.95	2.06	0.92–3.20
6	1	1.00 —	—	—
	2	— —	—	—
	3	0.97; 0.84	0.90	0.01–1.79
	4	0.87; 0.84	0.86	0.61–1.11
	5	0.77; 0.71	0.74	0.36–1.12

Fig. 3.

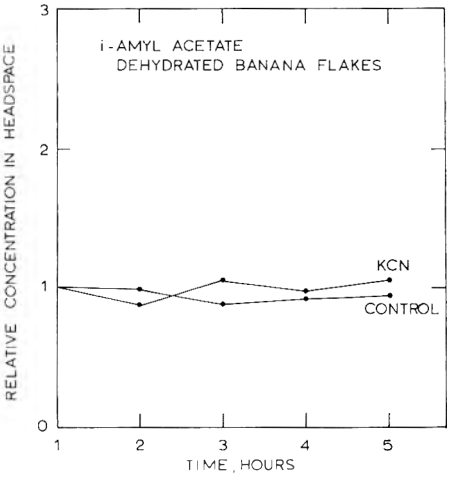

Fig. 4—Effect of KCN on the concentration of iso-amyl acetate in vapor over rehydrated banana flakes (control). Duplicate samples taken at each time interval.

slightly riper (R.I. 6) slices over a 5 hr period.

The areas of the *iso*-amyl acetate peaks at each ripeness stage after 1 hr incubation were 0.72 cm^2 at R.I. 3.5, 8.0 cm^2 at R.I. 5, and 100 cm^2 at R.I. 6. The large increase in *iso*-amyl acetate always occurred with unripe slices. At ripe or almost ripe (R.I. 5–6) stages often no increase was observed. *Iso*-amyl alcohol was not detected in the vapor of unripe slices and its concentration did not change during incubation of ripe or overripe slices.

Although the increases in *iso*-amyl acetate concentration were believed to result from its production by the slices during incubation, the possibility existed that they reflected gradual equilibration of the incubation mixtures. Unripe slices are hard and have a high degree of cellular integrity which decreases as ripening progresses (Sacher, 1962; Bain et al., 1964; Bauer et al., 1964). Hence, equilibration of the vapor composition over unripe slices might require more time than for ripe slices.

To determine whether biosynthesis or equilibration had been responsible, less than ripe (R.I. 4) slices were incubated in the presence of several metabolic inhibitors. The inhibitors were potassium cyanide, which affects cytochrome oxidase as well as many other enzymes; arsenate, which inhibits glyceraldehyde-3-phosphate dehydrogenase and uncouples oxidative phosphorylation; and 2,4-dinitrophenol (DNP), another uncoupler of oxidative phosphorylation. In addition, cyanide and DNP are known to inhibit ester formation in yeasts (Nordstrom, 1964).

Before inhibitor experiments were undertaken, however, it was necessary to determine what changes (if any) occurred with time in the amount of *iso*-amyl acetate in headspace over rehydrated banana flakes (Trop-Bana), the inactive reference material. The effect of adding cyanide to this medium was also observed. As shown in Figure 4 neither the cyanide-treated nor the control samples exhibited significant changes in the amount of *iso*-amyl acetate present.

Having established "baseline" effects in the inactive system, the effects of inhibitors on *iso*-amyl acetate concentration in the headspace vapor was determined. The results shown in Figure 5 indicated that arsenate and DNP may be inhibitory. There was little doubt, however, that cyanide had inhibited. The increase in *iso*-amyl acetate noted during a 5-hr incubation of untreated slices (control) was almost 7-fold while the concentration of *iso*-amyl acetate over cyanide-treated slices remained constant. The vapor concentration of *iso*-amyl alcohol remained constant in all samples.

Due to the time required (30 min) for separation of a single headspace sample, only 4 different treatments of the slices could be studied in a single experiment. Hence, duplicate evaluations of each treatment could not be carried out. The results of vapor analyses over slices incubated in the presence and absence of 0.01 *M* KCN in buffer are given in Table 2. In every case the relative concentration of *iso*-amyl acetate in the vapor over cyanide-treated slices decreased. Untreated slices showed an increase or no change in its vapor concentration. Thus it was concluded that the increases observed in the concentration of *iso*-amyl acetate over less-than-ripe slices were due to biological production and not to equilibration, and that this production was inhibited by 0.01 *M* cyanide.

The effect on the relative vapor concentration of acetate of crushing slices before their incubation is illustrated in Figure 6. Production of *iso*-amyl acetate was inhibited almost as much as by cyanide. As

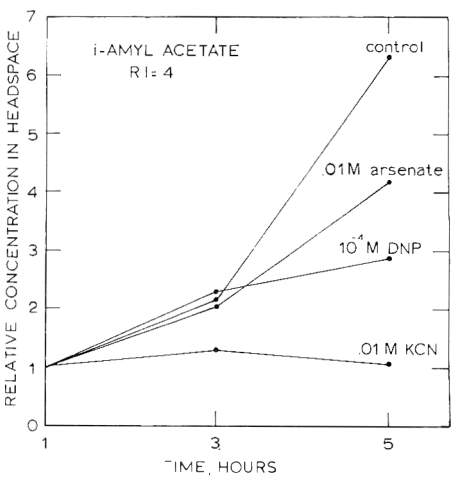

Fig. 5—Effect of metabolic inhibitors on the relative concentration of iso-amyl acetate in vapor over incubating banana slices (R.I. 4).

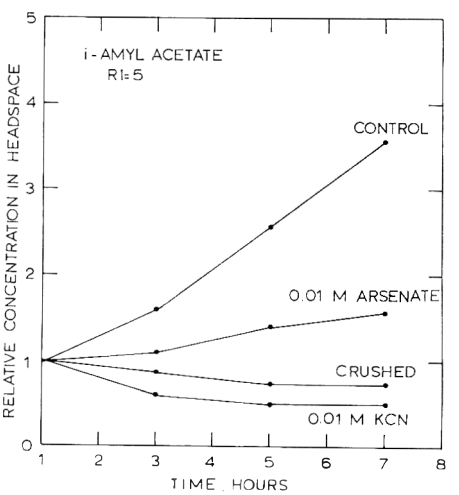

Fig. 6—Effect of metabolic inhibitors and crushing on the relative concentration of iso-amyl acetate in vapor over incubating banana slices (R.I. 5).

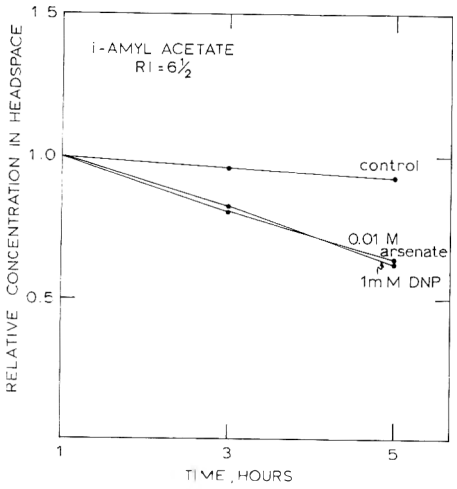

Fig. 7—Effect of metabolic inhibitors on the relative concentration of iso-amyl acetate in vapor over incubating banana slices (R.I. 6½).

Table 2—The effect of 0.01 M KCN on the relative amount of iso-amyl acetate in the vapor over incubating slices.

Stage of ripeness	Relative change vs time (hr) 1	2	3	4	5
Less than Ripe					
R.I. 3½–4					
Slices (Control)	1.00	—	2.50	3.40	4.30
Slices in KCN	1.00	—	1.00	1.00	1.00
Slices (Control)	1.00	—	2.14	—	6.25
Slices in KCN	1.00	—	1.35	—	0.90
R.I. 4½					
Slices (Control)	1.00	1.16	1.32	1.38	1.29
Slices in KCN	1.00	1.10	0.95	0.81	0.68
Slices (Control)	1.00	1.13	1.24	1.26	—
Slices in KCN	1.00	0.73	0.73	0.57	—
Ripe					
R.I. 5–6					
Slices (Control)	1.00	—	1.16	—	1.29
Slices in KCN	1.00	—	0.73	—	0.49
Slices (Control)	1.00	1.27	1.24	1.19	1.13
Slices in KCN	1.00	1.19	1.02	0.78	0.59
Slices (Control)	1.00	1.24	1.20	1.10	1.09
Slices in KCN	1.00	1.00	0.85	—	0.54
Overripe					
R.I. 7½					
Slices (Control)	1.00	1.00	0.95	0.91	0.84
Slices in KCN	1.00	0.81	0.63	0.44	0.34

before *iso*-amyl alcohol concentration was not affected. This result was interesting because homogenized banana pulp is the source of all odor concentrates used in investigations of the identity of volatile flavor constituents. Since Young (1965) has reported that tannins which inhibit enzymes are released in crushed banana pulp, this may have caused the inhibition of *iso*-amyl acetate production.

The effect of inhibitors on ripe slices (R.I. $6^1/_2$) is shown in Figure 7. The vapor concentration of *iso*-amyl acetate over untreated slices (control) remained constant or, in some cases, decreased by approximately 20%. Treatment of slices with cyanide (see Table 2), arsenate, and DNP resulted in headspace amounts of *iso*-amyl acetate that were 35 to 50% of the initial concentrations. In no experiment did the presence of inhibitors affect the concentration of *iso*-amyl alcohol in the headspace. It remained constant throughout the course of incubations.

In summary, vapor analyses showed that slices from unripe bananas produced *iso*-amyl acetate when incubated for relatively short lengths of time (5 hr). Production of *iso*-amyl acetate by ripe slices was not demonstrated since the amount of *iso*-amyl acetate in the headspace remained essentially constant over the 5-hr period. This might have resulted because production of *iso*-amyl acetate had stopped (similar data had been obtained from the "Trop-Bana" system in which no biological production of *iso*-amyl acetate was possible), or because steady-state production of the ester had occurred. In the latter case, a balance would have been reached between synthesis and degradation in which net changes in the amount of *iso*-amyl acetate were not detectable by vapor analysis.

The vapor concentration of *iso*-amyl alcohol remained essentially constant whether or not the slices had been treated.

Incubation of ripe slices in the presence of 0.1 mM, 1 mM or 0.01 *M* or D,L-leucine caused no change in the relative vapor concentration of either *iso*-amyl acetate or *iso*-amyl alcohol (Fig. 8). If leucine were a precursor to these compounds it was conceivable that their vapor concentration might have increased. It was also possible that the slices already contained enough endogenous leucine that the rate of formation of the alcohol and ester was not precursor-limited. Addition of more precursor would, therefore, not cause their increased production. Buckley had indicated the presence of about 2.5 μM of free leucine per gram (fresh wt.) in ripening banana pulp (Personal communication; Wick et al., 1966).

Based on the above evidence direct vapor analyses clearly showed that *iso*-amyl acetate was produced by less than ripe banana slices in the in vitro system. No precursor-product relationship between leucine and *iso*-amyl acetate and *iso*-amyl alcohol was observed.

The subsequent investigation of L-leucine-U-^{14}C as a precursor for these volatile constituents is described in detail elsewhere (Myers et al., 1970). It involved the incubation of less than ripe, ripe and overripe banana slices in the presence of isotopic leucine. Direct vapor analyses over the slices confirmed the results shown in Figures 5, 6 and 7.

Iso-Amyl acetate and *iso*-amyl alcohol were trapped as indicated in Figure 2 and their radioactivity determined. Leucine was, in fact, demonstrated to be a precursor for both *iso*-amyl acetate and *iso*-amyl alcohol, as well as for other volatile constituents at all three ripeness stages. Determination of the specific activity of both *iso*-amyl acetate and *iso*-amyl alcohol throughout the incubation periods at each ripeness stage showed conclusively (see Table 3) that these components were continually produced even though this had not been demonstrated by vapor analyses. It also showed that cyanide inhibited their production at all ripeness stages.

The striking difference between the increasing specific activities of the acetate and alcohol and their essentially unchang-

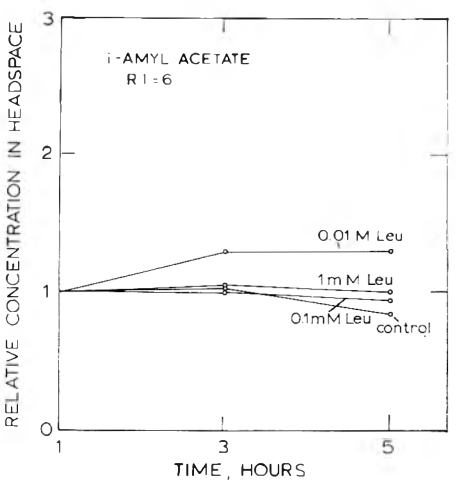

Fig. 8—Effect of several concentrations of DL-leucine on the relative vapor concentration of iso-amyl acetate over ripe banana slices (R.I. 6).

Table 3—Comparison of specific activities (dpm/nmole) of iso-amyl acetate and iso-amyl alcohol.

	Less than ripe R.I. 4–5		Ripe R.I. 5–6		Overripe R.I. 8–9	
Incubation time	Control	0.01 *M* KCN	Control	0.01 *M* KCN	Control	0.01 *M* KCN
Iso-amyl acetate						
10 min	—	—	4	—	—	—
20 min	—	—	9	—	—	—
30 min	—	—	14	—	—	—
1 hr	359	[1]	46	—	17	[1]
2 hr	—	—	103	26	—	—
3 hr	234	93	159	—	39	10
4 hr	—	—	200	—	—	—
5 hr	312	171	232	—	123	57
Iso-amyl alcohol						
10 min	—	—	50	—	—	—
20 min	—	—	79	—	—	—
30 min	—	—	111	—	—	—
1 hr	910	34	188	—	60	18
2 hr	—	—	274	65	—	—
3 hr	527	128	366	—	83	76
4 hr	—	—	382	—	—	—
5 hr	504	353	367	—	175	143

[1] Not statistically significant at the 99% level.

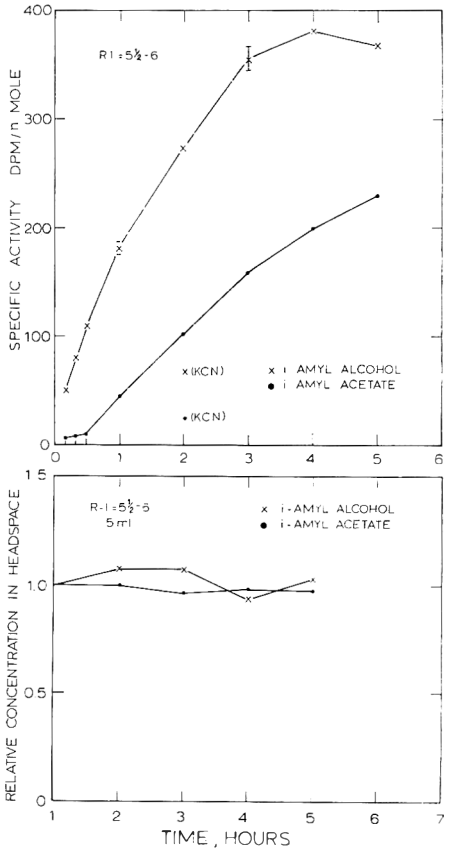

Fig. 9—Comparison of changes in the specific activity of iso*-amyl acetate and* iso*-amyl alcohol isolated from ripe slices (R.I. 5½–6) incubated with L-leucine-U-^{14}C, with changes in their concentration in the vapor over the slices.*

ing concentration in the vapor over ripe slices in the presence of isotopic leucine is illustrated in Figure 9. Not only was the specific activity of *iso*-amyl alcohol greater than that of *iso*-amyl acetate at all ripeness stages studied but the alcohol also contained the largest proportion of the radioactivity in the volatile odor concentrates.

CONCLUSIONS

DIRECT vapor analyses were carried out over banana slices which ranged in ripeness over the stages in which normal flavor and aroma develop, and which were incubated in an in vitro system for periods up to 5 hr. Biological production of *iso*-amyl acetate by unripe slices was demonstrated based on studies with metabolic inhibitors. The behavior of ripe and overripe slices was inconclusive since the vapor concentration of the acetate was essentially constant. In the presence of inhibitors it decreased.

The vapor concentration of *iso*-amyl alcohol remained unchanged at all ripeness stages studied. Either these compounds were not produced by the slices or they were utilized in some manner as fast as they had been formed.

Incubation of slices with leucine had no effect on the vapor concentrations of either *iso*-amyl acetate or the alcohol. Therefore, direct vapor analyses could not demonstrate a precursor-product relationship between these substances under the conditions used.

Incubation with L-leucine-U-^{14}C, isolation of *iso*-amyl acetate and *iso*-amyl alcohol and determination of their specific activities was necessary to show conclusively that leucine was, in fact, a precursor and that both compounds were continually produced at all ripeness stages. The implications of these results for research on the biochemical development of flavor in ripening fruit or vegetables are important since they demonstrate that interpretation of direct vapor analyses must be made with great caution. A constant vapor composition provides no evidence that components are not being synthesized.

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Pectin Changes in the Ripening of Irradiated and Stored Strawberries

SUMMARY—Strawberries of the "Red Gauntlet" variety picked at two different stages of maturation were irradiated with a ^{60}Co source at 200 krads. After irradiation the fruits were stored in a refrigerated cell at a temperature varying from 0 to 3°C and at a R.H. between 85–90%. During the storage a part of the control berries was irradiated at different times to find out differences in irradiation response at successive stages of ripeness in storage conditions. The effects of irradiation on the pectic substances, pectin-methylesterase activity and anthocyanin content were investigated. In addition, changes in softening and extent of fungal growth were followed. A different response of strawberries irradiated at two different stages of ripeness was found. The different ripeness in storage conditions also influences the radiation response of the strawberries. The possible practical applications of these findings are discussed.

INTRODUCTION

THE ROLE of the pectic substances in the ripening of fruits has been studied by several authors (Haller, 1929; Stier et al., 1956; Rouse et al., 1962; Rouse et al., 1964; McCready et al., 1964).

Fruit pectins consist of esterified anhydrogalacturonic acid units in unbranched chains with different amounts of galactose and arabinose. They may be divided into three general types (Kertesz, 1951; Doby, 1965): protopectins, pectins and pectic acids.

The protopectins are insoluble in water, consisting of methyl-poly-galacturonide chains partly linked to cellulose and hemicellulose. The pectins are water soluble and usually 80% of the carboxyl groups esterified with methyl alcohol and the remaining 20% of free acids or salts. The pectic acids are soluble in water, free from methyl ester groups and they form salts with metals. Another pectic substance is known. Derived from mild hydrolysis of the protopectins, the pectinic acid, soluble in water, has a considerable amount of methyl ester groups.

Protopectins are found in the primary cell wall and particularly in the walls of parenchymatous tissues, while the calcium and magnesium salts of the pectic acids are found in the middle lamella (Kertesz, 1951; Miller, 1957; Doby, 1965).

It is known that, during the ripening of fruits, part of the insoluble pectic substances is converted into a soluble form. In fact, the insoluble protopectins are hydrolyzed to soluble pectins by the protopectinase; then the pectins are demethylated by the pectin-methylesterase (P.E.) and hydrolyzed by the pectin-polygalacturonase to smaller acid residues, namely pectic acids (Haller, 1929; Stier et al., 1956; McCready et al., 1964). A 4th enzyme, pectin-depolymerase hydrolyzes pectic acid occurring in tomatoes (Kertesz, 1951; Doby, 1965).

These processes are related to the softening of fruit tissues during ripening.

The degradation of pectins by ionizing radiations has been observed through the decrease in viscosity of pure pectin in water solution (Dzamic et al., 1966) and in dry pectin as well (Kertesz et al., 1956; Glegg et al., 1956).

Other authors (McArdle et al., 1956; Kertesz et al., 1964; Somogyi et al., 1964; Rogachev, 1966; Maxie et al., 1968; Massey, 1968) observed that, when the fruits are irradiated, the viscosity decreases and the total pectic substances also generally decrease by increasing radiation exposure and a breakdown of protopectins to pectates and soluble pectins occurs.

The purpose of this work was to investigate the effects on the pectic substances of acute gamma irradiation applied at two successive stages of fruit ripening and at different times during the storage period of strawberries.

A similar investigation on the storage properties of some irradiated fruits was carried out by Clarke (1959 and 1968).

Chemical and biochemical analyses of the pectic substances and of the P.E. activity were carried out. The modifications of color and softening and fungal infection were also observed.

MATERIALS & METHODS

Sampling and irradiation

Strawberries of the "Red Gauntlet" variety were picked at two different stages of ripeness.

1. 40 Kg of strawberries of about equal size; 40–50% of their surface pink-red colored for a visual observation.
2. 30 Kg of strawberries of about equal size; 70–80% of their surface pink-red colored for a visual observation.

The berries of each group were divided into three parts. The first part was irradiated 2 hr after harvesting and stored at 0–3°C. The first analysis was made 2 hr after irradiation. The second part of berries was used for irradiation at various times during the storage to find out differences in irradiation response at successive stages of ripeness. The third part was used as control.

The irradiation was made at the Industrial Chemistry Laboratory of the Casaccia Nu-

clear Center in a Gamma Cell (^{60}Co source) at an exposure rate of 700 krads/hr, the total exposure being 200 krads. This exposure was chosen because, as indicated by several authors (SRIA 30, 1961; Cooper et al., 1963; Herregods et al., 1963; Maxie et al., 1964; Ben Yehoshua et al., 1966), it is the highest exposure at which no taste modifications were observed.

The fruit samples where put in a container of the Gamma Cell, in which the temperature was maintained during the irradiation at 26°C by a water circulation in a copper coil.

The irradiated and control samples were put in small plastic crates covered by pierced polyethylene and stored in a refrigerated cell at a temperature varying from 0 to 3°C and at a R.H. between 85 and 90%. During their storage period, these samples were observed at weekly intervals for color change, by anthocyanin determinations, for appearance and fungal infection (mainly *Botrytis cinerea* Pers.). They were analyzed nearly daily for pectic substances contents and P.E. activity.

The values obtained from all analyses are the average of three determinations on representative samples from each treatment. The t-values testing the significance of differences between irradiated samples and control are calculated. Three irradiated and three control samples of the second group of strawberries were stored at room conditions (23°C and 70–90% R.H.). This material was observed only for fungal infection (mainly *Botrytis cinerea* Pers).

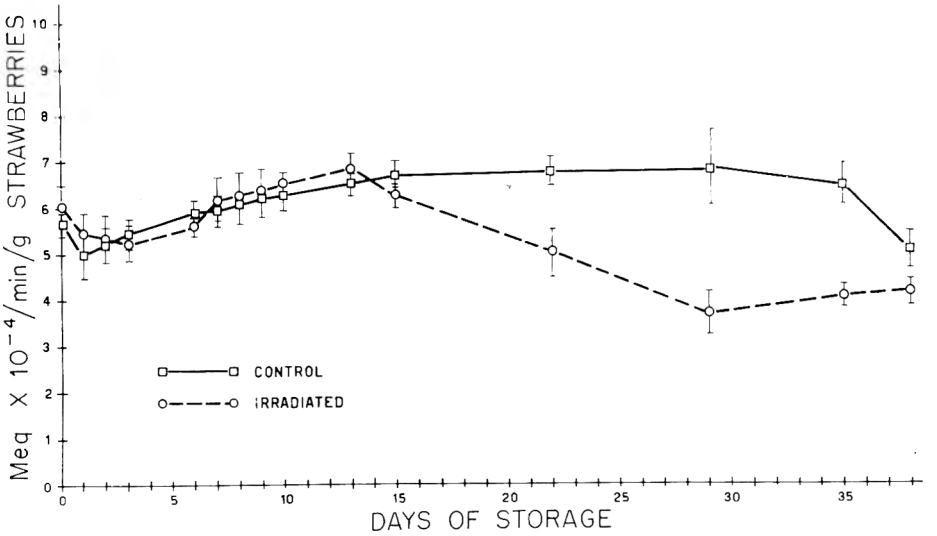

Fig. 2—Changes in the P.E. activity during the storage at 0–3°C and 85–90% R.H. of strawberries picked on May 2, 1967.

Anthocyanins determination

The method of Herregods et al. (1963), partly modified, was used. 100 g of strawberries were homogenized with 750 ml of H_2O and shaken. To 85 ml of this mixture, 180 ml of 95% ethanol and 45 ml of glacial acetic acid were added. The mixture was shaken again and centrifuged. The supernatant was collected and its spectrum was measured with a Beckmann DK 2 spectrophotometer. The anthocyanin content was expressed in optical density measured at 520 mμ, absorption maximum.

Extraction and analysis of pectic substances

Pectic substances were extracted and precipitated according to the method of Kertesz (1951). A 250-g sample of strawberries was

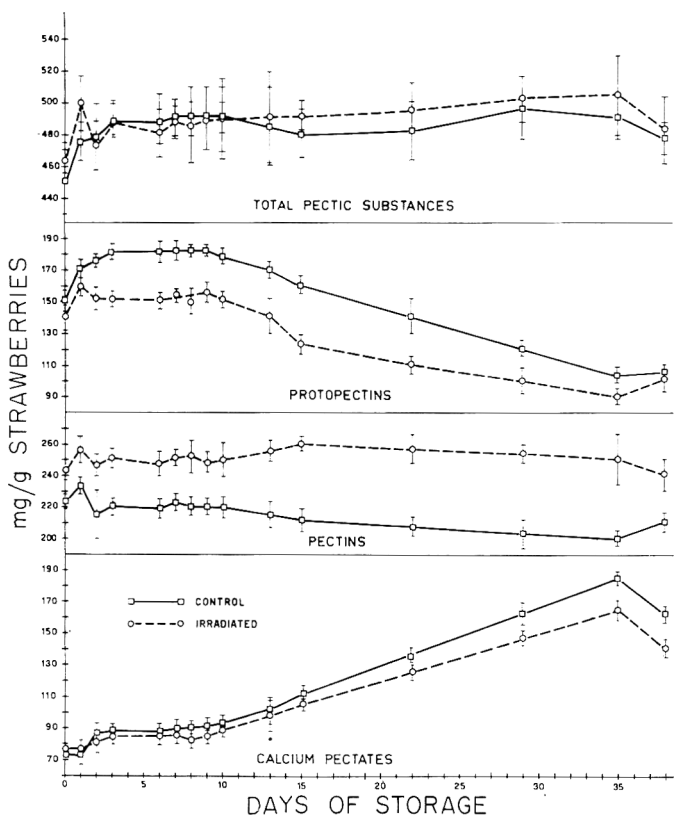

Fig. 1—Changes in the pectic content during the storage at 0–3°C and 85–90% R.H. of strawberries picked on May 2, 1967.

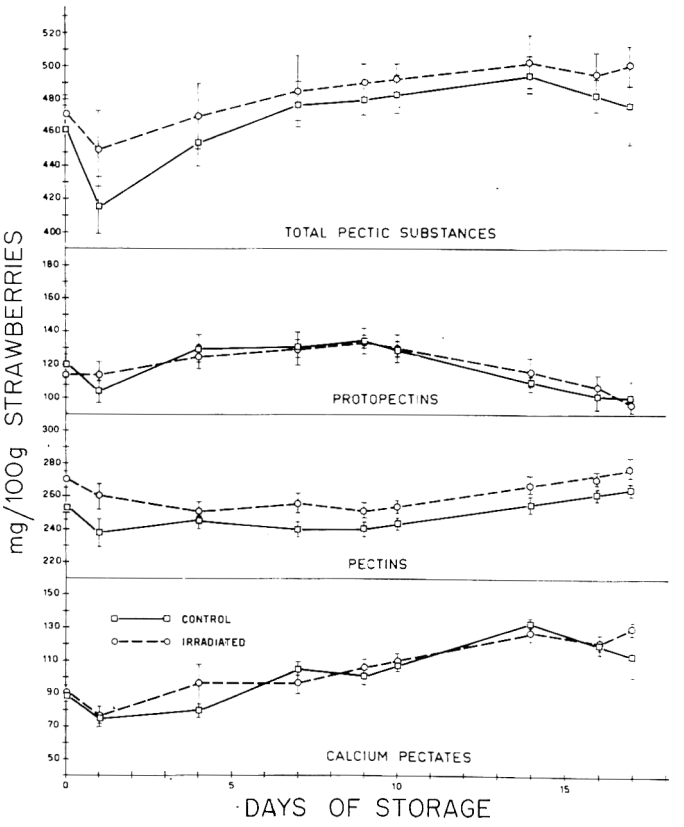

Fig. 3—Changes in the pectic content during the storage at 0–3°C and 85–90% R.H. of strawberries picked on May 22, 1967.

homogenized in boiling 95% ethanol. The mixture was simmered for 5 min and allowed to stand for about 20 hr. The filtrate was washed with 95% ethanol, ethanol and ether (1:1), and ether. The solid was dried at 60°C and pulverized. This alcohol insoluble residue was extracted to remove pectin. It was then extracted with 0.5% ammonium oxalate to remove pectate and finally extracted with 0.05*M* hydrochloric acid at 85°C to remove protopectin.

Each extract was precipitated by adding 95% ethanol (containing a quantity of hydrochloric acid calculated to make the total volume 0.05*M* HCl), so that in the final volume there was the 55% ethanol concentration. Afterwards, it was washed with 70% ethanol, 70% ethanol-ether (1:1) and ether, dried at 70°C and then weighed.

The pectic substance contents of strawberry tissues are expressed as mg per 100 g of fresh material.

Extraction and assay of the P.E. activity

The methods described by MacDonnel et al. (1945), Somogyi et al. (1964) and modified by the present authors were used.

A 100-g sample of strawberries was comminuted in a Waring Blendor with 100 ml M Na-acetate for 1 min. The pH was adjusted at 8 and the mixture was allowed to stand 1 hr at 0–5°C to facilitate extraction. Then the mixture was centrifuged at 5000 G for 15 min and the supernatant was separated to assay the P.E. activity. The P.E. activity as liberation of —COOH groups from pectin was determined by continuous titration with a Zeromatic pH meter of carboxyl groups at a selected pH and temperature for a fixed time.

The conditions adopted to assay P.E. were: 5 ml of enzyme extract with 50 ml of 1% pectin (from *Citrus decumana* produced by Fluka) solution in 0.2*M* NaCl incubated in a thermostated bath at 30°C. The pH of the reaction mixture was adjusted to 7.5 and kept at such value for a reaction period of 30 min by adding 0.02*N* NaOH.

Results were expressed in milliequivalents of ester hydrolyzed per min, per g of fresh tissue (P.E. units).

RESULTS

THE LESS RIPE strawberries of the first group, showed a progressive wrinkled appearance which was more evident in the irradiated material than in the control. The riper berries of the second group, showed a progressive loss of texture finally becoming spongy and water logged, without a valuable difference between irradiated and control samples.

The fruit color increased both for the control and the irradiated sample, but in the riper fruits a small increase was observed for the irradiated samples (Table 1).

Botrytis cinerea Pers. infection appeared in the control of the first group after 6 days of storage in the refrigerated cell. After 38 days, 50% of the strawberries were infected, while no infection was observed in the irradiated first group.

In the second group of strawberries stored in the refrigerated cell, the fungal infection appeared after 4 days in the control and after 15 days in the irradiated sample.

Table 1—Effect of irradiation (200 krads) on the content in anthocyanins of strawberries at two stages of ripeness at picking and after 2, 9, 15, 35 days of storage, at 0–3°C and at 85–90% R.H.

Days of storage	Optical density at 520 mμ: 40–50% of pink-red fruits		70–80% of pink-red fruits	
	Control	Irradiated	Control	Irradiated
0	0.18	—	0.23	—
2	0.18	0.20	0.26	0.25
9	0.20	0.23	0.32	0.27
15	0.23	0.25	0.41	0.32
35	0.31	0.30		

In the berries stored at room conditions the fungal infection appeared after 3 days in the control and after 8 days in the irradiated sample.

As seen from Figure 1, the total pectic substances of the strawberries of the first group obtained by addition of the three fractions did not change with time, neither in the irradiated nor in the control samples. The protopectin values increased slightly during the first days of storage and decreased rapidly after the values of the irradiated samples were always lower than the control ones.

The values of the pectins decreased very slightly during the storage and the values of the irradiated samples were significantly higher than the control. The pectate values increased greatly and in the last two weeks the control values were higher than those of the irradiated sample. The values of the P.E. activity for the irradiated samples were equal (Fig. 2) to those of the control during the first days, then they significantly decreased. The significance of differences between control and irradiated values are shown in Table 2.

In the second group of strawberries a slight progressive increase in the total pectic substances occurred (Fig. 3). The protopectin amounts were almost constant and presented no differences between the control and the irradiated samples (Table 3). The pectin amounts were almost constant, but the irradiated samples were higher than the control ones. The pectate values increased, and no differences between the control and the irradiated samples were observed.

The P.E. activity of the control and irradiated samples of the second group of strawberries increased slightly and then decreased in the last three days of storage (Fig. 4).

In the histograms of Figure 5 a remarkable decrease in protopectins of strawberries of the first group, irradiated at various intervals, is observed. In contrast, a clear increase in the pectins is gradually promoted. The pectate values are lower than the control ones. Irradiation had no effect on the P.E. activity of the fruits for the first 15 days of storage; after this period a noticeable reduction in P.E. activity was detected. Its significance is reported in Table 3.

In the histograms of Figure 6 a marked

Table 2—t Test values of the difference, at various days of storage, between irradiated and control samples of strawberries picked on May 2, 1967. A—samples picked on May 2, irradiated 2 hr after picking and analyzed after different periods of storing. B—samples picked on May 2, stored for different periods, then irradiated and analyzed.

	Days of storage: 0		1		6		10		13	
	A	B	A	B	A	B	A	B	A	B
Protopectins	1.00	1.00	1.39	1.49	3.26[a]	4.18[a]	3.89[a]	5.00[b]	2.79[a]	5.71[b]
Pectins	2.78[a]	3.28[a]	2.13	2.02	2.80[a]	4.69[a]	2.98[a]	3.37[a]	4.35[a]	5.68[b]
Calcium pectates	0.95	2.74	0.83	1.39	0.54	2.78[a]	0.54	1.67	0.23	1.67
P. E. activity	0.52	0.47	0.71	0.33	1.07	1.50	0.52	0.60	0.60	0.01

	Days of storage: 15		22		29		35		38	
	A	B	A	B	A	B	A	B	A	B
Protopectins	4.26[a]	3.20[a]	2.84[a]	4.00[a]	2.00	3.60[a]	1.77	3.89[a]	0.75	1.51
Pectins	5.00[b]	10.00[b]	3.91[a]	7.41[b]	5.00[b]	9.54[b]	2.94[a]	7.92[b]	2.78[a]	1.11
Calcium pectates	0.95	1.58	2.43	4.44[a]	2.87[a]	4.54[a]	2.83[a]	3.73[a]	2.82[a]	3.33[a]
P. E. activity	0.83	1.43	2.93[a]	2.84[a]	2.79[a]	3.80[a]	5.55[b]	3.28[a]	3.20[a]	1.10

[a] Means significance at 0.05 level.
[b] Means significance at 0.01 level.

Table 3—t Test values of the difference, at various days of storage, between irradiated and control samples of strawberries picked on May 22, 1967. A—samples picked on May 22, irradiated 2 hr after picking and analyzed after different periods of storing. B—samples picked on May 22, stored for different periods, then irradiated and analyzed.

	Days of storage							
	0		1		4		7	
	A	B	A	B	A	B	A	B
Protopectins	0.70	0.83	0.97	5.59[b]	0.47	2.89[a]	0.09	3.36[a]
Pectins	2.11	2.05	1.95	0.13	0.83	3.20[a]	2.98[a]	2.00
Calcium pectates	0.10	0.10	0.14	0.83	1.60	0.69	1.12	0.91
P. E. activity	1.00	2.86[a]	0.83	0.43	1.21	1.67	0.31	2.10

	Days of storage									
	9		10		14		16		17	
	A	B	A	B	A	B	A	B	A	B
Protopectins	0.10	3.72[a]	0.11	5.20[b]	0.56	4.54[a]	0.65	—	0.43	—
Pectins	2.14	2.22	2.97[a]	2.83[a]	2.94[a]	3.00[a]	2.78[a]	—	2.67	—
Calcium pectates	1.07	1.39	0.18	1.40	0.97	1.89	0.15	—	1.17	—
P. E. activity	0.50	2.81[a]	0.28	3.39[a]	0.71	4.17[a]	0.47	—	0.64	—

[a] Means significance at 0.5 level.
[b] Means significance at 0.01 level.

increase in the protopectins of strawberries of the second group, irradiated at various intervals, is noticed. The radiation produced a less noticeable increase for the pectins and no significant changes for the pectate values. The irradiations made from the 7th to the 14th day decrease the P.E. activity.

DISCUSSION

OUR RESULTS concerning the changes in pectic substances of control strawberries during their storage, agree with data on ripeness of detached and stored fruits obtained by other authors (Kertesz, 1951; Stier et al., 1956; Doesburg, 1957; Esau et al., 1962; Biale, 1964; McCready et al., 1964).

As to results of the irradiation it is clear that in the first group of strawberries a breakdown of the protopectins was induced by radiation. Meanwhile, an increase in new H_2O-soluble pectins was induced and in the last two weeks it was more evident than the decrease in protopectin. In fact, on the 35th day, 1 mg of degraded protopectin corresponded to 3.5 mg of pectin. This may be explained by assuming a smaller degradation of pectins into pectates: in the last two weeks of storage, the pectate values of the irradiated samples were lower than the values of the control.

The smaller content of pectates (Fig. 2), may be related to smaller P.E. activity in the irradiated samples. The decrease in the extractable pectates, because of a modified cell wall permeability, may also be due to a modified ionic equilibrium of the internal cell sap. This results in a reduction of the available calcium, which in turn causes a reduction in the amount of calcium pectate. This fact is supported by the work of Al-Jasim et al. (1968).

In the second group of strawberries the protopectin content of the irradiated samples was equal to that of the controls, while the pectin content was increased by irradiation. This may be explained by admitting that the irradiation of riper strawberries releases some pectins from the protopectins bound with cellulose and hemicellulose of the cell wall. According to this hypothesis, it must be also admitted that radiations are able to release the protopectins bound to the cell wall compounds, either as such, or degraded to pectins. In this case, the amount of free protopectins degraded to pectins is replaced by that part bound to the cell walls and released by radiations. The pectic acids, like the P.E. activity, are not modified by irradiation. This confirms the probable correlation between the enzymatic activity and the pectate content.

To explain the different effects of irradiation on the two groups of berries detached and irradiated at different times of ripeness, other equilibria, on which the pectic metabolism depends, must be taken into account. Doesburg (1957) and Esau et al. (1962), reported a correlation between changes in pectins and calcium ions solubility, changes in the pH of the cell wall and changes in cell permeability during the ripeness of fruits. The different behavior of the strawberries of the two groups toward irradiation might be due to these factors.

Concerning the radiation effect on the control samples of strawberries irradiated at different times of ripeness in storage conditions, it was found that the radiosensitivity of the less ripe detached berries was not modified during their maturation in a refrigerated cell. In contrast, the irradiation of the more ripe strawberries caused a breakdown of the cell wall structure as shown by the remarkable increase in the protopectins. Hence the modified fruit radiosensitivity depended on the overripeness stage at which the fruits were irradiated.

The different degree of ripeness influenced the radiation response of the strawberries also in respect to color and fungal infection. Irradiation slightly changed the anthocyanin contents in less ripe fruits

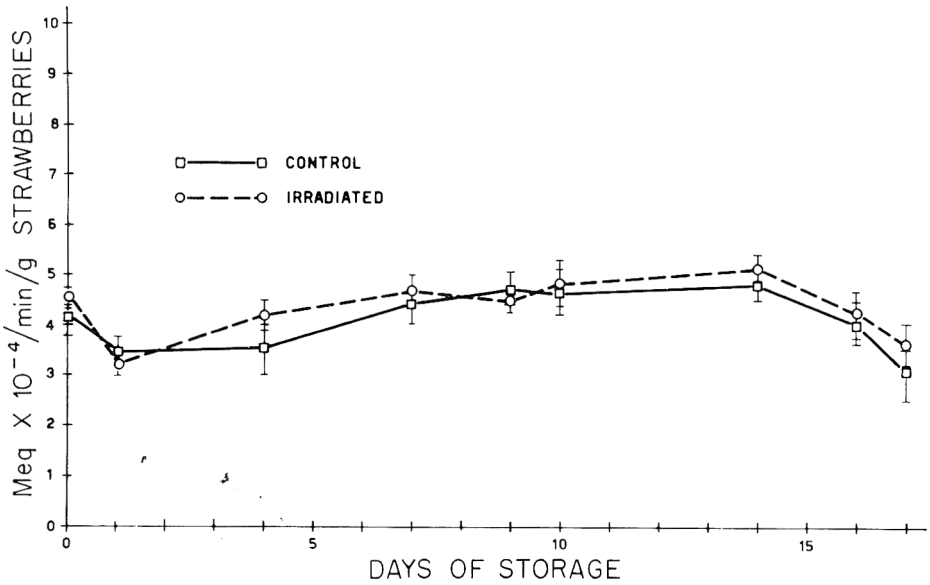

Fig. 4—Changes in the P.E. activity during the storage at 0–3°C and 85–90% R.H. of strawberries picked on May 22, 1967.

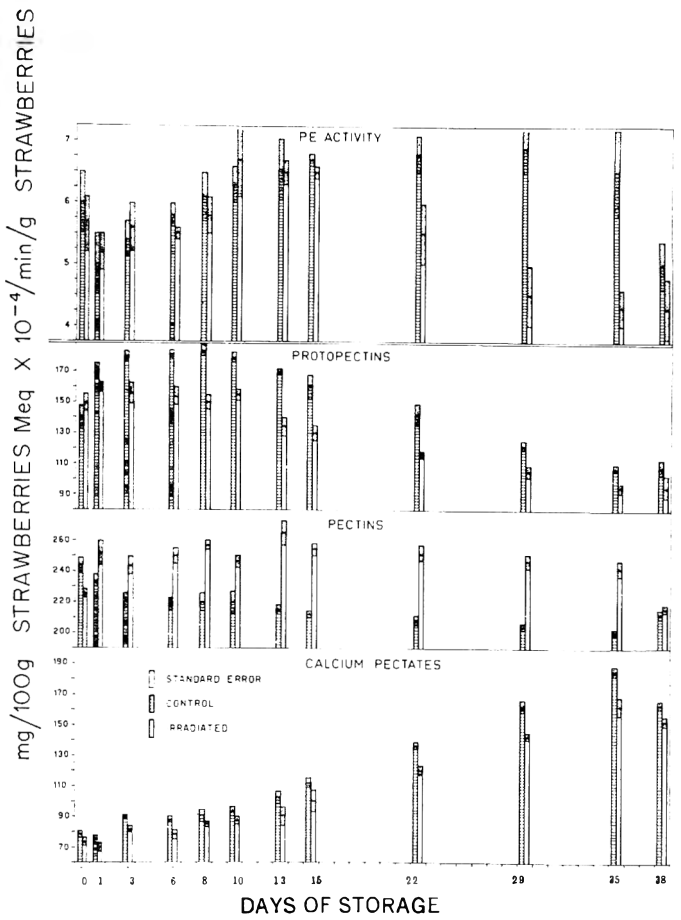

Fig. 5—Changes in the pectic content and in the P.E. activity of strawberries picked on May 2, 1967 and irradiated at different times of storage.

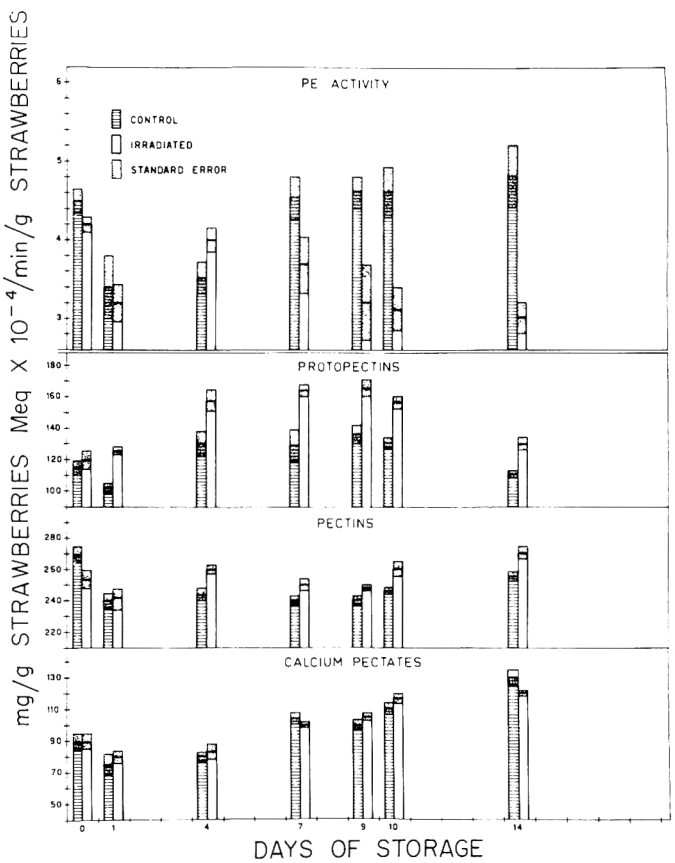

Fig. 6—Changes in the pectic content and in the P.E. activity of strawberries picked on May 22, 1967 and irradiated at different times of storage.

while it slowed down remarkably the same process in the riper fruits. The radiopasteurization was very efficient both in ripe and less ripe fruits but, in the first case, irradiation did not completely prevent fungal infection. Differences in the initial spore load on fruits cannot be excluded.

As to the fungal and color modifications, the above mentioned results agree with those of several authors (MacDonnel et al., 1945; Nelson et al., 1959; SRIA 30, 1961; Cooper et al., 1963; Maxie et al., 1964; Rogachev, 1966).

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Epimysial Connective Tissue Scores as Related to Beef Tenderness

SUMMARY—Steaks from 24 bulls 20 months old, 89 bulls 14 months old and 109 steers 20 months old were used to relate thickness of epimysial tissue to the amount of intramuscular connective tissue, and to correlate these factors with Warner-Bratzler shear values in muscles cooked to 140 or 160°F. The thickest portion of the LD epimysium was greater in lighter weight bulls than it was in heavier steers. Epimysial extension and thickness varied according to muscle location but at the same location it was not significantly different in steaks from tough versus tender steers. Differences in hydroxyproline content and connective tissue of steers and young bulls were small but older bulls had slightly greater amounts of both. The LD epimysium was tougher in older bulls than in younger bulls.

Shear values of epimysial tissue varied considerably within animals of the same age, especially when roasted to 140°F. Most connective tissue differences due to line of cattle were small. Correlations within groups of cattle between thickness of epimysial tissues of different muscles or between epimysial thickness and intramuscular connective tissues were low. With few exceptions neither hydroxyproline, epimysial thickness nor connective tissue scores were significantly related to shear force of muscle or shear force of epimysial tissue itself. Correlations between shear force of the LD and BF muscles were not significant ($P < 0.05$) in animals of the same age. There was a tenderness interaction between age of animal and type of muscle which suggested that collagen gelatinization occurred at a faster rate in the LD than in the BF as age increased.

INTRODUCTION

RESEARCH which relates quantity, thickness and character of the epimysium to intramuscular connective tissue and beef tenderness is lacking. However, numerous publications are available on the relationship between intramuscular connective tissue and tenderness. Larger amounts of intramuscular connective tissue are associated with less tender meat according to Mitchell et al. (1928), Mackintosh et al. (1936), Ramsbottom et al. (1945), Harrison et al. (1949), Husaini et al. (1950), Hiner et al. (1955) and Miller et al. (1956).

Recently researchers (Goll et al., 1964, Herring et al., 1967 and Hill, 1966) have suggested that variation in collagen solubility may be more closely related to tenderness in meat than amount of connective tissue. It appears that under some conditions both quantity of intramuscular connective tissue and collagen solubility may be related to meat tenderness. Since epimysial tissue is referred to as gristle by the consumer, it may also be a factor in consumer selection of meat at the time of purchase.

The objectives of this project were to relate thickness of epimysial tissues on different muscles to amount of intramuscular connective tissue and to correlate these factors with tenderness in two different muscles cooked to 140 or 160°F. Differences in characteristics of epimysial tissue, intramuscular connective tissue and tenderness due to sex, age and line of beef cattle were also investigated.

EXPERIMENTAL

STEAKS from 89 bulls approximately 14 months of age, and 24 bulls and 109 Hereford steers approximately 20 months of age were cut $1^1/_4$ in. thick after carcasses had aged 7 days at 40°F. The animals within each of the above 3 groups were killed at a constant live weight. The bull carcasses were from a line of inbred Herefords which had been intensively selected for rate of gain for 13 years; a line of Angus; a second line of Herefords with moderate selection for rate of gain; and a line from a Shorthorn-Charolais bull bred to cows from the Angus and Hereford line selected for moderate gain.

The bull carcasses possessed traces to small amounts of marbling while steer carcasses ranged from slight to moderate. Pictures were taken of the caudal side of the first steak cut from the 12 rib section (picture 1, Fig. 1). On 69 of the steers, pictures were also taken of the caudal side of an adjacent rib steak (picture 2, Fig. 1) Epimysial measurements obtained were compared to those from the 12th rib steak to test differences in characteristics of epimysial tissue according to muscle location.

Measurements on the rib steaks included thickness of the longissimus dorsi (LD) epimysium 2 cm lateral to the acorn or peninsula (Marugg, 1965) and length of the epimysial extension from the peninsula ventrally into the LD (Fig. 1, pictures 1 and 2). Additional pictures from the bulls included porterhouse steak (picture 5, Fig. 1); bottom round steak (picture 3, Fig. 1) and arm pot roast (picture 4, Fig. 1). The epimysium in each of these cuts was measured at the points described in Figure 1. All measurements were made from enlarged pictures which were projected on a screen.

Subjective connective tissue scores for collagen, elastin and total connective tissue were determined for samples taken from the center of the LD and from the center of the biceps femoris (BF). The samples were removed and fixed in 10% formalin until they could be sectioned approximately 25 microns thick with a freezing microtome. At least 3 sections approximately $^1/_2$ in. square from different areas of the center portion of each muscle were mounted on slides and stained with Weigherts elastic tissue stain and Van Gieson's collagenous tissue stain.

Two judges gave subjective scores ranging from 1 to 5 for size, distribution and frequency of stained elastic and collagenous fibers on each of the sections at a magnification of 450 times as described by Hiner

et al. (1955). Each of the size, distribution and frequency subjective scores were totaled for the 2 judges and reported as collagen or elastin score. Thus, total scores could range from 6 for samples on which each of the 2 judges gave scores of 1 for size, distribution and frequency to 30 for samples on which each of the 2 judges gave scores of 5 for size, distribution and frequency. In addition each slide was scored from 1 to 5 for total connective tisue by each of the 2 judges at a magnification of 10 times and total score was reported.

The epimysium was removed from LD and BF steaks after removal of muscle samples for histological study. The remaining portion of each steak was ground 3 times through a 1/8 in. plate and frozen 4 to 6 months. The muscle samples were prepared for hydroxyproline analysis according to the method described by Parrish et al. (1961). The Neuman et al. (1950) procedure was used in determining hydroxyproline content of the muscle tissue.

Rib and bottom round steaks, adjacent to those used for hydroxyproline and subjective connective tissue scores, were cut 1 1/4 in. thick and frozen 4 to 6 months before they were thawed at room temperature and roasted in a 350°F oven to an internal temperature of 160°F. A third rib steak adjacent to the cranial side of the steak cooked to 160°F was roasted to 140°F internal temperature. Three 1 in. cores cut parallel with the predominate muscle fibers were taken from the lateral, central and medial positions of the cooked LD or BF muscles and each core was sheared 3 times. In addition, one 1/2 in. LD core similar to those in picture 6 of Figure 1 was removed and sheared 3 times. Warner-Bratzler shear values on 1/2 in. cores containing epimysial tissue were compared with 1/2 in. cores without epimysial tissue.

Analysis of variance procedures and the t-distribution as described by Snedecor (1961) were used to test differences in unadjusted means. Least-squares analysis (Harvey, 1960) was used to estimate the line of cattle effect on the variables studied. Differences between least-squares means were tested for significance using the method described by Kramer (1957). Simple correlations were calculated within groups of cattle because of sex and age differences.

RESULTS & DISCUSSION

UNADJUSTED means and standard deviations for characteristics of the epimysium, intramuscular connective tissue and Warner-Bratzler shear values for steers and bulls of two age groups are given in Table 1. Significant ($P < 0.05$) differences in thickness of LD epimysium 2 cm lateral to the peninsula reflected differences in carcass weight. However, the thickest portion of the LD epimysium was higher in younger, lighter weight bulls that it was in steers. The epimysium extended significantly further into the LD muscle in bulls than in steers. Age of bull did not significantly influence epimysial extension. Epimysial thickness of the LD at the 5th lumbar; epimysial thickness of the BF at the thickest portion; and epimysial thickness of the triceps brachii (TB) at the thickest portion were thicker in older bulls than in younger bulls.

Means and standard deviations fo- epimysial measurements on the 12th rib steak and an adjacent rib steak from steers of the same age and weight are given in Table 2. This was done to measure variation in epimysial thickness and extension at slightly different locations on the LD. The adjacent rib steak cut cranial to the 12th rib steak had thicker epimysial tissue and the epimysium extended further into the LD than in the 12th rib steak.

Characteristics of the epimysium and intramuscular connective tissue in the LD of 29 choice grade steers selected as the 14 most tender and 15 least tender from a group of 109 steers are shown in Table 3. Mean differences for shear force of the epimysium, thickness of the epimysium and extension of the epimysium into the LD were not significant but significantly greater amounts of hydroxyproline were found in muscle from the least tender steers.

Subjective scores for collagen and elastin in the LD (Table 1) indicated that the heavy older bulls contained greater amounts of these tissues than younger bulls or steers. Hydroxyproline in the LD followed the same pattern but differences between young and old bulls were not significant.

Warner-Bratzler shear values showed that muscles from steers were significantly more tender than those from 14 or 20 month-old bulls, but Warner-Bratzler shear values were about the same for muscles from the two groups of bulls. This agrees with earlier work by Field et al. (1966) who reported no significant differences in muscle tenderness between 400 and 699 days of age. Epimysial cores

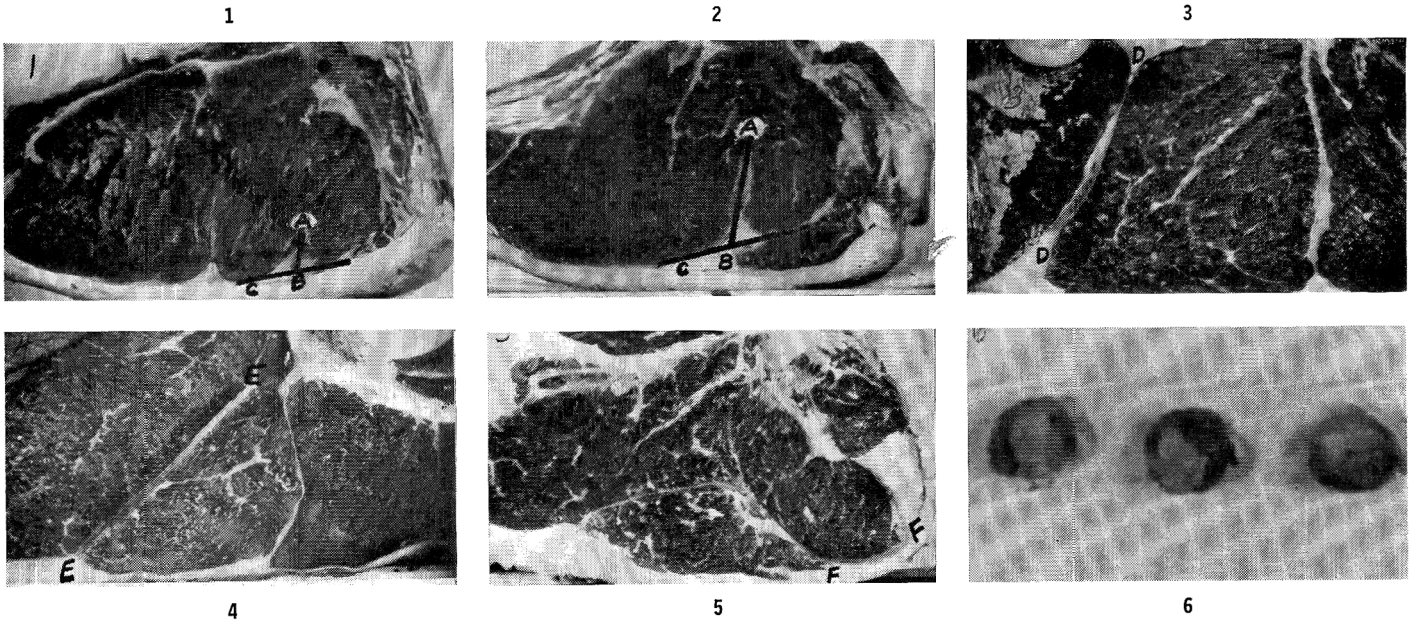

Fig. 1—Points A to B in pictures 1 and 2 show variation in the length of the epimysial extension into the LD at the 12th rib. The thickest portion of the LD epimysium at the 12th rib was measured at the thickest epimysial area between points C and A. Thickness of the epimysium 2 cm lateral to the peninsula is located at point C. The thickest portion of the epimysium between points DD, EE and FF was measured in pictures 3 (biceps femoris); 4 (triceps brachii) and 5 (longissimus dorsi at 5th lumbar) respectively. Picture 6 is a transverse section of ½ in. diameter cores taken near point A in pictures 1 and 2 after the steaks were cooked.

Table 1—Means and standard deviations for characteristics of the epimysium, intramuscular connective tissue and Warner-Bratzler shear values.

Variable	109 steers 20 mo old		89 bulls 14 mo old		24 bulls 20 mo old	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
Cold carcass weight, lb	639[b]	63	542[a]	16	728[c]	68
Epimysial thickness, mm						
LD at 12th rib, 2 cm	1.08[b]	0.35	0.96[a]	0.29	1.48[c]	0.41
LD at 12th rib, thickest portion	1.51[b]	0.40	1.85[a]	0.65	2.46[c]	0.62
LD at 5th lumbar	—	—	3.61[a]	0.74	3.98[b]	0.66
BF at thickest portion	—	—	4.16[a]	1.00	5.17[b]	1.08
TB at thickest portion	—	—	2.36	0.57	2.56	0.46
Epimysial extension, mm	16.57[a]	4.49	18.11[b]	5.22	19.58[b]	4.42
Connective tissue scores[1]						
Collagen, LD	14.66[a]	2.15	14.22[a]	2.36	16.16[b]	2.28
Elastin, LD	13.92[a]	2.03	13.52[a]	2.45	15.56[b]	2.31
Collagen, BF	—	—	15.47[a]	3.00	17.00[b]	2.66
Elastin, BF	—	—	15.23	3.24	16.24	2.40
Connective tissue, LD	4.60	1.64	5.00	1.74	5.14	1.42
Connective tissue, BF	—	—	5.64	1.96	5.86	1.50
Hydroxyproline, LD[2]	3.37[a]	1.01	3.77[a,b]	0.93	4.11[b]	1.21
Hydroxyproline, BF	—	—	5.37	1.36	5.52	1.89
W-B Shear on LD, lb						
Cores, 1 in., 160°F	15.38[b]	2.84	19.56[a]	4.73	18.31[a]	2.68
Cores 1/2 in., 160°F	6.39[b]	1.37	6.99[a]	1.22	6.61[a,b]	1.72
Epimysial core 1/2 in., 160°F	8.07	1.95	7.79	2.10	8.16	2.10
Cores 1 in., 140°F	—	—	17.05	3.33	16.83	3.06
Cores 1/2 in., 140°F	—	—	5.72	1.41	5.95	1.47
Epimysial cores 1/2 in., 140°F	—	—	12.86	9.30	15.23	6.29
W-B Shear on BF, lb						
Cores 1 in., 160°F	—	—	18.65[a]	2.97	24.08[b]	5.77

[1] Subjective scores of two judges. 14-month old bulls — n = 65.
[2] Expressed as mg/g dry tissue. Steers — n = 29, and 14 month-old bulls — n = 34.
[a,b,c] Means on horizontal lines bearing different superscript letters differ significantly ($P < 0.05$).

Table 2—Means and standard deviations for measurements on L.D. epimysium of two adjacent steaks from 69 steers.

Variable	Twelfth rib steak		Adjacent rib steak	
	Mean	S.D.	Mean	S.D.
Epimysial thickness, mm				
LD at 12th rib, 2 cm	1.11[a]	0.37	1.27[b]	0.35
LD at 12th rib, thickest portion	1.57[a]	0.42	1.71[b]	0.38
Epimysial extension, mm	16.40[a]	4.02	19.07[b]	4.59

[a,b] Means between twelfth and adjacent rib steaks are significant ($P < 0.05$).

Table 3—Means for characteristics of the epimysium and intramuscular connective tissue in the LD of 14 most tender and 15 least tender steers.

Variable	Least tender steers		Most tender steers	
	Mean	S.D.	Mean	S.D.
Warner-Bratzler Shear, lb[1]				
Cores, 1 in.	20.73[a]	2.27	12.36[b]	1.21
Cores, 1/2 in.	8.13[a]	1.83	5.41[b]	0.46
Epimysial cores, 1/2 in.	8.33	1.67	7.77	2.62
Epimysial extension	18.63	5.35	18.35	6.05
Epimysial thickness, mm				
LD at 12th rib, thickest portion	1.63	0.42	1.44	0.47
LD at 12th rib, 2 cm	1.05	0.37	1.12	0.37
Hydroxyproline[2]	3.60[a]	0.11	2.12[b]	0.09
Connective tissue[3]	5.46	2.32	5.14	2.16

[1] Steaks roasted to 160°F internal temperature.
[a,b] Means between more tender and less tender steers differ significantly ($P < 0.05$).
[2] Expressed as mg/g dry tissue.
[3] Subjective scores of two judges.

cooked to 160°F were as tough in steers as they were for the bulls. When adjacent rib steaks from bulls in both age groups were cooked to 140°F internal temperature they were slightly more tender than those cooked to 160°F in bulls of both age groups (Table 1).

As shown in Table 1, the epimysial cores cooked to 140°F were much tougher than the epimysial cores cooked to 160°F. This suggests that epimysial tissue continued to gelatinize after a temperature of 140°F was reached. Paul (1963) cited several research workers who reported that collagen is partially to completely solubilized during cooking, the extent of change depending on the duration of heating and the internal temperature reached.

Shear force values of epimysial cores cooked to 140°F or 160°F were slightly lower for younger bulls than for older bulls. It is probable that, at the same temperature, epimysial tissue in younger bulls gelatinized to a greater extent than it did in older bulls, thus causing the cores to be more tender. The work of Hill (1966), Goll et al. (1964) and Herring et al. (1967) showing that collagen solubility decreased with increasing age is evidence for this probability.

A few steaks were cooked to an internal temperature of 130°F. At this temperature 1/2 in. epimysial cores had Warner-Bratzler shear values over 30 lb. Paul (1964) stated that the initial change in collagenous tissue appears to occur as the tissue passes through the 131 to 140°F temperature range. The extremely tough cores with shear values over 30 lb resulted because the collagenous tissue of the epimysium had not passed through this range. The high standard deviations for the shear force of epimysial tissue cooked to 140°F indicated that shear force of epimysial tissue varied considerably within similar groups of animals. This data supports the work of Machlik et al. (1963) who pointed out the large biological variation in collagen shrinkage between samples of the same muscle from different animals.

The interaction of Warner-Bratzler shear values between age and muscle in the bulls is noteworthy (Table 1). No significant shear differences were found in the LD of bulls 14 or 20 months of age. Nevertheless the BF in younger bulls had a much lower shear force value than in older bulls. The slightly lower shear value for the BF when compared with the LD in 14 month-old bulls was not in agreement with Ramsbottom et al. (1945). However, Cover et al. (1956) and Ritchey et al. (1965) also reported that the BF was slightly more tender than the LD in young animals.

The small differences in tenderness of BF and LD muscles in younger bulls and

the wide differences in tenderness of BF and LD muscles in older bulls could be attributed to greater amounts of less soluble collagen in the BF when compared to the LD. This change in collagen solubility could have occurred to a greater extent in the BF than in LD as age increased. Cover et al. (1962) strengthened the above hypothesis as they found that connective tissue in the LD was tender at 142°F and increased only slightly in tenderness as temperature increased; whereas in the BF muscle it was scored tough at 142°F and became progressively more tender at 176 and 212°F.

Least-squares means for connective tissue and shear measurements by line of bulls holding age constant are listed in Table 4. Thickness of epimysial tissues was generally slightly lower for the Angus line than for the other three lines of cattle. However, the epimysium extended significantly further into the LD of the Angus line when compared with the other three lines of cattle. Intramuscular hydroxyproline was not significantly different for the Hereford, Angus and inbred Hereford lines in either muscle. The Charolais crosses exhibited lower subjective connective tissue scores than the other lines. The inbred Herefords tended to possess lower shear force values for the LD and higher shear force values for the BF than the other lines. However, with the exception of the epimysial cores cooked to 160°F these differences were not significant.

Table 4—Least-squares means for connective tissue and Warner-Bratzler shear measurements by line of bulls holding age constant.

Variable	Line of cattle			
	Herefords 29	Angus 18	Inbred Herefords 24	Charolais crosses 18
Epimysial thickness, mm				
LD at 12th rib, 2 cm	1.18	0.98	1.07	1.06
LD at 12 rib, thickest portion	1.38	1.16	1.24	1.23
LD at 5th lumbar	3.76	3.58	3.69	3.66
BF at thickest portion	4.70[a]	3.90[b]	4.73[a]	4.29[a,b]
TB at thickest portion	2.37	2.18	2.66	2.45
Epimysial extension, mm	18.46[b]	22.45[a]	15.98[b,c]	17.73[b,c]
Hydroxyproline, LD[1]	3.67	3.63	3.98	—
Hydroxyproline, BF[1]	5.03	5.73	5.21	—
Connective tissue, LD[2]	5.06[b]	6.10[a]	5.06[b]	4.34[b]
Connective tissue, BF[2]	6.10[a]	6.04[a]	5.96[a]	5.18[b]
W-B Shear on LD, lb				
Cores 1 in., 160°F	18.44	19.46	17.85	19.74
Cores 1/2 in., 160°F	7.08	7.57	6.78	7.34
Epimysial cores 1/2 in., 160°F	8.80[a]	7.34[b]	6.99[b]	7.73[a,b]
Cores 1 in., 140°F	16.81	17.22	16.19	17.10
Cores 1/2 in., 140°F	5.72	6.41	5.19	5.69
Epimysial cores 1/2 in., 140°F	12.68	13.64	12.61	12.09
W-B Shear on BF, lb				
Cores 1 in., 160°F	20.29	19.41	21.91	18.83

[a,b,c] Means in the same row bearing different superscript letters differ significantly ($P < 0.05$).
[1] Expressed as mg/g dry tissue, n = 58.
[2] Subjective scores of 2 judges n = 65.

Nine bulls sired 68 of the 89 bull carcasses used in this study. When characteristics of the epimysium were analyzed by least-squares analysis according to sire, holding age constant, only the epimysial extension was significantly different ($P < 0.05$) among sires. Thickness of the LD epimysial at 2 cm approached significance. When ranked according to greatest epimysial extension into the muscle, the three Angus bulls ranked 1st (greatest extension into LD), 4th and 6th. The only Shorthorn-Charolais crossed bull and a bull from the Hereford line had progeny which tended to possess the thinnest epimysial tissues in most parts of the carcass. Differences between line of cattle and between sires may exist for the epimysial characteristics studied. However, these differences were small and generally not significant ($P < 0.05$) in the population analyzed.

All possible correlations for each of the variables in Table 1 were calculated within groups of steers, 14 month-old bulls and 20 month-old bulls. Thickness of the various epimysial tissues listed were positively correlated with each other but the correlations were low (r = 0.20 to 0.30). There was a lack of association between thickness of epimysial tissues and intramuscular connective tissue as judged by amount of hydroxyproline or by subjective scores for collagen and elastin. The significant ($P < 0.05$) correlations between hydroxyproline in the BF and hydroxyproline in the LD were 0.45 and 0.34 for 20 and 14 month-old bulls, respectively. With few exceptions neither epimysial thickness, hydroxyproline, nor any subjective connective tissue score was significantly related to shear values of muscle or shear values of epimysial tissue at either 140 or 160°F internal temperature.

These low correlations support statements by Wilson et al. (1954), Goll et al. (1963), Wierbicki et al. (1954) and McClain et al. (1965) that total collagen or connective tissue has little relationship to meat tenderness. It should be pointed out that most of the earlier workers (Mitchell et al., 1928; Mackintosh et al., 1936; Ramsbottom et al., 1945 and Hiner et al., 1955) who found that larger amounts of connective tissue were associated with less tender meat, based their results on cattle of a wide age range. They often used differences between means and not correlations as the basis for their conclusions. A similar conclusion could have been made in this study if data from the 20 month-old bulls had been used to compare more connective tissue in the tougher BF with less connective tissue in the more tender LD.

Simple correlations among Warner-Bratzler shear values for 1 in. and 1/2 in. cores taken from the same steak cooked to 160°F were highly correlated (range = 0.74 to 0.81) but the 1 in. core had low correlations (range = 0.16 to 0.39) when related to the 1/2 in. epimysial core removed from the same steak. Perhaps the most surprising correlations were the low relationships (r = 0.22 and 0.03) between 1 in. cores from the LD and 1 in. cores from the BF of 14 and 20-month old bulls, respectively. Knutson et al. (1966) found a nonsignificant correlation of 0.29 between Warner-Bratzler shear values for BF and LD muscles from steers.

Doty et al. (1961) reported a correlation of 0.50 between tenderness of the LD and semitendinosus with no aging, but after four weeks aging the correlation between the tenderness of the two muscles dropped to 0.20. In this study epimysial cores cooked to 160°F had a correlation of 0.26 with epimysial cores cooked to 140°F. As shown in Table 1, standard deviations of shear values were much higher at 140°F than at 160°F. This would indicate that the extent of collagen gelatinization was much more variable at 140°F than it was at 160°F, resulting in these low correlations.

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Comparison of Survivor Curves of Bacillus subtilis Spores Subjected to Wet and Dry Heat

SUMMARY—Survivor curves for spores of Bacillus subtilis *were determined in wet and dry heat over a wide range of temperatures. Wet heat tests were determined using a thermoresistometer and thermal death time cans. Dry heat tests were conducted in a closed system using thermal death time cans. There were major differences in the shape of the wet vs. dry heat survivor curves. Wet heat resulted in convex curves at low temperatures, but a straight line at higher temperature. Dry heat resulted in concave curves at all temperatures. These results suggest that physiological differences exist between wet and dry heat destruction of bacteria. Several possible explanations for the difference in the shape of the survivor curves were discussed.*

INTRODUCTION

RATE OF DEATH of bacteria may be conveniently described by a survivor curve. Rahn (1945) proposed that when the logarithm of the number of remaining viable organisms is plotted vs. time, a straight line survivor curve results. The basis of Rahn's theory was that the bacterial cell would die when a single critical molecule was inactivated. Thus he assumed that death rate should follow the same equation as a monomolecular decay reaction.

Considerable evidence appears to support the theory of the logarithmic order of death, however, many investigators obtained survivor curves which were not linear. A number of workers obtained wet heat survivor curves which were convex (Amaha et al., 1957; El-Bisi et al., 1956; Licciardello et al., 1963). El-Bisi et al. (1956) obtained wet heat survivor curves which were convex at low temperatures but linear at high temperatures. Other investigators found wet heat survivor curves which were concave (Alderton et al., 1964; Frank et al., 1957; Walker et al., 1961).

Most of the available data on survivor curves was obtained using wet heat, however, Murrell et al. (1966) reported dry heat survivor curves that were concave. These data suggest that there may be differences between wet and dry heat survivor curves.

Shull et al. (1962) found that the survivor curve of dried *B. stearothermophilus* spores heated in saturated steam had three phases: (1) an initial rise; (2) a low rate of death which increased slowly; and (3) a logarithmic death phase. These workers attributed the first phase to spore activation and the second and third phases to normal logarithmic death. These workers found also that the rate of death was lower when superheated steam conditions prevailed. Pflug (1960) previously reported that rate of death of bacterial spores when subjected to superheated steam was lower than when the spores were subjected to saturated steam.

The research described in this paper was undertaken to determine if there are differences in the shape of survivor curves obtained when spores of *B. subtilis* are exposed to wet vs. dry heat.

MATERIALS & METHODS

Preparation of spores

The *B. subtilis* spores strain 5230 were

grown as described by Pflug (1960). A stock suspension was prepared at a concentration of approximately 1×10^7 spores per ml and stored in M/15 phosphate buffer at pH 7.0. This culture was essentially 100% spores. The stock suspension was stored at 3°C (38°F) throughout the test period.

A 0.01 ml aliquot of spore suspension was dispensed as needed from a micrometer syringe into small, 11 mm O.D. × 8 mm deep, tin plate cups. Prior to adding inoculum, cups were washed, placed in petri dishes and autoclaved at 121°C (250°F) for 30 min. Cups containing inoculum were dried under a vacuum of 29 in. of mercury for 24 hr and stored in a desiccator containing anhydrous calcium chloride until used. Cups with spores were prepared in the same manner for experiments in both wet and dry heat

To determine the initial number of organisms for each test, a cup containing approximately 1×10^5 spores was dropped into each of five screw-cap tubes containing 10 ml of sterile distilled water. The tubes were placed in boiling water for 10 min. After cooling, the tubes were shaken to remove spores from the surface of the cups and to distribute spores throughout the suspension. A 1:100 dilution was made from each tube and 1 ml of the dilution was plated using dextrose, tryptone, starch (DTS) agar. A thin layer of DTS agar was poured over the surface to prevent spreading of surface colonies. Plate counts were made after 48 hr incubation at 37°C (99°F). The average count of five plates was taken as the initial number of organisms.

Each point of the survivor curve represents the average number of organisms surviving in 10 replicate samples heated as indicated below.

Dry heat

Cups (containing 1×10^5 spores each) were placed in a thermal death time (TDT) can (Townsend et al., 1938) and the can closed on an automatic machine. To assure that the can was completely sealed, a thin layer of epoxy resin was spread around the double seam. The epoxy was allowed to cure for at least eight hours before testing was initiated. Cans of spores tested at 129°C (265°F) and at 152°C (305°F) were heated in miniature retorts in the manner described by Augustin (1964). Cans of spores tested at 113°C (235°F) and 95°C (203°F) were submerged in a preheated, constant temperature oil bath.

Wet heat

Cups containing the dried spores were heated at 121°C (250°F) for predetermined times in saturated steam in the thermoresistometer described by Pflug (1960). Wet heat at 104°C (220°F), 95°C (203°F) and 77°C (170°F) was obtained by placing the cups containing the dried spores into TDT cans to which was added a disc of sterile filter paper and 2 ml of sterile distilled water. The cans were closed in air at atmospheric pressure on an automatic closing machine and heated in the oil bath described above.

Subculturing technique

The TDT cans were removed from heating units, wiped clean, dried, opened aseptically and each of the 10 cups was dropped into a screw-cap test tube containing 10 ml of sterile distilled water. When the thermoresistometer was used, cups were deposited directly into the tubes. Tubes were shaken vigorously for 10 min to remove spores from the cup surface. Appropriate dilutions were made and 1 ml was plated using DTS agar. When the agar had solidified, a thin overlay of DTS agar was added.

Analysis of data

At each temperature-time heating condition the numbers of surviving organisms in all replicates were averaged to obtain the mean number of survivors and the results analyzed statistically to determine the 95% confidence limit of this mean. Means with their confidence limits are plotted on the survivor curve graphs.

D values (decimal reduction time, defined as time required to reduce the microbial

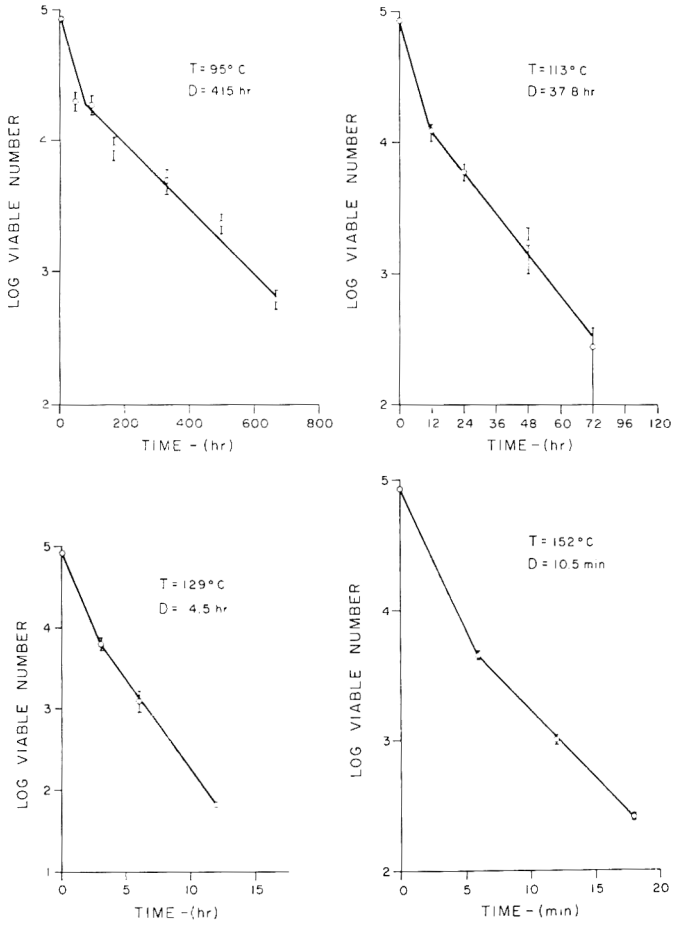

Fig. 1—Dry heat survivor curves. (95% confidence limits are shown for each point.)

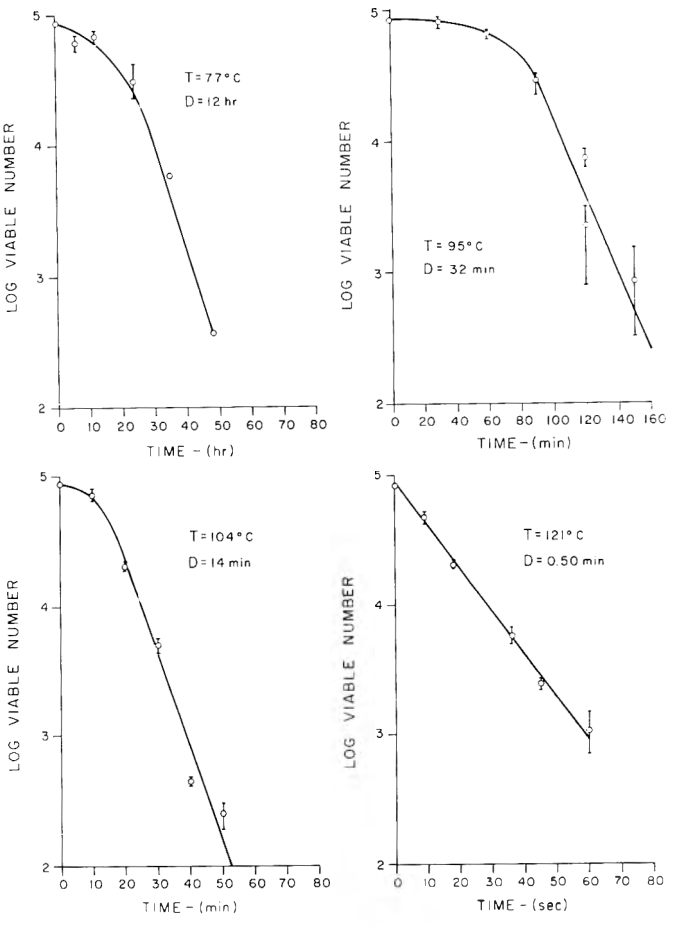

Fig. 2—Wet heat survivor curves. (95% confidence limits are for each point.)

Table 1—D values of Bacillus subtilis determined from wet and dry heat survivor curves.

Temperature °C	Temperature °F	D value (min) Wet heat	D value (min) Dry heat
77	170	720	—
95	203	32	24,900
104	220	14	—
113	235	—	2,268
121	250	0.5	—
129	265	—	270
152	305	—	10.5

population by 90%) were plotted vs. temperature to obtain the thermal resistance curve. The z values (defined as the change in temperature required to produce a tenfold change in D) were also determined.

RESULTS AND DISCUSSION

DRY HEAT survivor curves determined at 95, 113, 129 and 152°C (203, 235, 265 and 305°F) are shown in Figure 1. All of these curves appear to have two slopes with their second slope having a larger D value. Wet heat survivor curves for 77, 95, 104 and 121°C (170, 203, 220 and 250°F) are shown in Figure 2. The curves for 77, 95 and 104°C are all convex with the curve at 121°C being very close to a straight line.

The D values reported in Table 1 were calculated from the slopes of the second straight line portion of the two slope curves, or the slope of the final straight line portion of the curves that consist of a non-linear curve followed by the linear segment. The D values for dry and wet heat shown in Table 1 were plotted as dry and wet heat thermal resistance curves and are shown in Figures 3 and 4, respectively. The D values for dry heat fall on a straight line as shown in Figure 3 where the z value was 17°C (31°F). The wet heat thermal resistance curve shown in Figure 4 has a z value of 14°C (25°F) and was drawn straight although some of the points do not fall on the line.

Destruction of microorganisms by dry heat differs in magnitude and may also differ in mechanism from destruction by wet heat. Unlike wet heat which is specifically defined as heating in a medium consisting of 100 percent relative humidity, dry heat is not uniquely defined and is usually taken to refer to the destruction of microorganisms in an environment where the relative humidity is less than 100 percent. Dry heat therefore includes heating under numerous different relative humidities.

Rate of dry heat destruction depends on a number of conditions. Some of these are: (1) initial water content of organisms before dry heating; (2) nature of the medium or menstruum from which the organisms are dried; (3) relative composition of the gas atmosphere in contact with organism during dry heating; (4) physical nature of material in contact with or supporting the dry spores.

Furthermore, dry heat systems can be divided into two categories (Fox et al., 1968): An "open system" refers to a system for heating microbial spores where water can be gained or lost without limit during heating. In an infinite time the spores will be in equilibrium with the water condition of the environment. This definition places no restriction on the rate of water transfer; the spores may lose or gain water either rapidly or slowly. A "closed" system is a system where water movement or availability is restricted. The quantity of water that can be transferred is limited by the quantity of water initially present in the environmental volume.

The dry heat survivor curves described in this paper were of the closed system type. These spore suspensions may have behaved as two population systems because of some change in spore moisture content during heating. Fox et al. (1968) studied open system dry heating and found the same shape survivor curves as reported here for closed system experiments. However, differences in magnitude of D values between open and closed system dry heating of *B. subtilis* were observed.

In testing heat resistance of six species of spores at water activities (a_w) ranging from 0 to 1, Murrell et al. (1966) obtained survivor curves for two species which were similar in shape to our dry heat survivor curves. Initial decreases in viable numbers were attributed to damage during freeze drying of spores. We do not believe that drying damage is the cause of the initial drop in viable number in our experiments because both the dry heat and the wet heat experiments were conducted using pre-dried spores. Wet heat survivor curves showed no evidence of drying damage.

Since spores of *B. subtilis* do not germinate and grow readily unless given some type of heat shock, initial number is a difficult quantity to establish. To determine the initial number of organisms for both wet and dry heat experiments, all spores were wet heat shocked in boiling water for 10 min. At each time-temperature condition in the wet heat tests, including the initial point, spores received a wet heat shock by consequence of being heated in wet heat. However, at each time-temperature condition in the dry heat tests, except the initial point, spores received a dry heat shock by consequence of being heated in dry heat. It is possible that wet heat shock is more effective in activating spores than dry heat shock; this would account for the observed drop in the initial portion of dry heat curves.

The shape of wet heat survivor curves determined at 77, 95 and 104°C fit the explanation of Rahn (1945) as being the result of clumping. However, this theory is not acceptable as an explanation for these wet heat curves, since the dry heat curves did not show any evidence of clumping. The most satisfactory explanation of the shape of the wet heat survivor curves is that of Stumbo (1965). His theory states that this type of survivor curve would result if during the initial part of the heating cycle some spores were being destroyed at the same rate as others were being activated for germination.

The initial number of organisms for both wet and dry heat studies was de-

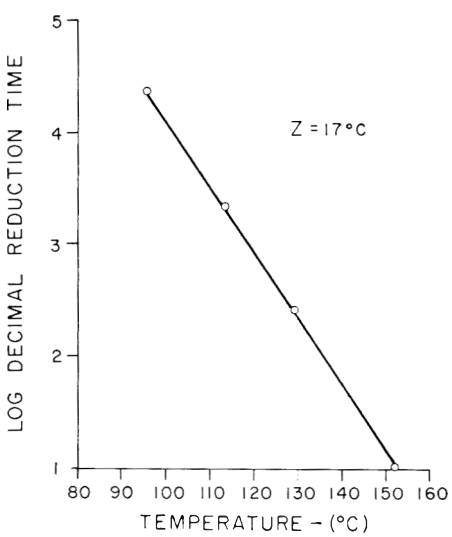

Fig. 3—Dry heat thermal resistance curve.

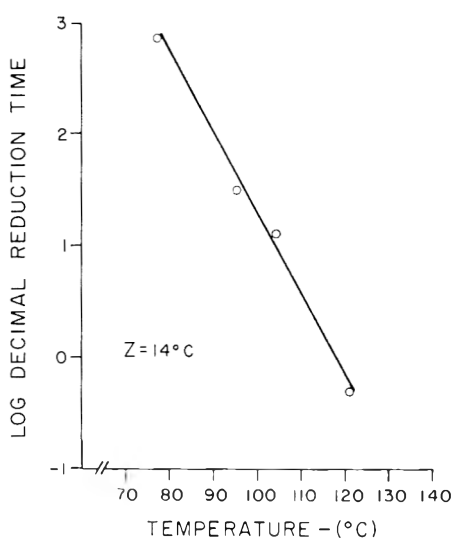

Fig. 4—Wet heat thermal resistance curve.

termined by heat shocking spores for 10 min at 100°C. Assuming the z value for wet heat to be 14°C (25°F) as found in these experiments, 10 min at 100°C is equivalent in lethal effect to approximately 23 min at 95°C (203°F). Therefore, we would expect and did find the point at 23 min on the 95°C (203°F) survivor curve (Fig. 2) to be the same order of magnitude as the initial point. The effect of the heat shock partially explains deviations from linearity of wet heat survivor curves determined at 77, 95 and 104°C (170, 203 and 220°F). This same result would be expected for wet heat at 121°C (250°F) but was not observed. El-Bisi et al. (1956) also observed convex wet heat survivor curves at low temperatures and suggested that at short heating times some spores were being activated as fast as others were destroyed.

Yokoya et al. (1965) studied deviations from exponential rates of thermal death caused by differences in states of sporulation, recovery medium, sporulation medium and pH of the suspending medium during thermal treatment. They found survivor curves for *B. coagulans* varied in shape from convex to linear to concave. These authors stated that "an initial lag or lower initial rates of thermal destruction can most often be explained by experimental errors, for example lags in heat penetration, clumping of cells, protection of spores by vegetative cells and contamination by spores of a different bacterial type."

Dry heat survivor curves in Figure 1 are the shape characteristic of the type of curve that results from a mixed culture of two different heat resistance levels. Since our data indicating wet heat destruction rate (Fig. 2) gave no evidence of a spore population with two levels of resistance, it seems most logical to assume that the initial inoculum was of a single heat resistance level, however, it may be possible to have a spore suspension that behaves as a single population when subjected to wet heat conditions but behaves as two populations when subjected to dry heat conditions.

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Terpene and Sesquiterpene Hydrocarbons in the Essential Oil from Fresh Celery

SUMMARY—The terpene and sesquiterpene hydrocarbons in celery essential oil that was recovered by two different methods were separated from the oxygen containing materials by column chromatography and analyzed by temperature-programmed gas chromatography. The essential oils were prepared from celery puree by a batch distillation procedure, and by vaporization in a Votator Turba-Film evaporator and rectification of the vapors in a packed distillation column. The terpenes α-pinene, β-pinene, myrcene, limonene, γ-terpinene and cymene; and the sesquiterpenes β-elemene, β-caryophyllene, α- and β-humulene and β-selinene were identified as constituents of the essential oils. The qualitative and quantitative relationships of the hydrocarbons between the two different oils were established and discussed.

INTRODUCTION

DURING RECENT investigations at this laboratory, procedures for recovering the essential oil from fresh celery in high yield were developed (Wilson et al., 1967). Development of these procedures made possible, for the first time, an extensive study on the volatile flavor constituents of the essential oil obtained from the celery plant. The purpose of this investigation was to isolate and identify the terpene and sesquiterpene hydrocarbons and to estimate the quantity of these constituents in celery essential oil.

Previous investigations at this laboratory, conducted on relatively small quantities of oil obtained by a modified vacuum recovery technique, showed that myrcene and limonene were present in celery (Gold et al., 1961, 1963). However, to the author's knowledge, no other

terpenes or sesquiterpenes have been reported as constituents of the celery plant.

EXPERIMENTAL

CELERY essential oil was recovered by two methods. In the first, the essential oil was recovered from celery puree by a batch essence recovery procedure (Wilson et al., 1967). In the second, distillation was accomplished in a continuous atmospheric essence recovery unit designed at this laboratory.

A positive displacement pump set at five gal per hr pumped the celery slurry into a Turba-Film evaporator of 1 sq ft evaporation surface with the rotor operating at 1290 rpm. Steam at 43 psig was supplied to the jacket to provide 20% evaporation.

The vapors from the evaporator entered a glass fractionating column consisting of a glass tee with 30 in. × 3 in. packed columns above and below, both filled with $^5/_8$ in. stainless steel "Pall" rings. The lower portion served as a stripping section and had a reboiler at the bottom made of a coil of stainless steel tubing heated with steam under controlled pressure. Sufficient steam was introduced to compensate for heat losses to the atmosphere in the stripping section and tee. The upper section served as a fractionating column and above it was a stainless steel reflux condenser of 22.5 sq ft. Sufficient tap water was admitted to the cooling side of this condenser to remove most of the water vapor, but still let through some water along with the volatile flavoring material. These remaining vapors passed into another condenser of 4.5 sq ft that was cooled to 55°F with chilled water. The condensate drained directly into an oil trap where the oil layer and aqueous layer were collected separately. The aqueous layer was set aside for use at some future date.

The hydrocarbons in celery essential oil were separated from the oxygenated materials by column chromatography (Hunter et al., 1965). 20 ml of each oil was placed on 2 cm × 30 cm neutral alumina columns (Fisher A-90, 80–200 mesh). The hydrocarbons were eluted with about 150 ml n-hexane and concentrated in vacuo in a rotary evaporator.

The terpene hydrocarbons were separated from the sesquiterpene hydrocarbons by vacuum distillation at 45°C and 0.5–1.0 mm Hg. The terpene and sesquiterpene fractions were separated into individual components by temperature-programmed gas chromatography with an F&M, Model 700, dual column chromatograph that was equipped with an F&M, Model 240, temperature programmer. The separations were achieved on an 18 ft × $^1/_4$ in. OD stainless steel column packed with 25% Carbowax 20M on 60–80 mesh Gas-Chrom P. Samples were temperature programmed from 100–225°C at 1°C/min, and the helium flow rate was 50 ml/min.

Identifications were made by comparing retention temperatures and the infrared and mass spectra with known compounds. Infrared spectra were taken on a Perkin Elmer 137 Infracord and the mass spectra with a Bendix Time-Of-Flight, Model 3012, mass spectrometer.

RESULTS & DISCUSSION

ORGANOLEPTIC differences were noted in the essential oil prepared by the two recovery methods. The essential oil prepared by the batch essence recovery method possessed a cooked celery odor. The essential oil obtained from the continuous essence recovery unit possessed an odor that closely resembled fresh celery, but also had a slight terpene odor. The improved organoleptic properties of the essential oil obtained from the continuous essence recovery unit were attributed to the use of the turbulent-film evaporator. Since the celery puree was subjected to heat for only a few min in this evaporator, rather than 1 hr in the still pot, it is reasonable to expect that less cooked and other objectionable odors would be produced.

Column chromatography indicated that the hydrocarbons comprised approximately 80% of the essential oil. A yield of 16 ml of hydrocarbons was obtained from 20 ml of the essential oils. Little difference was noted in the total quantity of hydrocarbons in the essential oil prepared by the two recovery methods. Vacuum distillation of the hydrocarbon fraction yielded about 15 ml of terpene and 1 ml of sesquiterpene hydrocarbons.

Figure 1 shows gas chromatograms of separate 10 μl injections of the vacuum distilled terpene and sesquiterpene hydrocarbons that were obtained from the essential oil prepared by the continuous essence recovery units. α-Pinene, β-pinene, myrcene, limonene, γ-terpinene and cymene were identified as constituents of the terpene hydrocarbon fraction. Limonene, the most abundant single constituent in celery essential oil, represents about 95% of the terpene hydrocarbon fraction. α-Pinene, β-pinene, myrcene, γ-terpinene and cymene were present in concentrations of approximately 0.5, 0.10, 2.0, 0.2 and 1.5% respectively.

Comparison of the gas chromatograms of the terpene hydrocarbon fractions from the essential oils recovered by the two different recovery methods showed that the two essential oils were qualitatively but not quantitatively identical. Examination of the essential oil obtained from the batch essence recovery unit indicated that α-pinene, β-pinene, myrcene, γ-terpinene and cymene were present in concentrations of about 0.05, 1.0, 1.0, 0.05 and 2.0%, respectively. The quantity of limonene did not change appreciably.

Two additional compounds that eluted between the terpene and sesquiterpene hydrocarbons were isolated from the essential oil prepared in the batch essence recovery unit. These two compounds were found in trace quantities in the essential oil prepared by the continuous es-

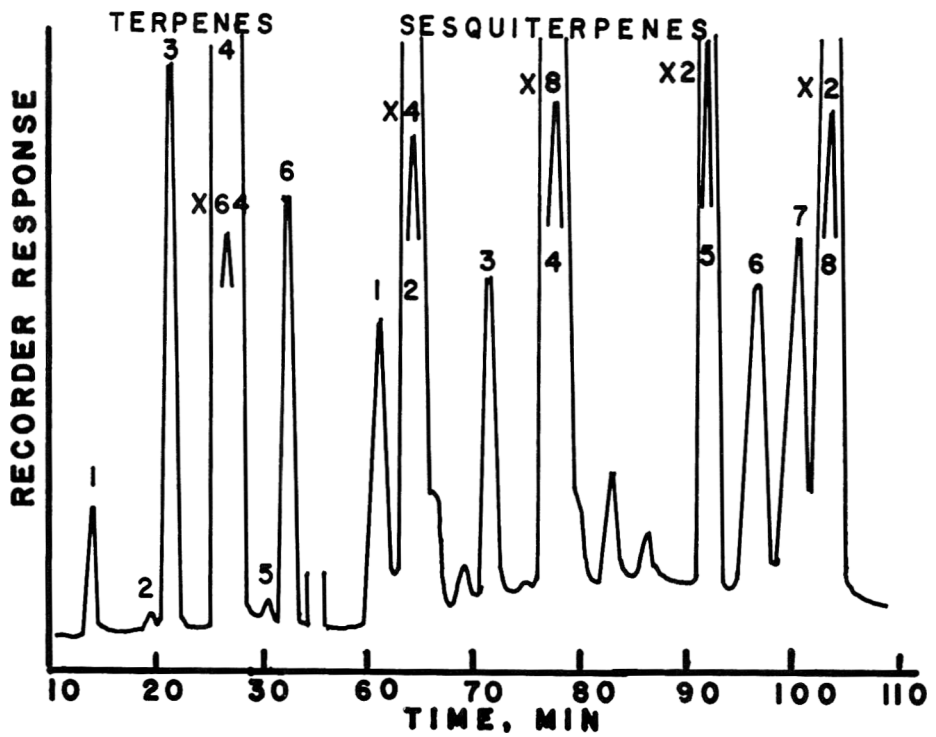

Fig. 1—Gas chromatograms of terpene and sesquiterpene hydrocarbons from continuous essence recovery unit—Terpenes: 1. α-pinene, 2. β-pinene, 3. myrcene, 4. limonene, 5. γ-terpinene, 6. cymene—Sesquiterpenes: 1. β-elemene, 2. β-caryophyllene, 3. α- and β-humulene, 4. β-selinene, 5. $C_{15}H_{24}$, 6. $C_{15}H_{24}$, 7. $C_{15}H_{24}$, 8. MW 219.

sence recovery unit and are not included in Figure 1. It is speculated that these compounds arose from thermal decarbonylation of a heat labile carbonyl compound.

The first compound was characterized by infrared, ultraviolet and mass spectra as an unsaturated cyclic hydrocarbon containing an n-C_5H_{11} side chain. The molecular weight, determined from its mass spectrograph, was 150. The ultraviolet spectrum ($UV_{max}254$) suggested that the compound contained conjugated double bonds. Further attempts to isolate and purify more materials for NMR analysis were not successful. It was found that, when in the pure state, the first compound underwent disproportionation or dehydrogenation and was identical in all respects to the second compound isolated. The second compound was identified as n-pentyl benzene from spectral data by comparison with the authentic compound. The first compound was assigned an empirical formula $C_{11}H_{18}$ and tentatively identified as 5-pentyl-1,3-cyclohexadiene on the basis of its UV spectrum, since the 1- and 2-pentyl isomer should absorb at longer wavelengths.

The first sesquiterpene peak shown in Figure 1 was tentatively identified as β-elemene plus another unidentified sesquiterpene hydrocarbon. This peak represents about 1% of the sesquiterpene fraction. The second peak was identified as β-caryophyllene, and constitutes about 25% of this fraction. The next major constituent is a mixture of α- and β-humulene. These two isomers are present at a concentration level of about 1%. The next and largest peak shown in the sesquiterpene fraction is β-selinene. This one compound represents about 50% of the total sesquiterpenes.

The fifth major constituent shown in Figure 1 has not been identified. It is thought to be an isomer of β-selinene since its infrared and mass spectra closely resembles that of β-selinene. The concentration of this compound is estimated to be about 10%. The next two sesquiterpenes, peaks 6 and 7, were not identified. These two compounds are present in concentrations of about 1% each. The last major constituent, about 10% of the total sesquiterpene fraction, was not characterized as a sesquiterpene hydrocarbon. The molecular weight of 219 suggests either a nitrogenous compound or a halocarbon.

The sesquiterpene hydrocarbons in the essential oil prepared by the batch essence recovery unit were, as it was in the case of the terpenes, qualitatively but not quantitatively identical when compared to the sesquiterpene hydrocarbons in the essential oil prepared by the continuous essence recovery unit. The concentration of β-elemene was reduced to about 0.5%. The concentration of β-caryophyllene was estimated to be about 10%, or about a twofold reduction in concentration. The concentration of α- and β-humulene remained at approximately 1.0%. The largest change was noted in the amount of β-selinene, which increased from about 50 to 75% concentration. The remaining four unidentified peaks, which correspond to the last four peaks shown in Figure 1, are estimated at concentrations of 5.0, 0.75, 0.25 and 5.0%, respectively. The last four unidentified constituents were reduced two fold in concentration.

The terpenes myrcene and limonene were previously reported as constituents of the essential oil obtained from fresh celery (Gold et al., 1961, 1963). This paper reports, for the first time, the identification of the terpenes α-pinene, β-pinene, γ-terpinene and cymene; and the sesquiterpenes β-elemene, β-caryophyllene, α- and β-humulene and β-selinene as constituents of the essential oil from fresh celery.

The contribution of the hydrocarbons toward flavor does not appear to be appreciable even though they constitute about 80% of the total essential oil. The aroma of the hydrocarbons was examined by smelling the effluent from the gas chromatograph and with one exception these compounds could not be described as having a pleasant aroma. The one exception was β-selinene, which possessed an aroma that was characteristic of celery.

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References to specific products of commercial manufacture are for illustration and do not constitute endorsement by the U.S. Department of Agriculture.

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The Relation of Force to Sample Dimensions in Objective Measurement of Tenderness of Poultry Meat

SUMMARY—Relations between maximum shear resistance and cross-sectional dimensions were established for cooked turkey meat samples of various shapes and sizes with a Warner-Bratzler type shearing device mounted in an Instron tensile tester. The resistance to shear was proportional to the equivalent diameter raised to a power of about 1.2, over a wide range of sizes, shapes and intrinsic toughness. Degree of compaction before shearing was significantly correlated ($r = 0.65$, significant at 0.1% level) with the force at first failure per unit of equivalent diameter, and the compaction naturally increased with increasing intrinsic toughness. These data will permit comparison of shear force measurements from meat samples of different sizes and shapes.

INTRODUCTION

THE FORCE required to shear cooked poultry meat by any of several mechanical methods is reasonably well correlated with subjective estimates of toughness (Szczesniak et al., 1965). However, comparison of results from different laboratories is impractical because of lack of standardization of procedures. Even with a particular apparatus and procedure there is no established way of comparing shear force values obtained from samples of differing cross-sectional areas.

Different sized samples are encountered when samples are cut before cooking, when small muscles difficult to trim to a fixed size are used or when paired muscles or muscle strips which contract and thicken to different extents during aging or cooking are compared.

The literature provides little enlightenment on the relation of size of sheared specimen to the force required to shear. Paul et al. (1955) measured the Warner-Bratzler shear resistance of 0.5 and 1.0 in. diameter samples of beef semimembranosus and adductor muscles. They found that 1 in. samples required about 2.5 times the shearing force needed for 0.5 in. samples. Correspondence of this relation to those found in the present study is discussed below.

This investigation was designed to reveal any quantitative relationship between cross-sectional dimensions of sheared samples and shear resistance of cooked poultry muscle and also any relation between degree of compaction before shearing and forces applied.

PROCEDURE

FOUR TYPES of material were studied:

1. Tender turkey breast (pectoralis superficialis) from thoroughly aged 25 lb young tom turkeys cooked in a rotating electric oven to an internal thigh temperature of 85°C;
2. Tough turkey breast from 40 lb old tom turkeys cooked within 2 hr of slaughter in boiling water to an internal temperature of 85°C;
3. For comparative purposes, beef semimembranosus muscle cooked in a mold in boiling water to an internal temperature of 85°C; and
4. Fibrous regenerated soy protein of food grade used as received without further preparation.

All samples for shearing were cut so the shear would be at a right angle to fiber direction. From the tender turkey, samples of circular, square or equilateral triangular cross section with areas from less than 1 cm^2 to more than 5 cm^2 were prepared. From the other materials only cylindrical specimens with diameters ranging from 0.85 to 2.54 cm were cut.

A shearing knife and jaws fabricated identically to those of a Warner-Bratzler meat shearing machine were fitted to an Instron Universal testing instrument (Fig. 1). This assembly provides a graphic analog record of the translation of the jaws and the force applied to the knife. The record enables the measurement of the maximum force exerted, the slope of the curve of force vs. distance, the area of the sample enclosed by the edges

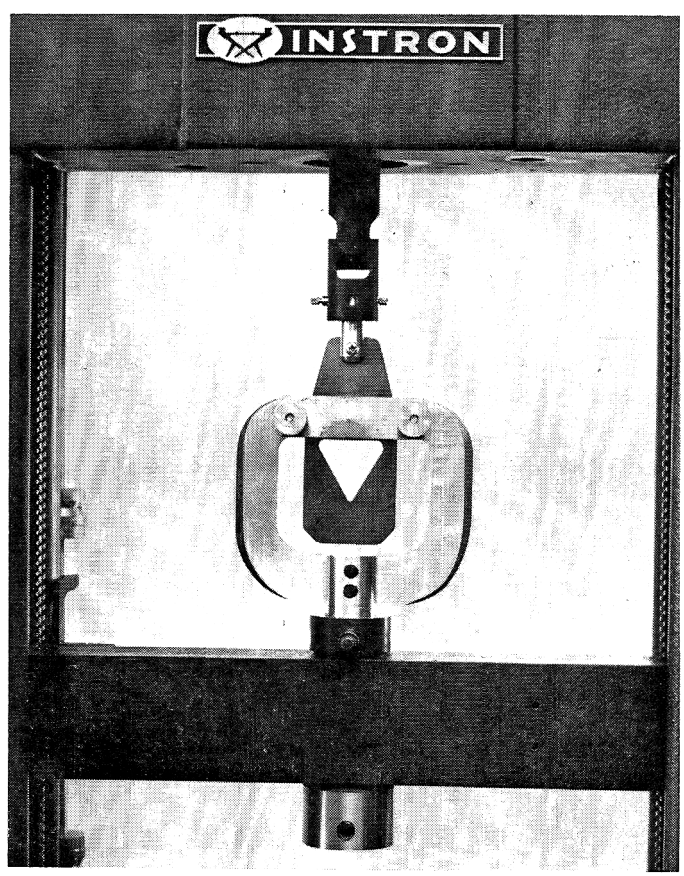

Fig. 1—Knife and jaws identical to Warner-Bratzler design adapted to Instron testing instrument.

of the knife and the moving jaws at any instant and the position of the jaws at the instant at which the first failure of a portion of the sample occurs. A typical record is shown in Figure 2.

After cooking, meats were held overnight at 3°C with precautions against dehydration, then cut into the various shapes and sizes mentioned above. Each region of the muscle was represented as nearly as possible by an equal number of samples of each size. Cut samples were wrapped in plastic film to minimize dehydration and sheared as soon as possible. At least two and usually three cuts were made and shear resistance results averaged for each sample.

RESULTS & DISCUSSION

SHEAR RESISTANCE values for all samples of tender turkey meat are shown plotted against sample diameter or equivalent diameter in Figure 3. Equivalent diameter is the diameter of a cylinder having the same cross-sectional area as that of the square or triangular sample used. Also shown in the figure are the calculated regression lines for the four types of material. The additional slightly curved line in the figure is the exponential regression curve for the tender turkey meat and is the same as that shown on the log-log graph of Figure 4.

Log-log regression lines shown in Figure 4 were calculated to determine the simple exponential function of sample diameter which best expressed its relation to resistance to shear. The linear regression curve of the form $\log F = m \log D + \log b$ is readily transformed to the exponential form $F = b D^m$. The exponential equations developed are listed in Table 1.

Table 1—Simple exponential equation of best fit for relation of shear force F to equivalent diameter D.

Sample	Equation	95% confidence limits for exponent
Tender turkey meat	$F = 0.108\ D^{1.17}$	1.10–1.24
Beef semimembranosus	$F = 0.189\ D^{1.27}$	1.14–1.41
Tough turkey meat	$F = 0.432\ D^{1.15}$	1.06–1.23
Fibrous soy protein	$F = 0.377\ D^{1.24}$	0.92–1.55

Sufficient data are provided in the publication of Paul et al. (1955) to enable an estimate of the corresponding equation for the beef used in their work. As calculated from the 2 points their data provide it is: $F = 0.109\ D^{1.35}$.

With all four rather diverse types of material the exponent of the diameter is much nearer to 1.0 than to 2.0. This suggests that a comparison of shear forces of different sized pieces is more accurate when expressed as force per unit of linear dimension, such as a diameter or side of a square, than as force per unit of cross-sectional area.

In tenderness measurements on meat shear resistance or shear strength is a very small or negligible part of the resistance to separation manifested by a sample in a tenderness measuring device. A flexible or ductile material subjected to shearing stress is immediately distorted and a portion of the force is transformed into a component of tensile stress in the stressed fibers. When they separate it is largely because of the force applied in tension rather than in shear. In technical engineering discussions of shear force it is assumed that the force is applied uniformly across the shear plane. This is not possible with a rather fluid material such as meat.

A force applied to surface fibers of a meat sample, as by the knife of a Warner-Bratzler device, is transmitted in turn, not across the shear plane, but into the volume of the sample below and adjacent to the line of stress. For this reason only those fibers in immediate contact with the shearing member receive the maximum vertical component of the stress. The area to be considered then in calculating stress, or force per unit area, is the area directly under the shearing blade, which would be equal to the thickness of the blade times the distance over which it is bearing on the meat. Since the thickness of the blade is constant, the force per unit of area becomes the force per unit of a transverse linear dimension.

The application of force to a piece of meat undergoing a test results in compression of the region under the knife and the expulsion of fluid. The area of the

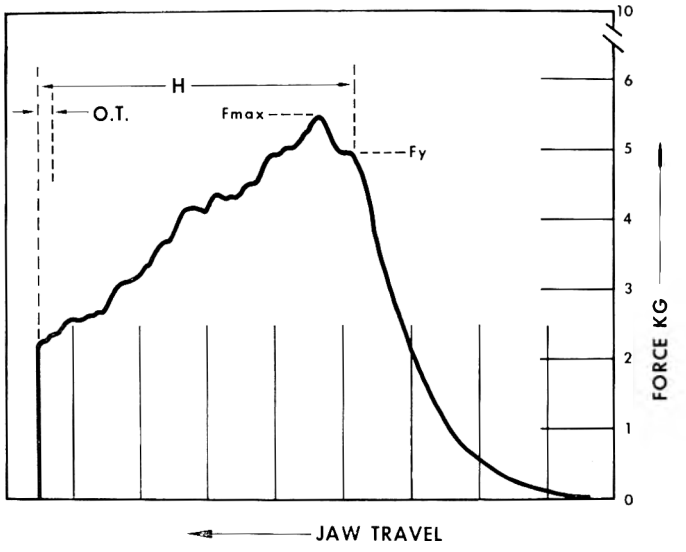

Fig. 2—Typical graphic record showing maximum force exerted, F_{max}; force applied when first failure of specimen occurs, F_y; the corresponding distance remaining for jaw to travel, H; and the distance, O.T., the jaws travel beyond the opening in the knife. The area of the sample at point of first yielding can be calculated by the relation $A_y = (H - O.T.)^2 \tan 30°$ and the equivalent diameter $D = (4A_y/\pi)^{1/2}$.

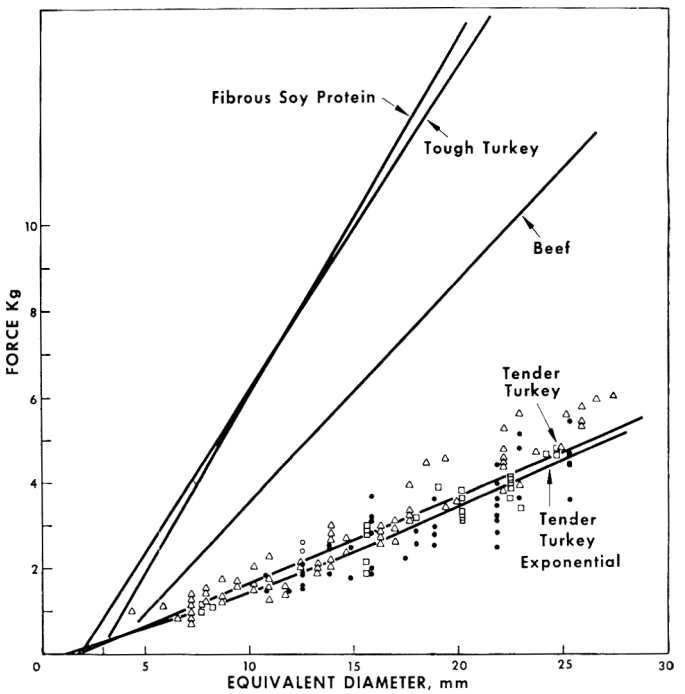

Fig. 3—Linear plots of shear resistance versus diameter showing linear regression curves for the 4 types of material, the individual points for tender turkey meat in which the shapes of the plotted points indicate shape of the sample sheared, and the exponential curve for the tender turkey.

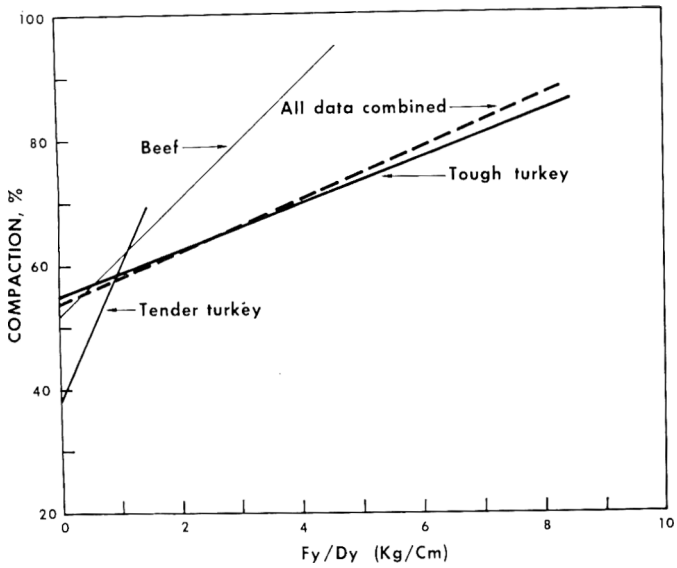

Fig. 5—Regression lines showing sample compaction as a function of the force per unit of compacted equivalent diameter applied at the instant the specimen first begins to fail.

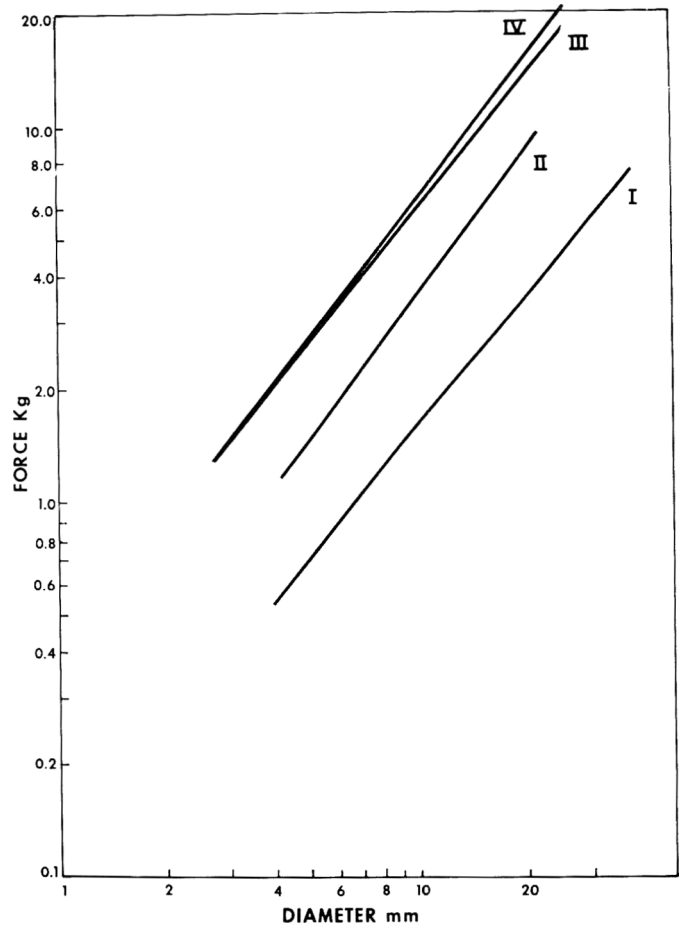

Fig. 4—Regression lines of shear resistance versus sample diameter on log-log coordinates. I. Tender turkey; II. Beef; III. Tough turkey; IV. Fibrous soy protein.

section being compressed is considerably reduced before the failure of any of the meat fibers and can be estimated by examining the record.

Observations made during the shearing of samples varying considerably in tenderness indicated that less tender meat samples were compacted to a greater extent than more tender. To check this, measurements were made on most of the records of individual cuts on the cylindrical samples.

From these measurements the area of the compacted region at the time of failure of the first fibers was obtained. From this the equivalent diameter, D_y was calculated. From this equivalent diameter and the initial diameter, D_I, the fractional diameter reduction or compaction was calculated. The relation of compaction $[1 - (D_y/D_I]$ to force at first failure per unit of equivalent diameter F_y/D_y is shown as regression lines for each of the three meats and for all three combined in Figure 5.

A calculation of the correlation constant for 687 pairs of measurements in the data from Figure 5 gave the result r = 0.65 which is significant at a level of $P \ll 0.001$. No implications were drawn from these data regarding the magnitude of the force required to compact the sample compared to the maximum force. Qualitatively it is obvious that much of the work done on the system is devoted to squeezing fluid out of the compacted region.

The data reported here were obtained with a Warner-Bratzler type device and the preceding discussion applied strictly to that instrument. However, all the biting, cutting or "shearing" machines depend on forcing a blunt edge through the sample and these remarks apply in a general way to all such devices.

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Polygalacturonase Content of Dates and Its Relation to Maturity and Softness

SUMMARY—The changes in polygalacturonase (PG) activity during ripening and the relationship between its activity and the quality of Deglet Noor dates were studied. PG activity, which was virtually absent at the green stage, began to develop as maturity progressed. The greatest part of the development occurred at the late red stage and activity reached its maximum when the fruits commenced softening. Among the four grades of dates used in this study the activity was highest with natural, followed by waxy, number 1 dry and number 2 dry grades. The dates contained 0.9–1.3 units of PG per g of fresh tissue. The results of chemical and physical analyses related to the texture are also reported.

INTRODUCTION

PECTIC ENZYMES undoubtedly play an important role in changes in the texture of plant tissues during ripening and maturation. Polygalacturonase (PG) is one of these enzymes. It catalyzes the hydrolysis of α-1,4-glycosidic linkages of pectic substances and results in a decrease in firmness of the tissues. The presence of PG in certain plant tissues is well established (Demain et al., 1957; Patel et al., 1960; Hobson, 1962 and 1967; Hatanaka et al., 1964; Reymond et al., 1965).

Texture is perhaps the most important factor which controls the quality of dates. The formation of firm (dry) Deglet Noor dates has been recognized for many years in the date industry. The mechanism involved in this undesirable process is not well understood. It is known, however, that firm dates contain less moisture and a relatively lower ratio of reducing to total sugars than soft dates (Sinclair et al., 1942; Cook et al., 1953; Maier et al., 1961; Coggins et al., 1968).

Research has mainly concerned chemical and physical analyses related to the quality and ripening of dates. Studies on the biochemistry of dates and biochemical changes with ripening have been very limited. The primary purpose of this study was to determine the quantitative changes in PG activity during ripening of dates and also to determine how PG activity is related to the quality of dates. Some chemical and physical analyses were also carried out.

EXPERIMENTAL

Materials

The dates (Deglet Noor variety, *Phoenix dactylifera L.*) used for determination of PG activity during ripening were grown at the USDA Date and Citrus Experiment Station, Indio, California. Samples were harvested during August and September 1967, sorted into 6 maturity groups as follows: (1) Green—fruits approaching maximum size but still green in color; (2) Early Red—color greenish yellow to red; (3) Late Red—color faded red, texture firm; (4) 50% Soft—color light brown, softened to the equatorial region; (5) 100% Soft—color brown, entire fruit softened; (6) Soft—Ripe—color dark brown, smaller in size, wrinkled.

The dates were kept at −20°C until used. The different grades of dates employed were from the 1967 crop of the same variety and were obtained from a commercial source. Wichmann pectic acid used for the substrate of PG assay was prepared according to the procedure of Newbold et al. (1952). Galacturonic acid was recrystallized from an acetone-water solution.

Extraction of PG

Six dates taken randomly from storage were weighed, pitted, sliced into small discs and blended for 2 min in an Omnimixer with 150 ml of 4.0% NaCl solution containing 0.5% polyvinylpyrrolidone (PVP). The mixture was centrifuged at 12,100 *g* for 15 min. The residue was suspended again in 100 ml of the same solution and the extraction was repeated. The combined extracts were dialyzed against running tap water for 18 hr. A small amount of cloudy material which formed during dialysis was removed by filtration through fluted filter paper. The volume of the filtrate was measured and aliquots were taken for assay of PG activity.

In preliminary studies, extractions, as described above, were tried also with various concentrations of NaCl solution. It was found that maximum activity was extracted at a salt concentration of 4.0%. PVP was used to complex tannins and other phenolic compounds to prevent their inhibition of enzymes (Hathaway et al., 1958; Loomis et al., 1966). A level of 0.5% in the solution gave consistent results.

Assay methods

Routinely, enzymic reaction mixtures (10 ml) contained 0.125% Wichmann pectic acid; 0.1 *M* sodium acetate buffer, pH 5.0; and 2.5 or 5.0 ml of the enzymic preparation (0.05–0.5 unit). The mixture was incubated at 29°C. A preliminary study indicated that PG had its optimal activity at pH 5.0.

Release of aldehyde groups during enzymic hydrolysis of pectic acid was measured colorimetrically by the procedure of Hobson (1962). 1 ml of sample was pipetted at 10-min intervals for determination of reducing power. Under these conditions, the rate of reaction was proportional to the enzymic concentration and was linear with time. The activity was determined by measuring the slope of the plot of optical density versus time. One unit of PG activity was defined as the amount of enzyme which catalyzes the production of 1 μmole of reducing groups per minute under the above conditions.

Determination of proteins

Proteins were measured by the micro-Kjeldahl method (Association of Official Agricultural Chemists, 1965). Total proteins were determined by measuring 80% alcohol insoluble nitrogen, whereas soluble proteins were determined by measuring 4.0% NaCl soluble nitrogen.

Moisture

Approximately 5–10 g of the homogenates obtained from 10 pitted dates were placed in a tared aluminum dish, weighed and dried in a vacuum oven at 65°C. The sample was considered dry when the loss of moisture between two successive weighings (4 hr) was not more than 0.1%.

Soluble solids

The remainder of the homogenate from the moisture determination was squeezed through two layers of gauze and the soluble solids of the juice was measured with a Bausch & Lomb Abbe-3 L refractometer.

Sugars

Ten fruits were pitted, ground in a food chopper and mixed. A 10-g sample was weighed and macerated with 200 ml of 80% alcohol and the mixture filtered. The filter cake was re-extracted with 200 ml of the aqueous alcohol and the combined filtrates were used for sugar determinations. Reducing sugars were determined by the hypoiodite method described by Patel et al. (1959). Total sugars were run in the same manner after inversion with invertase. Sucrose was then determined by difference between total and reducing sugars.

Table 1—Description of Deglet Noor dates used for determination of PG activity at different stages of maturity.[1]

Maturity	Fresh wt, g/date	Moisture, %	Soluble solids, %	Protein, mg/date	
				Soluble	Total
Green	12.7	82.3	14.3	12	87
Early Red	12.3	66.0	29.9	24	127
Late Red	14.0	56.4	41.5	39	119
50% Soft	13.4	45.4	54.0	60	109
100% Soft	12.5	41.9	56.4	53	91
Soft-ripe	9.8	28.2	68.6	49	83

[1] Each figure is based on the means of two determinations.

Table 2—Polygalacturonase activity in Deglet Noor dates at different stages of maturity.

Maturity	Units/date[1]	Units/g dry wt	Specific activity	
			Unit/mg soluble protein	Unit/mg total protein
Green	trace	—	—	—
Early Red	0.75	0.18	0.313	0.006
Late Red	14.0	2.3	0.359	0.118
50% Soft	18.2	2.5	0.304	0.167
100% Soft	18.2	2.5	0.344	0.200
Soft-ripe	5.68	0.81	0.165	0.069

[1] Figures are based on the mean of 4 determinations.

Table 3—Description of four different grades of Deglet Noor dates used for determination of PG activity.[1]

Grades	Fresh wt, g/date	Moisture, %	Sugars,[2] %		
			Reducing	Sucrose	Total
Natural	10.5	25.0	37.6	42.4	80.0
Waxy	9.4	19.5	32.7	45.6	78.3
No. 1	7.9	16.4	29.8	48.4	78.2
No. 2	6.9	15.6	30.8	49.5	80.3

[1] Figures are based on the means of 2 determinations.
[2] Dry weight basis.

Table 4—Comparison of polygalacturonase activity in the four grades of Deglet Noor dates.[1]

Grades	Polygalacturonase activity		
	Units/date	Units/g dry wt	Relative activity
Natural	13.2 ± 0.444[1]	1.68	100
Waxy	10.5 ± 1.64	1.39	83
No. 1 dry	7.7 ± 0.775	1.17	70
No. 2 dry	6.2 ± 0.353	1.06	63

[1] Figures are expressed as means plus or minus standard errors of 6-8 determinations.

RESULTS

THE RESULTS of analyses of dates used for determination of PG activity are shown in Table 1. Moisture was highest at the Green stage and decreased as the fruits matured. Soluble solids, on the other hand, increased as maturity progressed. These changes are comparable to those obtained by Sinclair et al. (1941) and Rygg (1946).

The total protein content reached its maximum at the Early Red stage at which time the fruits contained 127 mg per fruit or 8.5 mg/g of fresh weight. As the fruit matured beyond the Early Red stage, the total protein decreased steadily. The soluble protein content, on the other hand, increased as maturity progressed.

The changes in PG activity as a function of maturity are shown in Table 2. PG activity was virtually absent at the Green stage. The activity was low until the fruit reached the Late Red stage at which time the activity rose very sharply and reached its maximum at the 50% Soft stage. The dates contained 18.2 units of PG per fruit at the maximal level.

The specific activity, the ratio of PG activity to total protein, continued to increase from the Early Red stage to 100% Soft stage, whereas the specific activity expressed on a soluble protein basis remained fairly constant during ripening, except the Soft-Ripe stage where a decrease was observed.

To determine whether the tissues after extraction of soluble PG possess any activity due to bound PG, the residue, which had been extracted with 4.0% salt solution, was incubated under the standard conditions and assayed for PG activity. It was found that the extracted residues obtained from the Early Red and 50% Soft stages had no activity.

Softening of dates commences at the apical end and progresses toward the basal (stem) end until the whole fruit is ripe. To determine how PG activity is related to this progression of softening, dates were divided at the equater and the apical and basal halves were analyzed separately for PG activity. Dates which were approximately 10% soft were chosen for this purpose. The apical half contained 0.91 unit per g of fresh weight while the basal half contained only 0.043 unit.

The grades of dates used in this study are described in Table 3. The better grades of dates had a higher percentage of moisture than the lower grades. These values, 25.0–15.6%, are in good agreement with those obtained by Sinclair et al. (1942) and Rygg (1946).

The total sugars comprised 78.2–80.3% of the dry weight. These values are quite comparable to those reported by Sinclair et al. (1942), Rygg (1946) and Cook et al. (1953). The reducing sugars showed greater variation than the total sugars. The reducing sugar content tended to be higher in the higher grades than in the lower grades as noted previously by others.

The results of PG analyses of the four grades of dates are shown in Table 4. Since there were large differences in fresh weight among the dates used, the activity was expressed also on a dry weight basis. PG activity was much higher in soft (natural and waxy) dates than in firm (numbers 1 and 2) dates. The activity in the waxy grade was 83% of that of the natural grade while the number 1 and 2 dry grades were 70 and 63%, respectively.

DISCUSSION

THE DATA SHOW that the Deglet Noor dates used in this study possess polygalacturonase (PG). Although there are slight differences in activity levels among dates used, the values, 0.9–1.3 units per g of fresh weight are higher than those of avocados, pineapples and pears (Hobson, 1962; Reymond et al., 1965), but lower than those of tomatoes (Patel et al., 1960; Hobson, 1962 and 1967) and carrots (Hatanaka et al., 1964).

As shown in Table 4, there was a significant increase in PG activity with ripening. The greatest part of the increase occurred during the period when the fruit ripened from the Early Red to the Late Red stage. This period immediately precedes softening.

This increase in PG activity is most likely due to the conversion of inactive, insoluble protein to soluble, active protein rather than due to a direct synthesis of enzyme. This interpretation is suggested by the data on specific activity which show a direct correlation between PG activity and soluble protein. Also, total protein accumulation ceased before PG activity reached its maximum. However, the possibility that the increase in PG activity might be due to a direct synthesis of en-

zyme cannot be definitely ruled out until further evidence is obtained. Also the possibility cannot be excluded that the development of PG activity might be caused by the disappearance of substances inhibitory to PG.

The total protein content, 127 mg per data at the maximal level, is slightly less than the data reported by Sinclair et al. (1941), but it is in agreement with Mizuno et al. (1958).

PG activity and softening are closely related. This was demonstrated by using samples taken from a single fruit. The slightly softened apical half had PG activity approximately 20 times higher than that of the firm basal half. Reymond et al. (1965) have also observed with avocado fruit that the development of PG activity and softening occurred simultaneously. Hobson (1967) has suggested that PG is the controlling factor in loss of firmness in tomatoes. Although other factors are undoubtedly involved, the present study emphasized the importance of PG in the ripening (softening) of dates.

The mechanism involved in the formation of dry dates during ripening has not been clearly demonstrated. Coggins et al. (1968) have suggested that the creation of dry dates is due to the lack of water supply through the stems to maintain moisture levels high enough to allow enzymes to complete the ripening processes. Data reported by many workers favor this suggestion. According to Sinclair et al. (1941) there is a direct relation between moisture levels and inversion of sugars. That is, the reducing sugars decrease with a decrease in moisture. This was demonstrated by Maier et al. (1961). When dates were treated by an increase in moisture and temperature, the hydrolysis of sucrose by natural and added invertase was accelerated and the dates became much softer.

In addition to the differences in moisture and the composition of sugars between soft and firm dates, Coggins et al. (1967) have shown significant differences in structure of cells and cell walls. Their histological study revealed that firm dates were similar to immature tissues, having rigid and intact cell walls, whereas soft dates lost considerable cell wall structure and contained many broken cells.

It is not clear how PG activity is related to those structural changes and consequently to the creation of different qualities of dates. However, since there is the close correlation between the quality of dates and their PG activity, it appears that this enzyme might be involved, in a way, in controlling the texture of dates.

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Salt and Aging Time Effects on the Viability of Trichinella spiralis in Heavy Dry-cured Hams and Shoulders

SUMMARY—28 pigs weighing approximately 100 lb were dosed with 10,000 to 15,000 excysted trichina larvae, grown to 285 lb and sacrificed. Fresh lean samples from one ham and shoulder per carcass were digested in a pepsine-HCl solution and microscopically examined to determine infection rates. 24 of the more heavily infected hams and shoulders then were selected for further observations. The hams and shoulders were dry-cured for 2 days per lb, using an 8% cure applied in 3 equal applications at 5-day intervals. The cure contained salt, sugar, potassium nitrate and sodium nitrite. After curing, the meat was placed in salt equalization for 30 days, smoked for 24 hr at 100°F and aged at 75°F until termination of the study. Cored lean samples were taken from 8 hams and shoulders at weekly intervals after salt equalization and analyzed for salt concentrations and viability. Viable trichinae persisted throughout curing, salt equalization, smoking and 2 weeks of aging. Samples taken from hams and shoulders after 3 weeks of aging were found to be free of any live trichinae. Similar lean samples were taken at this time and force fed to rats for 5 days. After 8 weeks on a commercial ration the rats were sacrificed, artificially digested and examined. No trichina larvae could be recovered.

INTRODUCTION

CONSIDERABLE interest has developed in areas of the United States in producing quick-aged country style hams on a commercial basis. Acceptable, uniform hams can be produced in periods of 3 to 6 months by using controlled aging temperatures, controlled humidity and less stringent curing procedures than previously used in home-curing methods (Cecil et al. 1954; Kemp et al., 1961; and Skelley et al., 1964).

Federal inspection (U.S.D.A., 1960) requires that dry-cured hams which are not ready-to-eat be processed under regulations which require a severe curing process. This process insures complete destruction of any trichinae by the action of salt in conjunction with time and temperature. These regulations are based on work done by Ransom et al. (1920) and are not conducive to modern commercial production of highly acceptable products. Therefore, this study was designed to evaluate a method of curing and aging which will produce high quality hams and shoulders that are free from disease-producing trichinae.

METHODS

THERE WERE 28 pigs infected by orally dosing each with 10,000 to 15,000 excysted trichina larvae. The hogs were raised to 285 lb and sacrificed. The hams were removed from the carcass and trimmed regular style (skin on). The shoulders were removed from the carcass between the 5th and 6th rib, the neckbone removed, and the shoulder trimmed. After removal from the carcass, each ham and shoulder was individually identified. A lean sample was obtained from the butt of one ham and trimmings of one shoulder of each carcass to determine trichinae infection rates. 24 of the more heavily infected hams and 24 of the more heavily infected shoulders (5 or more larvae per g of fresh lean) were then selected for further studies.

The hams and shoulders were individually weighed and dry-cured at 38°F for 2 days per lb using curing ingredients in the amount of 8% of the fresh weight. The curing mixture contained 73.6% salt, 24.5% white sugar, 1.2% potassium nitrate and 0.6% sodium nitrite and was applied in 3 equal portions at 5-day intervals. After curing, the meat was hung 30 days at 45°F for salt equalization. The hams and shoulders were then soaked for 30 min in lukewarm water to remove excessive salt and mold growth, placed in stockinettes, allowed to dry, and smoked for 24 hr at 100°F. They were then placed in an aging room and aged at 75°F with a relative humidity of 60–65% until the trial was terminated.

Eight hams and 8 shoulders were sampled after salt equalization and at weekly intervals during aging. Lean samples were taken as described and illustrated by Gammon et al. (1968). This procedure consisted of coring into the cushion of the ham and the thickest part of the picnic with a 1⅝" coring device. The outer portion of the sample was discarded. The center portion was designated the outer sample, while the innermost portion adjacent to the bone was designated the inner sample. The holes were then refilled with melted lamb fat to prevent excessive drying and microbial growth. The samples were physically defatted and a 25-g inner sample and a 25-g outer sample were taken for artificial digestion. The remainder was frozen in plastic containers for subsequent salt analysis.

The trichina larvae in the infected meat samples were examined and counted by first excysting them by artificial digestion. A 25-g portion of a defatted sample was finely chopped and placed in a Mason-type quart jar. 50 ml of powdered pepsin, 700 ml of tap water and 8 ml of concentrated HCl were added. The containers then were incubated in a hot water bath at 98°F for 24 hr. The mixture was constantly agitated by forced air during digestion.

After digestion, the mixture was allowed to sit for 30 min in the water bath to allow the excysted larvae to settle to the bottom. Approximately two-thirds of the supernatant was carefully siphoned off the top and the jar refilled to volume with tap water. The mixture was allowed to sit another 30 min. The supernatant was again carefully siphoned off until approximately 100 ml containing the trichina larvae remained. The remainder was shaken well and transferred to volumetric test tubes, allowed to sit for 30 min and siphoned until 25 ml remained.

The test tubes were shaken vigorously to distribute the larvae evenly throughout the mixture. A 1-ml aliquot was then quickly withdrawn from the center of the tube by a pipette and placed on a ruled Syracuse watch glass for microscopic examination. The ex-

Table 1—Mean values and standard deviations for ham samples.

Period	No. tested	Wt. loss %	Wt. loss S.D.	Live trichinae/g of fresh ham No.	Live trichinae/g of fresh ham S.D.	Inner sample Salt %	Inner sample Salt S.D.	Inner sample Live trichinae/g No.	Inner sample Live trichinae/g S.D.	Outer sample Salt %	Outer sample Salt S.D.	Outer sample Live trichina/g No.	Outer sample Live trichina/g S.D.
After salt equalization	8	9.2	1.4	21	18	1.5	0.4	19	14	2.6	1.1	10	8
1 Week aging	8	13.2	2.1	25	13	2.4	0.5	16	13	3.1	0.5	9	11
2 Weeks aging	8	14.8	0.9	24	18	2.6	0.4	8	6	2.7	0.6	3	1
3 Weeks aging	8	15.7	3.4	25	13	2.5	0.6	0	0	4.1	1.7	0	0
4 Weeks aging	8	20.9	3.4	29	21	3.5	1.2	0	0	4.3	1.7	0	0
5 Weeks aging	8	17.9	0.7	23	18	3.8	0.7	0	0	4.9	1.7	0	0

cysted larvae were examined and counted under a Stereozoom Microscope of 10-to 20-power magnification. This procedure was repeated 3 times and the average expressed as the number of larvae per g of lean meat.

Salt concentrations were determined by weighing 15 g of lean meat and blending in a Waring Blendor with 150 ml distilled water for 3 min. The mixture was then filtered through no. 1 filter paper and the filtrate stored in duplicate $2^1/_2$ ml capped vials. Samples were then analyzed on a Technicon Autoanalyzer by modifying an automated procedure by Zall et al. (1956).

When trichinae larvae appeared to be dead or their vitality questionable upon microscopic examination, similar samples of lean were fed to rats. The lean samples were soaked for 2 hr in lukewarm water to remove excessive salt and stop the action of the salt on the larvae. Commercial feed was removed from the rats 18 hr prior to feeding meat samples. Diced meat was then fed for a period of 5 days during which the rats had access only to the meat and water. The animals were than placed back on their commercial ration for 8 weeks, sacrificed, eviscerated and their carcasses artifically digested and examined for trichina larvae.

RESULTS & DISCUSSION

INDIVIDUAL mean values for periods of sampling are given in Tables 1 and 2. The data are given for ham and shoulder inner and outer samples.

The viability of trichinae was not appreciably affected prior to aging in the inner areas of the hams and shoulders. However, the outer samples showed a decline of approximately 48% in viable larvae during these periods. This may be attributed to the higher salt concentrations in the outer samples. The marked increase in percent live trichina larvae in the shoulder inner samples prior to this period can be attributed only to errors in sampling or variations of infections in different muscles. Prior to this period, the hams and shoulders were held at moderately low tempratures (36 and 45°F); and as indicated by previous studies, moderately low temperatures do not decrease appreciably the viability of trichinae (Brandly, 1964; Ransom et al., 1920; Gammon et al., 1968).

Ham and shoulder samples taken after one week aging showed a marked decrease in viable larvae. Ham inner samples decreased to 64% viable larvae, and ham outer samples decreased to 18%. Percent salt concentration for ham inner and outer samples for these periods was 2.4 and 3.1%, respectively. Viable larvae in inner and outer samples of the shoulders during this period decreased to 20 and 4% with respective salt concentrations of 3.3 and 4.3%.

Table 2—Mean values and standard deviations for shoulder samples.

Period	No. tested	Wt. loss %	Wt. loss S.D.	Live trichinae/g fresh shoulder No.	Live trichinae/g fresh shoulder S.D.	Inner sample Salt %	Inner sample Salt S.D.	Inner sample Live trichinae/g No.	Inner sample Live trichinae/g S.D.	Outer sample Salt %	Outer sample Salt S.D.	Outer sample Live trichinae/g No.	Outer sample Live trichinae/g S.D.
After salt equalization	8	7.1	1.2	23	11	2.5	1.0	45	21	4.5	1.2	11	0
1 Week aging	8	14.5	3.5	25	22	3.3	1.2	5	5	4.3	1.5	1	0
2 Weeks aging	8	19.1	0.3	34	2	3.9	0.6	12[1]		5.2	0.9	9[1]	
3 Weeks aging	8	18.0	2.0	22	11	4.6	1.2	0	0	6.0	1.0	0	0
4 Weeks aging	11	18.9	3.4	27	20	6.9	0.7	0	0	7.4	1.4	0	0

[1] Viable larvae in only one shoulder.

Temperatures during this period were increased to 100°F for 24 hr during smoking and 75°F for 1 week during aging. This increase in temperature and increase in salt concentration due to shrinkage are believed to have been largely responsible for the destruction of viable larvae. These results agree closely with those of Gammon et al., (1968) who also noted an increase in percent salt accompanied by a shrinkage of the hams at higher temperatures during smoking which resulted in a decrease of viable larvae.

After 2 weeks aging, the period when live trichinae were last observed, salt concentration, accompanied by shrinkage of hams, increased to 2.6% in the ham inner samples and decreased from 3.1 to 2.7% in the outer samples. This decrease can be attributed only to experimental error. Shoulder inner and outer samples increased in salt concentration to 3.9 and 5.2%, respectively. The percent live trichinae in hams decreased to 33 and 13% for inner and outer samples. The percent viable larvae remained relatively higher in the ham inner samples. Viable larvae were found in only one shoulder during this period.

No viable larvae were found in either hams or shoulders after 3 weeks aging. The mean salt concentrations of inner and outer samples of hams were 3.3 and 4.5% for shoulders. This closely agreed with Gammon et al. (1968) who observed salt concentrations of 3.32 and 4.37% for lighter weight hams and shoulders respectively at one month aging when no viable larvae were observed.

Trichina larvae from lean samples of hams for periods of 3, 4 and 5 weeks' aging produced no infections in rats. Similarly, lean samples from shoulders for periods of 3 and 4 weeks produced no infections in rats after having been force-fed the meat for 5 days.

Trichina larvae excysted from fresh samples and after salt equalization were tightly coiled and considered generally very viable. However, after one week of aging the larvae began to appear in an uncoiled "figure 6" appearance and were considered inanimate or incapable of producing an infection. These characteristics were illustrated by Gammon et al. (1968). After 2 weeks' aging there was a sharp reduction in the total number of inanimate or viable larvae. Many of the dead larvae appeared to contain vacuoles, and the body tissues appeared to be segmented. This was also observed by Ransom et al. (1920). After 3 weeks' aging only a few degenerated larvae could be found.

After 3 weeks' aging the shoulders were sampled an additional week and the hams an additional 2 weeks to confirm prior observations. The remaining larvae appeared to continue to degenerate until it was impossible to find any larvae in many of the meat samples.

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Relationship between Meat Swelling, Viscosity, Extract-release Volume, and Water-holding Capacity in Evaluating Beef Microbial Quality

SUMMARY—This research was undertaken in order to determine the relationship between the phenomena of meat swelling (SW), viscosity (η), extract-release volume (ERV), and water-holding capacity (WHC) as simple and rapid techniques of determining beef microbial quality. These techniques were compared by analyzing beef samples held from freshness to spoilage at refrigerator temperatures, fresh beef homogenized with urea, and beef adjusted to different pH values. In addition, irradiated fresh ground beef samples were inoculated with the normal flora of spoiled beef and analyzed similarly.

With the microbially spoiled meats, the highest degree of correlation occurred between SW and ERV, followed by ERV & WHC, SW & η, η & ERV and SW & WHC. These findings indicate that ERV and SW are quite similar and equally reliable in determining meat microbial quality while the relationships between η and ERV, and η & WHC were of lower orders of significance.

When fresh beef was homogenized with urea at levels between 0.5 and 5M, η correlated best with SW followed by ERV & η, and ERV & SW. Over the pH range 3–11, SW and ERV correlated best followed rather closely by SW and η, and ERV & η.

INTRODUCTION

LABORATORY techniques for determining meat freshness (or degree of spoilage) were first proposed around 1912. Over 40 methods have been proposed since that time based on bacteriological, biochemical, physical and physiochemical conditions and changes brought about by the spoilage flora.

From research carried out in this laboratory, two simple and rapid techniques have been reported, both of which are based upon the change in meat hydration capacity as meat undergoes microbial spoilage.

The first technique is based upon the volume of aqueous extract released by a meat homogenate allowed to filter through Whatman No. 1 paper for 15 min under standard conditions. By this technique, meat of good microbial quality releases large volumes of extract, while meat that is obviously spoiled releases smalled quantities or none under the same conditions. This technique has been designated extract-release volume (ERV), and the effect of various parameters upon ERV have been reported (Jay, 1964; Cook, 1968).

The second technique is based upon the water-holding capacity (WHC) of meats as measured by the modified filter-paper press method of Wierbicki et al. (1958). By this method, fresh beef produces large free-water areas (low water-holding capacity) while spoiled beef produces progressively smaller free-water areas or none (increased water-holding capacity). These two techniques have been compared as meat freshness tests, and with respect to the effect on them of various meat conditions or treatments (Jay, 1966).

Wierbicki et al. (1962, 1963) presented a method of measuring the water-binding capacity of muscle proteins with low water-holding forces which they referred to as meat swelling (SW). Since these authors did not address themselves to the use of this procedure as a meat freshness test, and since this method appears to bear some relationship to ERV, it seemed desirable to investigate the use of this relatively simple method toward these ends.

With respect to the sensory evaluation of meat freshness, a previous study from this laboratory showed that tactile response correlated better with bacterial numbers than either odor or color in spoiling beef (Jay et al., 1964). Since the phenomenon of increased tackiness appeared to be related to beef homogenate viscosity (η), it seemed desirable to investigate η measurements as another possibility of determining meat freshness or spoilage.

EXPERIMENTAL

MEATS EMPLOYED in this study consisted of fat-free portions of semimembranous (SM) and biceps femoris (BF) muscles from a Holstein heifer slaughtered four days earlier and fine-ground using plate with $^1/_8$ in. diameter openings.

Samples of 25 g each were put in 100-ml beakers, covered with aluminum foil and stored at 5°C. Portions of muscle that were not immediately used were frozen to −20°C upon removal from the carcass and later thawed and ground just prior to use. In order to control the numbers of spoilage bacteria in meat samples, appropriate samples were subjected to 0.78 Mrad employing the 10,000 Curie cobalt-60 source at the

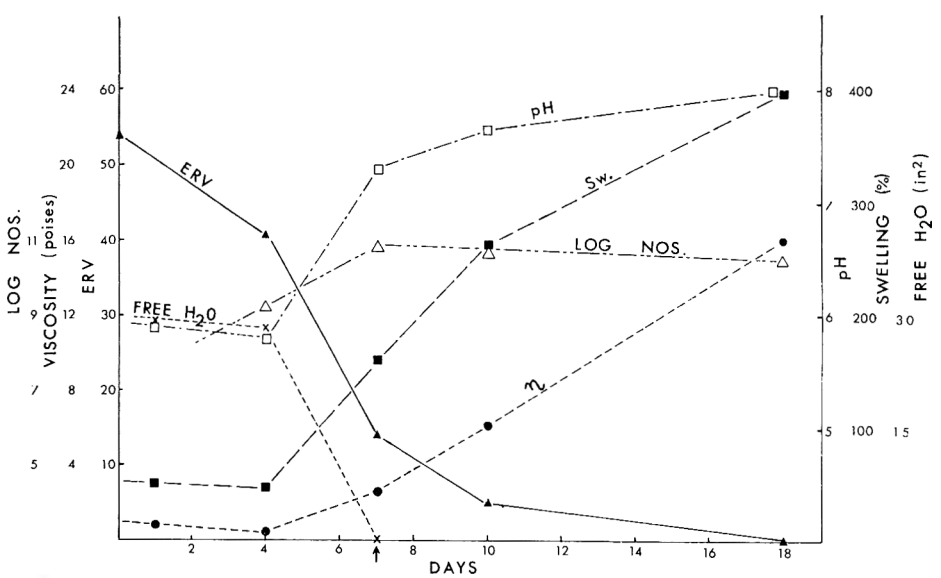

Fig. 1—ERV, WHC, SW, η, log bacterial numbers and pH on ground SM muscle stored at 5°C for 18 days. The day in which off odors were first detectable is indicated by the arrow.

University of Michigan. The irradiated samples were inoculated with the normal flora of spoiled beef by employing spoiled beef homogenates and needle and syringe.

Beef swelling was determined in the following manner, which is essentially the procedure of Wierbicki et al. (1963). Beef samples of 25 g each were blended with 100 ml deionized water for 1 min. Meat particles adhering to the blender walls were swabbed down and blending was continued for an additional one-half min. The water : meat ratio of 4 : 1 was found most suitable for covering the range of SW in beef from freshness to frank spoilage.

Two 35-g samples of the homogenate were weighed into 40 ml centrifuge tubes and centrifuged at 2000 rpm for 15 min. The supernatant was collected in a graduated cylinder and the ml volume recorded. By taking 1 ml supernatant = 1 g, and neglecting the weight of the meat solubles lost with the supernatant, percent swelling was determined as follows:

$$\% \text{ swelling} = \frac{(35 - S - 7)}{7} \times 100 = 400 - 14.3S$$

where S denotes supernatant.

Viscosity was determined by blending 25-g samples of beef for 1½ min (as in SW) and transferring the homogenate to a 160-ml beaker (d = 4.5 cm, L = 11 cm). The beaker was then immersed in a constant temperature bath (Cannon Instruments Co., Model MI) set at 30°C for 8 min. The η of homogenates was determined by means of a Brookfield Model LVT viscometer with readings every minute for five consecutive minutes. The η of the homogenates was taken as the mean value of the five readings.

ERV, WHC, bacterial numbers and pH were determined as previously reported (Jay, 1964, 1965).

RESULTS & DISCUSSION

THE RELATIONSHIP between the techniques of assessing beef freshness is presented in Figure 1 along with meat pH. All data on this and subsequent illustrations represent duplicate determinations. In the case of SW and η, the percent difference between duplicate samples was 0–2.5 and 4–9 respectively. In Figure 1 the meat consisted of SM muscle held for 18 days at 5°C. Between the 4th and 7th days of holding, ERV and free-water area decreased sharply while the other phenomena increased. Organoleptically, this meat was first judged to be undesirable on the 7th day.

A similar relationship is presented in Figure 2 for SM muscle which was subjected to 0.78 Mrad of gamma radiation, and then inoculated with spoiled meat flora. Organoleptically, this meat first displayed signs of microbial spoilage on the 8th day. Of particular interest with this series of samples was the sharp increase in hydration capacity between the 6th and 8th days of holding. The same general magnitude of change was reflected by all six assay methods including pH changes.

Figure 3 presents data showing the same general relationship of ERV, WHC, SW and η to each other employing BF muscle which was kept frozen after removal from carcass, thawed and ground just prior to use. The meat displayed signs of spoilage after 5 days at 5°C, compared to 7 days for the unfrozen portion of the same muscle.

The same general relationship between ERV, WHC, SW, η, pH and bacterial numbers as presented in Figures 1–3 has been shown to hold for fresh and frozen commercial ground beef. While the meats

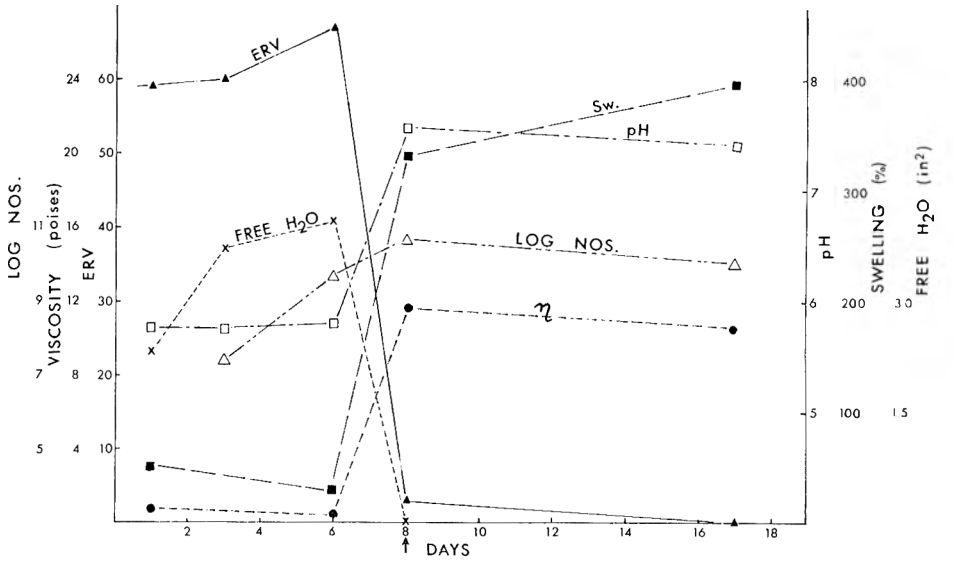

Fig. 2—ERV, WHC, SW, η, log bacterial numbers and pH on irradiated SM muscle inoculated with normal beef spoilage flora and held at 5°C for 17 days. The day in which off odors were first detectable is indicated by the arrow.

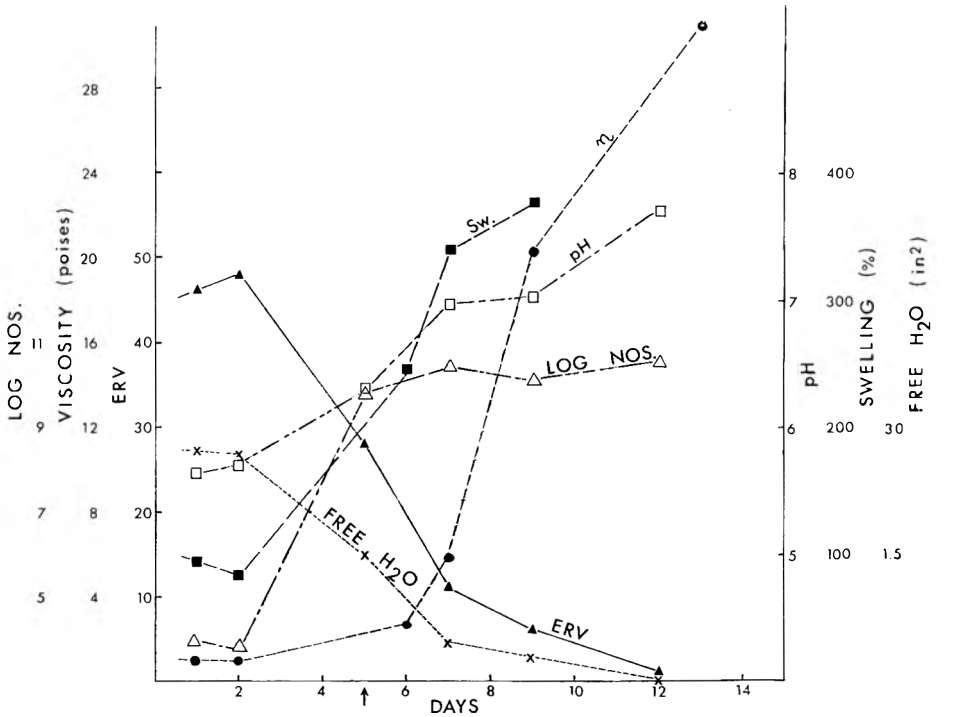

Fig. 3—ERV, WHC, SW, η, log bacterial numbers and pH on frozen BF muscle held at 5°C for 12 days. The arrow denotes the day in which off odors were first detectable.

employed in the present study were all trimmed of visible fat, attempts were made to determine the effect of varying percentages of fat on SW and η. Results were inconsistent due to the fact that fat floats on the surface of SW homogenates making it difficult to accurately measure its volume. With η determinations, large quantities of fat interfere with the viscometer spindle thereby giving readings which fluctuated. The effect of fat on ERV and WHC has already been presented (Jay, 1966).

To determine the specific effect of pH on ERV, SW and η, frozen BF muscle was thawed, ground and blended with dilute HCl and NaOH over the pH range 3–11. The pH of control meat was 5.6. Figure 4 presents plots of ERV, SW and η versus pH which show a singular point that corresponds to the isoelectric point range of meat proteins (*ca.* 5.4). At this point, meat hydration was lowest with around 40% of the bound water usually still retained by the meat. Viscosity at this point was at its minimum (around 10 cp), whereas ERV was at its maximum of around 65.

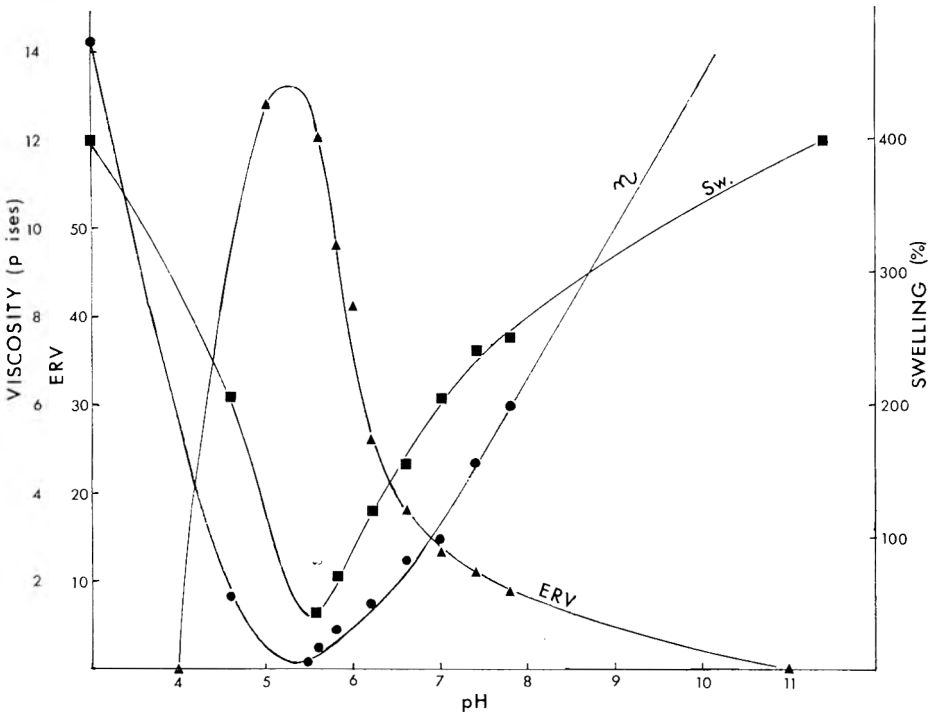

Fig. 4—Effect of beef pH on ERV, SW and η employing fresh SM muscle.

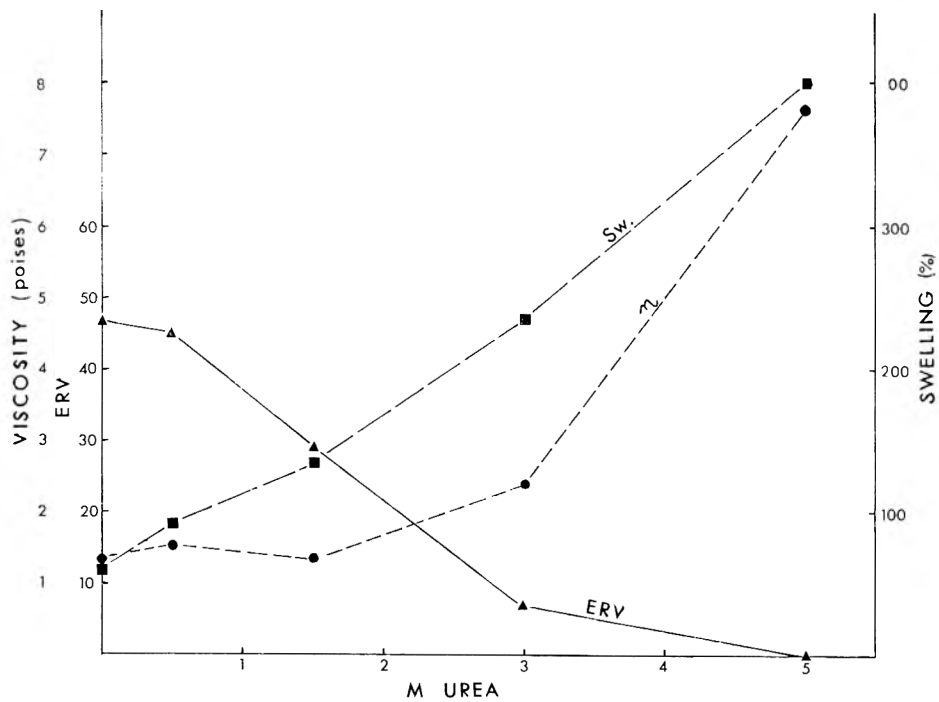

Fig. 5—Effect of urea on ERV, SW and η employing fresh SM muscle. Urea was added to phosphate buffer at pH 5.8.

It is likely that increased dissociation of basic or acidic ionizable meat proteins results in increased SW and η, and decreased ERV values. This behavior may be explained on the assumption that repulsion between protein groups with the same charge causes the space between peptide chains to enlarge and therefore more water can penetrate into the protein matrix (Hamm, 1960).

While pH alone has definite effects upon meat hydration capacity, it can be seen from Figure 4 that at pH 6.0, ERV was around 40 and decreased to around 10 at pH 8.0. From Figure 1 where natural refrigeration spoilage occurred, it can be seen that ERV was around 30 at pH 6.0 but was zero at pH 8.0, thus ruling out the possibility that hydration changes that occur when beef undergoes refrigeration spoilage are due solely to the increase in pH that normally occurs in such meat.

The relationship between ERV, SW and η employing urea-treated beef is presented in Figure 5. All determinations were made by employing urea in phosphate buffer at pH 5.8. As the Molar concentration of urea increased, there was a simultaneous increase in beef hydration capacity as measured by ERV, SW and η. Since urea is known to break hydrogen bonds, it seems plausible that with this action on beef proteins, an increased protein net charge occurred thereby making for the increase in meat hydration.

The statistical correlations (r) between η & SW, and between these phenomena and ERV, WHC, bacterial numbers and pH presented in Figures 1–5 is presented in Table 1. A highly significant negative r was obtained between SW & ERV, suggesting that these two methods measure or respond to the same general changes in meats, although inversely. The r between SW and η was almost as good as the previous. The statistical r between η-pH, and SW-pH was significant above the 1% level in all cases but one. However, no statistical significance was found to exist between η or SW and log bacterial numbers as the first two phenomena continue to increase even after numbers of viable bacteria have reached the de-

clining phase.

Although there is little doubt that bacterial growth is the major cause of meat spoilage, it is possible that the biochemical changes caused by the total number of bacteria are responsible for the increase in SW and η, and that the change in meat hydration is a function of all bacterial cells in spoiling meat, both living and dead.

The use of SW, then, appears to provide a reliable index of meat freshness similar to ERV although not enough data are available at this time to establish an SW value above which beef might be considered microbially spoiled. The technique is rather simple to perform and requires only around 20 min for duplicate samples.

On the other hand, the measurement of η of beef homogenates appears not to be as useful as SW in determining meat freshness. As incipient spoilage sets in, η values continue to increase over a much wider range of time, considerably beyond the time when ERV is zero or SW values are maximum at the water: meat ratio employed in this study. It is conceivable that η of meat homogenates might prove useful in studying various other aspects or treatments of meats.

Table 1 Statistical correlations between η and SW, and between these phenomena and ERV, WHC, bacterial numbers, and pH for meats presented in Figures 1–5.

	Fig. 1	*Fig. 2*	*Fig. 3*	*Fig. 4*	*Fig. 5*
Viscosity					
SW	0.955*	0.976**	0.778**	0.932*	0.918***
ERV	−0.770	−0.992***	−0.842*	−0.801	−0.882***
WHC	−0.642	−0.946**	−0.814	—	—
Log bact. nos.	0.493	0.738	0.631	—	—
pH	0.803	0.997***	0.888	—	—
Swelling					
ERV	−0.882*	−0.994***	−0.999***	−0.940*	−0.796**
WHC	−0.827	−0.947*	−0.996***	—	—
Log bact. nos.	0.683	0.144	0.897*	—	—
pH	0.938*	0.979**	0.997***	—	—
ERV					
WHC	0.965**	0.960**	0.998***	—	—

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
— = no data.

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Identification and Quantitative Estimation of Alcohols in Celery Essential Oil

SUMMARY—The alcohols in celery essential oil obtained from fresh celery by two essence recovery methods were separated by gas chromatography and identified by spectroscopic methods. Thirteen alcohols were reported. Twelve of the 13 alcohols have not been previously reported as constituents of celery essential oil. A semiquantitative estimation of each constituent was made.

INTRODUCTION

CELERY is used for its unique texture and appetizing flavor. Commercially available celery flavor is obtained from celery seed oil. This is different from the flavor of the essential oil derived from the celery plant. Recent advances in techniques, made at this laboratory, made it possible to recover essential oil from fresh celery in high yield, and to conduct a thorough study of its chemical composition for the first time (Wilson et al., 1967).

The purpose of this investigation was to identify and quantitatively estimate the alcohols present in the essential oil obtained from fresh celery.

Gold et al. (1961, 1963) reported results of chemical composition studies on celery essential oil obtained in small quantities by a vacuum essence recovery process. They identified isoamyl alcohol, hexanol and heptanol, and through functional group analysis and GLC retention times indicated the presence of C-3, C-4, C-8, C-9, C-10, C-11, and C-12 normal alcohols.

EXPERIMENTAL

ESSENTIAL OILS used in this investigation were recovered by two different methods. In the first, a batch essence recovery unit was used. Batches of celery puree were steam distilled and the vapors rectified in a packed distillation column (Wilson et al., 1967). In

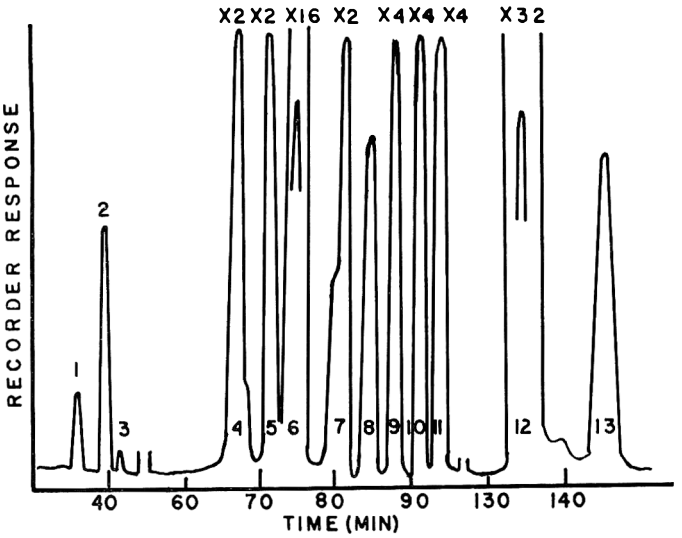

Fig. 1—Gas chromatogram of 30 μl injection of alcohol fractions from essential oil recovered in a continuous essence recovery unit. 1. n-hexanol; 2. cis-3-hexene-1-ol; 3. trans-2-hexene-1-ol; 4. trans-2,8-p-menthadiene-1-ol; 5. cis-2,8-p-menthadiene-1-ol; 6. α-terpineol; 7. dihydrocarveol; 8. trans-1(7)8-p-menthadiene-2-ol; 9. trans-carveol; 10. cis-carveol; 11. cis-1(7)8-p-menthadiene-2-ol; 12. 8(9)-p-menthene-1,2-diol; 13. (isomer) 8(9)-p-menthene-1,2-diol.

the second, a continuous essence recovery unit was employed. Slurried celery puree was pumped into a Turba-Film evaporator and the vapors were rectified in a packed distillation column of different design than the first (Wilson, 1969). The essential oils were separated into functional groups by column chromatography and each fraction was then analyzed separately.

20 ml of each oil was placed in 30 × 2 cm neutral alumina columns (Fisher A-90, 80–200 mesh). The hydrocarbons were eluted with hexane and the carbonyls with 1:1 v/v hexane-diethyl ether. The alcohols were stripped with absolute ethanol. The solvent was removed in vacuo in a rotary evaporator. The alcohol fraction was separated into individual components by temperature-programmed gas chromatography with an F&M, Model 700, dual column chromatograph that was equipped with an F&M, Model 240, temperature programmer. An 18 ft × 1/4 in. OD stainless steel column packed with 25% Carbowax 20M on 60–80 Gas-Chrom P was used to achieve separations.

Samples were temperature-programmed from 100–225°C at 1°C/min. The helium flow rate was 60 ml/min. Identifications were based on comparisons of the isolated compounds with infrared and mass spectra of authentic compounds (Hunter et al., 1966) and (Mitzner et al., 1968). Infrared and mass spectra were taken on a Perkin Elmer, Model 137 Infracord, and the mass spectra were taken on a Bendix Time-of-Flight, Model 3012, mass spectrometer.

RESULTS AND DISCUSSION

COLUMN chromatography indicated that alcohols comprised about 10–15% of the two essential oils. Approximately 3.0 and 2.0 ml of alcohols were separated from the oils recovered by the batch and continuous recovery units, respectively.

Figure 1 is a chromatogram obtained from a 30 μl injection of the alcohol fraction separated from the essential oil recovered in the continuous essence recovery unit. The first three peaks were identified as *n*-hexanol, *cis*-3-hexene-1-ol and *trans*-2-hexene-1-ol. The concentration of these compounds was estimated to be about 0.25, 0.50 and 0.10%, respectively. These three alcohols were also present in essential oil recovered in the batch essence recovery unit. However, the concentration was much higher. The concentration of *n*-hexanol was about 1.0%. *Cis*-3-hexene-1-ol, one of the two most abundant compounds in this fraction, and *trans*-2-hexene-1-ol were estimated at concentrations of 20.0 and 1.0%, respectively.

The difference in concentration of these alcohols in the two essential oils is thought to be caused by their increased solubility in water. In the batch essence recovery method, the aqueous phase was returned to the column head and the more dilute water soluble compounds were redistilled. During the concentration process these water soluble materials were extracted into the organic phase. However, in the continuous essence recovery method, enrichment and extraction of more water-soluble alcohols into the organic phase was not as probable since the aqueous material was continually withdrawn from the oil separator.

Terpene alcohols in the essential oil recovered in the continuous essence recovery unit are represented in Figure 1 by peaks 4 through 13. The fourth and fifth major components were identified as trans-2,8-*p*-menthadiene1-ol and *cis*-2,8-*p*-menthadiene-1-ol. The estimated concentration of each of these two alcohols was about 4.0%. The sixth peak, estimated at approximately 20% concentration, was identified as α-terpineol. The seventh peak, also present in about 4.0% concentration, was identified as dihydrocarveol.

The next four compounds identified, peaks 8, 9, 10 and 11 were *trans*-1(7)8-p-menthadiene-2-ol, *trans*-carveol, *cis*-carveol and *cis*-1(7)8-*p*-menthadiene-2-ol. These alcohols were present in concentrations of approximately 1.5, 8.0, 8.0 and 8.0%, respectively. The last two alcohols shown in Figure 1 were identified as 8(9)-*p*-menthene-1,2-diol isomers. Estimated concentration of these two compounds was about 35.0 and 1.0%, respectively.

The terpene alcohols, represented by peaks 4–12 in Figure 1, were also present in the essential oil recovered by the batch essence recovery unit. The *trans*- and *cis*-2,8-*p*-menthadiene-1-ol isomers were estimated at concentrations of about 2.5% each. The concentration of terpineol, about 0.5%, was reduced about 40-fold. Dihydrocarveol was present in about 1.0% concentration. *Trans*-1(7)8-*p*-menthadiene-2-ol, *trans*-carveol, *cis*-carveol and *cis*-1(7)8-*p*-menthadiene-2-ol were estimated at concentrations of 2.0, 5.0, 7.0 and 8.0%, respectively.

Peak 12, 8(9)-*p*-menthene-1,2-diol, was the most abundant compound in this fraction and constituted about 50% of the alcohols in essential oil recovered by the batch essence recovery unit. Its isomer peak 13 was not present. Compounds reported in this paper, with the exception of *n*-hexanol, were not previously reported as flavor constituents of celery essential oil.

Alcohols reported previously were not isolated from the oil used in this investigation (Gold et al., 1963). However, this does not preclude the existence of these compounds as flavoring constituents of celery since these alcohols were isolated in trace quantities from more volatile fractions collected from a vacuum essence recovery unit. It is quite possible that these compounds will be found in the aqueous essence removed from the continuous recovery unit, or that due to their volatility they were lost during the recovery processes.

The relationship of the alcohols in essential oils to flavor was not established. However, the aroma of alcohol fractions separated by column chromatography was pleasant and the effluent from the gas chromatograph that contained the individual components did not indicate the

presence of undesirable aromas. Although individual compounds did not possess an aroma characteristic of celery per se, it is speculated that the alcohols do complement the flavor and aroma of celery.

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A Comparison of Four Methods for Pastry Tenderness Evaluation

SUMMARY—Four types of pastry were evaluated for tenderness by a trained sensory panel, a Bailey shortometer, a L.E.E.-Kramer shear press, and a "tenderpen" (an objective device designed to evaluate the tenderness of pastry). All the methods of evaluation investigated could detect significant differences in tenderness between the types of pastry used in the study. Highly significant correlations were found between the subjective and objective methods of evaluation examined; the best agreement existed between the results of the organoleptic panel and those of the shear press. The panel and the shear press rated all four pastry types in the same order, the panel and shortometer disagreed on the tenderness of one type, and the tenderpen and panel ranked two of the four types similarly. The shear press was the most precise and the shortometer the least precise of the four methods investigated.

INTRODUCTION

MEASUREMENT of food quality must be both precise and valid in order to evaluate any food attribute fairly. Because of human involvement, subjective methods are often difficult to carry out and tend to lack precision. Objective measurements are usually considered to be more reliable, but can be misleading if results do not agree with those of an organoleptic panel. This investigation was undertaken to compare the evaluation of pastry tenderness by a sensory panel and three mechanical methods.

The Bailey shortometer (Davis, 1921; Bailey, 1934) has been traditionally used for the evaluation of pastry tenderness (Fisher, 1933; Harvey, 1937; Lowe et al., 1938; Hornstein et al., 1943; Swartz, 1943; Rose et al., 1952; Briant et al., 1957; Hirahara et al., 1961; and Matthews et al., 1963).

Harvey (1937) and Briant et al. (1957) reported that the sensory measurement of pastry tenderness was less sensitive than the estimation of tenderness with the Bailey shortometer. Rose et al. (1952) found their sensory panel could detect differences between pastries when the breaking strength was greater than 3 or 4 oz. Hornstein et al. (1943) obtained poor correlation coefficients between the evaluations by an organoleptic panel and those by the shortometer, but Matthews et al. (1963) found high correlation values between these methods.

The L.E.E.-Kramer shear press (Kramer et al., 1951) is a versatile instrument that has been used successfully to evaluate the texture of a variety of products, such as meat (Sharrah et al., 1965), poultry (Wilkinson et al., 1967), fruits (Kanujoso et al., 1967) and vegetables (Lebermann et al., 1968).

Matthews et al. (1963) report good agreement between the shear force of whole biscuits and sensory scores on crumb tenderness. Funk et al. (1965) and Gruber et al. (1966) found high correlations between panel evaluations and shear press measurements of angel cakes and butter cakes, respectively.

EXPERIMENTAL

Preparation of pastry samples

Four different pastries, designated as standard, kneaded, oil and extra fat, were prepared using a standard formula and procedure. The formula used follows: flour, all purpose, 130 g; fat, 65 g; water, 30 g; salt, 2.5 g.

For the oil variation, 58.5 g sunflower seed oil (Sunberta, Western Canada Seed Processors Ltd.) was substituted for 65 g hydrogenated rapeseed oil (Vream, Swift Co. Ltd.), and the extra fat pastry contained an additional 30 g hydrogenated rapeseed oil. Conditions were maintained at 22°C, and sufficient amounts of all ingredients were purchased to complete the 10 replicates carried out.

Pastry samples were prepared by blending the fat or oil and the dry ingredients for 20 sec using an electric mixette (General Electric, Model M7B), low speed. Water was added all at once, and the mixture was combined for 15 sec at low speed. At this point, the kneaded variation was gently squeezed from one hand to the other 25 times.

All doughs were rolled directly onto an aluminum baking sheet (16 × 14 × 1/4 in.), with eight strokes of a stainless steel rolling pin (13 1/2 × 2 in.), to a thickness of 1/8 in. Stainless steel frames ensured consistent thickness of the dough before baking. The pastry was cut into wafers and each wafer was pricked uniformly to prevent blistering.

Baking was accomplished in a standard

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electric range equipped with a temperature controller (Honeywell Corp.) and the baking temperature was 425°F. After baking 17.0 min, the pastry wafers were placed on wire racks to cool. All samples were evaluated within 2 hr of baking.

Sensory evaluation of the samples

A panel of eight, trained to recognize slight differences in pastry tenderness, evaluated the coded samples. Ratings were converted into measurements of tenderness by determining the distance from the lower edge of the scorecard to a horizontal line drawn by the panel member. The greater the distance, the higher the score, and the more tender the sample. The panelists were requested to evaluate tenderness only.

Shortometer evaluation

Eight pastry wafers (1 × $2^1/_2$ in.) of each type were broken on a Bailey shortometer and the results averaged for each replicate. Readings on the shortometer, unlike those of the tenderpen, were found to be independent of sample cross-sectional area; thus, no preliminary measurements were carried out on the samples to be broken by the shortometer.

Shear press evaluation

Pastry samples, $2^1/_2$ × $2^1/_2$ in., were sheared on the L.E.E.-Kramer shear press using the 3,000-lb test ring and a speed of descent of 0.1 mm/sec. Approximately five wafers of each type were sheared. Since the peaks that recorded maximum force were symmetrical, peak heights were converted to lb force/g pastry.

Tenderpen evaluation

The tenderpen, devised to measure pastry tenderness, is shown in Figure 1. When disengaged, the pendulum of the tenderpen swings into the 2 × $^5/_8$ in. pastry sample and breaks it. If the sample is tough, the arc through which the pendulum can travel is decreased. A tender sample will inhibit the swing of the pendulum to a lesser degree. Tenderness values were obtained by subtracting the number of degrees through which the pendulum swung from 100. Therefore, as with the shortometer and shear press, the tougher samples had higher readings.

Preliminary investigations revealed that tenderpen values were highly dependent upon the cross-sectional area of the pastry sample. Therefore, the thickness and width of all pastry wafers to be tested on the tenderpen were measured using a micrometer, and tenderpen values per in. were recorded. Ten wafers of each type were broken and the mean recorded.

Table 1—Tenderness evaluation by the sensory panel and three mechanical instruments for standard, kneaded, oil and extra fat pastries. Means of ten replicates.[1]

Pastry type	Sensory, cm	Shortometer, oz	Shear Press, lb/g	Tenderpen, deg/sq in.
Standard	8.7	8.6	20.7	261.1
Kneaded	9.2	13.7	16.2	299.4
Oil	5.5	14.2	37.1	275.9
Extra fat	12.6	4.4	5.2	213.3

[1] For sensory evaluation and the shortometer, 8 readings comprised 1 replicate, for the shear press, 5 readings made up 1 replicate, and for the tenderpen, 10 readings comprised 1 replicate.

RESULTS & DISCUSSION

EVALUATIONS of the four pastry types by the sensory panel, shortometer, shear press and tenderpen are summarized in Table 1. Analysis of variance and Duncan's multiple range test (Steel et al., 1960) were carried out on the data collected for each of the methods of evaluation; these revealed no significant differences between replicates and highly significant differences between pastry types. Thus, all of the methods of tenderness evaluation under scrutiny were found to be able to significantly differentiate between each of the pastry types used in the study.

Both the sensory and objective methods of evaluation rated the pastry with the extra fat as the most tender. The pastry made with oil was considered to be the toughest by the panel, shortometer and shear press. This is in disagreement with the findings reported by Lowe et al. (1938) and Matthews et al. (1963). The latter reported that pastry containing oil at a level of 45% of the weight of the flour compared in tenderness to pastry with fat at a level of 51% of the weight of the flour; the standard and oil pastries reported here followed these proportions, but all four methods of evaluation rated the oil pastry as being tougher than the standard.

Both the panel and the shear press indicated that the kneaded pastry was more tender than the standard. Hirahara et al. (1961) report the reverse of this, and the shortometer and tenderpen ratings agree with them.

Highly significant correlations (Table 2) were obtained between each of the methods of evaluation examined, but the relationship between the panel and the shear press was the best with a correlation coefficient of −0.92. Examination of the data of Table 1 shows that the panel and

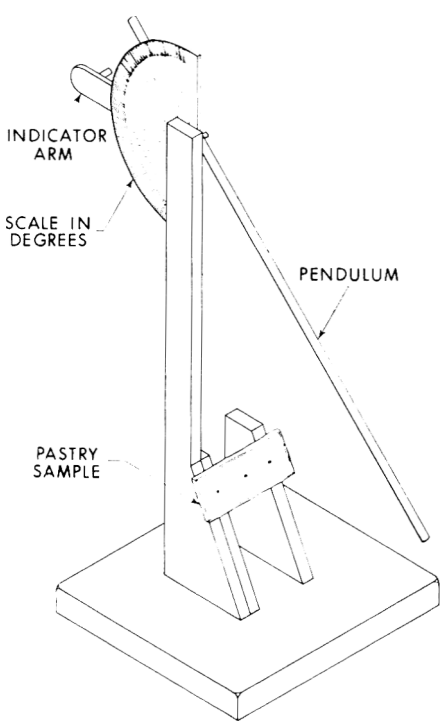

Fig. 1—Tenderpen apparatus showing pendulum in motion.

Table 2—Correlation coefficients between the sensory and objective methods of evaluation for standard, kneaded, oil and extra fat pastry.

Correlation	Correlation coefficient
sensory vs. shortometer	−0.76***
sensory vs. shear press	−0.92***
sensory vs. tenderpen	−0.54***
shortometer vs. shear press	+0.68***
shortometer vs. tenderpen	+0.65***
shear press vs. tenderpen	+0.40**

*** Significant at 0.1% level.
** Significant at 1% level.

Table 3—Relative precision[1] of the sensory panel and three mechanical instruments for the evaluation of pastry tenderness based on 40 paired means.

	Relative precision, %
Sensory	7.9
Shortometer	18.0
Shear press	11.2
Tenderpen	14.6

[1] Relative precision = sd/Rs, where sd = standard deviation of the difference between two duplicates, and Rs = range of the means of the duplicates. A smaller value indicates greater recision.

Table 4—Coefficient of variability[1] of the evaluations by the sensory panel and three mechanical instruments on four pastry types based on each reading obtained over 10 trials.

Pastry type	Sensory	Short-ometer	Shear press	Tender-pen
Standard	27.2	40.2	17.7	20.6
Kneaded	24.9	26.4	11.3	26.1
Oil	48.9	34.8	16.5	21.6
Extra fat	5.5	31.4	25.9	19.7

[1] Coefficient of variability = 100s/x.

shear press ranked all of the pastry types similarly. The correlation coefficient of −0.76 between the panel and the shortometer reflects the disagreement of these two methods in the tenderness of the kneaded pastry samples. The tenderpen readings did not correlate with the panel as well as the other instruments because the tenderpen rated both the kneaded and oil samples differently from the panel.

Hornstein et al. (1943) reported that their judges' scores did not agree with the breaking strengths found by the shortometer. Matthews et al. (1963), however, reported high correlation coefficients between panel scores and shortometer readings. Preliminary investigations and the results of this study indicate that subjective and shortometer ratings seem to agree when a series of related pastry samples is evaluated. Agreement is not always possible, however, when pastries of different textures, such as those used in the present study, are compared.

The relative precision of the sensory panel and each of the three mechanical instruments (Table 3) was determined as outlined by Kramer et al. (1966). The means of the replicates for each pastry type were paired in a predetermined manner and the resulting estimation of relative precision indicates the variability between the means of each replicate. Table 3 shows that of the four methods investigated, the organoleptic panel was the most precise, and measurements with the Bailey shortometer the least. Of the three mechanical instruments, the shear press demonstrated the greatest precision.

Table 4 indicates the variability which occurs in tenderness measurements from one wafer to another for each pastry type and each method of evaluation. The calculation of coefficient of variability is based on the standard deviation and the mean of all the values obtained throughout the ten replicates. It is evident from these data that some methods of evaluation were more variable in the measurement of certain pastry types. For example the shortometer exhibited the greatest degree of variability in the measurement of standard pastry, the panel showed the greatest variability in the measurement of oil pastry, and the shear press was most variable in the measurement of the pastry that contained extra fat.

The panel had no trouble evaluating the tenderness of samples made with extra fat because this product tended to be quite uniform; the oil pastry, however, exhibited greater heterogeneity which made it more difficult to evaluate. The shear press and the tenderpen seemed to show the least variability from sample to sample, the shortometer the most.

It is difficult to compare the results of Tables 3 and 4 because the former measures the variability between means, and information in the latter table shows the variability between individual readings. However, it does appear that evaluations by the shortometer (whether individual or averages) tend to be more variable, whereas those of the shear press are less variable.

All of the mechanical instruments used in this study were destructive in nature. One nondestructive test, using a puncturing device for the estimation of the physical properties of fruits and vegetables (Engineering Research Service, Canada Department of Agriculture) was investigated. Textural differences in the pastry samples were evident in the graphs obtained, but the interpretation of these graphs was difficult. This method requires further investigation.

It appears that of the three objective methods investigated, the L.E.E.-Kramer shear press is the superior device because of its excellent agreement with the sensory panel and its high degree of precision. The use of either the shortometer or tenderpen could be justified in certain circumstances. The shortometer could be used to determine the tenderness of those types of pastry where good agreement with a sensory panel was found.

The tenderpen is inexpensive to construct and might be useful in some type of quality control work where the tenderness of one type of pastry would be measured from day to day or batch to batch. The tenderpen requires only a small sample, and because of its precision, it might prove to be successful in these circumstances.

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Autoxidation of Methyl Linoleate in Freeze-Dried Model Systems. 4. Effects of Metals and of Histidine in the Absence of Water

SUMMARY—*Autoxidation of methyl linoleate dispersed on filter paper was studied in the dry state in the presence and absence of additives. Oxidation was followed volumetrically and by measuring the increase in conjugated dienes. Evaluation of the rate data indicates that added histidine is antioxidant in the very early stages of the oxidation and becomes prooxidant in the later stages. Increasing the concentration of histidine promoted an earlier appearance of its prooxidant effect. Cobalt chloride and manganese sulfate had a pronounced catalytic effect on the oxidation reaction in the model systems buffered to a high (8.0 and 9.0) but not to a low (4.0) pH. Histidine increased the catalytic activity of manganese but eliminated that of cobalt. Thin-layer chromatography showed that conditions under which histidine increased the prooxidant effect of manganese also resulted in the appearance of some decomposition products of histidine. These decomposition products may have catalytic activity.*

INTRODUCTION

THE POROUS structure of a freeze-dehydrated food gives oxygen ready access to its components and promotes oxidative changes, in particular the oxidation of lipids.

Heavy metals, particularly those possessing two or more valency states with a suitable oxidation-reduction potential between them, generally increase the rate of oxidative deterioration of food lipids. The catalytic activity of metals is affected by other food components, including amino acids. Amino acids may also have direct effects on oxidation: Karel et al. (1966a) found that seven amino acids (including histidine) exhibited antioxidant activity in a freeze-dried model system containing methyl linoleate. Marcuse (1960 and 1962) showed that in aqueous systems pH had an influence on the activity of amino acids.

Oxidation and activity of catalysts and inhibitors are also affected by water. The protective effect of water against oxidation of lipid in dry systems has been widely recognized (Salwin, 1959; Koch, 1962; and Karel, 1963). This study of the effects of transition metals and of histidine on the autoxidation of methyl linoleate in a dry model system using filter paper as the solid support was conducted at controlled pH's and in the dry state. A subsequent paper will deal with the effects of water on this system.

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EXPERIMENTAL

Solid support

Ash-free filter paper was chosen as the solid support to simulate the hydrophilic porous structure of freeze-dried foods (Karel. 1960). The filter papers were 4.25 cm in diameter and weighed about 100 mg each. The amount of lipid dispersed on the filter paper was in the range of 80–100 mg/filter paper.

Methyl linoleate

Methyl linoleate of the highest purity commercially available (Hormel Institute, Minneapolis, Minn.) was further purified by double distillation on a 90 × 2.5 cm Vigreux column vacuum distillation apparatus at 5–20 μ Hg. In each of the two successive distillation steps only the constant boiling center cut was collected (Wallace, 1966).

Amino acid

l-Histidine was of the highest grade commercially available (Cal. Biochem., Los Angeles, Calif.). A solution of the amino acid (in base form) was freshly made up in twice-distilled deionized water at the desired concentration for each run. The concentration of histidine is expressed as moles/mole linoleate.

Metal catalysts

Manganese sulfate monohydrate and cobalt chloride hexahydrate were of reagent grade (Malinckrodt Chemical Works, St. Louis, Mo.). Solutions were made up of the metal salts in twice-distilled deionized water, and the concentrations were based on the amount of solution absorbed by each filter paper. The concentration of metal salts is expressed as ppm with respect to the weight of linoleate.

Preparation of the model system

The filter paper was dipped into a buffered aqueous solution containing water-soluble additives. It was then dried in air on a stainless steel wire tray for 10 min and placed in a beaker in a vacuum desiccator. The desiccator was evacuated by pumping with an aspirator for 8 hr. and was then connected to a vacuum pump through a liquid nitrogen trap and the filter paper further dried for 24 hr at a pressure of 5–10 μ. The dehydrated filter paper containing water-soluble additives was then weighed, dipped into the methyl linoleate solution in n-hexane (50–60%) for 10 sec, and dried on a stainless steel wire at room temperature for 5 min. The vacuum dehydration procedure was than repeated.

The weight of the methyl linoleate in each sample was obtained by weighing the filter paper after treating it with additives and drying, then reweighing it after the lipid had been deposited on the filter paper and dried. The average weight of aqueous solution per filter paper prior to dehydration was 352 mg, and the average amount of linoleate per filter paper was 100 mg.

While the amount of linoleate in each filter paper was actually determined, only average values were used for calculation of catalyst to be added. Concentration of catalyst varied up to ±5% between samples. The concentration of additives given in the Results Section is therefore subject to deviations of as much as 10%.

Buffering of additive solutions

Ten ml of additive solution were adjusted to the desired pH by adding a few drops of concentrated hydrochloric acid or 10 N sodium hydroxide solution, depending respectively on whether low pH (<7) or high pH (>7) was required. To this solution 0.5 ml (5%) of a buffer solution was added. The solution was then ready for application.

The buffers used were as follows:

for pH 4.0
12.29 parts of 0.1*M* citric acid
7.71 parts of 0.2*M* disodium phosphate
for pH 8.0
0.55 parts of 0.1*M* citric acid
19.45 parts of 0.2*M* disodium phosphate
for pH 9.0
50 ml of 0.025*M* borax
4.6 ml of 0.1*M* hydrochloric acid.

Table 1—Definitions of oxidation parameters.[1]

t_i (hr)	Induction time = time required to achieve absorption of 5 mM O_2 per mole of linoleate
$K_M \left(\frac{\text{moles } O_2}{\text{mole linoleate}}\right)^{1/2}$ (hr^{-1})	Rate constant in the monomolecular period
K_B (hr^{-1})	Rate constant in the bimolecular period

[1] An increase in t_i indicates inhibition of autoxidation. Increases in K_M and/or K_B indicate acceleration of autoxidation.

Table 2—Effect of the presence of histidine on the oxidation of methyl linoleate in a model system.[1]

	Control	Histidine added (moles/mole linoleate) 10^{-3}	10^{-2}	10^{-1}
K_M				
Average	0.015	0.016	0.023	0.026
Range	0.014–0.016	0.015–0.017	0.016–0.030	0.021–0.034
K_B				
Average	0.068	0.065	0.066	0.074
Range	0.060–0.073	0.060–0.072	0.060–0.083	0.071–0.084

[1] Average of 4 runs.

The pH of each solution was checked both before application to the filter and in a methanolic solution after extraction from the filter paper. Of course, the pH of the dry system in absence of water could not be defined or measured.

Oxidation

Reaction flasks containing the model systems were attached to the manometers of a Gilson differential respirometer, and the oxidation conducted under air at 37°C in a water bath. To eliminate the possibility of oxygen depletion, the reaction flasks were flushed with dry air at suitable intervals.

Oxidation was also followed, using similarly treated samples stored in 50-ml Erlenmeyer flasks, by recording the increase in conjugated hydroperoxides. The conjugated hydroperoxides were measured by UV absorption at 233 mμ (Privett et al., 1962). Extraction of the sample was done using dry methanol.

Thin-layer chromatography

1. Regular TLC chromatographic plates (20 × 20 cm), 250 μ thick, were made in the usual manner using Silica Gel G (Stahl, 1965), then activated for 30 min at 110°C and developed by the usual ascending technique.
2. 10 × 5 μl of 10 ml methanol extract of each sample containing histidine were spotted on the plates using a 5-μl glass capillary.
3. The solvent system consisted of (Oette, 1965) 25 ml of *n*-butanol, 6 ml of glacial acetic acid and 25 ml of redistilled deionized water. The upper layer of this mixture was used for development of the plates in a regular TLC chamber at room temperature (25 ± 1°C).
4. The dried plates were then sprayed with two spray reagents: a 0.3% solution of ninhydrin (1,2,3-triketohydrindene) in *n*-butanol (amino group reagent); and diazotized sulfanilic acid, which consists of 5 ml of 0.9% sulfanilic acid, 5 ml of 5% sodium nitrite and 10 ml of an aqueous solution of 10% sodium carbonate (imidazole group reagent).

RESULTS & DISCUSSION

OXIDATION of methyl linoleate dispersed on filter paper resulted in typical kinetic behavior (Bateman, 1954). There were three stages of autoxidation: an induction period, a period of monomolecular hydroperoxide decomposition, and a rapid oxidation period characterized by bimolecular hydroperoxide decomposition.

The effects of additives were compared primarily by comparing rates of oxidation between different treatments in the same run. In addition, kinetic parameters t_i, K_M and K_B were calculated for comparison between runs. These parameters are defined in Table 1, and the details of the calculation of K_M and K_B were published previously (Maloney et al., 1966).

Effects of histidine

Histidine added to the model system in concentrations of $10^{-2}M$ and $10^{-1}M$ acted as a prooxidant (Table 2), especially during the monomolecular period. Added at a concentration of $10^{-3}M$ it had some slightly prooxidant activity in the monomolecular period, but acted as an antioxidant very early in the oxidation. This trend has been observed throughout the investigation: low histidine concentrations show early antioxidant activity followed by slightly prooxidant activity; higher concentrations show an earlier and more powerful prooxidant effect.

Effects of histidine at very low hydroperoxide concentrations are illustrated by the UV absorption data presented in Table 3. These data also show that histidine at $10^{-3}M$ has antioxidant activity during at least the first 11 hr of oxidation, but at $10^{-1}M$ its prooxidant activity is already apparent at that time.

Table 3—Effect of histidine on the increase in diene conjugation during oxidation of model systems.

Time (hr)	Control	Diene conjugation[1] (mmoles/mole linoleate) 10^{-1}	10^{-3}
0	2.5	1.2	0.7
11	4.0	5.3	2.6
21	6.1	8.4	7.2
33	17.0	35.0	22.5

[1] Moles histidine/mole linoleate.

Karel et al. (1965; 1966a,b) studied the antioxidant effects of 11 amino acids, including histidine, on the oxidation of linoleate in freeze-dried model systems. They found that the overall effect of histidine during the early stages of oxidation was antioxidant, but in some experiments it became a weak prooxidant in later stages of the oxidation. They also observed that lower histidine concentrations showed greater antioxidant activity.

Similar concentration effects were also observed by Marcuse (1961). He found that histidine in emulsions containing linoleate was antioxidant at concentrations of $2 \times 10^{-5}M$ to $2 \times 10^{-3}M$ and prooxidant at concentrations of $2 \times 10^{-2}M$ or higher.

Effects of cobalt and manganese

Addition of cobalt and manganese salts to the model system gave the expected acceleration of the reaction compared to the corresponding control samples. However, this effect was strongly pH-dependent. Figure 1 shows that cobalt chloride had a much stronger catalytic effect on oxidation at pH 8.0 than at pH 4.0. Similar effects of pH on the prooxidant activity of manganese sulfate are shown in Figure 2.

In evaluating the results of several runs it became apparent that the catalytic activity of the metal salts was minimal at low pH and increased with increasing pH. In subsequent runs, therefore, the pH was maintained at the highest feasible level, pH 8.0 for cobalt chloride and pH 9.0 for manganese sulfate. These upper pH limits were set because above them the respective metals precipitated as hydroxides.

The adjustment of the pH of aqueous solutions added to the filter papers had only a slight effect on the rate of oxidation of control samples. The rate was somewhat higher at a pH of either 8 or 9 than at 4. There was no difference between the rates of oxidation at pH 8 and pH 9 in spite of the differences in buffers used (borax-HCl vs. citric-phosphate buffer).

Effects of metal-histidine combinations

In a series of runs the effects of the combined addition of metals and histidine

were studied. The results shown in Figure 3 are typical of those of a large number of other runs in the dry state. They show that histidine *promotes the catalytic activity of manganese* while it *negates the catalytic activity of cobalt.*

In their studies on catalysis of cyclohexane autoxidation by trace metals in the presence of chelating agents Chalk et al. (1957) found that manganese used as heptoate was not affected by sexidentate chelating agents. This is rather unusual since sexidentate chelating agents are able to block catalysis by other transition metals, such as copper and cobalt, by forming octahedral complexes.

However, Calvin et al. (1962) suggested that manganese can form a transient 7 coordinated complex, and this is probably connected with the existence of vacant d- and p-orbitals in ionic manganese. A hybrid dp-orbital would have strong directional properties which probably extend beyond the group in the first coordination sphere. This might allow transient bonding of a hydroperoxide for a period long enough for electron transfer to be probable. In the case of cobalt, iron and copper compounds, d- and p-orbitals are completely filled so that a transient 7 coordination state would not be expected.

It appears, therefore, that the suppression of catalytic activity of cobalt by chelation with histidine and the lack of such suppression in the case of manganese may be explained by the electron configurations of the two metals. This explanation also receives some support from work in humidified systems (Tjhio, 1968), which will be presented in a later paper.

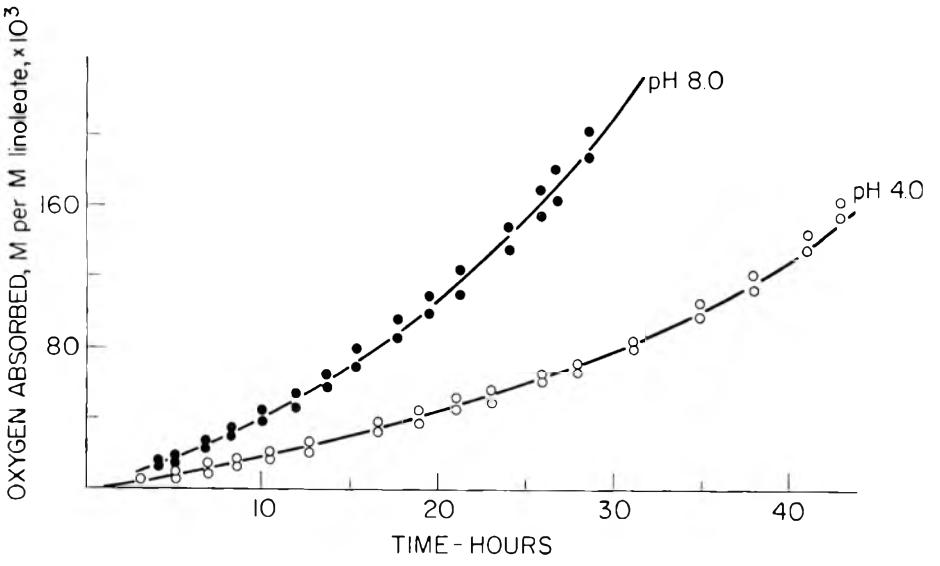

Fig. 1—Effect of pH on the catalytic activity of cobalt chloride (100 ppm Co).

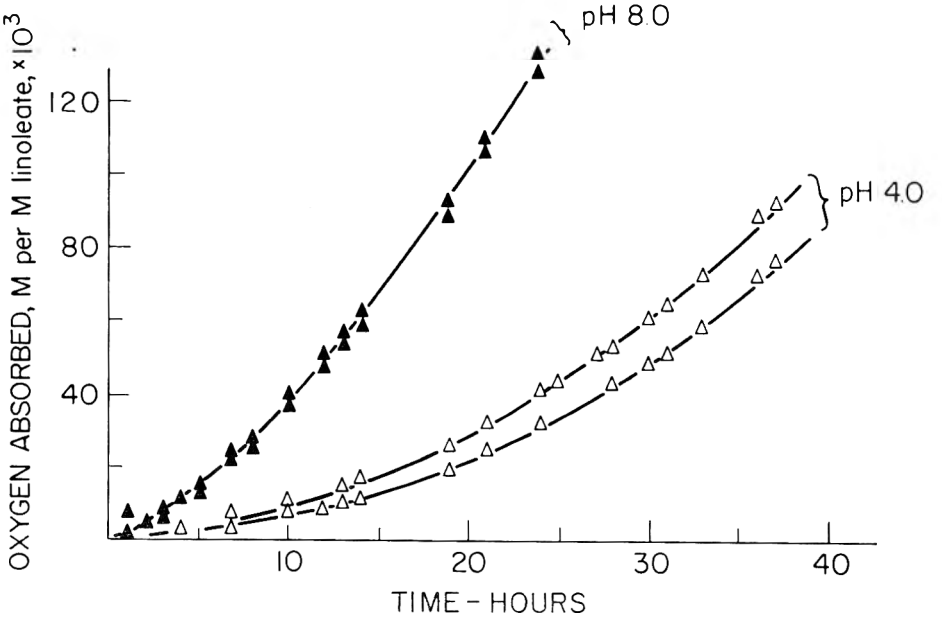

Fig. 2—Effect of pH on the catalytic activity of manganese sulfate (100 ppm Mn).

These differences in properties of the two metals do not, however, explain the observed increase in catalytic activity of manganese as a result of addition of histidine. Manganese-histidine showed a stronger prooxidant effect than manganese by itself. The difference in the catalytic effect may be due to a histidine decomposition product in the manganese-histidine-treated systems. This decomposition product has been qualitatively characterized by thin-layer chromatography.

A schematic diagram of a TLC plate after development is presented in Figure 4. This typical plate shows that manganese-histidine-containing systems at a high pH gave imidazole-containing decomposition products while histidine- and cobalt-histidine-containing systems did not. It is possible that this decomposition product has an additive effect on the prooxidant activity of the manganese-histidine complex.

With respect to the effect of histidine on the catalytic activity of cobalt, Saunders et al. (1962) and Marcuse (unpublished data) found, in their investigations of the effects of histidine in the presence of cobalt on the oxidation of linoleate in emulsion, that histidine in combination with cobalt acted as an antioxidant compared to histidine alone. Hence, their findings are very similar to the results obtained in this study.

Saunders et al. (1962) suggested that cobalt as a histidine complex may form chain-terminating, non-radical decomposition products when it reacts with peroxy radicals; thus, it appears to inhibit the prooxidative action of cobalt. Similar results were also found by Karel et al. (1967).

The results of this study lead to the following conclusions:

1. Histidine in low concentrations has an antioxidant effect in the very early stages of oxidation followed by a slight prooxidant effect in the later stages.
2. At higher concentrations of histidine the prooxidant effect appears earlier and is more pronounced. Therefore, at high concentrations the early antioxidant effect of histidine may often not be apparent.
3. High pH enhances the catalytic activity of manganese and of cobalt in the model system.
4. Histidine promoted the prooxidant activity of manganese.
5. Histidine completely eliminated the catalytic activity of cobalt.
6. The different effects of histidine on cobalt and on manganese may be due to directional properties of the cobalt-histidine and the manganese-histidine com-

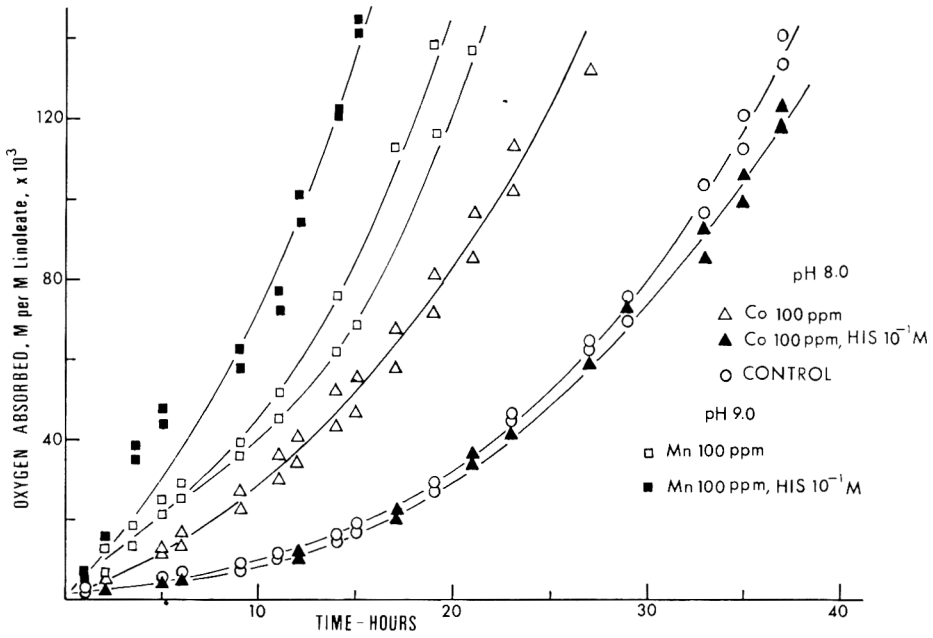

Fig. 3—Effect of histidine on the catalytic activities of cobalt chloride and of manganese sulfate.

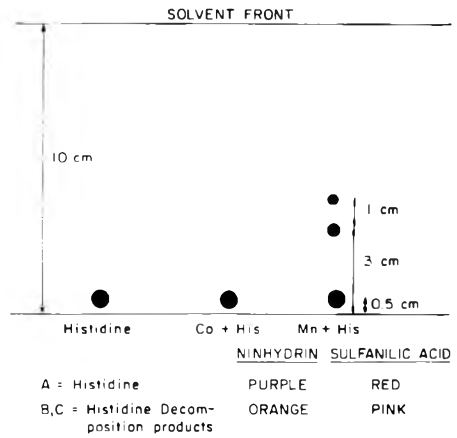

Fig. 4—Schematic representation of TLC plates of histidine and derivatives recovered from oxidized model systems.

plexes.

7. A decomposition product of histidine was formed during oxidation carried out in the presence of manganese. The appearance of this decomposition product coincided with strong prooxidant activity of manganese in combination with histidine.

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Composition of Montmorency Cherry Essence. 2. High-boiling Components

SUMMARY—Volatile components of commercial Montmorency cherry essence boiling above ethanol were extracted from the essence by ether and concentrated by distillation. This concentrate was fractionated by gas chromatography. Individual components were identified using the methods of functional group analysis, gas co-chromatography, infrared analysis and mass spectrometry. The concentrations of the components in the original essence were estimated. The major components identified were n-propyl alcohol, isobutyl alcohol, isoamyl alcohol, and benzaldehyde. Minor components identified include n-butanol, n-hexanol, a hexenol, benzyl alcohol, α-terpineol, furfural, isoprene, myrcene and numerous higher terpenes, methyl benzoate, ethyl benzoate, benzyl acetate, ethyl caprylate, ethyl caprate, n-propyl benzoate, isobutyl benzoate, isoamyl benzoate and di-butyl phthalate.

INTRODUCTION

PREVIOUS WORKERS have identified several compounds with distinctive odors in cherries or cherry products. Mohler (1934) isolated an intermediate boiling fraction from cherry brandy that was considered to have the characteristic aroma of the brandy, as well as a high-boiling fraction that contained high boiling alcohols, benzaldehyde, coumarin and vanillin. Wasser et al. (1937) found terpineol in cherry wine. Nelson et al. (1939) isolated benzaldehyde from Montmorency cherry juice and also a yellow oil with a "lemon" aroma that they considered suggestive of geraniol.

In the first paper of this series Stinson et al. (1969) discussed the composition of the fraction of cherry essence boiling below ethanol. Various components found in this fraction had perceptible odors in the concentrations in which they were present in the original cherry essence. However, the fraction as a whole did not possess the distinctive aroma of cherry essence. This observation led to the belief that much of the distinguishing aroma would be found among the higher boiling components. This paper describes the isolation and identification of some of these higher boiling compounds.

MATERIALS & METHODS

SEPARATION and identification of trace amounts of higher boiling compounds was complicated by the excessive amounts of methanol and ethanol present. This complication was overcome in the present investigation by solvent extraction with ether and concentration by distillation followed by preparative gas chromatography. Identification was chiefly by tandem gas chromatography and mass spectrometry supplemented by chemical methods of analysis and gas co-chromatography.

Cherry essence

The cherry essence used in this study was the same as the material described previously (Stinson et al., 1969). It was a commercial grade 150-fold Montmorency cherry essence with a strong pleasant "cooked cherry" aroma. The product was shipped in 1 gal polyethylene bottles and stored in these containers at 34°C until used.

Preparation of extract for qualitative gas chromatography

Sufficient reagent grade NaCl was added to 1500 ml portions of cherry essence to insure saturation. The mixture was extracted with 400 ml of freshly distilled reagent grade ether in a 2000-ml separatory funnel. The phases were separated and the extraction was repeated using two 300-ml portions of fresh ether. The aqueous layer, after removal of residual ether by distillation, had no perceptible cherry aroma.

The ether extracts were combined, dried with anhydrous Na_2SO_4 and distilled to $^1/_4$ their volume using a 10 plate Oldershaw column. The distillation was continued with a 36 in. glass spiral Widmer column until the temperature at the head of the column reached 36°C. The residue at this point was largely ethanol with smaller amounts of the high-boiling components. Most of the remaining ether and much of the ethanol were removed by continued distillation using an 18 in. spinning band column (Nester/Faust Manufacturing Corp., Newark, Del.). The distillation was stopped when the temperature of the distillate reached 78°C.

The weight of the residue obtained in this manner from 11.7 kg of essence was 7.2 g. or 615 ppm of the original essence. This amount was smaller than the total of the quantities reported in Table 1 (approx. 1000 ppm) due to incomplete removal of low molecular weight alcohols during solvent extraction. The concentrations of the major alcohols listed in Table 1 were based upon analysis of the original essence.

Gas chromatography

Analytical determinations were made with an F & M 720 gas-liquid chromatograph equipped with the F & M 700 module (F & M Corporation, Avondale, Pa.). This permitted the use of either thermal conductivity or flame ionization detectors. Analytical determinations were carried out primarily with the flame ionization detectors and the following columns:

(a) 10% Carbowax 20-M on 80–100 mesh Chromosorb Z, 6 ft × $^1/_8$ in. O.D. (paired columns)
(b) 10% SE-30, ditto
(c) Carbowax 20-M, solid support not known, 50 ft × $^1/_8$ in. O.D. (standard "Hi-Pak" column distributed by the F & M Corp.)

Preparative scale gas chromatographic separations using the thermal conductivity detectors were made to facilitate analysis with the mass spectrometer. The following column was used for this purpose: 20% SE-30 on 45–60 mesh Chromosorb W support, 8 ft × $^1/_4$ in. O.D. The organic compounds emerging from the exit port of the gas chromatograph were divided into five fractions in the basis of order of emergence from the column. The material was condensed by passing the gas through U-tubes immersed in a dry ice-acetone bath. The gas chromatograph (an F & M 810) used in conjunction with the mass spectrometer was equipped with a flame ionization detector and a 1:100 stream splitter. The following columns were used:

(a) 20% SE-30 on 60–80 mesh Chromosorb Z, 8 ft × $^1/_4$ in. O.D.
(b) 20% Carbowax 20-M, ditto

Identification methods

The components of the fractions isolated by gas chromatography were identified by qualitative organic classification tests, mass spectrometric analysis, and gas co-chromatography.

Qualitative organic classification tests were used to indicate the presence of various functional groups. The mixtures were examined by gas chromatography before and after the reaction. Change in the relative peak heights indicated the presence of a particular functional group in the compound responsible for that peak.

The following classification tests described by Howard (1967) were used: carbonyl compounds, 1% hydroxylamine hydrochloride; unsaturated compounds and aldehydes, $KMnO_4$. The acetyl chloride test for alcohols was used to a certain extent but was not entirely satisfactory as extraneous peaks were introduced by the reagent and the ace-

tate derivatives that were formed.

The mass spectrometer used was model 21-103C manufactured by Consolidated Electrodynamics, Inc., Monrovia, California. This was used for the analysis of either pure samples isolated by gas chromatography or for the analysis of compounds present in the gas stream emerging from the gas chromatograph. These were conducted to the mass spectrometer inlet through heated conduit tubes. Identifications were made by matching the spectra of the unknown compounds with the reference spectra published by ASTM and API (Project 44). Only strong spectra showing positive identity were used. The mass spectra of known compounds obtained under similar conditions were compared with the spectra of the unknown compounds in several instances.

Additional information on identity was obtained by gas co-chromatography on both polar (Carbowax 20M) and nonpolar (SE-30) columns.

Quantitative Determinations

The concentrations of the high-boiling components present in the Montmorency cherry essence were determined from their peak heights from direct gas chromatography of the commercial essence. Peak heights rather than peak areas were the parameters measured due to the small concentrations present. The components sufficiently abundant to be determined using the original essence included *n*-propyl alcohol, isobutyl alcohol, *n*-butyl alcohol, isoamyl alcohol, and benzaldehyde. *n*-Pentanol was used as an internal standard for determining the concentrations of these compounds.

Two solutions were prepared, one consisting of cherry essence and a known concentration of standard, and the other consisting of a synthetic mixture containing the compounds known to be present and the same standard. The ratios between the peak height of the compound and the internal standard were calculated for both the cherry essence and the synthetic mixture, and the concentrations were estimated by comparing the values of these two ratios.

Additional concentration was necessary to permit estimation of the concentrations of the minor components indicated in Table 1. A fresh concentrate was prepared under carefully controlled conditions from 100 ml of cherry essence using three successive ether extractions of 35, 25 and 25 ml of solvent. The extracts were combined, dried and concentrated to 1.5 ml by distillation. A standard solution was prepared containing known amounts of *n*-hexyl alcohol, furfural, *n*-heptyl alcohol, and isoamyl benzoate in ethanol.

n-Heptyl alcohol served as an internal standard for the determination of *n*-hexyl alcohol and furfural. Isoamyl benzoate was used as a reference peak for the estimation of the concentrations of the minor components of Fraction V. The concentration of isoamyl benzoate in Fraction V was estimated by comparing its peak height with the peak height of this compound in the standard solution described above. The concentrations of the remaining compounds of Fraction V given in Table 1 were estimated by comparing their peak heights with isoamyl benzoate. The assumption was made that the response of the system to equivalent amounts of the other compounds would be roughly proportional to the response to this compound.

Table 1—Neutral high-boiling components in Montmorency cherry essence.

Fraction no.	Peak no.	Identity	Est. conc. in orig. essence, ppm	Method of identification[2]
1	1	acetaldehyde[2]		MS
	2	acetone[2]		MS
	3	ethyl acetate[2]		MS
	4	ethanol[2]		MS
	5	isoprene (tentative)	(Variable)	MS
	6	1-propanol	120[3]	MS, GC, CHEM
	7	2-methyl-1-propanol (isobutyl alcohol)	90[3]	MS, GC, CHEM
	8	1-butanol	6[3]	MS, GC, CHEM
2	9	3-methyl-1-butanol (isoamyl alcohol)	290[3]	MS, GC, CHEM
3	14	1-hexanol	1.7	MS, GC
	16	a hexenol	Approx. 1	MS
	19	furfural	1.6	MS, GC
4	20	benzaldehyde	490[3]	MS, GC, CHEM
5	23	myrcene	0.1	MS, GC
	25	benzyl alcohol	Combined,	MS, GC
	26	methyl benzoate	Approx. 1.6	MS, GC
	27	Mixt. of terpenes	3.8	MS
	28	ethyl benzoate + benzyl acetate + high mol. wt. material	1.1	MS, GC
	30	α-terpineol + ethyl caprylate + unident. terpenes	1.8	MS, GC
	32	a benzyl alcohol derivative	0.3	MS
	33	*n*-propyl benzoate	0.3	MS, GC
	35	isobutyl benzoate	0.2	MS, GC
	37	ethyl decanoate	0.3	MS, GC
	43	isoamyl benzoate	0.8	MS, GC
	45	"BHT"-(butylated hydroxy-toluene)	1.5	MS
	60	di-butyl-phthalate	0.40	MS

[1] MS = mass spectroscopy; GC = gas chromatography; CHEM = chemical derivatives.
[2] Low-boiling previously identified (see Stinson, et al., 1969).
[3] Concentrations obtained from original cherry essence.

RESULTS & DISCUSSION

A GAS CHROMATOGRAPHIC analysis on the original essence was desired to avoid possible alteration in the ratios of the components present due to the extraction and concentration methods. Although most components were present in too small amounts to be detected, some compounds were sufficiently abundant to be measured without preliminary concentration. Figure 1 indicates the relative quantities of the most abundant materials in the Montmorency cherry essence emerging after ethanol. The conditions selected for this analysis achieved both adequate separation of the first components emerging after ethanol and volatilization of the higher-boiling compounds so that they emerged within a reasonable time.

The first two peaks observed in Figure 1 contained compounds earlier identified in cherry essence by Stinson, et al. (1969): Peak 1, acetaldehyde + other low boiling components; Peak 2, ethanol + methanol. The subsequent peaks were identified as Peak 3, *n*-propyl alcohol; Peak 4, isobutyl alcohol; Peak 5, isoamyl alcohol; and Peak 6, benzaldehyde. Due to the prominence of their peaks, the concentrations of these compounds could be determined from gas chromatography of the original essence.

A minor peak between isobutyl alcohol and isoamyl alcohol (not visible in this diagram) was *n*-butyl alcohol. The diminutive character of this peak as well as the other minor peaks between isoamyl alcohol and benzaldehyde required preliminary concentration (described in Materials and Methods) for their quantitative determination. The heights of the peaks emerging immediately after ethanol are slightly distorted due to trailing by ethanol. Subsequently, the quantitative analysis of these components were conducted at a lower temperature (110°) where separation was more complete and the distortion less significant.

The concentrate prepared for qualitative gas chromatography (7.9 ml of concentrate from 11.7 kg of essence) was separated by preparative gas chromatography into five fractions to facilitate isolation and identification of the components. The fractions were collected in the order in which they emerged from the SE-30 column upon programming at 2°/min between 70° and 230°C. Two very large peaks were collected as Fractions II

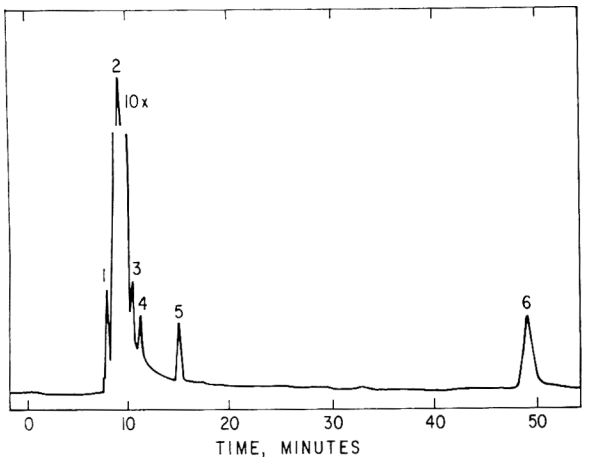

Fig. 1—Chromatogram of original Montmorency cherry essence showing high-boiling components. 50 ft × 1/8 in. Carbowax 20 M column at 140°C.

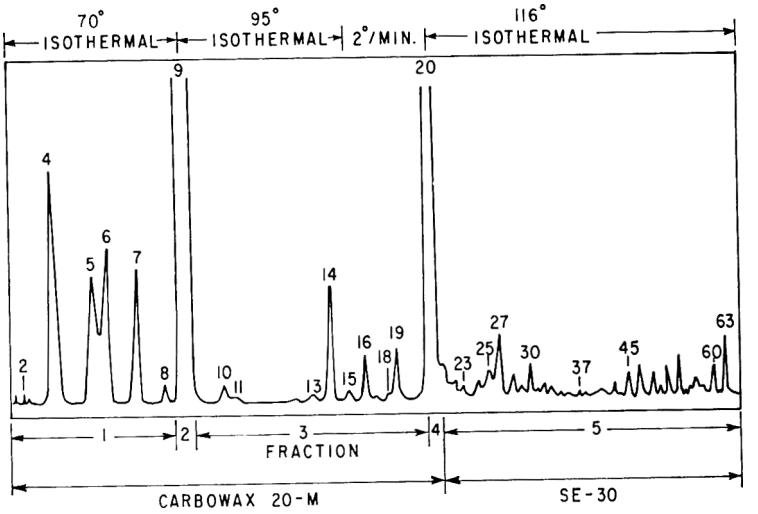

Fig. 2—Composite chromatogram of fractions from concentrated extract of Montmorency cherry essence. 6 ft × 1/8 in. Carbowax 20 M column for Fractions I–IV; 6 ft × 1/8 in. SE-30 column for Fraction V. Temperatures as indicated.

and IV, respectively. Fractions I and V consisted of those materials emerging before the first major peak and after the second major peak, respectively, while Fraction III consisted of the material emerging between the two major peaks. Fractions I to IV were colorless liquids. Fraction V was a yellow oil with an aroma resembling "lemon" or "cooked cherry."

Figure 2 is a composite showing the results of the analysis of these fractions on analytical columns. Columns containing Carbowax 20-M liquid phase were used for analysis of the first four fractions, while columns containing SE-30 were used for the analysis of the fifth fraction. The conditions used are given at the top of Figure 2.

The identification of the various peaks is indicated in Table 1. Trace amounts of acetaldehyde, acetone, ethyl acetate and ethanol were observed in Fraction I. These and other low-boiling compounds were reported earlier by Stinson et al. (1969). The main components of Fraction I were *n*-propyl alcohol, isobutyl alcohol, and isoamyl alcohol, together with a small quantity of *n*-butyl alcohol. Peak 5 gave a mass spectral pattern suggestive of methyl butadiene (isoprene). This peak was absent in the gas chromatography of the original cherry essence. This compound may be the result of thermal degradation of heat sensitive materials, possibly terpenes, during distillation and preparative gas chromatography. Brewster (1953) described the pyrolysis of a terpene to yield isoprene. No estimate was made of the concentration of the hydrocarbon in cherry essence.

Fraction II consisted almost entirely of isoamyl alcohol, but also contained trace amounts of the components of the preceding and following fractions.

Fraction III contained the components emerging between isoamyl alcohol and benzaldehyde. The compounds definitely identified in this region included *n*-hexyl alcohol and furfural. Another peak was tentatively identified on the basis of mass spectral analysis as a hexenol-1.

Fraction IV consisted almost entirely of benzaldehyde with trace amounts of the components of the preceding and following fractions. Gas chromatographic analysis of Fraction V revealed 42 peaks. Mass spectral analysis indicated that many, if not most, of these peaks contained more than one component.

Benzoic acid, not shown in the table, was identified in the benzaldehyde fraction (Fraction IV) where it may have been formed as the result of oxidation of the benzaldehyde. The presence of this compound in the original essence has not been confirmed. Two compounds, butylated hydroxytoluene (BHT) and di-butylphthalate, peaks 45 and 60, may be artifacts as these compounds are used in industry as an anti-oxidant and a plasticizer for plastics. Current investigations in our laboratory on untreated cherry juice is expected to establish whether these compounds are present in the original fruit.

It is not possible at this point to assign responsibility for the characteristic Montmorency cherry flavor to specific compounds. Many of the minor components of Fractions III and V were observed to have intense odors. Identification of these minor components is in progress. Work is also in progress on changes in composition during processing.

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Mention of company or trade names does not imply endorsement by the U.S. Department of Agriculture over others not named.

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Water Extractable Proteins of Porcine Muscle

SUMMARY—*The study was performed on 31 samples of porcine muscle (m. longissimus dorsi) of different qualities, ranging from extremely pale, soft and exudative (PSE) to dark red in color, firm in structure and dry in appearance (DFD) in order to determine the water solubility of muscle proteins 24 hr post mortem. It was found that the water solubility of muscle proteins depends on the quality of the muscle, and is lowest in extremely PSE muscle. It was demonstrated, with the aid of electrophoresis on agar and acrylamide gels, that some sarcoplasmic proteins which undergo precipitation 30 min after adding a citrate-phosphate buffer (pH 4.6) at temperature of 20°C, are extracted in smaller amounts from PSE muscle than from normal muscle. One of these fractions was benzidine positive.*

INTRODUCTION

THE APPEARANCE of pale, soft and exudative (PSE) porcine muscle is connected with a rapidly progressing glycolysis after slaughter (Wismer-Pedersen, 1959; Lawrie, 1960; Briskey et al., 1961; Bendall et al., 1963; Charpentier et al., 1963; Sayre et al., 1963b; Fujimaki, 1964; Kastenschmidt et al., 1968).

A low pH and high temperature in muscles post mortem is accompanied by a considerable loss of solubility of sarcoplasmic and of myofibrillar proteins (Bendall et al., 1962; McLoughlin, 1963; Sayre et al., 1963a; Scopes, 1964; Topel et al., 1967).

Bendall et al. (1962) suggest that the PSE muscle morphology is connected with the denaturation and precipitation of sarcoplasmic proteins on the myofibrils, causing them to become insoluble in buffers with a high ion concentration. The results obtained by McLoughlin et al. (1963) and Goldspink et al. (1964) also indicate that in the conditions of low pH and high temperature in the muscles post mortem the sarcoplasmic proteins may be subject to denaturation and precipitation.

It is not yet known whether all sacroplasmic proteins are subject to partial denaturation at the same rate, or whether only some of these are especially susceptible to denaturation. Charpentier et al. (1963), studying electrophoretically the sarcoplasmic protein composition of PSE and normal muscle, did not observe any differences in the obtained electropherograms.

Scopes et al. (1963) demonstrated that a protein fraction of the sarcoplasm with creatine phosphotransferase activity decreased as a result of high temperature and low pH in the muscle. Charpentier et al. (1964) also showed differences in the density of isoenzymes of the lactate dehydrogenase of the sarcoplasm of PSE muscle in comparison with the sarcoplasm of normal muscle.

Hart (1962) ascertained that 30 min after treating the water extract of normal muscle with a citrate-phosphate buffer (pH 7.6) a considerable turbidity resulted from the coagulation and precipitation of protein, while the turbidity of the water extract of PSE muscle was small. There were very significant differences between the turbidity rate of the water extract of normal and PSE muscle. Vold (1965), Kolczak (1966) and Ockerman et al. (1968) obtained similar results.

The aim of the present work was: (1) to investigate the water solubility of muscle proteins, from extremely PSE to DFD muscle; (2) to find what percentage of water soluble proteins are precipitated in a citrate-phosphate buffer (pH 4.6); (3) to ascertain which of the water extractable protein fractions are precipitated; (4) to study qualitatively the composition of proteins in the water extract of PSE and normal muscle.

EXPERIMENTAL

Material

The experiments were carried out on muscles of pigs from the Progeny Testing Station in Chorzelów. The samples were taken from Large White and Landrace pigs. No distinction between these two breeds was made in this paper. The only consideration was the quality of meat. The methods of feeding, slaughter and of preserving the carcass were patterned after that of the Progeny Testing Station and were the same for all pigs (Kielanowski et al., 1957). The pigs were slaughtered after reaching a weight of 96 kg. Samples of muscle (m. longissimus dorsi) of different qualities, from extremely pale, soft and exudative (PSE) muscle to dark red in color, firm in structure and dry in appearance (DFD) muscle were collected from 31 pigs, 24 hr post mortem.

The quality of the muscle was determined on the basis of the following tests and measurements: (a) of the relative turbidity of water extract of the muscle in a citrate-phosphate buffer (pH 4.6) as outlined by Hart (1962) according to the procedure of Kolczak (1966); (b) of the pH of the longissimus dorsi muscle 45 min post mortem, using the Radiometer 12 b with spear electrodes (glass type G 213 C, and calomel type K 4111); (c) an expressible juice determination according to the method presented by Briskey et al. (1959); (d) subjective texture of PSE in the muscle 24 hr post mortem, according to Kolczak (1966).

The samples of muscle were collected behind the last thoratic vertebra, then frozen and kept on solid carbon dioxide for 4 days at the most.

Procedure

Frozen samples of muscle of a determined quality were cut into sections 30 μ thick on a freezing microtome. 10-g pieces of the frozen sections were extracted with distilled water filled up to a total volume of 30 ml, during 24 hr in a temperature of 2°C. They were subsequently centrifuged for 20 min at 800 G and filtered. Thus a muscle water extract was obtained.

In the water extract the amount of the total and nonprotein nitrogen was determined. The quantity of nonprotein nitrogen was measured after trichloroacetic acid (20 %) precipitation. The quantity of the protein nitrogen was calculated as the difference between the amounts of total and nonprotein nitrogen.

Two test tubes were filled with 1 ml of water extract of the muscle and 5 ml citrate-phosphate buffer (pH 4.6), and left for 30 min in a temperature of 20°C. Both test tubes filled with the turbid solution were cooled in ice water. The value of turbidity was noted for one of the test tubes. The second was centrifuged 3 min at 1,500 G in a low temperature. The supernatant was poured off, the sediment rinsed twice in a citrate-phosphate buffer (pH 4.6) and then treated with H_2SO_4 after which the nitrogen content was determined.

The supernatant was prepared for electrophoretic investigation in the following manner: 4 ml of citrate-phosphate buffer (pH 4.6) was added to 4 ml of the muscle water extract and treated as above. However, immediately after cooling and centrifuging the supernatant was poured off and frozen on solid carbon dioxide. Before the electrophoretic run the supernatant was defrosted

Table 1—Nitrogen compounds of the water extract of porcine muscle (m. longissimus dorsi).

Relative[1] turbidity	pH[2]	Number of pigs	mg Nitrogen/ml of water extract				
			Total	Protein			Non-protein
				Total	Precipitated[3]	Non-precipitated[3]	
<300	5.15	4	3.40	1.93	0.06	1.87	1.47
300–600	5.62	5	3.69	2.25	0.14	2.11	1.44
600–900	5.80	4	3.71	2.23	0.23	2.00	1.40
900–1200	6.24	2	3.97	2.56	0.32	2.24	1.41
1,200–1,500	6.48	4	4.23	2.71	0.41	2.30	1.52
1,500–1,800	6.44	6	4.14	2.68	0.46	2.22	1.46
1,800–2,100	6.50	4	4.26	2.78	0.56	2.22	1.48
>2100	6.76	2	4.32	2.95	0.59	2.36	1.37

[1] Relative turbidity as outlined by Hart (1962) according to the procedure of Kolczak (1966), [2] average pH value of m. longissimus dorsi, 45 min post mortem, [3] protein precipitated during 30 min with a citrate-phosphate buffer (pH 4.6) at a temperature of 20°C.

Table 2—Correlation coefficients between the relative turbidity and nitrogen compounds of the water extract of porcine muscle (31 pigs).

	Correlation coefficient
N Protein precipitated	+0.99**
Total N protein	+0.91**
Total N	+0.90**
N Protein nonprecipitated	+0.67**
N Nonprotein	−0.05

** $P < 0.01$.

and immediately analyzed.

The amount of nitrogen was determined by Kjeldahl's micromethod. In some cases (for acrylamide gel disc electrophoresis) the protein level was measured according to the biuret reaction with Benedict's reagent (Bailey, 1962).

Electrophoresis

The electrophoretical analysis of proteins of the water extracts and of the supernatants of 8 samples from extremely PSE muscle and of 7 samples from normal muscle were carried out. Electrophoresis was performed using two kinds of gels, i.e., agar and acrylamide gels.

Agar gel electrophoresis was carried out according to the method of Grabar et al. (1953, 1955) with Scheidegger's modification (1955). Bacto-agar (Difco, Detroit) was purified using the technique described by Grabar et al. (1960). 1% agar gel in sodium veronal buffer (pH 8.2, ionic strength 0.025) was used. Electrophoresis was carried out during 2½ hr, employing in the reservoirs the same buffer but of double concentration, the gel potential differences being of the order of 6 to 8 V/cm. The glass plates covered with agar were stained after the electrophoresis with a 0.1% solution of amino-black 10 B, or with 1% alcohol solution of benzidine, according to the method of Uriel (1960). The density of protein fractions of the electropherograms was delineated by means of an automatic densitometer MGF, Berlin.

Acrylamide gel disc electrophoresis was carried out by means of the Ornstein (1964) and Davis (1964) method. For the polymerization of 7.5% acrylamide gels a set of reagents from K. Light, Colnbrook, Bucks, was used. The solutions subjected to an electrophoretic run were placed on the surface of the gel in quantities from 10 to 40 µl (in relation to the protein concentration). The electrophoresis was carried out during 1 hr in a temperature of 4°C, with a potential difference of the order of 200 V. After the electrophoresis, the gels were removed from the tubes and stained with a 1% solution of amido-black 10 B in 7% acetic acid for more than 10 hr. They were rinsed with water and 7% acetic acid, and the excess coloring matter was electrophoretically removed by means of 7% acetic acid (Reisfeld et al., 1962).

RESULTS & DISCUSSION

Water solubility of muscle proteins

The nitrogen compounds of the water extract of muscle of different quality is presented in Table 1. Results were related to the relative turbidity and to the pH of the muscle. The amount of nonprecipitated protein in the water extract was calculated by subtracting the protein precipitated in a citrate-phosphate buffer (pH 4.6) from the value of total protein in the water extract of the muscle.

The water solubility of muscle protein depends on the quality of the muscle and is lowest in the extremely pale, soft and exudative (PSE) muscle. The amount of protein subject to precipitation during 30 min in a citrate-phosphate buffer (pH 4.6) at a temperature of 20°C has a considerable influence on the total amount of protein extracted from the muscle. While about 20% of protein of the muscle water extract with a good morphology is subject to precipitation, only a very small amount of protein is precipitated from the water extract of PSE muscle in the same conditions.

The amount of nonprecipitated protein in a citrate-phosphate buffer (pH 4.6)

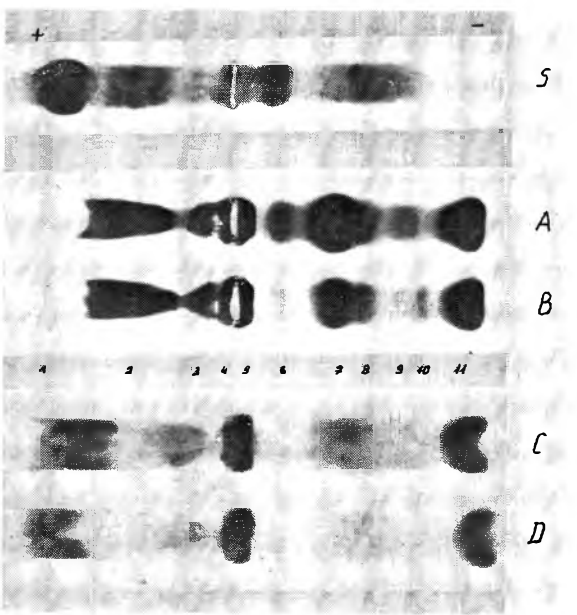

Fig. 1—Agar gel electrophoretic protein patterns. S—pig serum, A, B, C, D—water extracts of porcine longissimus dorsi muscles 24 hr post mortem. Equal volumes of solutions were disposed. A—water extract of normal muscle (relative turbidity = 1852, muscle pH, 45 min post mortem = 6.79). B—water extract of PSE muscle (relative turbidity = 197, muscle pH, 45 min post mortem = 5.03). C and D—water extracts of normal muscle (sample A) and PSE muscle (sample B), respectively, after precipitation of protein during 30 min with an equal volume of citrate-phosphate buffer (pH 4.6) at a temperature of 20°C. Electropherograms stained with amido-black 10 B.

during 30 min also depends on the quality of the muscle, although to a much smaller degree. This interdependence was confirmed by calculating simple correlation coefficients between the nitrogen compounds of water extract of muscle and relative turbidity (Table 2).

Hence, it is plausible to assume that the proteins of water extract of muscle which are subject to precipitation in a citrate-phosphate buffer (pH 4.6) are not extracted from PSE porcine muscle. To verify this supposition, electrophoresis on agar and acrylamide gels was carried out.

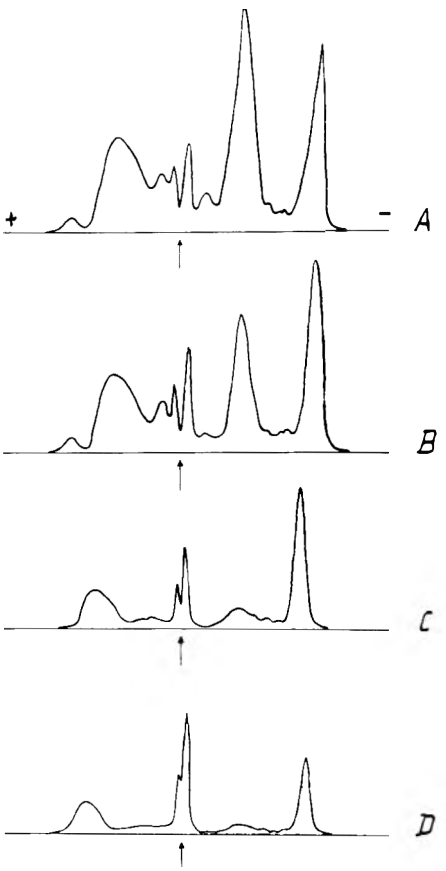

Fig. 2—The densitometric curves of the electropherograms of water protein extracts of muscle presented in Fig. 1. A and B—water extracts of normal and PSE muscle, respectively, C and D—water extracts of normal muscle (sample A) and PSE muscle (sample B), respectively, after precipitation of protein with an equal volume of citrate-phosphate buffer (pH 4.6).

Agar gel electrophoresis

Identical volumes of solutions were subjected to electrophoretic migration. Electrophoretic patterns are presented in Figure 1. The proteins of muscle water extracts were compared with pig serum proteins.

Eleven distinct protein fractions were observed in the water extract of the muscle, four of them migrating toward the anode and seven toward the cathode. No qualitative differences in the electrophoretic protein patterns between the water extract of normal muscle and that of PSE muscle were observed, although the total amount of protein subjected to electrophoretic migration was considerably smaller in the water extract of PSE muscle. However, the relative densities of protein fractions migrating toward the cathode and marked as numbers 6 and 7, were considerably smaller in all investigated water extracts of PSE muscle.

When comparing the electrophoretic distributions of proteins of the water extracts of normal and PSE muscle, before and after protein precipitation in a citrate-phosphate buffer (pH 4.6), the protein fraction No. 6 usually disappears and the extensive protein fraction No. 7 is always in a much smaller amount.

The densitometric curves of protein fractions in electropherograms of water extracts of normal and PSE muscle, before and after protein precipitation during 30 min in a citrate-phosphate buffer (pH 4.6) at a temperature of 20°C, are presented in Figure 2.

The protein fractions migrating toward the cathode, marked 6 and 7, appear in the water extract of PSE muscle in considerably smaller quantities than in the water extract of the normal muscle, and they are subject to precipitation in the citrate-phosphate buffer (pH 4,6).

After electrophoresis, some agar gels were stained with the benzidine technique to localize benzidine positive substances in the electropherograms of proteins. The presence of two fractions was established (Fig. 3), one of them extensive and possessing the mobility of the protein fraction No. 6 of the muscle water extract and the second one, not noticeable in Figure 3, present in a small amount, with a slightly greater mobility, between fractions 6 and 7. Wieme (1965) obtained similar results when localizing the benzidine positive substances in water extracts of human skeletal muscles. Water extracts of normal and PSE muscle after protein precipitation in citrate-phosphate buffer (pH 4.6) demonstrated only a slight positive reaction with benzidine.

Acrylamide gel disc electrophoresis

Solutions with a similar concentration of protein were subjected to an electrophoretical distribution. The electrophoretic patterns are presented in Figure 4. A diagram of relative densities of protein fractions is given with each electropherogram.

As in the electrophoresis on agar gel,

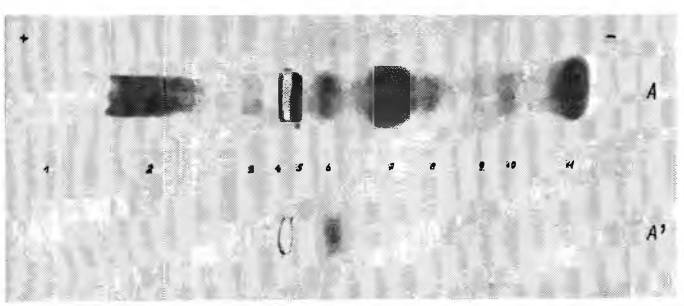

Fig. 3—Agar gel electrophoretic patterns of water extractable proteins of normal porcine longissimus dorsi muscle. A—stained with amido-black 10 B. A'—stained with benzidine.

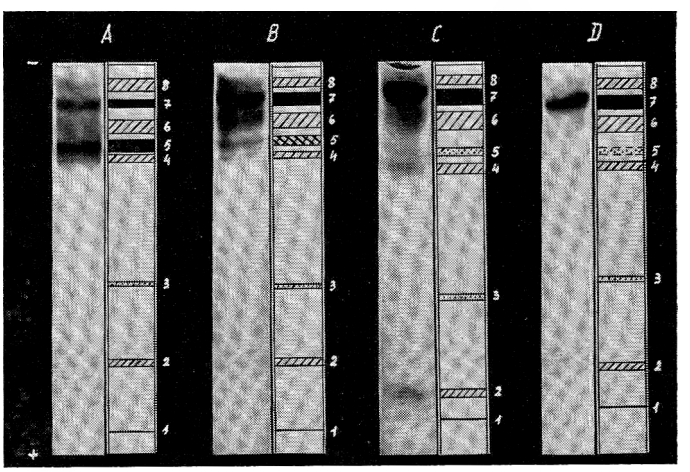

Fig. 4—Acrylamide gel disc electrophoretic patterns of water protein extracts of porcine longissimus dorsi muscle, 24 hr post mortem. Solutions with a similar concentration of protein were analyzed. A—water extract of normal muscle (relative turbidity = 1949, muscle pH 45 min post mortem = 6.81). B—water extract of PSE muscle (relative turbidity = 217, muscle pH 45 min post mortem = 5.21). C and D—water extracts of normal muscle (sample A) and PSE muscle (sample B), respectively, after precipitation of protein during 30 min with an equal volume of citrate-phosphate buffer (pH 4.6) at a temperature of 20°C. Electropherograms stained with amido-black 10 B.

nonqualitative differences were observed in the disc electrophoretic protein patterns of the water extracts of normal and PSE muscle. In a similar concentration of protein a much smaller density of the protein fraction No. 5 in the water extract of PSE muscle can be seen. Variations in the densities of fraction No. 5 were observed in all investigated water extracts of PSE muscle. The precipitation of a part of the protein of the water extract during 30 min in a citrate-phosphate buffer (pH 4.6) at the temperature of 20°C demonstrated that the protein fraction No. 5 was subjected to precipitation.

The results obtained in the present paper indicate that the water solubility of sarcoplasmic protein of PSE muscle is considerably diminished. The differences in the turbidity value (transmission value) between water extracts of normal and PSE muscle in the citrate-phosphate buffer (Hart, 1962; Vold, 1965; Kolczak, 1966; Ockerman et al., 1968) may result from denaturation and a loss of solubility of certain sarcoplasmic proteins, especially susceptible to denaturation, which are not extracted from PSE porcine muscle.

It is suggested that a reduced solubility of sarcoplasmic proteins may be the result of their denaturation and precipitation as a consequence of low pH and high temperature in muscles post-mortem (Bendall et al., 1962; Lawrie et al., 1963; Goldspink et al., 1964) or a low pH at onset of rigor (Sayre et al., 1963a; Briskey, 1964; Sayre et al., 1964).

Considering results obtained in the present work, it seems that not all of the sarcoplasmic proteins are subject to denaturation to the same degree in PSE muscle. This is in accordance with results obtained by Scopes et al. (1963). The sarcoplasmic protein fractions, which are precipitated with a citrate-phosphate buffer (pH 4.6) according to the method of Hart (1962), are extracted in a much smaller degree from PSE muscle. One of these fractions is benzidine positive. The second fraction, however, which is one of the basic protein fractions of the sarcoplasm, was not identified.

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Influence of Organophosphate Insecticides on Ascorbic Acid Oxidation in Aqueous Systems

SUMMARY—Aqueous systems at four pH's containing ascorbic acid and various levels of organophosphate insecticides were prepared. The rate of ascorbic acid oxidation was measured polarographically. Organophosphate insecticides tested exhibited antioxidant activity. The antioxidant activity of malathion in the presence of copper ions was lost when EDTA was added. Oxygenated, oxidized and hydrolyzed samples of organophosphates still had antioxidant activity. Experimental evidence indicates that the phosphate moiety of malathion is responsible for this antioxidant activity. It is postulated that the antioxidant activity might be due to the phosphate's ability to chelate metal ions.

INTRODUCTION

BIODEGRADABILITY or inactivation of pesticides can be categorized into oxidation, reduction and hydrolysis (Metcalf, 1966). These reactions are catalyzed by enzymes, metals, as well as oxidizing and reducing agents. Rowlands (1964) proposed a possible breakdown of malathion on stored corn which involved oxidation and hydrolysis. Coffin (1964) suggested that the oxidation of carbophenothion formed its oxygen analog.

Copper salts greatly increase the rate of hydrolysis of parathion and EPN at slightly alkaline pH's (Ketelaar et al., 1956) but do not catalyze the hydrolyses of paraoxon (Heath, 1961). Catalytic activity in paraoxon is attributed to a copper phosphate complex formation.

The purpose of the present study is to determine if organophosphate insecticides and their breakdown products effect ascorbic acid oxidation in vitro.

METHODS

ALL EXPERIMENTS were carried out in an aqueous system at pH 2.2, 4, 6, and 8 in a phosphate-citrate buffer. Varying amounts of 0.2 *M* Na_2HPO_4 and 0.1 *M* citric acid were used to obtain the desired pH.

Samples were prepared using 180 ml of buffer solution; 10 ml of ascorbic acid solution to give a final concentration of 1.4 mg/ml and 10 ml of ethanol or an ethanolic insecticide solution to give indicated ppm of pesticide. Glass distilled water was used. No attempt was made to keep it oxygen free. Controls in all cases were treated exactly as treatment samples, but contained no insecticide.

Organophosphate insecticides used in all experiments were obtained from the Entomological Society of America for use as analytical standards.

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Effect of pesticides on ascorbic acid oxidation

The following experiments were carried out as previously described. Samples were placed in 250 ml beakers, covered with parafilm and stored at constant temperature. There was no attempt to maintain sterile conditions; however, no microbial growth was apparent in any of the samples analyzed. Ascorbic acid was measured periodically to determine its rate of oxidation by the polarographic procedure reported by Pratt et al. (1954). Aliquots were taken for analysis immediately after the samples were prepared, daily for the first four days and at two-day intervals, thereafter.

Experiment I. The effect of 3 levels of malathion on ascorbic acid oxidation was studied. Aqueous solutions of ascorbic acid and three levels of malathion (0, 10, 100 ppm) were prepared.

Experiment II. The effect of 3 levels of malathion (0, 10, 100 ppm) on ascorbic acid oxidation in the presence of chelated and nonchelated copper was studied. All samples contained 0.5 ppm of copper. Chelated samples at each pH and pesticide level contained 3.0 ppm of EDTA.

Experiment III. This experiment was to determine if oxygenation or oxidation of the pesticide would change its effect on ascorbic acid oxidation. Oxygen was bubbled through an ethanolic solution of malathion from which samples were prepared. Samples containing 10 ppm oxygenated, 10 ppm nonoxygenated and a control containing no pesticide were run concurrently.

Oxidation of malathion with peracetic acid was achieved by the method of Archer (1963). Samples containing 10 ppm of oxidized malathion, 10 ppm of nonoxidized malathion and a control containing no malathion were run concurrently. In both cases above the ethanol, from which all ethanolic additions were made, was treated with bubbled oxygen or the peracetic acid treatment.

Experiment IV. The effect of malathion and malathion breakdown products on ascorbic acid oxidation was studied. Malathion was hydrolyzed to give a phosphate moiety and fumaric acid (Jura, 1955), and used to prepare samples at a concentration of 30 μM. Samples were run concurrently containing no pesticide, 30 μM fumaric acid and 30 μM unhydrolyzed pesticide.

RESULTS AND DISCUSSION

THE PESTICIDES acted as an antioxidant by slowing down the rate of oxidation of the ascorbic acid in all *in vitro* studies. In Experiment I, as shown in Table 1, the effect of 10 and 100 parts of malathion was compared. In all cases 100 ppm had an equal to or greater antioxidant activity than 10 ppm. In no case was the increase linearly proportional to the amount of increase in malathion. The greatest antioxidant activity appears to be at pH 4 and 6. The least appears to be at pH 2.2 and 8.

The following organophosphate pesticides were tested similarly to malathion and showed antioxidant activity in regard to ascorbic acid oxidation: carbophenothion, parathion, diazinon and ethion.

Experiment II indicates that the antioxidant activity of malathion on ascorbic acid is lost when EDTA is added to a sample containing 0.5 ppm of copper (Table 2; Figs. 1 and 2). Without the addition of EDTA, malathion possessed appreciable antioxidant activity.

EDTA appeared to exhibit little antioxidant activity at the concentration used except at pH 2. In a study by Butt et al. (1961) at a ratio of 1 mole of EDTA to 1 g atom of copper, the rate of ascorbic acid oxidation was inhibited by as much as 85% at pH 5.5. At a 0.5:1 ratio there

Table 1—The effect of various levels of malathion on the rate of ascorbic acid oxidation at 37°C + 1

pH	Hours for destruction of ascorbic acid in control	Antioxidant index[1] 10 ppm	Antioxidant index[1] 100 ppm
2.2	140	1.3	1.4
4.0	89	2.1	2.1
6.0	91	1.4	1.6
8.0	87	1.3	1.5

[1] Antioxidant index = (hours for 50% destruction of sample)/(hours for 50% destruction of control).

Table 2—The effect of copper and EDTA on antioxidant properties of malathion on ascorbic acid at 35°C + 1.

Sample	pH	Hours for 50% destruction of ascorbic acid in control	Antioxidant index[1] 10 ppm	Antioxidant index[1] 100 ppm
0.5 ppm copper	2.2	144	1.2	1.5
	4.0	78	1.5	2.2
	6.0	84	1.3	2.0
	8.0	60	2.5	2.7
0.5 ppm copper	2.2	No destruction of any sample		
	4.0	102	1.0	1.0
3.0 ppm EDTA	6.0	120	0.9	1.0
	8.0	90	1.0	0.9

[1] Antioxidant index = (hours for 50% destruction of sample)/(hours for 50% destruction of control)

was 16% inhibition. In an equimolar reaction three ppm of EDTA would tie up approximately 0.5 ppm of copper in a 1:1 ratio.

Maximum contamination from analytical reagents used in preparing buffers could be as high as 0.8 ppm of heavy metals, some of these metals might not be chelated and could result in less antioxidant activity than reported by Butt et al. (1961).

Lack of additional antioxidant activity when malathion was added might be due to a copper, EDTA and malathion complex. Two organophosphates (Sarin & DEP) have been indicated to form a complex with EDTA chelated copper (Courtney et al., 1957).

In certain cases the existence of a highly stable 2:1 copper chelate results in disproportionation of the 1:1 chelate with simultaneous precipitation of half of the metal in the form of its hydroxide (Martell et al., 1957). As metal hydroxides would be more readily formed at the higher pH, this might also help explain why less antioxidant activity was observed at pH 8, 6 and 4 than at pH 2.

The antioxidant properties of pesticides are probably of little significance unless pesticides actually get into the food product. Much evidence indicates loss or removal of pesticides on field crops and during food processing. At harvest time one week after spraying with a 0.1% active malathion solution there was less than 1 ppm of active malathion on raspberries, strawberries and logenberries (Tew et al., 1960). Koivistoinen et al. (1964) investigated the stability of residues during the storage and processing of various fresh fruits and vegetables. Losses of malathion varied with the processing methods used and the product being processed. Losses were between 31 and 100%.

It appears that breakdown products would be present in raw products and processed food even if toxicity were lost. Rowlands (1964) studied the breakdown of malathion in stored corn. Dimethyl phosphorothiolic acid, malathion monoacid and malathion di-acid were identified by thin layer chromatography. The breakdown of malathion in this study appeared to be mostly hydrolytic rather than oxidative.

Experiments III and IV were conducted to see if oxidized, oxygenated and hydrolyzed breakdown products of malathion have antioxidant activity. Malathion is not stable under the conditions of experiments I and II (Burchfield et al., 1965). These experiments are apparently measuring the antioxidant activity of malathion and its breakdown products.

In experiment III, malathion was oxygenated by bubbling oxygen through it or oxidized by peracetic acid. Results in Tables 3 and 4 show that antioxidant

Table 3—The effect of oxygenated malathion on the rate of ascorbic acid oxidation at 35°C + 1.

pH	Hours for 50% destruction of ascorbic acid in control	Antioxidant index[1] 10 ppm	Antioxidant index[1] 100 ppm
2.2	280	1.0	1.1
4.0	140	2.9	3.0
6.0	114	1.5	1.7
8.0	120	1.2	1.3

[1] Antioxidant index = (hours for 50% destruction of sample)/(hours for 50% destruction of control).

Table 4—The effect of oxidized malathion on rate of ascorbic acid oxidation at 35°C + 1.

pH	Hours for 50% destruction of ascorbic acid in control	Antioxidant index[1] 10 ppm	Antioxidant index[1] 100 ppm
2.2	230	1.0	1.0
4.0	122	1.4	1.5
6.0	112	1.2	1.2
8.0	104	1.1	1.1

[1] Antioxidant index = (hours for 50% destruction of sample)/(hours for 50% destruction of control).

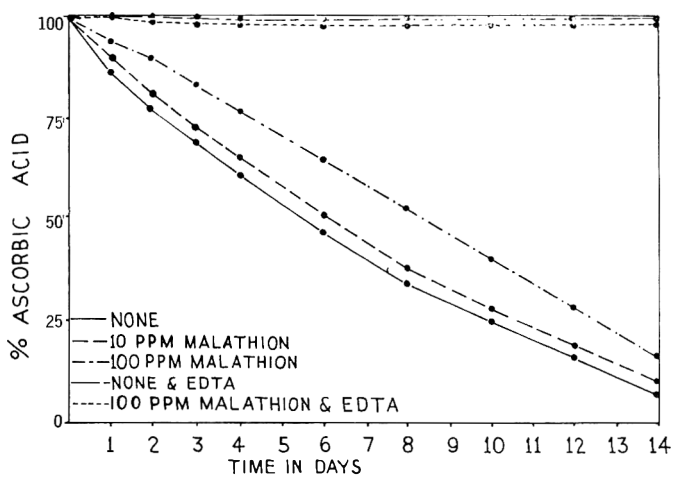

Fig. 1—The effect of malathion on the oxidation of ascorbic acid in the presence of copper with and without EDTA at pH 2 and 35°C ± 1.

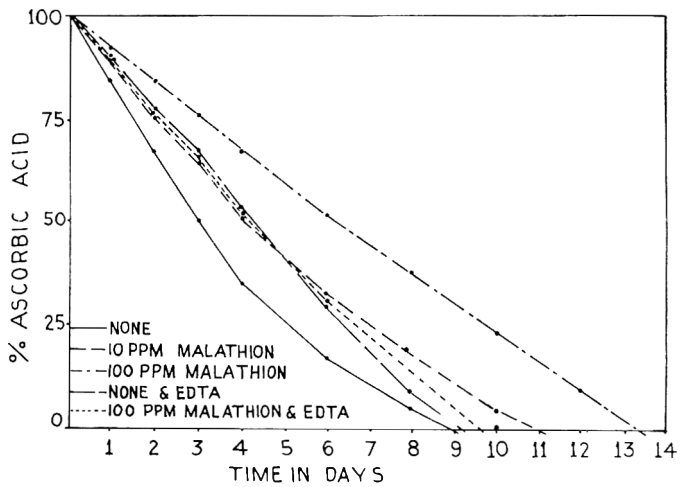

Fig. 2—The effect of malathion on the oxidation of ascorbic acid in the presence of copper with and without EDTA at pH 6 and 35°C ± 1.

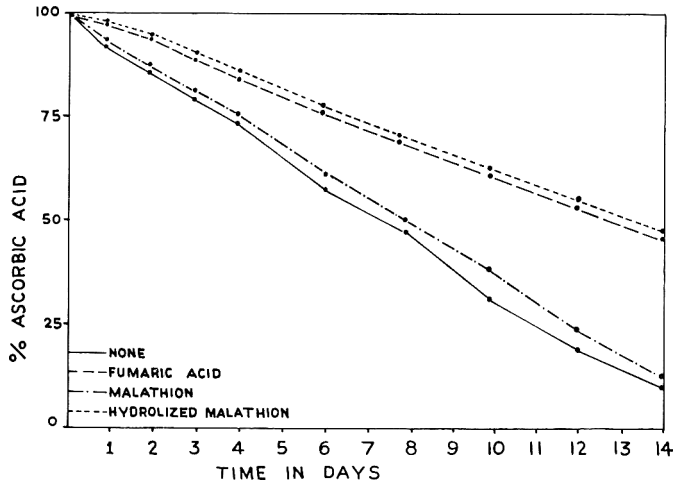

Fig. 3—The effect of malathion, hydrolyzed malathion and fumaric acid on ascorbic acid oxidation at pH 4 and 35°C ± 1.

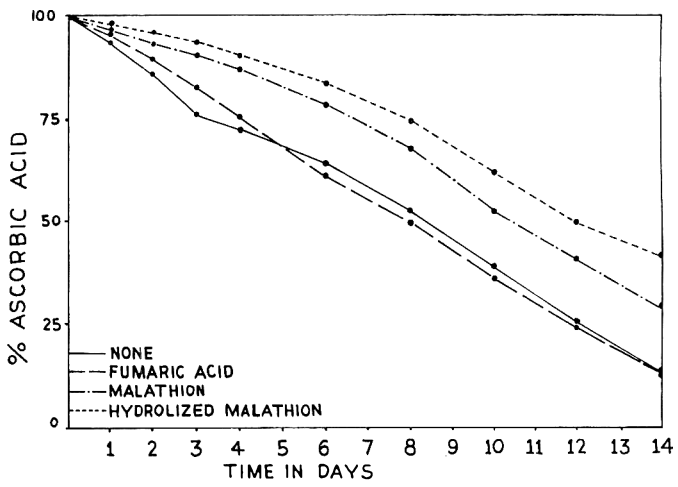

Fig. 4—The effect of malathion, hydrolyzed malathion and fumaric acid on ascorbic acid oxidation at pH 6 and 35°C ± 1.

properties were still present. The peracetic acid treatment of Archer (1963) forms malaxon, the oxygen analogue of malathion. This reaction would remove oxygen from the system. No attempt was made to keep aqueous solutions oxygen free. Therefore, the effect of this reaction in Experiments I and II where non-degraded malathion was used is not known. In oxygen free or oxygen limited systems this reaction might be important. Coffin (1964) suggested oxidation and the formation of the oxygen analogue of carbophenothion as a possible breakdown product. Rowlands (1964) did not detect malaoxon in his analysis of malathion treated stored corn.

In Experiment IV malathion was hydrolyzed and still maintained its antioxidant activity (Figs. 3 and 4).

The results of Experiments III and IV show that the breakdown products of malathion due to oxygenation, oxidation and hydrolysis have antioxidant properties. Studies on parathion, diazinon and carbophenothion showed they also retained their antioxidant properties when oxygenated. Carbophenothion also showed antioxidant activity when oxidized with peracetic acid.

According to Jura (1955) malathion forms a thiophosphate moiety and fumaric acid. In Experiment IV fumaric acid showed little or no antioxidant activity. Therefore, it would appear that the thiophosphate moiety is responsible for the antioxidant activity of malathion and its breakdown products.

The orthophosphate ion is a relatively good complexing agent (Van Wazer, 1958). Perhaps organophosphates act as antioxidants in aqueous systems by complexing metal ions which accelerate ascorbic acid oxidation. This would explain why malathion showed antioxidant activity in the presence of 0.5 ppm of copper and the activity was not present when a chelator was added. The phosphate present in the buffer did not affect the oxidation of ascorbic acid. The groupings within the phosphate moiety of the organophosphate insecticides may be significant.

As all insecticides studied contain similar phosphate groupings, chelation of metals by this moiety might explain why all possessed antioxidant properties.

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External Fat Cover Influence on Raw and Cooked Beef. 1. Fat and Moisture Content

SUMMARY—The fat content of beef semitendinosus muscle, raw and cooked at two oven temperatures, with and without the external fat cover, has been determined. Each muscle was divided into two, an anterior and a posterior roast. The external fat cover was removed from half of the total number of roasts, and all roasts were then cooked to an internal temperature of 58°C at an oven temperature of either 163° or 218°C. Two types of samples were analyzed for their moisture and fat content. These were a separable lean sample and a total sample. The total sample was composed of both the separable lean and the external fat cover. The moisture content of the lean samples was not affected by the oven temperature, the external fat cover or the end of the muscle used. The total samples had moisture contents that reflected the amount of crude fat present. The yield of crude fat from raw or cooked meat was not significantly altered by the use of a polar solvent in place of a nonpolar one. Both of the fat determination methods extracted significantly more fat from the cooked lean samples than from the comparable raw ones. The lean samples from meat roasted at 163°C contained significantly more extractable fat than the samples roasted at 218°C. The presence or absence of the external fat cover did not affect the amount of fat extracted from the cooked lean.

INTRODUCTION

IT IS USUALLY found that the amount of crude fat in the separable lean of cooked beef is greater than the amount in the raw, even when results are converted to a dry basis to allow for the moisture lost during cooking. Many workers, using a variety of types of meat and methods of cooking, have reported this increase (Batcher et al., 1960; Batcher et al., 1962; Dawson et al., 1959; Gilpin et al., 1965; Griswold, 1955; Iyengar et al., 1965; Paul, 1962; Paul, 1964; Paul et al., 1964; Satorius et al., 1938; Thille et al., 1932; Toepfer et al., 1955; and Weir et al., 1962).

The increase of fat in the lean is usually attributed to the infiltration of melted fat into the muscle tissue during heating. Weir et al. (1962) report that pork chops cooked after the removal of the external fat cover had a fat content of the cooked lean less than that of the chops cooked with the fat on, and nearer that of the raw lean. Using trimmed beef cuts, Kincaid (1966) obtained variable results.

Other possible reasons given in the literature (Weir, 1962; Paul, 1965; Kincaid, 1966) for an increase in the fat content on cooking include: (1) a difference in the fat content of the two cuts used as comparable raw and cooked samples; (2) improved extractability of fat from the cooked meat due to an alteration in the muscle structure and/or breakdown of lipid-protein complexes by heat; (3) masking of fat loss by proportionally greater losses of other components; (4) inaccurate determination of moisture and fat content.

The method used to determine the fat content in raw and cooked meat could also be important. The most commonly used solvents for extracting fat from meat are anhydrous diethyl ether or petroleum ether. However, since much of the lipid in muscle tissue is complexed with protein, ether does not extract all the lipid from meat (Giam et al., 1965).

The two most widely used polar solvent fat extraction methods are the ones described by Folch et al. (1957) and Bligh et al. (1959). These methods have been used on raw meat but the only reports of methanol-chloroform extraction on meat cooked by a conventional method are those by Weir et al. (1962) and Cole (1966). The purpose of this investigation was to study the fat content of beef semitendinosus muscle, raw and cooked at two oven temperatures, with and without the external fat cover. Two extraction techniques were used to determine the fat content.

EXPERIMENTAL

27 CHOICE GRADE semitendinosus muscles were purchased from a local supermarket. The previous history of the meat was unknown. Each muscle was divided into an anterior and a posterior roast, and these roasts alternated between the four prepara-

Table 1—The average percent moisture content of raw and cooked beef.

Oven temperature, °C	Fat cover[1]	Total, %		Lean, %	
		Raw	Cooked	Raw	Cooked
218	On[2]	64.08	59.35	73.99	66.08
	Off[3]	73.06	66.15	73.85	67.23
163	On[3]	63.01	55.36	73.89	66.80
	Off[2]	73.36	66.71	73.94	67.81

[1] "On"—intact cut used; "Off"—external fat cover removed before cooking.
[2] Mean of 13 replications.
[3] Mean of 14 replications.

Table 2—Means and standard deviations for crude fat extracted by petroleum ether (dry basis).

Oven Temperature, °C	Fat cover	Muscle section	No. of replications	Total				Lean			
				Raw		Cooked		Raw		Cooked	
				Mean, %	S.D.	Mean, %	S.D.	Mean, %	S.D.	Mean, %	S.D
218	On	Ant	8	42.54	6.82	30.10	5.77	11.83	2.10	16.48	2.57
		Post	5	44.80	4.65	43.71	7.59	13.55	3.79	17.57	8.64
	Off	Ant	7	17.02	3.94	17.48	4.23	14.70	2.79	16.64	2.83
		Post	7	17.01	2.48	15.24	2.86	13.47	2.57	18.04	5.86
163	On	Ant	6	47.01	2.74	41.63	8.87	15.22	3.69	20.77	5.19
		Post	8	48.12	2.93	54.30	6.16	14.40	3.56	19.58	6.33
	Off	Ant	6	17.60	4.69	21.99	4.66	15.03	3.48	19.68	5.55
		Post	7	16.32	3.63	18.43	4.68	14.54	3.18	20.14	5.27

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tion procedures. The external fat cover was removed from half the total number of roasts before they were cooked. The cuts were roasted in household ovens at either 163° or 218°C to an internal temperature of 58°C (rare).

A total sample, composed of both the separable lean and the external fat layer, and a separable lean sample were analyzed for moisture and fat content. Slices for the analyses were taken from either end of each roast both before and after cooking. The samples were triple ground before analysis.

Moisture determinations were made by drying 10-g samples at 120°C until there was less than 0.5% change in weight in 30 min. The two methods used for crude fat determination were ether extraction and chloroform-methanol extraction. In the first, dried samples were extracted for 4 hr with petroleum ether (b.p. 30°–60°C). The procedure described by Folch et al. (1957) was used for the chloroform-methanol extraction.

RESULTS AND DISCUSSION

Moisture content

The moisture content of the samples are presented in Table 1. These results support the generally accepted fact that the total moisture content decreases as the fat content increases. The roasts with the fat cover contained less moisture than the comparable roasts without the fat layer. Cooking also caused a decrease in the moisture content, as expected.

Fat determinations

The mean values for the fat content of the samples are listed in Tables 2 and 3. All values given are calculated on the dry basis to allow for the moisture lost during cooking. The F ratios from analysis of variance are given in Table 4.

Table 3—Means and standard deviations for crude fat extracted by methanol-chloroform (dry basis).

Oven temperature, °C	Fat cover	Muscle section	No. of replications	Total				Lean			
				Raw		Cooked		Raw		Cooked	
				Mean, %	S.D.	Mean, %	S.D.	Mean, %	S.D.	Mean, %	S.D.
218	On	Ant	8	41.82	5.94	30.76	4.92	14.97	2.38	18.24	2.64
		Post	5	43.42	4.34	43.00	5.90	15.68	4.10	19.02	7.40
	Off	Ant	7	17.82	4.20	18.85	3.36	16.62	3.81	18.43	2.76
		Post	7	18.24	2.66	17.10	2.87	15.56	2.69	19.45	5.03
163	On	Ant	6	46.05	4.18	39.75	9.35	16.25	3.65	20.74	4.81
		Post	8	46.42	3.33	50.94	6.18	17.25	3.41	20.23	5.08
	Off	Ant	6	20.02	3.63	22.82	4.68	17.42	3.18	21.15	5.27
		Post	7	17.05	2.07	19.83	2.52	16.12	3.21	21.18	3.32

Table 4—F ratios from analysis of variance, for the percent fat extracted.

Source of variation	F Ratios			
	Petroleum ether extract[1]	MeOH-$CHCl_3$ extract[1]	Total cooked sample	Lean cooked sample
Treatment (raw or cooked)	25.16**	18.90**	—	—
Solvent	—	—	N.S.	N.S.
End of muscle	N.S.	N.S.	29.67**	N.S.
Temperature	7.02*	4.20*	58.65**	7.66**
External fat cover	N.S.	N.S.	632.06**	N.S.

[1] Lean samples.
** Significant at the 1% level.
* Significant at the 5% level.
N.S.: Nonsignificant.

Comparison of extraction solvents

Although the methanol-chloroform procedure extracted more fat from the raw lean samples than did petroleum ether, the difference was not significant. The data from the cooked samples also showed that the solvent used did not cause a significant difference in the amount of fat extracted. Cooking the meat had a greater effect on the amount of fat extracted from the samples than did the type of solvent used.

The two solvents performed differently at different fat concentrations. As the fat

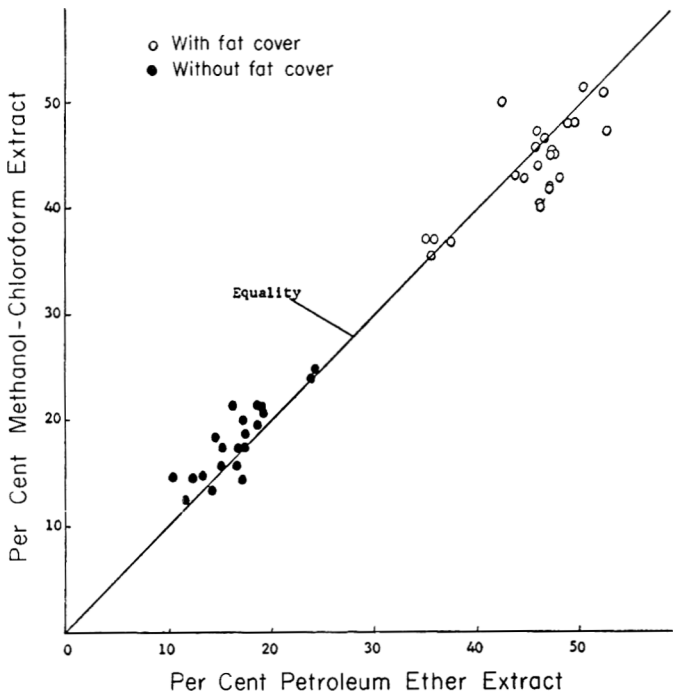

Fig. 1—Comparison of extraction solvents on the raw total samples.

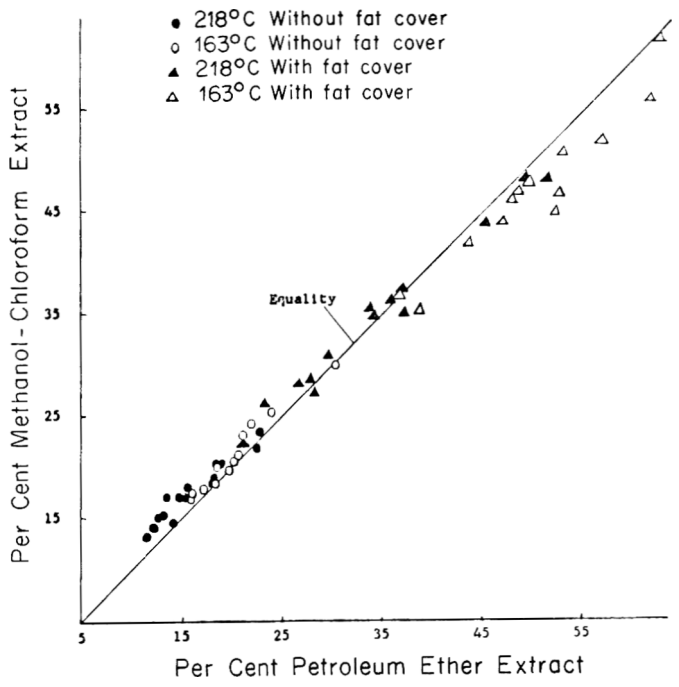

Fig. 2—Comparison of extraction solvents on the cooked total samples.

concentration of the samples increased, the petroleum ether technique extracted more fat than the methanol-chloroform technique. Both raw and cooked samples showed this effect, as illustrated in Figures 1 and 2. As the crude fat concentration of a sample increases the types of lipid making up the total fat change. Lipid in the lean or muscle tissue is largely polar, whereas the lipids of adipose tissue are nonpolar. Thus, as the amounts of adipose tissue increased, the nonpolar extraction solvent, petroleum ether, extracted more crude fat than the polar methanol-chloroform mixture.

Fat content of the total roast

Roasts cooked with the external fat cover contained less total fat after cooking than before because some fat was lost as drip. The roasts cooked at 218°C lost more fat in the drip than did those cooked at 163°C. There was no significant difference between the total amounts of fat extracted from the raw or cooked roasts from which the fat cover had been removed.

Fat content of the separable lean

Significantly more crude fat was extracted from the cooked than from the raw lean samples ($P < 0.01$). Both petroleum ether and methanol-chloroform extracted more from cooked than from raw lean. Significantly higher values for percent fat were obtained from the roasts cooked at 163°C than from those cooked at 218°C. There was no significant effect due to cooking with or without the external fat cover, or due to the end of the muscle used.

These results indicate that in this study the increased amount of fat in the cooked lean sample was not due to infiltration of fat from the exterior, nor was the increase of fat eliminated by the use of a polar solvent extraction technique. The cooking process must in some way cause this apparent increase of fat in the lean. When the meat is cooked at an oven temperature of 163°C, more fat is extracted from the lean than when it is cooked at 218°C.

It is reported in the literature that there is a difference in the degree of doneness of meat cooked to the same internal temperature but at a different oven temperature (Lowe, 1955). The piece of meat cooked at the lower temperature is more evenly cooked throughout and appears more well done. This phenomenon was observed in the present study and led to the conclusion that, as the denaturation of muscle protein by heat increases, lipid material, which previously was inaccessible to both polar and nonpolar solvents, is released from a complex with protein.

The samples used for comparison of the raw meat to the cooked were from adjacent regions of the muscle. Although this muscle is fairly uniform throughout its length, there are certain places in the lean where fat and connective tissue deposits are present. These deposits are around the major blood vessels entering the muscle and occur in the same place in each muscle. Taylor (1959) analyzed the chemical composition of adjacent steaks from the semitendinosus. However, these analyses were conducted on the combined separable lean and external fat cover so do not give information on the variation to be expected in the lean. A visual assessment of the raw samples and the roasts to be cooked led to the conclusion that the raw sample was representative of the roast.

It has been suggested that the apparent increase of the fat content in the lean after cooking is caused by undetected losses of other constituents (Kincaid, 1966). In this study the fat content is reported on a dry basis to allow for the moisture lost during heating. However moisture is not the only substance lost. Other volatile constituents, such as aromatic substances and decomposition products of fat and protein, are lost by evaporation, and there are also drip losses. If the fat content of a cooked lean sample is 4% greater than that of the raw sample(on a dry basis), a loss of moisture-free volatiles and drip forming approximately 9% of the cooked weight must have occurred to cause the difference in the fat content of the two samples. The increase in fat content of the lean after cooking was found, in the study, to be approximately 4%.

An error in the determination of moisture in the raw lean would have to be on the large side by approximately 1.5% to cause an apparent increase in the amount of fat present after cooking. It is improbable that errors of this type would be consistent enough to cause the definite increase of fat found when meat is cooked.

It is concluded from the results of this study that, during heating, the lipid present in the muscle tissue was made more accessible to extraction techniques. Heating caused denaturation of the protein and the lipid complexed with protein may then have been released. It is possible that at the beginning of the cooking process the slow increase in temperature activates enzymes which in turn release bound fat. If there is fat in the raw tissue which is not extracted by chloroform-methanol then it must be bound in the tissue very strongly.

In this study a dry heat method of cooking was used. Kincaid (1966) cooked beef by both dry and moist heat methods and concluded that when the external fat cover was trimmed from beef cuts before cooking, the samples cooked by moist heat methods showed a greater increase in the fats than those cooked by dry heat. She used steaks for dry heat cookery whereas roasts were used in the present study. Raw muscle contains almost three-quarters water and so the interior cooks in a moist heat environment. Roasts are larger than steaks, so cook more slowly. In large cuts of meat this moist interior may be more important in determining the medium that heats the muscle constituents than the method of cooking. A greater amount of fat is extracted from cooked lean than from raw lean. If this is due to release of lipids from complexes, both moisture and heat may be necessary for the process.

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Studies on Some Physical Properties of Bovine Skeletal Muscle

SUMMARY—The physical properties of the major muscles of the round from 30 crossbred beef and 20 straightbred dairy carcasses were studied. Sex, sire and body type were related to these properties. Generally, the steers had longer, heavier and lighter-colored muscles than the heifers. A negative relationship was noted between the specific gravity and reflectance values for the crossbred muscles. The semimembranosus muscle was heaviest and accounted for the highest percent of the rough round in certain beef and dairy sire groups. In the dairy cattle, the weight of the biceps femoris muscle was also affected by sire. Heritability estimates indicated that the weight of the semimembranosus was highly heritable and its percent of the rough round moderately heritable in both beef and dairy cattle. The dairy b. femoris weight and circumference were also moderately heritable. Color reflectance, specific gravity, pH, length and circumference/length ratio were not observed to be heritable physical properties.

INTRODUCTION

PHYSICAL properties of skeletal muscle are useful indicators of carcass value and meat quality. Increasing consumers' demand for uniform, economical, lean meat products has focused attention on these characteristics of skeletal muscle.

Orme et al. (1960) found that the weights of the individual muscles of the bovine round, especially the biceps femoris, were quite accurate in predicting total carcass separable lean. Ramsbottom et al. (1948) observed that these bovine round muscles varied considerably in the amount of intramuscular fat (marbling). The b. femoris contained 5.1% intramuscular fat whereas the adductor contained only 2.5%.

The specific gravity of a carcass or cut was shown to be highly related to the amount of fat it contained (Brown et al., 1951). As the fat content increased, the specific gravity value decreased. Chemical analyses supported this observation and the validity of using specific gravity measurements.

In 1939, the relationship between muscle pH and color (Winkler, 1939a) and between muscle pH and tenderness (Winkler, 1939b) was reported. These reports indicated that as the pH decreased the muscle becomes lighter and less tender. However, below pH 5.5 the opposite relationship was observed. The pH of muscle is also of interest since it influences various processing characteristics (Sayre et al., 1964).

In spite of these investigations, knowledge concerning the phenotypic interrelationships of the physical properties of the bovine round muscles is not very extensive. It has not been well established if these physical properties are completely compatible with each other in contributing to carcass value and meat quality. Also, the influence of sire, sex and body type on these various muscle characteristics have not been sufficiently studied. Estimates of heritability for the physical properties of the economically important muscles are urgently needed for the breeding research programs and the development of more accurate selection indexes. The purpose of this research was to pursue these challenges.

Table 1—Beef cattle sex means of muscle properties.

Sex	Wt[1]	% Rd[2]	Sp Gr[3]	pH	Refl[4]	Length	Circ[5]	C/L[6]
				Semimembranosus				
Steers	3.47	11.2	1.0688	5.30	16.3	30.3	41.8	1.37
Heifers	3.21	11.4	1.0715	5.29	13.8	30.6	41.1	1.35
	0.26**				2.5**			
				Biceps Femoris				
Steers	4.06	13.1	1.0653	5.34	17.3	40.0	38.4	0.96
Heifers	3.65	13.0	1.0670	5.29	14.7	38.3	36.4	0.95
	0.41**			0.05*	2.6*	1.7*		
				Semitendinosus				
Steers	1.73	5.6	1.0727	5.31	20.5	35.0	25.8	0.74
Heifers	1.51	5.4	1.0727	5.30	19.0	32.3	25.5	0.79
	0.22**					2.7**		0.05*
				Adductor				
Steers	1.23	4.0	1.0699	5.32	17.0	18.1	30.4	1.68
Heifers	1.11	3.9	1.0729	5.28	13.8	17.4	28.6	1.65
				Quadriceps Femoris				
Steers	3.19	10.3	1.0627	5.34	17.2	21.0	49.5	2.36
Heifers	2.68	9.6	1.0627	5.32	15.9	19.6	47.1	2.41
	0.51**	0.7**				1.4**	2.4**	

* $P < 0.05$. ** $P < 0.01$.
[1] Weight of muscle (kg).
[2] Percent of rough round.
[3] Specific gravity.
[4] Reflectance at 485 mμ.
[5] Circumference of muscle (cm).
[6] Circumference-to-length ratio.

Table 2—Beef sire means of muscle properties.

Sires	Wt[1]	%Rd[2]	Sp Gr[3]	pH	Refl[4]	Length	Circ[5]	C/L[6]
			Semimembranosus					
1	3.26	10.9	1.0711	5.28	14.9	30.2	41.2	1.36
2	3.44	11.6	1.0707	5.28	15.9	30.5	42.0	1.38
3	3.15	11.0	1.0671	5.32	14.9	29.9	40.8	1.36
4	3.49	11.7	1.0717	5.30	14.6	31.6	42.0	1.33
Difference*	0.20	0.6						
			Biceps Femoris					
1	3.88	12.9	1.0664	5.32	15.8	39.0	35.7	0.92
2	3.95	13.3	1.0661	5.30	15.4	39.7	38.4	0.97
3	3.64	12.7	1.0666	5.32	16.2	38.2	37.0	0.97
4	3.94	13.2	1.0655	5.32	16.6	39.7	38.5	0.97
			Semitendinosus					
1	1.64	5.4	1.0741	5.30	18.3	33.1	25.1	0.77
2	1.58	5.3	1.0694	5.28	19.5	33.9	25.0	0.74
3	1.54	5.4	1.0715	5.33	20.5	32.9	25.8	0.79
4	1.72	5.8	1.0757	5.31	20.5	34.7	26.7	0.77
			Adductor					
1	1.15	3.8	1.0683	5.30	14.8	17.6	30.3	1.73
2	1.23	4.1	1.0646	5.29	16.3	18.0	29.9	1.67
3	1.08	3.8	1.0744	5.32	14.7	17.7	27.5	1.56
4	1.22	4.1	1.0784	5.30	15.9	17.7	30.3	1.71
			Quadriceps Femoris					
1	2.94	9.7	1.0618	5.34	16.4	20.1	48.9	2.44
2	3.01	10.1	1.0628	5.30	16.5	20.5	48.4	2.38
3	2.84	9.9	1.0616	5.35	16.8	20.5	46.7	2.28
4	2.96	9.9	1.0647	5.33	16.6	20.2	49.2	2.44

* Difference between sires required to be significant ($P < 0.05$). When values are not given there were no significant differences.
[1] Weight of muscle (kg).
[2] Percent of rough round.
[3] Specific gravity.
[4] Reflectance at 485 mμ.
[5] Circumference (cm).
[6] Circumference-to-length ratio.

Table 3—Dairy sire means of muscle properties.

Sire	Wt[1]	%Rd[2]	Sp Gr[3]	pH	Refl[4]	Length	Circ[5]	C/L[6]
			Semimembranosus					
1	3.58	11.1	1.0712	5.38	16.2	33.4	41.6	1.25
2	3.79	11.3	1.0722	5.37	18.0	34.0	41.6	1.23
3	3.98	12.2	1.0685	5.39	16.4	33.8	43.0	1.27
4	4.07	12.0	1.0705	5.35	16.8	34.3	43.6	1.27
Difference*	0.31	0.8						
			Biceps Femoris					
1	4.48	13.9	1.0705	5.44	15.8	44.3	37.2	0.84
2	4.73	14.1	1.0707	5.43	16.2	43.7	39.3	0.90
3	4.73	14.4	1.0734	5.43	17.6	44.1	39.2	0.89
4	4.89	14.4	1.0735	5.39	18.0	45.3	38.4	0.85
Difference*	0.29							
			Semitendinosus					
1	1.95	6.0	1.0718	5.35	21.8	37.4	26.0	0.72
2	2.08	6.2	1.0731	5.42	23.2	38.1	27.7	0.73
3	2.11	6.4	1.0724	5.42	21.9	37.3	28.4	0.76
4	2.13	6.0	1.0743	5.39	22.0	37.3	27.9	0.75
			Adductor					
1	1.18	3.7	1.0782	5.38	15.0	19.6	28.5	1.46
2	1.33	4.0	1.0678	5.42	16.2	19.0	30.6	1.61
3	1.33	4.1	1.0728	5.38	15.8	19.9	30.3	1.53
4	1.35	4.0	1.0724	5.38	15.8	20.7	29.4	1.42
			Quadriceps Femoris					
1	3.44	10.7	1.0665	5.49	20.0	22.2	49.2	2.22
2	3.77	11.2	1.0669	5.48	20.2	22.8	51.7	2.27
3	3.55	10.8	1.0666	5.43	18.7	22.3	49.3	2.21
4	3.86	10.8	1.0660	5.42	18.7	23.3	50.8	2.19

* Difference between sires required to be significant ($P < 0.05$). When values are not given there were no significant differences.
[1] Weight of muscle (kg).
[2] Percent of rough round.
[3] Specific gravity.
[4] Reflectance at 485 mμ.
[5] Circumference (cm).
[6] Circumference-to-length ratio.

MATERIALS & METHODS

Experimental animals

Two groups of animals (*Bos taurus*) representing different body types were used. The first or crossbred beef group included 14 steers and 16 heifers. These calves were the progeny of four purebred Polled Hereford sires selected for outstanding achievement in National and International show ring competition. The dams of these calves were Angus-Holstein crossbred cows that were randomly mated to four bulls. Each sire was represented by eight calves, four steers and four heifers, with the exception that sire 4 was represented by only two steers and four heifers.

The second or dairy-type group consisted of 20 straightbred Holstein bulls. These were the progeny of four purebred Holstein sires, selected for their ability to sire daughters of high milk-producing ability. Each sire group consisted of five progeny.

When the steers and the bulls weighed about 450 kg and the heifers 420 kg, they were fasted for 24 hr and then slaughtered. There was no significant ($P > 0.05$) difference between the ages of the steers (444 days) and the heifers (441 days). However, the dairy animals were significantly ($P < 0.01$) younger (386 days) than the crossbreds. All of the animals were fed a similar finishing ration.

Carcass cutting methods

After a 48-hr chilling period at 5°C, the carcasses were processed into wholesale cuts according to the procedures recommended by Schoonover et al. (1967).

Physical measurements

After weighing, the round was trimmed and the following muscles excised: semimembranosus, biceps femoris, semitendinosus, adductor and quadriceps femoris. The external fat was completely removed from these muscles. Each muscle was weighed and its percent of the untrimmed or rough round was calculated. Length of the muscle was measured from the width midpoint of one end to the width midpoint of the other end of the muscle. Circumference was measured at the midpoint of the length of the muscle. A circumference to length ratio was then calculated.

Specific gravities were obtained by hydrostatic weighing of the muscles according to Brown et al. (1951). The pH of each muscle was recorded after dissection using a Beckman Zeromatic pH meter equipped with a combination probe electrode. Three separate readings were taken and their average was reported as the value for the muscle.

The reflectance (color) of each muscle was determined for a fresh muscle sample (3 × 2 × 2 cm) using a Bausch and Lomb Spectronic 20 spectrometer standardized at a wavelength of 485 mμ and operated according to the method of Sayre et al. (1964).

Statistical analyses

Analysis of variance and correlation methods (Snedecor, 1956) were used as appropriate. When significant ($P < 0.05$) differences occurred among sire groups in the analysis of variance, Duncan's (1955) multiple range

test was used.

Heritability estimates

The following mathematical model was used to estimate the components of variance:

$$y_{ijl} = \mu + b_i + s_j + bs_{ij} + r_{ijl}$$

where μ is the over-all mean, b_i is the effect of the i*th* bull, s_j is the effect of the j*th* sex, bs_{ij} is the interaction of the ij*th* subclass, and r_{ijl} is the error term. The heritability estimates and the standard errors were computed by the method of Hazel et al. (1945).

RESULTS & DISCUSSION

Muscle properties

Table 1 is a listing of the means of muscle properties for the steer-heifer comparison.

The steers had significantly ($P < 0.01$) heavier semimembranosus, b. femoris, semitendinosus, adductor, and q. femoris muscles than heifers. However, only the q. femoris was significantly ($P < 0.01$) larger when expressed as percent of the rough round. The weight difference between the other muscles could be attributed to the larger rounds noted on the steers. An interaction due to inconsistent sex differences among sires was significant ($P < 0.05$) for the semitendinosus expressed as percent of the rough round.

There were no significant differences between the specific gravity values of the various muscles for steers and heifers.

The b. femoris was the only muscle in which a significant ($P < 0.05$) sex effect on pH was noted. Heifers were observed to have a lower pH than steers for this muscle. The effect of sex on reflectance values indicated significant differences for semimembranosus ($P < 0.01$) and b. femoris ($P < 0.05$) muscles. Both muscles were darker in heifers than in steers. The relation of lower pH and darker color is consistent with the results of Winkler (1939a) who observed that this relationship existed in beef muscle between a pH of 4.5 and 5.5.

The b. femoris ($P < 0.05$) semimembranosus ($P < 0.01$), and q. femoris ($P < 0.01$) muscles were longer in steers than in heifers. The circumference of the q. femoris was also greater ($P < 0.01$) in steers than in heifers. As expected from the above, the circumference to length ratio of the semimembranosus was significantly ($P < 0.05$) higher for heifers than for steers.

The comparison of muscle properties (Table 2) indicates only one significant ($P < 0.05$) beef sire group difference. Sire groups 2 and 4 were different from sire groups 1 and 3 for semimembranosus muscle weight and percent of rough round.

The comparison of the muscle properties of the dairy sires is listed in Table 3. Dairy sire group 4 was significantly ($P < 0.05$) different from sire group 1, but not from sire groups 2 and 3 for semimembranosus weight. When this muscle was expressed as percent of the rough round, sire groups 3 and 4 were significantly ($P < 0.05$) different from sire groups 1 and 2. In addition, the b. femoris was significantly ($P < 0.05$) heavier in the sire group 4 cattle than in those of sire group 1.

Table 4—Significant ($P < 0.01$) phenotypic correlations between beef cattle muscle properties.[1]

SM wt		SM sp gr[3]		BF refl[5]	
BF wt	0.83	SM pH	−0.47	ST refl[5]	0.88
BF % rd[2]	0.53	SM refl[5]	−0.55	AD % rd[2]	−0.63
BF Length	0.62	BF sp gr[3]	0.53	AD refl[5]	0.55
BF circ[4]	0.57	BF pH	−0.60	AD length	−0.49
ST wt	0.66	ST refl[5]	−0.71	QF refl[5]	0.74
ST length	0.65	AD wt	0.74	SM sp gr[3]	−0.62
QF wt	0.59	QF pH	−0.56	SM refl[5]	0.77
QF circ[4]	0.55	QF refl[5]	−0.57	BF sp gr[3]	−0.61
SM refl[5]		**SM circ[4]**		**BF pH**	
BF sp gr[3]	−0.56	BF wt	0.52	BF refl[5]	0.54
ST refl[5]	0.69	BF % rd[2]	0.59	AD pH	0.56
AD refl[5]	0.68	BF circ[4]	0.47	QF wt	0.49
QF refl[5]	0.67	ST length	0.50	QF pH	0.58
SM % rd[2]		**SM pH**		**ST wt**	
BF % rd[2]	0.62	BF pH	0.65	QF wt	0.53
QF sp gr[3]	0.48	QF pH	0.56	QF cric[4]	0.52
ST pH		**ST sp gr[3]**		**ST C/L[6]**	
AD pH	0.64	QF refl[5]	−0.58	AD sp gr[3]	0.57
BF sp gr[3]		**ST refl[5]**		**AD refl[5]**	
ST circ[4]	−0.56	SM pH	−0.53	QF wt	0.52
ST C/L[6]	−0.48	AD % rd[2]	−0.62	QF refl[5]	0.64
AD % rd[2]	0.57	AD length	−0.47	QF length	0.57
AD refl[5]	−0.46	QF refl[5]	0.69	ST refl[5]	0.48
AD length	0.50	BF pH	0.50	BF pH	0.50
QF refl[5]	−0.70	BF sp gr[3]	−0.63	SM sp gr[3]	0.49
BF wt		**ST length**		**ST circ[4]**	
ST wt	0.69	BF length	0.59	AD sp gr[3]	0.63
AD refl[5]	0.54	QF length	0.50	ST % rd[2]	−0.54
QF wt	0.65	QF wt	0.50		
QF circ[2]	0.57	BF wt	0.57		
AD % rd[2]		**AD length**		**AD circ[4]**	
QF refl[5]	−0.53	QF refl[5]	0.47	QF C/L[6]	0.42
SM sp gr[3]	0.60				

[1] SM = semimembranosus, BF = biceps femoris, ST = semitendinosus, AD = adductor, QF = quadriceps femoris.
[2] % rd = Percent of rough round.
[3] sp gr = Specific gravity.
[4] circ = Circumference of the muscle (cm).
[5] refl = Reflectance at 485 mμ.
[6] C/L = Circumference-to-length ratio.

Muscle correlations

Each beef muscle property that was significantly ($P < 0.01$) correlated with another trait is listed in Table 4. The omitted exceptions were traits directly and obviously related to each other.

The semimembranosus, b. femoris, semitendinosus, and q. femoris muscle weights were all positively related with each other. Therefore, an increase in round size would be expected to make approximately the same proportional weight increase in each of these four muscles. Since the muscles are related by weight, it is also reasonable to find them size-related. Generally, the dairy muscles were related by weight and size as were the crossbred muscles (Table 5).

Generally, the round muscles, in both crossbred and dairy animals, were positively related with each other in regard to specific gravity, reflectance, and pH values. The interrelationships between these various physical properties were essentially positive in both groups of animals, except that the correlations between specific gravity and reflectance and between specific gravity and pH were negative in the crossbred muscles.

Heritability estimates

The weight of the semimembranosus was observed to be highly heritable in both beef (58%) and dairy (76%) cattle. Rapid progress can be expected if

Table 5—Significant ($P < 0.01$) phenotypic correlations between dairy cattle muscle properties.[1]

SM wt		BF wt		SM % rd[2]	
BF wt	0.84	ST wt	0.69	SM circ[4]	0.64
BF % rd[2]	0.62	AD wt	0.81	BF % rd[2]	0.76
ST wt	0.71	AD % rd[2]	0.59	AD wt	0.64
ST circ[4]	0.63	AD circ[4]	0.55	AD % rd[2]	0.71
AD wt	0.78	QF wt	0.64	BF circ[4]	0.62
AD % rd[2]	0.63	QF length	0.57		
AD circ[4]	0.58	SM circ[4]	0.59		
SM circ[4]	0.68				
BF circ[4]		**ST circ[4]**		**SM circ[4]**	
ST wt	0.67	BF circ[4]	0.72	BF % rd[2]	0.56
AD wt	0.78	SM % rd[2]	0.61	ST wt	0.64
AD % rd[2]	0.82	AD wt	0.72	ST circ[4]	0.73
AD circ[4]	0.78	AD % rd[2]	0.70	ST C/L[6]	0.65
SM wt	0.60	AD circ[4]	−0.68		
ST wt		**BF C/L[6]**		**SM refl[5]**	
AD wt	0.76	ST circ[4]	0.67	BF sp gr[3]	−0.57
AD % rd[2]	0.61	AD wt	0.58	ST refl[5]	0.64
AD circ[4]	0.63	AD % rd[2]	0.68	QF refl[5]	0.68
ST circ[4]	0.80	AD circ[4]	0.60		
BF % rd[2]		**AD circ[2]**		**BF length**	
AD wt	0.67	AD wt	0.75	QF length	0.56
AD % rd[2]	0.77	AD % rd[2]	0.72	QF C/L[6]	−0.58
BF circ[4]	0.60				
QF length		**BF pH**		**ST refl[6]**	
SM length	0.63	SM pH	0.56	QF refl[5]	0.62
QF wt	0.61	SM C/L[6]	−0.56	BF sp gr[3]	0.59
QF wt		**QF pH**		**ST sp gr[3]**	
QF circ[4]	0.60	AD pH	0.57	ST pH	0.56
SM C/L[6]		**AD length**			
ST C/L[6]	0.56	QF refl[5]	0.73		

[1] SM = semimembranosus, BF = biceps femoris, ST = semitendinosus, AD = adductor, QF = quadriceps femoris.
[2] % rd = Percent of rough round.
[3] sp gr = Specific gravity.
[4] circ = Circumference of the muscle (cm).
[5] refl = Reflectance at 485 mμ.
[6] C/L = Circumference-to-length ratio.

mass selection for this trait were practiced. Moderate heritability values for semimembranosus muscle expressed as percent of the rough round were also noted in beef (34%) and dairy (45%) animals. In the dairy cattle, the weight and circumference of the b. femoris muscle were found to be 30 and 32% heritable, respectively. These moderate values indicate some progress can be made through mass selection for these traits.

All other muscle properties studied had low or zero heritability values, indicating that little or no progress will be obtained by mass selection for these characteristics and therefore should not receive appreciable emphasis in selection programs.

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Thermochemical Factors Influencing the Death Kinetics of Spores of Clostridium botulinum 62 A

SUMMARY—Death kinetics of spores of Clostridium botulinum, Type A, strain 62, were studied at sterilant gas temperatures in the range of 40°C (104°F) to 70°C (158°F). Hygroscopic carriers in the form of small discs of filter paper were used. The sterilant gas used was a mixture of dichlorodifluoromethane and ethylene oxide (88 and 12% by weight respectively). Pressures in the exposure atmosphere were adjusted to obtain an ethylene oxide concentration of 700 mg per liter at all temperatures. It was shown by gas-chromatographic analysis that an actual concentration of 700 ± 20 mg per liter was maintained at all exposure temperatures during all exposure periods. The effect of various moisture levels on spore death kinetics was also studied. The relative humidities employed in both the preconditioning and exposure of spores were 3, 23, 33, 53 and 73%, respectively. Thermochemical resistance parameters, D and z, were calculated as the reciprocals of the slopes of the survivor curves and thermochemical destruction time curves, respectively. Of the various environmental moisture levels studied, a preconditioning and exposure relative humidity of 3% for destruction of C. botulinum spores taken from an aqueous suspension, was found to be most effective. This effect held for all temperatures studied. Over the range studied, temperature was seen to have the greatest effect at 3% relative humidity (RH), second greatest at 33% and least effect at 73%.

INTRODUCTION

CLASSICALLY, sterilization is most often accomplished by the application of steam under pressure in a chamber, generally called an autoclave, or by the use of the liquid form of a chemical agent. Many other methods are also utilized. Due to the hypersensitivity of many materials to sterilization by either dry or moist heat alone, interest developed in the possibility of applying microbicidal chemicals in the vapor phase at temperatures which would not be injurious to such materials. However, the concept of sterilization by a chemical in the vapor phase is of long duration. Its efficiency has been found to be affected by a number of variables including water activity of the contaminant, environmental moisture levels, temperature and concentration of sterilant. Despite these many variables, the refinement of methods of chemical vapor phase sterilization would serve to fill a decided gap in many areas where sterilization is now being used.

The effects of variables influencing vapor phase sterilization have been studied by many workers during the past three decades. (Gross et al, 1937; Griffith et al, 1938; Yesair et al, 1942; Kaye et al, 1949; Phillips, 1949; Phillips, 1952; Opfell et al, 1959; Ernst et al, 1962; Vondell, 1962; Gilbert et al, 1964; Liu et al, 1968). Owing to its high microbicidal properties, and to its explosiveness in the pure form when mixed with oxygen, ethylene oxide in combination with an "inert" diluent gas seems the most promising of the numerous chemicals studied thus far. Halogenated hydrocarbons, nitrogen and carbon dioxide have proved the most suitable of the carrier gases.

The effects of time, temperature and concentration upon the rate of sterilization by ethylene oxide are generally straightforward. Doubling the sterilant concentration permits sterilization in roughly half the time (Phillips, 1949). Each 10°C (18°F) increase in temperature about doubles the activity of this agent (Phillips, 1949; Ernst et al, 1962; Vondell, 1962; Liu et al, 1968).

However, two additional factors merit a greater evaluation in their relation to vapor-phase sterilization. The first to consider is the microorganism of utmost importance that must be eliminated. This is the most resistant species normally in the material to be sterilized which will endanger the consumer and/or cause spoilage of the food or any foods into which the item is incorporated. The microorganism of major public health significance in low-acid canned foods is *Clostridium botulinum*.

The second important factor to consider is the effect of moisture, which presents a much more complicated picture. The high relative humidities needed with some other gases used in vapor-phase sterilization, such as formaldehyde and β-propiolactone, are not needed with ethylene oxide. Furthermore, optimum relative humidities for sterilization of different bacterial species have been reported (Kaye et al, 1949; Vondell, 1962; Gilbert et al, 1964; Shibasaki et al, 1964). Related to this problem are the hygroscopic characteristics of the carrier materials.

Since nothing has been reported heretofore regarding death kinetics of spores of *C. botulinum* subjected to sterilant gases, this investigation was undertaken to determine the death kinetics of spores of the microorganism on exposure to ethylene oxide at various preconditioning and exposure relative humidities. Since sterilization in this manner is relatively slow at ambient temperatures, the death kinetics were studied at slightly elevated temperatures. Hygroscopic carriers were used and a constant ethylene oxide concentration was maintained. It was felt that investigations of this design would lend further insight to the feasibility of macro-scale vapor-phase sterilization at temperatures below those injurious to certain materials.

MATERIALS & METHODS

Test microorganism

Clostridium botulinum Type A strain 62, was chosen as the test microorganism to be used throughout this study. The choice was due to the public health significance of this organism and to the reported use of ethylene oxide in the sterilization of some dried food ingredients. However, certain drawbacks were encountered in laboratory technique owing to the anaerobic nature of the genus and to the fastidious growth requirements regarding sporulation and recovery media.

Sporulation medium

One pound of fresh beef heart from which the fat had been removed was ground, mixed with 1000 ml of distilled water and the mixture was boiled slowly for 1 hr. The pH was then adjusted to 7.6, and the beef particles were removed by straining the material through cheesecloth. The volume of the broth was made up to 1 L, and the following ingredients were added: Isoelectric casein, 5.0 g; gelatin, 10.0 g; glucose, 0.5 g; K_2HPO_4, 4.0 g; sodium citrate 3.0 g.

The broth was then distributed into test

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tubes (15 ml) or screw-cap dilution bottles (50 ml), and then beef heart particles, previously removed, were added to a depth of about 1 in. These tubes/containers were then sterilized by autoclaving.

This medium was chosen for the production of spores of *C. botulinum* since previous work (Stumbo, 1965; Townsend et al, 1956) has established that spores of this microorganism obtained greatest heat resistance when produced in this medium. Since the thermochemical resistance of these spores was being ascertained in this study, it was felt justifiable to grow spores in a medium from which maximum heat resistance would be derived.

Recovery Medium

One pound of fresh pork from which the fat had been removed was ground, mixed with 1000 ml of distilled water, and the mixture boiled slowly for 1 hr. The pork particles were removed by filtration through layers of cheesecloth. The filtrate was cooled in the refrigerator, after which the fat was skimmed off and the volume made up to 1 L with distilled water. The broth was placed in a straight-walled container and the following ingredients were added: Peptone, 5.0 g; tryptone, 1.5 g; K_2HPO_4, 1.25 g; soluble starch, 1.0 g; sodium thioglycollate 0.5 g.

The medium was heated, with stirring, to dissolve these ingredients. The pH was adjusted to 7.6, 15 g of agar were added, and the medium was autoclaved for 25 min at 110°C (230°F) to liquefy the agar and promote precipitate formation. While still hot, the medium was placed in a refrigerator at 4°C (39.2°F) until solidified. The solidified medium was then removed from the beaker by loosening it from the wall with a spatula and inverting the beaker. The layer containing any settled precipitate was trimmed off and discarded. The remaining agar was cut into small pieces which were returned to the beaker, and the medium was reliquified by autoclaving for 25 min at 110°C (230°F). It was then dispensed in tubes and sterilized.

Incubation temperature and time

In all cases, the incubation temperature was 30°C (86°F) and the incubation period was 1–2 weeks.

Preparation of the spore suspension

Each of 20 tubes of beef heart casein broth which had been previously exhausted of air by heating at 5 psig for 15 min were inoculated with the stock cell suspension. These broth cultures were incubated at 30°C (86°F) and examined daily by malachite green spore stains with bright field microscopy and with phase contrast microscopy of wet mounts. When a high percentage of mature spores had been produced in the tube cultures, these were used for inoculating larger volumes of media in 150 ml screw-cap dilution bottles for spore production.

Just prior to inoculation, air was exhausted from the bottles of medium by autoclaving them for 15 min at 5 psig. After autoclaving, the bottles were placed in an 80°C (176°F) water bath and time was allowed for the temperature of the medium to approximate that of the bath before inoculation. After inoculation, the bottles of medium were left in the bath for about 10 min to activate the spores for rapid germination. The inoculated bottles were then incubated at 30°C (86°F) and again examined daily as described above until almost complete sporulation was indicated by a large portion of mature, refractile spores.

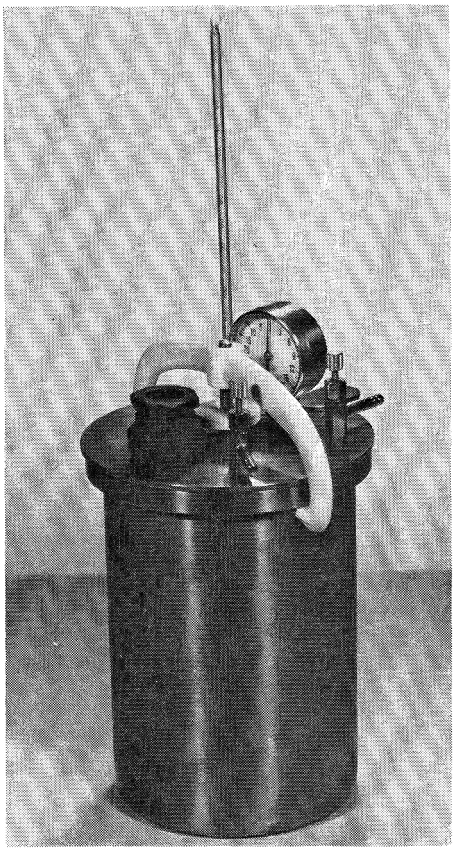

Fig. 1—The exposure chamber.

After incubation, the larger particles of undigested meat were removed by filtration through sterile layers of cheesecloth between which was a layer of glass wool. The crude spore suspension so obtained was then distributed into several sterile centrifuge bottles and the spores were sedimented by centrifugation at 1,000 *g* for 5 min at 4°C (39.2°F). After centrifugation, the supernatant was decanted and the spore pellet was redispersed in cold sterile distilled water and again centrifuged. This washing procedure was repeated seven times to reduce the amount of extraneous material carried over from the sporulation medium.

After the final washing the spores were resuspended in cold sterile distilled water in several 100-ml dilution bottles, each of which contained a layer of sterile No. 2 glass beads. This final stock spore suspension was stored at 4°C (39.2°F) until used in chemical vapor-phase resistance determinations.

Preparation of test microbial populations

A test spore suspension was prepared by appropriate dilution of the stock suspension to yield a spore concentration of about 10^7 per ml. However, this concentration was found intermittently to vary slightly from the desired density. This could have been due to clumping of spores, nonhomogeneity of the spore suspension, or error in the experimental sampling technique. Each test suspension was heat-shocked at 80°C (176°F) for 10 min to kill vegetative cells and to activate the spores for rapid germination and outgrowth when placed in the recovery medium after exposure. After heat-shocking, the test suspension was immediately cooled in an ice bath.

Hygroscopic carriers were used throughout this study. These consisted of highly absorbent circular paper discs used for the assay of penicillin and other antibacterial substances.

Uninoculated carrier discs were superimposed on Pyrex glass discs to prevent possible eventual inoculum loss by seepage, placed in a Petri dish, and the dish with contents sterilized by autoclaving. These were then thoroughly dried at 160°C (320°F) for 1 hr and 0.01 ml of the cold test suspension was deposited on each paper disc. The Petri dishes containing the samples were placed in a desiccator in which there was an abundance of a particular salt solution to maintain a desired relative humidity within the desiccator and dishes. A 3% RH was obtained more conveniently by the appropriate concentration of an aqueous sulfuric acid solution. However, to achieve all other relative humidities, saturated solutions of specific salts were used.

The various salts used to maintain the particular relative humidities (RH) were: 23% RH—potassium acetate ($KC_2H_3O_2$), 33% RH—magnesium chloride ($MgCl_2 \cdot 2H_2O$), 53% RH—magnesium nitrate ($Mg(NO_3)_2 \cdot 6H_2O$), and 73% RH—sodium chloride (NaCl) (Tech. Bull. (5), Hygrodynamics Inc., 1964). The desiccator containing the inoculated carrier discs was then stored overnight at 4°C (39.2°F) to achieve humidity equilibrium and to prevent germination of spores during this period.

Exposure system

The system essentially consisted of a battery of three stainless steel anaerobic jars (3.17 L capacity each) with clamp-on lids. (An external view of such a jar is presented in Figure 1.) These were equipped for either vacuumizing or pressurizing. Figure 2 is a schematic diagram of the heating and exposure chambers and their accessory fittings.

The first two jars were used as preheating chambers to bring the incoming gas to any desired temperature. These were immersed up to near the lids in a water bath, the temperature of which was thermostatically controlled. The third jar was used as the exposure chamber and it was immersed up to near its lid in a mineral oil bath, the temperature of which was also thermostatically controlled.

The heating media in the baths were actively circulated in order to promote better heat transmission and to maintain a uniform temperature throughout each bath. The lid of the exposure chamber was fitted by way of a male pipe thread pressure fitting with a narrow range humidity sensing element. These sensing elements were alternated, depending upon the relative humidity under consideration. The sensing element was connected to an electric hygrometer which indi-

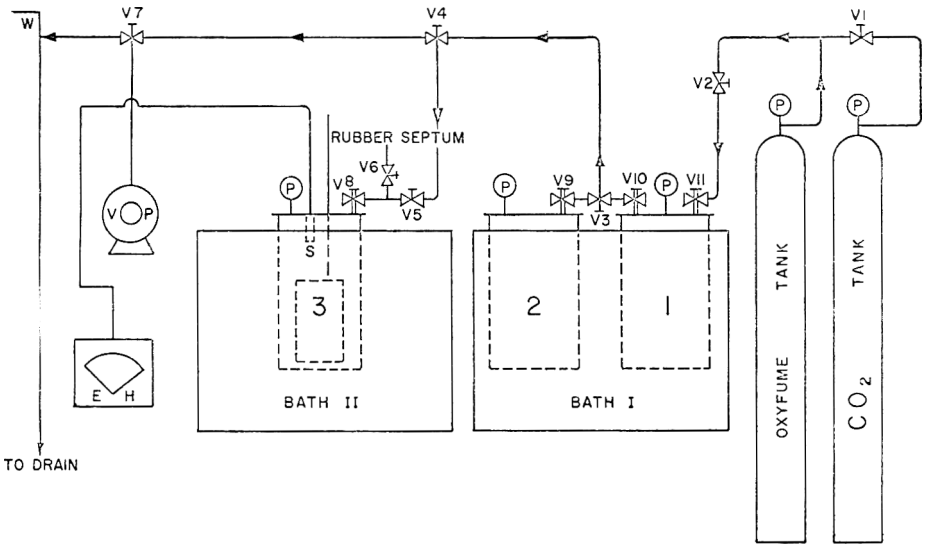

Fig. 2—Schematic diagram of ethylene oxide exposure system.

cated the humidity of the atmosphere inside the exposure chamber and could be quickly calibrated with a plug-in standard sensor.

A rubber septum water injection port and a standardized thermometer mounted directly over the test samples, were also fitted into the exposure chamber lid. All transport lines were glass with flush connections and rubber supports. Stopcocks were either glass or Teflon. The line connecting the preheating chambers with the water injection port and exposure chamber was wrapped with heating tape, the temperature of which was thermostatically controlled.

Carbon dioxide was used for flushing the system before introduction of the sterilant gas. This flushing procedure was done to free the system of any water condensate. Oxyfume Sterilant 12, a mixture of 12% ethylene oxide and 88% dichlorodifluoromethane by weight, was used as the sterilant gas. The system was evacuated to at least 29 in. Hg prior to introduction of the sterilant gas. A water aspirator was used to withdraw the sterilant after any exposure period.

The relative humidity of the exposure atmosphere was achieved by injecting a precalculated quantity of water through the rubber septum and valve as depicted in Figure 2.

Temperature, relative humidity and ethylene oxide concentration

Death kinetics of spores of *Clostridium botulinum*, Type A were studied at sterilant gas temperatures in the range of 40°C (104°F) to 70°C (158°F). No difficulty was encountered in maintaining constant temperatures durng exposure periods.

The effect of various moisture levels on spore death kinetics was also studied. The relative humidities employed in both the preconditioning and exposure of spores were 3, 23, 33, 53 and 73%, respectively.

Pressures were adjusted to obtain an ethylene oxide concentration of 700 mg per liter of sterilant atmosphere at all temperatures employed. This level was arbitrarily chosen since most commercial applications used from 400 to 1000 mg per liter (Bruch, 1961). The pressures ranged from 5 psig at 40°C (104°F) to 6.9 psig at 70°C (158°F). It was shown by gas-chromatographic analysis that a concentration of 700 ± 20 mg per liter was maintained at all exposure temperatures during all exposure periods.

Exposure procedure

(As described by Liu, et al, 1968, with some modifications.) The following stepwise procedure was used throughout the study:

1. The preheating and oil baths were brought up to and maintained at a desired temperature for at least 1 hr prior to the exposure operation.
2. The electric heating tape was turned on and the controller set at the desired temperature at least $^1/_2$ hr prior to the exposure operation.
3. The system was evacuated to at least 29 in. Hg.
4. Carbon dioxide was introduced to a pressure of 15 psig. This was withdrawn and the system re-evacuated. This flushing procedure was performed three times to free the system, especially the transport lines, of any water condensate.
5. The system was evacuated to 29 in. Hg.
6. The sterilant gas was introduced into the two preheating chambers to a pressure of 15 psig.
7. The vacuum of the exposure chamber was broken, the chamber lid removed, the test samples in a Petri dish were introduced, and the chamber lid was clamped back into place.
8. The exposure chamber was evacuated to at least 29 in. Hg. and sufficient sterile distilled water was injected through the rubber septum to give the desired relative humidity of the sterilant gas when it was introduced.
9. As soon as temperature equilibrium was attained, which was only a matter of seconds, heated sterilant gas from the preheating chambers was introduced into the exposure chamber to give the desired exposure pressure, and hence the ethylene oxide concentration. Only a few sec were required to attain any desired pressure in the exposure chamber.
10. Timing was started as soon as the desired exposure pressure was reached.
11. After any predetermined exposure period, the pressure in the exposure chamber was relieved by withdrawing the sterilant gas by way of a water aspirator.
12. The lid of the exposure chamber was removed, the exposed samples taken out and the samples dropped into tubes of sterile 1% Darvan No. 1 solution.
13. The tubes were placed on a reciprocating shaker in the cold at about 4°C (39.2°F) and shaken overnight to dislodge the spores from the carrier material and disperse them in the diluent.
14. These tubes were then agitated on a mixer until the paper discs were pulped.
15. Appropriate dilutions were then prepared and tubed in the recovery medium.
16. Counts of colonies developing from survivors were made after one or 2 weeks incubation at 30°C (86°F). Counts did not change significantly after 1 week incubation and so the shorter time was generally used.

Analysis of spore death rate data

As presented by Stumbo (1965),

$$t = D\ (\log a - \log b)$$

where t is the exposure time, D is the time necessary for 90% destruction of the bacterial population, and a and b are initial and final surviving populations, respectively.

Survivor curves were constructed by plotting the logarithms of the number of survivors on the ordinate versus time of exposure on the abscissa (Stumbo, 1965). The method of least squares (Snedecor, 1957) was used to locate the line of best fit for the straight line portions of the curves. D values were ascertained from the resulting regression coefficients.

Thermochemical destruction time (TCDT) curves were constructed by plotting on semilogarithmic paper, D values, in min, on the ordinate scale against corresponding temperatures in degrees Fahrenheit on the abscissa (Stumbo, 1965). These curves represent the times required at the respective temperatures to reduce the initial number of spores by 90%. Values of the term, z, may be taken directly from this plot or calculated by the least squares method. The term z, is defined as the number of °F required for a curve to traverse one log cycle. In reference to a TCDT curve, this value characterizes the relative heat and chemical resistance of a microorganism (Stumbo, 1965), in this case, *Clostridium botulinum*, Type A, strain 62.

Due to the exhaustive amount of data obtained from this study, location of the line of best fit calculations were programmed on a CDC 3600 computer system written in FORTRAN II. From the lines so obtained, D and z values were computed.

The centigrade temperature coefficient, Q_{10}, was computed by the following equation (Rahn, 1945):

$$z = 18/\log\ Q_{10}$$
$$Q_{10} = \log^{-1}(18/z)$$

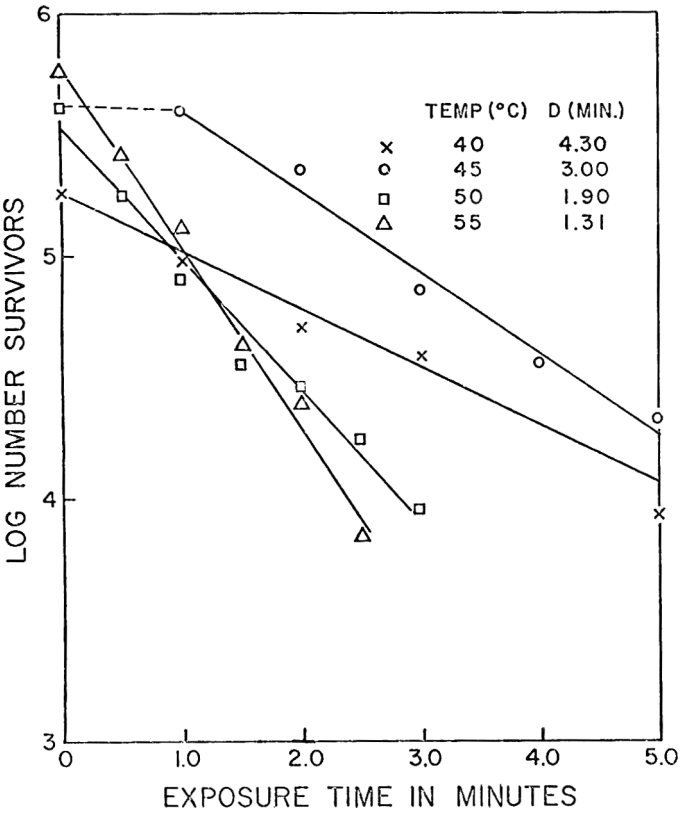

Fig. 3—Survivor curves for C. botulinum spores suspended in distilled water, deposited on paper discs, preconditioned at 3% RH and exposed to Oxyfume Sterilant 12 at specified temperatures, 700 mg ethylene oxide per liter, and 3% RH.

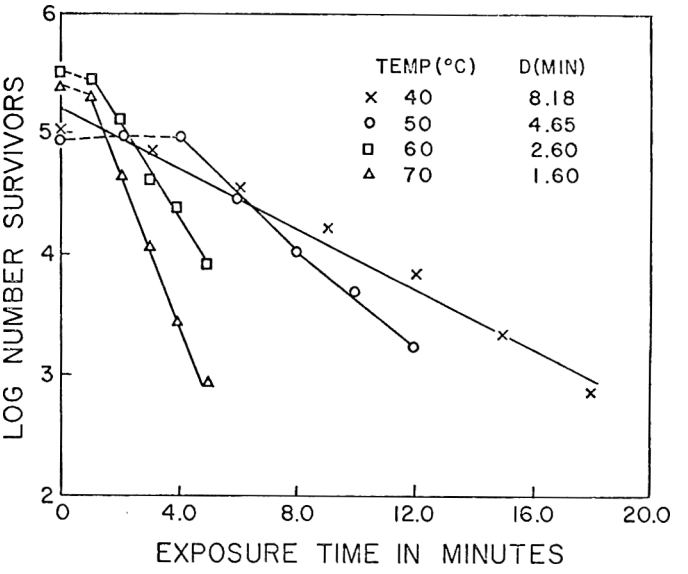

Fig. 4—Survivor curves for C. botulinum spores suspended in distilled water, deposited on paper discs, preconditioned at 23% RH and exposed to Oxyfume Sterilant 12 at specified temperatures 700 mg ethylene oxide per liter, and 23% RH.

RESULTS

SURVIVOR curves for *Clostridium botulinum* spores deposited on paper discs, preconditioned at the desired exposure relative humidity and exposed to Oxyfume Sterilant 12 at various temperatures and at relative humidities of 3, 23, 33, 53 and 73% appear in Figures 3, 4, 5, 6 and 7, respectively. The slightly lower temperatures used at 3% RH permitted convenient exposure periods which yielded significant survivor data. Each point used in these curves represents the average of three trials. In each trial, triplicate samples were exposed and each sample was cultured in at least triplicate tubes. Therefore, each point in the curves represents the average of at least 27 tube colony counts.

For those survivor curves in which an initial time lag appears, as denoted by the broken line, it is believed that some spores were being activated for germination by the additional heat of the treatment at nearly the same rate as others were being destroyed by the heat and sterilant gas. In cases where it did appear, the time lag caused by the heat activation requirement seemed to decrease as the temperature increased. It is also possible that some of these lags may have been caused, in part, by time required for the exposure chamber to equilibrate with respect to temperature and relative humidity.

Figures 3 to 7, inclusive, demonstrate that of the environmental moisture levels studied, a preconditioning and exposure relative humidity of 3% is most effective for destruction of *C. botulinum* spores by ethylene oxide in Oxyfume Sterilant 12. A reduction in D value of from about one-half to two-thirds was observed at this relative humidity, compared to those obtained at the other relative humidities studied. This decrease was observed over the entire temperature range used. This effect can be seen more easily by comparing D values at 40°C (104°F). At 3, 23, 33, 53 and 73% RH, the D values were found to be 4.3, 8.18, 13.75, 10.20 and 8.80, respectively. These values appear similar, especially in the 8.18–10.20 range. However, these data were subjected to a student's "t-test" (Mendenhall, 1964) and a statistical difference at the 1% level of significance was found to exist between the D values determined at all the different relative humidities, when taken in a paired comparison in all possible combinations except one. The only exception was between the D values determined at 23 and 73% RH, 8.18 and 8.80, respectively.

The differences in D values determined at the higher temperatures were also analyzed for a statistical significance by the "t-test." The results are given in Table 1.

At 50°C (122°F) no significant difference was found to exist between the D value determined at 23% RH (4.65)

Table 1—Student's "t-test" to ascertain a difference at the 1% level of significance between D values determined at various relative humidities and temperatures.

	Comparison of D values in pairs at various relative humidities															
Temp.	23/33[1]			23/53			23/73			33/53			33/73			53/73		
40°C (104°F)	8.18[2]	13.75	+	8.18	10.20	+	8.18	8.80	−	13.75	10.20	+	13.75	8.80	+	10.20	8.80	+
50°C (122°F)	4.65	5.75	+	4.65	5.60	−	4.65	5.10	+	5.75	5.60	−	5.75	5.10	+	5.60	5.10	−
60°C (140°F)	2.60	2.65	−	2.60	2.80	−	2.60	3.00	−	2.65	2.80	−	2.65	3.00	−	2.80	3.00	−
70°C (158°F)	1.60	1.74	+	1.60	1.80	+	1.60	1.95	+	1.74	1.80	+	1.74	1.95	+	1.80	1.95	+

Legend: + = a significant difference − = no significant difference.
[1] Relative humidity comparisons.
[2] D values determined at the particular relative humidity and temperature.

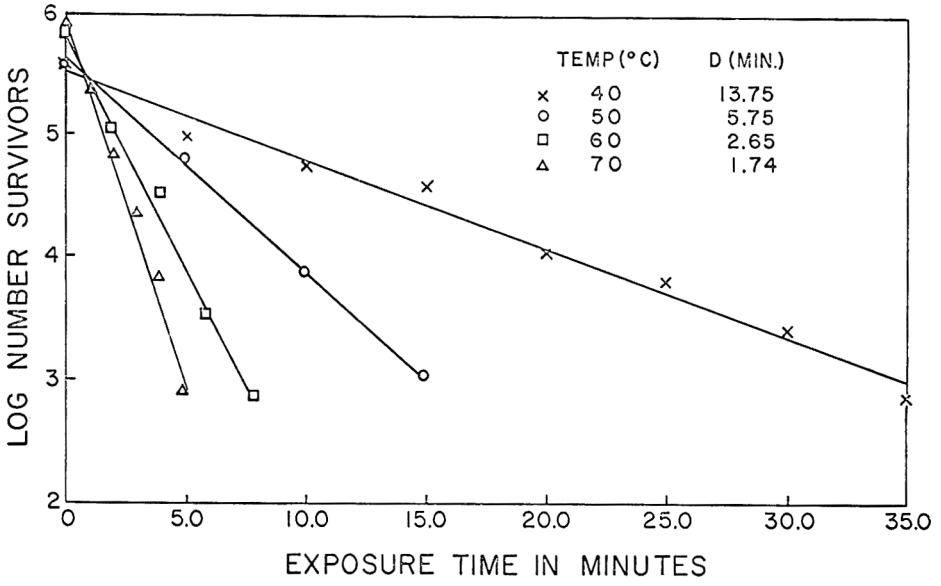

Fig. 5—Survivor curves for C. botulinum spores suspended in distilled water, deposited on paper discs, preconditioned at 33% RH and exposed to Oxyfume Sterilant 12 at specified temperatures, 700 mg ethylene oxide per liter, and 33% RH.

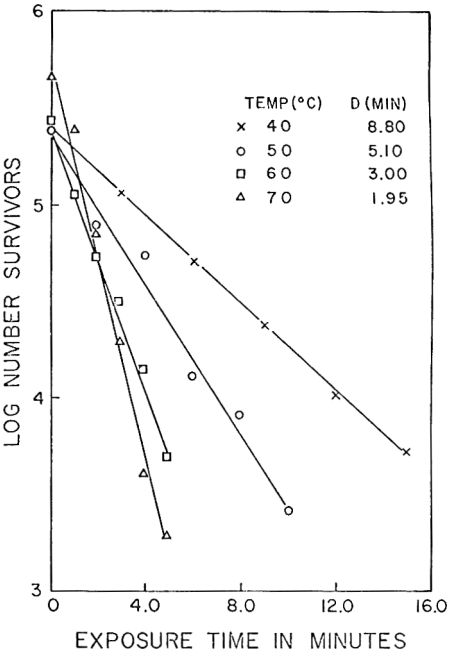

Fig. 7—Survivor curves for C. botulinum spores suspended in distilled water, deposited on paper discs, preconditioned at 73% RH and exposed to Oxyfume Sterilant 12 at specified temperatures, 700 mg ethylene oxide per liter, and 73% RH.

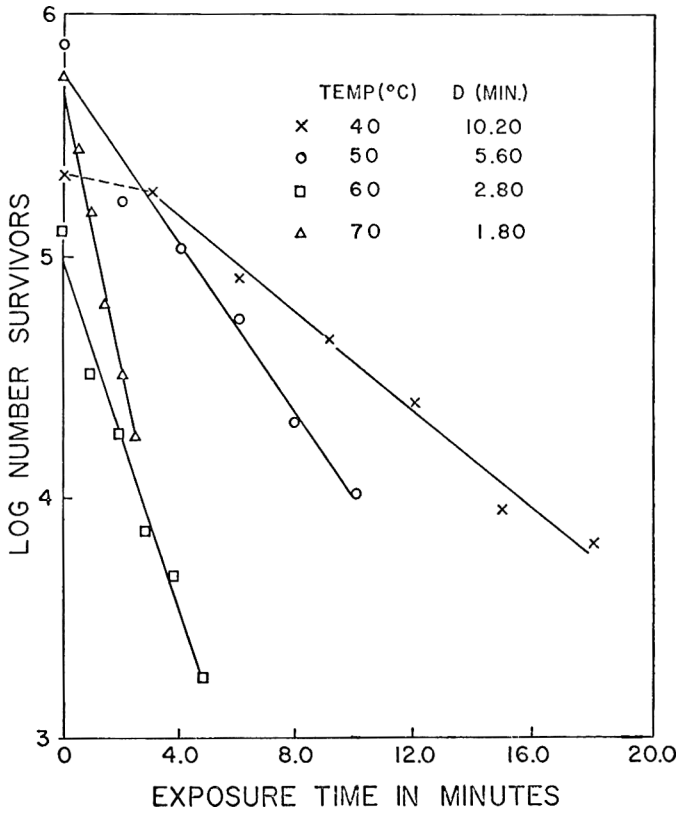

Fig. 6—Survivor curves for C. botulinum spores suspended in distilled water, deposited on paper discs, preconditioned at 53% RH and exposed to Oxyfume Sterilant 12 at specified temperatures, 700 mg ethylene oxide per liter, and 53% RH.

versus that at 53% RH (5.60). A similar relationship was found with the D value at 33% RH (5.75) versus that at 53% RH (5.60) versus that at 73% RH (5.10). The latter two paired comparisons seem reasonable, but on the other hand, that of the first pair seems difficult to explain, especially when contrasted with that of 23% RH vs. 73% RH. Perhaps in the 23 vs. 53% RH comparison, the lack of a significant difference was due to the spread of the D values determined at 53% RH, one of which approximated closely those determined at 23% RH. However, this doubtful observation could not be rejected with 90% confidence using the rejection coefficient (Q) test (Dean et al, 1951).

At 60°C (140°F), no significant difference was found between the D values determined at the relative humidities listed in Table 1. This indicated that at this temperature, an equivalent destructive effect was exerted on the spores of *C. botulinum* by Oxyfume Sterilant 12 at all tabulated humidities.

At 70°C (140°F) a significant difference was demonstrated between the D values determined at all relative humidities.

The thermochemical destruction time curves (TCDT) for *C. botulinum* spores suspended in distilled water, deposited on paper discs, preconditioned at varying relative humidities and exposed to Oxyfume Sterilant 12 at the same relative humidities appear in Figure 8.

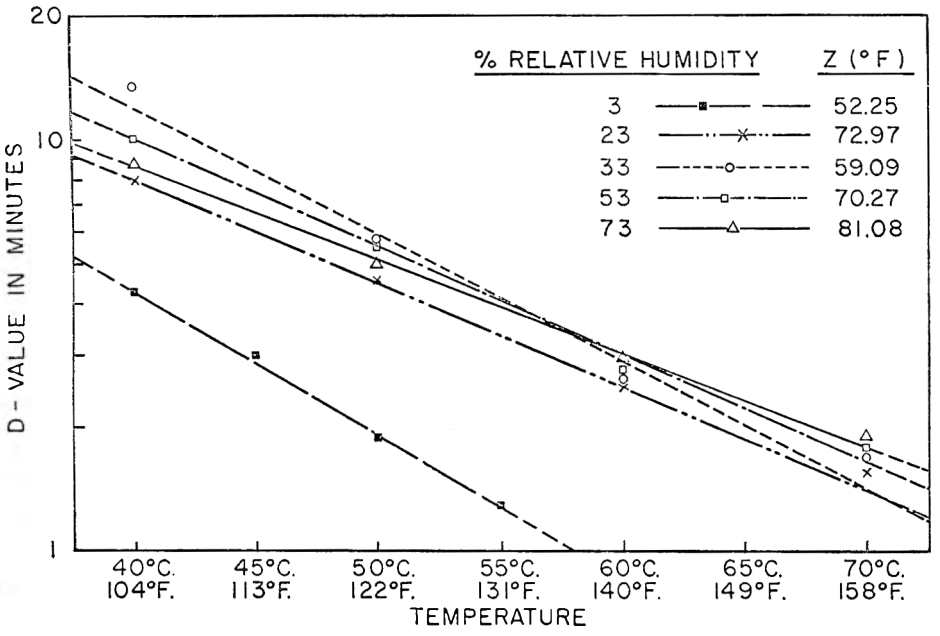

Fig. 8—Thermochemical destruction time curves for C. botulinum spores suspended in distilled water, deposited on paper discs, preconditioned at specified relative humidities and exposed to Oxyfume Sterilant 12 at the same relative humidities.

For a relative humidity of 3% a z value of 28.00°C (52.25°F) and a Q_{10} of 2.21, were determined. At 23, 33, 53 and 73% RH, respectively, the corresponding z values for these conditions were 40.50°C (72.97°F), 32.79°C (59.09°F), 39.00°C (70.27°F) and 45.00°C (81.08°F), while the Q_{10} values were 1.76, 2.02, 1.80 and 1.67, respectively. Table 2 summarizes the z and Q_{10} values calculated from results obtained at the various relative humidities of the exposure atmosphere.

DISCUSSION

A STUDY of the effect of varying environmental moisture levels on the resistance of *C. botulinum* spores taken directly from an aqueous suspension, equilibrated to a desired relative humidity, and exposed to a dichlorodifluoromethane-ethylene oxide mixture, revealed an optimal relative humidity for their destruction at slightly elevated temperatures. Of the relative humidities studied, namely, 3, 23, 33, 53 and 73%, spores preconditioned and exposed at 3% RH and subjected to Oxyfume Sterilant 12 at the same relative humidity, were most susceptible to the lethal effects of this sterilant. At the other environmental moisture levels, a correlation of resistance with relative humidity was not consistent at all temperatures, since TCDT curves were not parallel and, in some cases, intersected.

Table 2—z and Q_{10} values calculated for spores of C. botulinum, suspended in distilled water, deposited on paper discs, preconditioned at various relative humidities and exposed to Oxyfume Sterilant 12 at the same relative humidities.

Preconditioned and exposed: % R.H.	z value °C	z value °F	Q_{10}
3	28.00	52.25	2.21
23	40.50	72.97	1.76
33	32.79	59.09	2.02
53	39.00	70.27	1.80
73	45.00	81.08	1.67

It had been previously reported (Kaye et al, 1949; Gilbert et al, 1964) that preconditioning and exposure at 33% RH was optimal for destruction of *B. globigii* (*B. subtilis* var. *niger*) spores. However, this evidence resulted from work with pure ethylene oxide and at 25°C (77°F).

Sair (1966) reported on the use of propylene oxide, apparently in conjunction with carbon dioxide as the carrier, to destroy Salmonella cells and spores of microorganisms in dried egg whites. He found a relative humidity of from 3 to 5% optimal for destruction of bacterial spores at 48.9°C (120°F). Additional work in this laboratory with spores of *C. botulinum* Type B, Strain 213, and with *B. subtilis* (ATCC 9524), indicates a similar decrease in cell resistance at this low relative humidity (Private Communications, McCormick et al, 1967).

The results of Sair (1966) can be correlated, to a limited degree, with this study since the type of test samples, paper and dehydrated egg white, were both hygroscopic materials. At 3% RH, a one-half to two-thirds reduction in D value, in comparison to that determined at the other relative humidities studied, occurred at temperatures from 40°C (140°F) to 55°C (131°F). The linear nature of the TCDT curves obtained for 3% RH and given in Figure 8 may justifiably permit an extrapolation of death characteristics to the somewhat higher temperatures used for the remaining relative humidity values.

Death rate characteristics found at the higher relative humidities (23, 33, 53 and 73%) do not all exhibit distinct differences. Taken in paired comparisons, various differences were found at 50°C (112°F). However, at 60°C (140°F) a statistically significant difference between death rates was not demonstrable. This would imply that at this temperature, destruction of *C. botulinum* spores would proceed equally well at any of the relative humidities mentioned above.

At 70°C (158°F), two effects became more apparent. Even though the D values determined at this temperature were very close (1.60, 1.74, 1.80 and 1.95 for 23, 33, 53 and 73% RH, respectively), these were shown to be statistically different even at the 1% level of significance. The first point to be made is the decreasing death rate with increasing relative humidity at this temperature. The second is the occurrence of differences in TCDT curves.

Figure 8 gives the TCDT curves for spores of *C. botulinum* exposed to Oxyfume Sterilant 12 at temperatures from 40°C (104°F) to 70°C (158°F) for the different relative humidities. The z values from these curves indicate that over the ranges studied, temperature exerted the greatest effect at 3%, with that at 33% second, and that at 73% the least. This further demonstrates the diminishing sterilizing efficiency of the dichlorodifluoromethane-ethylene oxide mixture as the relative humidity of the sterilizing atmosphere increases.

This observation perhaps could be correlated with two isolated reports from the older literature. Puck (1947) observed the phenomenon of diminished bactericidal activity of propylene glycol vapors at high humidities. Church et al (1956) found that aerobic spores became more sensitive to ethylene oxide after mild lipid extraction. When added back, an emulsion of the extracted lipid had a protective effect for the spores treated with ethylene oxide. These workers speculated that removal of lipid might have sensitized a spore by exposing critical areas to the action of ethylene oxide.

In this experiment, *C. botulinum* spores used for thermochemical resistance studies were all produced in one lot in the same medium. It might be concluded that members of a random sample would all have approximately the same

fat content; and, consequently, would all have the same amount of this type of protection. Therefore, differences in spore resistance at high or low environmental relative humidities perhaps could not be attributed to this protective effect of the lipid.

However, as the relative humidity of the preconditioning and exposure atmospheres was increased, the moisture content around individual spores would seemingly also increase. This "wetting" of the spore perhaps would decrease, or in some way interfere with its permeability to Oxyfume Sterilant 12.

Another speculative explanation would utilize the property of ethylene oxide of infinite water-solubility. A monomolecular hydrolysis of ethylene oxide to ethylene glycol (a less effective bactericide) possibly occurred at the spore coats, the extent of which would vary with the amount of moisture around each spore. Such an occurrence would perhaps be reflected in the increase in D values at high relative humidities.

It was believed reasonable to extrapolate to higher temperatures at those relative humidities which were studied to give some indication of what might be expected. This may be done using the following general equation of thermal or thermochemical destruction time curves (Stumbo, 1965).

$$\log D_2 - \log D_1 = {}^1/_z (T_1 - T_2)$$

in which,

D_2 = D value corresponding to temperature T_2, and the time required to destroy 90% of the spore population when exposed temperature T_2

D_1 = D value corresponding to a higher temperature T_1, and the time required to destroy 90% of the spore population when exposed to temperature T_1.

Taking the D values determined at 40°C (140°F) for the different relative humidities as D_2, the D values expected with exposure temperatures of 100°C (212°F) and 110°C (230°F) were computed and these are given in Table 3. As anticipated, the lowest D values at these elevated temperatures were found to be at the 3% and 33% relative humidities. Taking those calculated at 3% as an example, the D value expected with an exposure temperature of 100°C (212°F) would be 0.036 min or 2.16 sec and similarly, at 100°C (230°F), the expected D value would be 0.017 min or 1.02 sec.

Table 3—Calculated D values at e evated temperatures, for spores of C. botulinum, suspended in distilled water, deposited on paper discs, preconditioned at various relative humidities and exposed to Oxyfume Sterilant 12 at the same relative humidities.

Preconditioned and exposed:	D value at 100°C (212°F)		D value at 110°C (230°F)	
% R.H.	Min	Sec	Min	Sec
3	0.036	2.16	0.017	1.02
23	0.271	16.26	0.154	9.24
33	0.205	12.30	0.101	6.06
53	0.296	17.76	0.164	9.84
73	0.410	24.60	0.246	14.76

In keeping with the 12 D concept for the reduction of spores of *C. botulinum*, under the conditions at 3% RH employed in this study, it should require 25.92 sec at 100°C (212°F) or 12.24 sec at 110°C (230°F) to reach this level of sterilization. This same concept could also be applied to spore population reduction at the other relative humidity values with the data listed in Table 3.

The study of temperature effect on the sporicidal action of ethylene oxide in this investigation helps form a sounder basis for commercial application of vapor-phase sterilization. The explosive nature of pure ethylene oxide can be circumvented by the use of a nonexplosive mixture such as Oxyfume Sterilant 12. This mixture has been shown to be active for vapor phase sterilization applications. However, the relatively high resistance observed for this one type and strain of *C. botulinum*, compared with resistance values reported for other bacterial species (Liu, et al, 1968), emphasizes an urgent need for studying resistance of more *C. botulinum* types and strains.

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External Fat Cover Influence on Raw and Cooked Beef. 2. Cooking Time, Losses, Press Fluid and Shear Force Values

SUMMARY—The semitendinosus muscle was used in a study of the influence of the external fat cover and the oven temperature on the characteristics of cooked beef. Roasts were cooked either with or without the external fat cover to an internal temperature of 58°C (rare). Two oven temperatures, 163° or 218°C, were used. The roasts cooked at 218°C required a shorter time in the oven than did the roasts cooked at 163°C but the presence or absence of the external fat cover had very little effect on the min per lb. Roasting at an oven temperature of 218°C caused significantly greater total cooking losses than roasting at 163°C. The cooking losses were increased significantly by cooking the roasts with the fat cover on, as compared with removing the external fat. The increase was mainly due to an increase in the drip losses. The presence or absence of the external fat cover had a significant influence on the quantity of press fluid obtained from the samples. A greater amount of fluid was pressed from the roasts without the fat cover. The posterior ends of the muscle were more tender (as indicated by Warner Bratzler shear force values) than the anterior ends. The lower oven temperature also caused significantly more tender roasts.

INTRODUCTION

THE EFFECTS of the external fat layer on the speed of roasting and shrinkage of beef were investigated by Thille et al. (1932). They concluded that when beef was roasted at 225°C to an internal temperature of 65°C, the exterior fat, because of the change in heat conductivity of fat as it passes from the solid to the liquid state, speeds up the rate of heat penetration, but interior fat may retard the rate of heat penetration. The purpose of the present investigation was to study the effects of the external fat cover and the oven temperature on the cooking time, cooking losses, press fluid content and shear force values of beef. The effects on the moisture and fat content of the roasts were reported in a previous paper.

EXPERIMENTAL

27 CHOICE GRADE semitendinosus roasts were prepared and roasted as described in the first paper of this series (Woolsey et al., 1969). Cooking losses were calculated as a percent of the raw weight. The drippings were removed from the pans with hot water and stored in beakers until the fat layer solidified. The fat cake was then weighed and the percent fat in the drip calculated. The cooked roasts were wrapped in aluminum foil and refrigerated overnight before being sampled.

The center portions of the roasts were used for press fluid and shear force determinations. Cores 1/2 in. in diameter were sheared on the Warner Bratzler apparatus. At least six measurements were taken on each roast. The press fluid content was determined on samples 1/2 in. in diameter. The slices, weighing between 0.65 g and 0.80 g, were placed between Whatman No. 1 filter paper and pressed between glass plates by a force of 45 g/cm² for 5 min. The press fluid was calculated as the per cent change in weight of the sample.

RESULTS & DISCUSSION

TABLE 1 GIVES the average results for the cooking time, cooking losses and shear force for each of the four treatments. Table 2 gives the F ratios from analysis of variance.

Table 1—Mean values for cooking time, cooking losses, press fluid and shear force.

	Fat cover on		Fat cover off	
Type of data	218° C N = 13	163° C N = 14	218° C N = 14	163° C N = 13
Total cooking time (min)	65	79	51	69
Cooking time (min/lb)	35	44	34	47
% Cooking losses				
Total	22.0	17.6	16.7	14.1
Evaporation	14.8	11.0	14.7	11.2
Drip	7.2	6.6	2.0	2.9
% fat in drip	63.2	52.9	2.9	1.7
% press fluid	34.8	35.8	41.3	37.7
Shear force (lb)				
Anterior	10.1	9.3	10.0	9.1
Posterior	9.0	8.0	8.4	8.3
Mean	9.7	8.6	9.2	8.7

Cooking time

All roasts were cooked to the same internal temperature of 58°C (rare) but two different oven temperatures (218° and 163°C) were used. The roasts cooked at 218°C required a shorter time than the ones cooked at the lower temperature. The presence or absence of the external fat cover did not greatly affect the min per lb required to cook roasts at either temperature. This is in contrast to work by Thille et al. (1932) who reported that lean surfaced roasts required a longer time in min per lb to reach the same internal temperature of 65°C than did fat surfaced roasts.

Until beef fat melts, at between 31° and 38°C, it is a poor conductor of heat. It seems that when meat is cooked to 58°C the external fat layer does not have an effect on the rate of heat penetration but at higher internal temperatures the external fat layer may reduce the cooking time. In this study the oven temperature had more effect on the rate of heat penetration than did the external fat cover.

Cooking losses

The roasts cooked at 218°C had significantly greater losses than did the roasts cooked at 163°C ($P < 0.01$). This was due mainly to an increase in evaporation losses at the higher temperature. The roasts with the external fat cover had significantly greater total losses than the roasts without the fat cover ($P < 0.01$), due to greater drip losses. The increase in the quantity of drip was composed of fat.

Table 2—F Ratios for total cooking losses, press fluid content and shear force values.

	F Ratio		
Source of variation	Total cooking losses	Press fluid content	Shear force values
External fat cover (F)	42.64**	35.96**	N.S.
Oven temperature (T)	18.26**	N.S.	6.24*
End of muscle (E)	N.S.	N.S.	14.27**
F × T	6.18*	10.10**	N.S.
T × E	N.S.	6.71*	N.S.
F × E	N.S.	N.S.	N.S.

** Significant at the 1% level.
* Significant at the 5% level.
N.S.: Nonsignificant.

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Press fluid content

Press fluid values (Table 1) for roasts cooked without the fat cover were significantly higher than for those cooked with the fat cover ($P < 0.01$). The oven temperature and the end of the muscle used did not cause significant differences in the amount of press fluid in the muscle. In the literature it is reported that the water holding capacity (measured by the percent press fluid) decreases as the extent of denaturation of the muscle protein increases (Hamm et al., 1960).

In this study the roasts were cooked to the same internal temperature but the different oven temperatures did cause a difference in the apparent doneness of the roasts. When cooked at the lower oven temperature, the interior appeared more well done. From this it was expected that the oven temperature might influence the press fluid content, but it did not. No reason why the presence or absence of the external fat layer should influence the amount of press fluid can be given unless it is indirectly through the amount of drip. The roasts with the greatest amounts of drip losses had the lowest press fluid contents, and vice versa.

Shear Force Values

The end of the roast and the oven temperature had significant effects on the shear force values whereas the presence or absence of the external fat layer did not. The roasts from the posterior end of the muscle were significantly more tender than the anterior roasts ($P < 0.01$). Ramsbottom et al. (1945) state that the semitendinosus is fairly uniform throughout its length. However both Ginger et al. (1958) and Taylor (1959) found variations in tenderness between anterior steaks. The posterior steaks were more uniform.

When the anterior and posterior shear force values reported by Taylor (1959) are compared, the posterior steaks have lower values than the anterior, in agreement with the results of the present study. The differences between anterior and posterior end of this muscle must be considered when setting up experiments to investigate tenderness.

The roasts cooked at 163°C had significantly lower shear force values than those cooked at 218°C ($P < 0.05$). It is generally accepted that lower oven temperatures give more tender meat.

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Cooking Methods and Heating Effects on DDT in Chicken Tissues

SUMMARY—DDT incorporated into chicken tissues during the growing period was reduced in concentration during cooking by either baking, frying, or steaming and during heating of tissues in closed containers for varying lengths of time. DDT was converted to DDD in each of the treatments, but the concentration of DDE was not altered significantly. Total losses of residue were greater when tissues were fried or steamed than when the samples were either baked or heated in closed containers. Losses of residue from chicken tissue occurred primarily through leaching of fat during the cooking process.

INTRODUCTION

A PREVIOUS report from this laboratory (Ritchey et al., 1967) recognized that concentrations of DDT and lindane in chicken tissue were reduced during the process of cooking. Liska et al. (1967) indicated that chlorinated hydrocarbon residues were reduced approximately 90 percent in chicken tissue heated rather severely. These reports, in addition to the previous work of Liska et al. (1965) and Carlin et al. (1966), are indicative of a growing effort to determine the effects of processing and preparation methods upon insecticide residues deposited in foods.

While numerous reports have emphasized the concentration of various insecticide residues found in foods, only a few have been concerned with the alteration in amounts of residues brought about during cooking. For example, several investigators, including Draper et al. (1950), Ivey et al. (1961), Liska et al. (1964), and Naber et al. (1961) reported the occurrence of residues in chicken tissues and eggs.

A major concern about the increased use of pesticides in production has expressed itself in research designed to determine methods of reducing residue concentration during processing. Thus, food products not acceptable for consumption might become usable because the insecticide residues had been decreased below established tolerance levels.

Since in the case of most animal products, foods are consumed after cleaning procedures and after cooking there is a need to investigate the effects of these processes on commonly occurring residues. This report discusses information on the effects of commonly used cooking methods and the effects of heating on

Table 1—Carcass weights, cooking losses and changes in dry matter and fat of chickens heated by different methods and for different times.

Method and Time of Heating	Carcass wt. (g)	Cooked wt. (g)	Cooking loss (%)	Dry matter (%)	Fat (%)
Raw	1116 ± 157[1]	—	—	26.3 ± 1.9	12.2 ± 1.7
Fried	1014 ± 194	694 ± 149	31.8 ± 3.1	34.8 ± 1.9	10.7 ± 4.1
Steam Pressured	1080 ± 184	741 ± 128	31.5 ± 1.3	35.4 ± 1.6	10.1 ± 3.0
Baked	1124 ± 139	876 ± 119	22.4 ± 3.4	30.7 ± 8.7	10.2 ± 2.3
30 min[2]	—	—	—	29.4 ± 2.6	10.2 ± 3.3
60 min[2]	—	—	—	32.3 ± 3.8	11.1 ± 2.9
90 min[2]	—	—	—	37.6 ± 5.8	12.9 ± 4.5

[1] Mean ± SD; 20 birds per group.
[2] These samples represent chicken tissue ground, placed in small closed pans and heated in an oven at 350°F for varying times.

Table 2—The amounts of DDT, DDE, and DDD in chicken tissues cooked by different methods and heated for varying times.

Method and Time of Heating	ppm in dry matter			
	DDT	DDE	DDD	Total
Raw	31.3 ± 7.7[1]	11.0 ± 2.5	3.0 ± 0.6	45.3 ± 9.9
Baked	20.7 ± 4.0	10.9 ± 1.4	11.6 ± 2.2	43.2 ± 6.6
Fried	14.4 ± 3.1	9.0 ± 1.8	9.5 ± 2.3	32.9 ± 4.1
Steam Pressure	7.8 ± 2.5	8.4 ± 5.1	11.8 ± 2.0	28.0 ± 4.9
30 min[2]	22.7 ± 7.2	9.9 ± 2.2	5.6 ± 2.2	38.2 ± 9.6
60 min[2]	14.7 ± 5.6	9.7 ± 1.7	11.8 ± 4.0	36.2 ± 9.1
90 min[2]	9.4 ± 3.9	9.8 ± 1.5	14.5 ± 3.8	33.7 ± 4.6

[1] Mean ± SD; 20 samples per group.
[2] These samples represent chicken tissue ground, placed in small closed pans and heated at 350°F for varying times.

Table 3—Concentrations of DDT, DDE and DDE in the fat portion of drippings from cooked chicken.

Treatment	ppm in fat drippings		
	DDT	DDE	DDD
Baked	69.4 ± 10.3[1]	21.5 ± 5.0	17.1 ± 4.6
Fried	37.8 ± 14.2[1]	21.6 ± 3.6	27.0 ± 4.7
Steam Pressure	52.0 ± 6.1[2]	22.7 ± 4.3	27.3 ± 4.5

[1] Mean ± SD of 16 birds.
[2] Mean ± SD of 14 birds.

DDT in chicken tissues.

EXPERIMENTAL

DAY-OLD VANTRESS, AA male chicks purchased from a commercial hatchery were housed in wire-screen batteries and were fed *ad libitum* commercial started and grower mashes to which had been added DDT at a concentration of 10 ppm. All birds were given the same ration for the growing period of 10 weeks, then were slaughtered, processed in the conventional manner, wrapped in freeze-paper and frozen at −20°F.

The frozen birds were divided randomly into experimental groups of 20 each. One group was used as a control (raw) and other groups were cooked, either by baking, frying or pressure cooking. Baking was accomplished by heating the whole carcass on a wire rack in an oven for 60 min at 350°F. Frying was done by cutting the birds into the usual anatomical parts and heating in an electric frying pan with 15 mls of corn oil for 40 min. Pressure cooking was done by heating the divided carcass with one-half cup of water for 15 min at 10 lbs pressure.

Cooking procedures were standardized so that individual carcasses within a group were treated alike. After cooking, the meat was removed from the bones, and the entire carcass was ground thoroughly. Portions of each carcass were used for subsequent analyses.

In addition to cooking by different methods, chicken tissues were heated in closed containers. Meat was removed from the control (raw) carcasses, ground thoroughly, and divided into four equal parts. One portion was used for the control. The other portions were placed in small pans, covered with a lid, and heated for either 30, 60 or 90 min in an oven at 350°F. Individual samples were mixed after heating so that all moisture and fat which had separated during the heating were returned to the sample. Subsequent analyses were made on portions of each individual sample.

Carcass weights of all birds were recorded and the amount of cooking loss was determined. The amounts of nitrogen, fat, and dry matter were determined by methods of the A.O.A.C. (1960). DDT, DDD, and DDE were determined in each sample by the method of Stemp et al. (1964) as modified slightly by Ritchey et al. (1967). Residues were calculated on a dry matter basis.

Drippings were collected from carcasses for each of the cooking methods. Samples of fat were taken from the drippings and the amounts of residue were determined by the methods mentioned previously.

RESULTS AND DISCUSSION

INFORMATION about the birds (Table 1), including average carcass weights of each group, average weight after cooking, cooking losses and average amounts of dry matter and fat remaining in the carcasses gave a good indication of the amount of heat applied in the various treatments. This was particularly true in the samples heated in containers having covers from which only the moisture and volatile components escaped. In those samples the moisture and fat remaining in the pans were added back to the meat sample prior to analysis.

Previously (Ritchey et al., 1967), the effects of cooking on DDT and its breakdown products had been examined in chicken tissues containing relatively small amounts of the residue. The amounts of residue deposited in the tissue of birds on this present study were much greater. This allowed for better comparisons of residue losses during cooking and heating, and yielded data showing variability between individual birds. Data on the raw tissue indicated considerable variation in amounts of residue deposited.

The amounts of DDT calculated on a dry matter basis decreased during the cooking and heating treatments (Table 2). The concentration of DDE remained fairly constant, but DDD increased as the tissues were heated. The relatively constant amount of DDE found in the chicken tissues indicated that heating or cooking had little effect upon the conversion of DDT to DDE and suggested that this occurred prior to the heating as reported by Liska et al. (1967). The concentration of DDT was reduced considerably by each of the three cooking methods with the pressure steaming bringing about the largest reduction.

The tissues were analyzed for residue content after heating in small closed pans for either 30, 60 or 90 min in order to study the effects of heating per se upon the residue. Losses of fat were minimized although there was considerable loss of water as evidenced by increasing amounts of dry matter during the heating (Table 1).

That heating causes considerable lowering of the DDT and increasing of the DDD concentration was apparent (Table 2). These particular data, coupled with the constant amount of DDE in the heated tissues, demonstrate clearly the conversion of DDT to DDD during the heating process. However, DDD was found in the raw tissues at a concentration of around 3 ppm and indicated that all DDD did not come from DDT during the heating process.

There were significant losses of residue during each of the cooking processes and these losses can very likely be attributed to losses of residue in fat. The concentrations of DDT, DDE, and DDD were determined in the fat portion of the drippings (Table 3). Although the data cannot be regarded as an exact measure of losses because of the manner in which drippings were collected, relatively high concentrations of DDT, DDE, and DDD were found in the fat.

Comparison of total amounts of residues in all treatments revealed that the greatest reduction in residue concentration occurred in the cooking procedures in which there were losses through leaching of fat. There was some reduction in total amount of residue in samples heated in closed containers as heating time increased. However, these studies indicate that the greatest losses occur because of leaching and not because of heat.

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Studies on Bovine Natural Actomyosin 2. Physico-Chemical Properties and Tenderness of Muscle

SUMMARY—Studies were made of physicochemical characteristics of natural actomyosin from bovine longissimus of different post-mortem ages and tenderness classifications. Reduced viscosity, ATP sensitivity, and "actin" content (polyethylene sulfonate treatment) were higher for natural actomyosin prepared from muscle 12–24 hr post-mortem than from pre-rigor muscle, which confirms previous reports for rabbit natural actomyosin. A higher actin to myosin ratio in actomyosin from muscle 12–24 hr was therefore postulated. A stronger interaction of actin and myosin in actomyosin from muscle 12–24 hr post-mortem than from pre-rigor or aged muscle was also suggested by reduced viscosity and ultracentrifugation data. Reduced viscosity differences between actomyosins from tough and tender muscle suggested a higher gel character in actomyosin from tough muscle. This possibly indicated a higher content of α-actinin. No consistent differences in ATP sensitivity, myosin and actin content of natural actomyosin of tough and tender muscle were found. Natural actomyosin from muscle aged post-mortem showed the appearance during analytical ultracentrifugation of an additional component which sedimented at about 11S to 12S. This component appeared in the actomyosin prepared from tender muscle after 24 hr but did not appear until 10 days in the actomyosin from tough muscle.

INTRODUCTION

BIOPHYSICAL and biochemical changes in muscle which occur with the onset and completion of rigor mortis as well as with subsequent aging may be reflected in certain properties of natural actomyosin prepared from that muscle. Differences have also been reported to exist in natural actomyosin which has been prepared from different sources (Seidel et al., 1964; Barany et al., 1965; Perry, 1960).

Fujimaki et al. (1958) observed differences in certain physicochemical properties of natural actomyosin prepared from bovine, equine, and rabbit muscle. Seidel et al. (1964) reported ATPase activity, reduced viscosity and ATP-sensitivity of natural actomyosin from red muscles were considerably less than in natural actomyosin from white muscles (rabbit).

It has also been shown that rabbit natural actomyosin, prepared after varying periods of aging post-mortem, varies in the ratio of myosin to actin (Fujimaki et al., 1965b). These authors showed that the myosin to actin ratio was high in natural actomyosin prepared from fresh muscle, low when prepared from 2 day old (aged) muscle, and intermediate when prepared from 7 day old muscle. It was also suggested that the interaction between or binding of actin and myosin became weaker when either muscle or natural actomyosin were aged (Okitani et al., 1967).

Fujimaki et al. (1958) have shown that aging rabbit and veal muscle 2–4 days increased the actomyosin ATPase activity. Herring et al. (1969a) showed that aging muscle post-mortem resulted in an increase in the ATPase activity of natural actomyosin to a maximum after 12–24 hr aging and a gradual decline in activity thereafter. Furthermore, results with superprecipitation of actomyosin suggested a stronger interaction of actin and myosin in 12–24 hr prepared acto-

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myosin and a weaker interaction in the actomyosin after the muscle had been aged for 5–10 days. Fundamental differences in superprecipitation suggested actomyosins prepared from tough and tender muscle were possibly different in inherent protein compositions.

Therefore, this study was designed to investigate the physicochemical characteristics of natural actomyosin with regard to (1) tenderization which occurs in muscle during aging post-mortem and (2) tenderness differences due to animal variation.

EXPERIMENTAL

Muscle source and sampling procedure

The muscle source (longissimus) and sampling procedures were similar to those described previously (Herring et al., 1969b).

Preparation of natural actomyosin

Natural actomyosin was prepared as described previously (Herring et al., 1969a).

Viscosity

Viscosity of natural actomyosin was measured in an Ostwald viscosimeter with an outflow time for water of 68 sec at 25°C. A 5-ml volume was used under the following conditions: 0.5 *M* KCl and 20 mM Tris-Acetate buffer (pH 6.8). Reduced viscosity (η red) was calculated as follows from the relative viscosity (η rel): η red $=$ (η rel $-$ 1)/c where c $=$ protein concentration in mg/ml.

ATP sensitivity

The adenosine triphosphate (ATP) sensitivity was determined by measuring the relative viscosity before and after addition of 1 mM ATP under the following conditions: 0.5 *M* KCl, 20 mM Tris-Acetate (pH 6.8), 1 mM $MgCl_2$, and 0.3 mg/ml of protein. By definition, ATP sensitivity is the change in viscosity produced by the addition of ATP and is expressed:

$$\frac{Z_\eta - Z_\eta \text{ ATP}}{Z_\eta \text{ ATP}} \times 100;$$

$$\text{where } Z_\eta = \lim_{c \to o} \frac{\eta \text{ sp.}}{c} = \frac{\ln \eta \text{ rel}}{c};$$

where c $=$ mg protein/ml.

Approximate content of myosin

The approximate content of myosin in natural actomyosin was estimated according to the procedure of Weber (1956). Natural actomyosin preparations (0.6 *M* KCl, 1 mM $MgCl_2$, 20 mM Tris-Acetate, pH 6.8) with various concentrations of PPi (inorganic tetra-sodium pyrophosphate) were centrifuged 4 hr at 78, 480 × G at 4°C. PPi was used instead of ATP, as PPi is not hydrolyzed during the centrifugation period (Martonosi et al., 1960).

The initial protein concentration was 4–6 mg/ml. After centrifugation, the tubes contained a clear supernatant and a small, dense pellet. The supernatant was separated from the pellet and the protein concentration and volume were determined. No corrections were made for sedimentation of myosin.

Content of "F-actin complex"

The procedure of Maruyama et al. (1962) was used for determining the content of the "F-actin complex" which will be called "actin" for purposes of simplicity. The preparation of natural actomyosin in 60 mM KCl, 20 mM Tris-Acetate (pH 6.8), 1 mM $MgCl_2$, 1 mM ATP, and 1×10^{-4} *M* PES (polyethylene sulfonate) was incubated 10 min at 25°C and then centrifuged (4°C) at 10,000 × G for 5 min. The supernatant was separated and its volume and protein concentration determined.

The interaction inhibitor (PES) combines with myosin and upon centrifugation, the "F-actin complex" is left in solution (Barany et al., 1960; Maruyama et al., 1962). Natural actomyosin is thought to contain the regulatory proteins, "native tropomyosin," α-actinin, and β-actinin (Ebashi et al., 1964, 1965), and therefore the estimation of F-actin, as accomplished in these experiments probably includes these regulatory proteins. No corrections for actin sedimentation were applied.

Protein concentrations

Protein concentrations were determined by the biuret method (Gornall et al., 1949) standardized against crystalline bovine serum albumin.

Analytical ultracentrifugation

The Spinco Model E analytical ultracentrifuge equipped with diagonal schlieren optics was used in these experiments. The temperature of the runs was about 20°C, since low temperature is known to have a dissociative effect on actomyosin (Laki et al., 1952), and temperature was measured and controlled by the Rotor Temperature Indicator and Control unit.

The sedimentation coefficients, $S_{20,w}$, were calculated from schlieren diagrams and the bar angle used was 65°. Both "wedge window" and "standard window" cells were

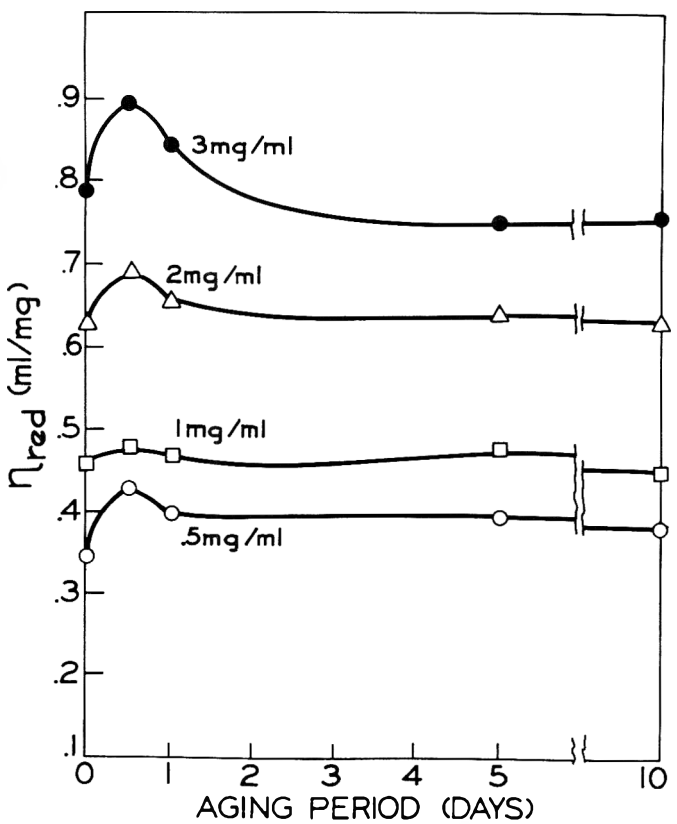

Fig. 1—Influence of aging muscle post-mortem on reduced viscosity of natural actomyosin. Final concentrations: 0.5 M KCl; 20 mM Tris-Acetate (pH 6.8); protein concentrations as shown. Temp. 25°C.

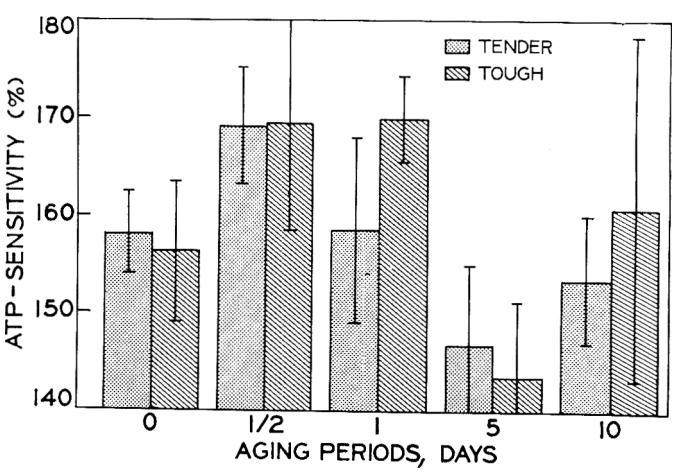

Fig. 2—Reduced viscosity of natural actomyosin from tough and tender muscle. Final concentrations: 0.5 M KCl, 20 mM Tris-Acetate (pH 6.8); 2 mg/ml of actomyosin. Temp. 25°C.

used in most cases. The areas of schlieren peaks were measured by enlarging and tracing the diagrams. The enlarged areas were estimated by counting squares. Areas were corrected for radial dilution.

Materials

All reagents used were analytical grade. ATP, as the disodium salt, was purchased from Sigma Chemical Co., St. Louis, Mo., and treated as previously described prior to use (Herring et al., 1969a). Polyethylene sulfonate was provided by the Upjohn Co., Kalamazoo, Mich.

RESULTS & DISCUSSION

Effect of post-mortem aging on reduced viscosity

The reduced viscosity of natural actomyosin is highly dependent on velocity gradient of flow and protein concentration and therefore is a qualitative measurement. However, differences in viscosity of natural actomyosin were found to be less variable than those for different synthetic actomyosins (Weber et al., 1952).

Figure 1 shows that the reduced viscosity of natural actomyosin was highly dependent on protein concentration. Natural actomyosin prepared from muscle 12-hr post-mortem had a higher reduced viscosity than that prepared from 0-hr muscle, and the reduced viscosity decreased slightly with aging. Thus, if one would plot the reduced viscosity versus protein concentration, the intrinsic viscosity would be lowest for natural actomyosin obtained from pre-rigor muscle, highest for that from muscle 12–24 hr post-mortem, and intermediate for that from aged muscle (5 to 10 days post-mortem).

Similarly, Fujimaki et al. (1965a) reported that the intrinsic viscosity was higher for natural actomyosin prepared from a 2-day aged muscle than from 0-hr muscle. Natural actomyosin from muscle aged for 12 hr may be more asymmetric or have a higher axial ratio than that obtained from 0-hr or 5- or 10-day aged muscle. An alternative explanation is that this natural actomyosin could have a higher ratio of actin to myosin which would result in a higher reduced viscosity. Weber (1960) found that the relative viscosity of synthetic actomyosin was higher when the ratio of myosin to actin by weight was one to one than when it was four to one.

Effect of tenderness classification on reduced viscosity

The reduced viscosity of natural actomyosin was higher for actomyosin from tough than that from tender muscle at all aging periods (Fig. 2). The reduced viscosity of actomyosin from tender muscle varied little with aging while reduced viscosity of actomyosin from tough muscle was quite variable.

When reduced viscosity was plotted against shear force (Fig. 3), the difference between the two tenderness groups was readily apparent. A higher intrinsic viscosity, according to Yang (1961), is due to higher particle asymmetry for rigid particles, or higher effective volume for flexible polymers. Which of these two descriptions contributes to the higher reduced viscosity of actomyosin from tough than from tender muscle is untenable.

Alternatively, the higher reduced viscosity observed may have been due to the more pronounced gel character that was evident for natural actomyosin from tough than from tender muscle. Maruyama et al. (1965) and Briskey et al. (1967) observed that α-actinin promoted gelation of actomyosin with possibly more cross-linking of the actin moiety resulting in the formation of a ternary complex in actomyosin. Herring et al. (1969b) observed that tender and tough muscle differed in properties of superprecipitation and they suggested a higher content of α-actinin for the tough group. The present findings on viscosity and gelation support the original suggestion regarding α-actinin.

ATP sensitivity

The effects of aging muscle and tenderness classification on ATP sensitivity of natural actomyosin are shown in Figure 4. ATP-sensitivity was highest in natural actomyosin from muscle 12-hr post-mortem, while natural actomyosin from pre-rigor and 5- and 10-day aged muscle had a considerably lower ATP-sensitivity. Szent-Györgyi (1951) suggested that a high specific viscosity for natural actomyosin denoted a higher ratio of actin to myosin, and when the ratio was 1:2.5, specific viscosity reached a maximum. When ATP was added to actomyosin with a high actin content a large depression of viscosity occurred. When myosin content was high, viscosity depression by ATP was small.

The present results showed a higher ATP-sensitivity for natural actomyosin from muscle 12–24 hr post-mortem and lower ATP-sensitivity for natural actomyosin from pre-rigor and aged muscle. These results were similar to those of Fujimaki et al. (1958) with bovine actomyosin except they reported high ATP-sensitivity at 2 days post-mortem. Therefore, the myosin to actin ratio was probably higher for natural actomyosin from pre-rigor and aged muscle than from muscle 12–24 hr post-mortem.

Interaction of actomyosin with ATP and PPi

The interaction of actomyosin with ATP was studied with viscometric methods and the interaction of actomyosin with PPi was investigated with ultracentrifugation. The reduction in viscosity caused by ATP is generally believed to be due to dissociation of actin and myosin

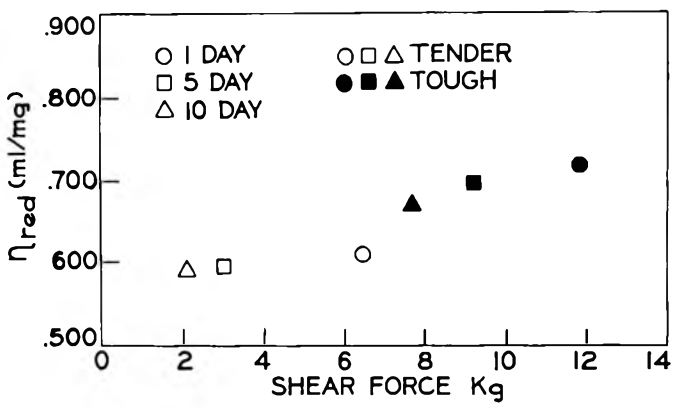

Fig. 3—Reduced viscosity of natural actomyosin versus shear force of muscle. Final concentrations: 0.5 M KCl; 20 mM Tris-Acetate (pH 6.8); 2 mg/ml of actomyosin. Temp. 25°C.

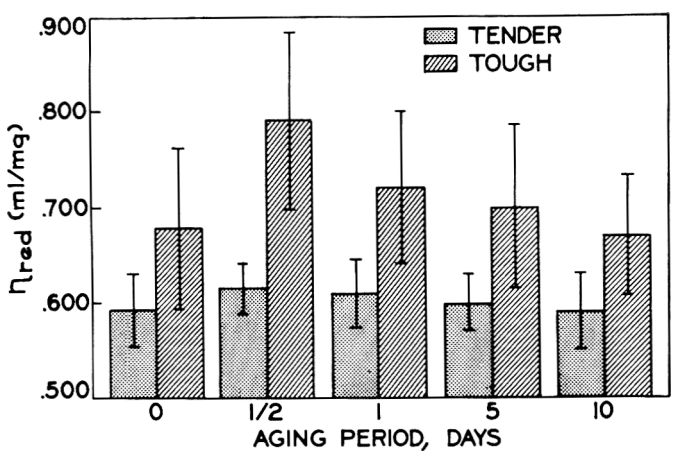

Fig. 4—Influence of aging muscle post-mortem on ATP-sensitivity of natural actomyosin. Final concentrations: 0.5 M KCl; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 1 mM ATP; 3 mg/ml of actomyosin. Temp. 25°C.

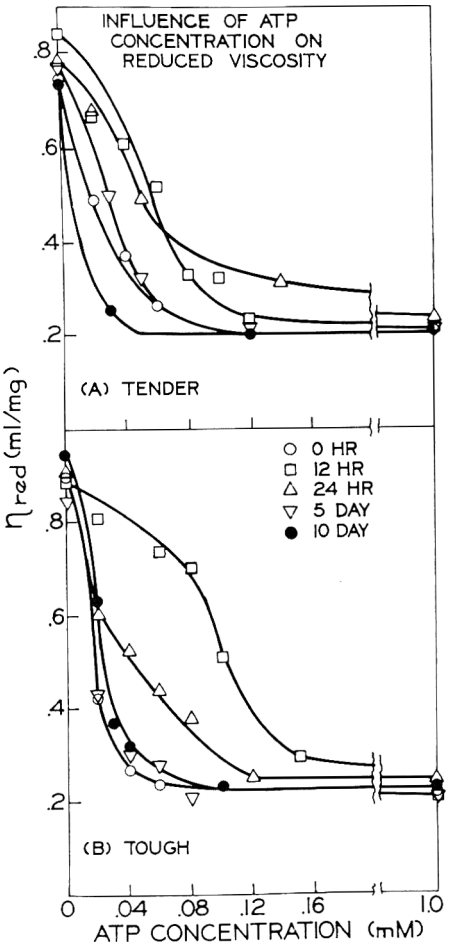

Fig. 5—Influence of ATP concentration on reduced viscosity of natural actomyosin from tough and tender muscle. Final concentrations: 0.5 M KCl; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 3 mg/ml of actomyosin. Temp. 25°C.

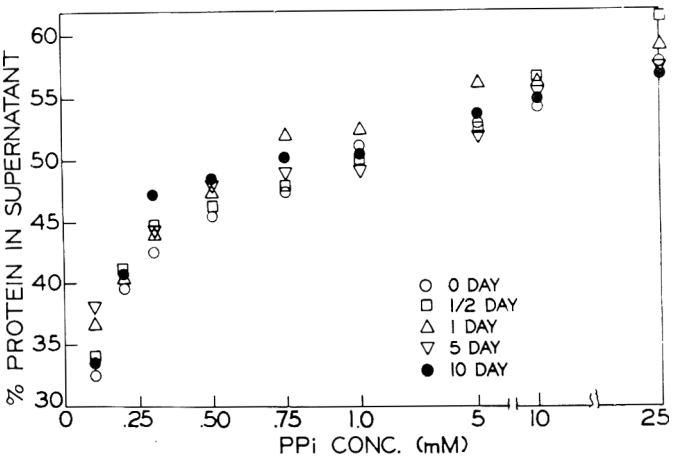

Fig. 6—Influence of PPi concentration on ultracentrifugal separation of myosin from bovine natural actomyosin. Final concentrations: 0.6 M KCl; 20 mM Tris-Acetate (pH 6.8); 1 mM Mg^{++}; 5 ± 1 mg/ml of actomyosin. Temp. 25°C.

(Mommaerts, 1948; Weber et al., 1952).

Figure 5a summarizes the effect of ATP on viscosity of actomyosin isolated from tender muscle at various times post-mortem. The interaction (as measured by viscosity decrease) appeared stronger for actomyosin from muscle 12–24 hr post-mortem than that from prerigor (0-hr) muscle, while actomyosin from 10 day old (aged) muscle was most easily dissociated by the lowest concentrations of ATP. Similar results were also obtained with actomyosin from tough muscle (Fig. 5b). However, actomyosin from 12-hr aged muscle of the tough group was much more difficult to dissociate than other preparations, suggesting a stronger interaction of its proteins, actin, and myosin.

Ultracentrifugal studies of natural actomyosin in the presence of PPi, as influenced by post-mortem age of the muscle at the time of extraction are summarized in Figure 6. The results obtained partially supplement those of Figure 5, except that natural actomyosin from 0-hr samples was hardest to dissociate, judging from percent protein in the supernatant, while natural actomyosin from aged muscle was somewhat easier to dissociate. Fujimaki et al. (1965b) similarly found, through ultracentrifugation, that natural actomyosin prepared from rabbit muscle immediately after death was hardest to dissociate, the 2-day preparation was intermediate, and the 10-day sample easiest to dissociate in the presence of ATP.

"Actin" and myosin contents of natural actomyosin

The "actin" and myosin contents of natural actomyosin are summarized in Figure 7. The results obtained for myosin content were somewhat of an enigma. While other physicochemical methods suggested a higher ratio of myosin to actin in natural actomyosin prepared from 0-hr muscles, ultracentrifugation in the presence of PPi presented conflicting data. Perhaps, since the actomyosin from the 0-hr muscle was shown to be harder to dissociate with increasing PPi (Fig. 6), more of the myosin sedimented with "actin" during ultracentrifugation. On the other hand, perhaps some protein other than myosin remained in the supernatant, was also measured and influenced the results obtained in a peculiar manner. The results for myosin content, as affected by aging and tenderness classification (Fig. 7a), were extremely variable.

The results for content of "actin" from natural actomyosin (Fig. 7b), as determined by use of PES treatment, agreed more closely with results obtained with viscosity and ATP-sensitivity. The content of "actin" in natural actomyosin prepared from the 0-hr muscle was lower than that from natural actomyosin prepared from the 12- to 24-hr post-mortem muscle. However, no differences between tough and tender muscle in "actin" content were found.

Fujimaki et al. (1965b) reported about 20–25% of the protein (actin) was found in the supernatant after PES treatment and 45–50% of the protein (myosin) was found after centrifugation in the presence of ATP. This directly accounted for only up to 75% of the protein of natural actomyosin. The results shown in Figure 7 account for up to 90% of the protein of natural actomyosin. These procedures for estimating the amounts of actin and myosin in the actomyosin preparations are at best only approximate indications of total quantities.

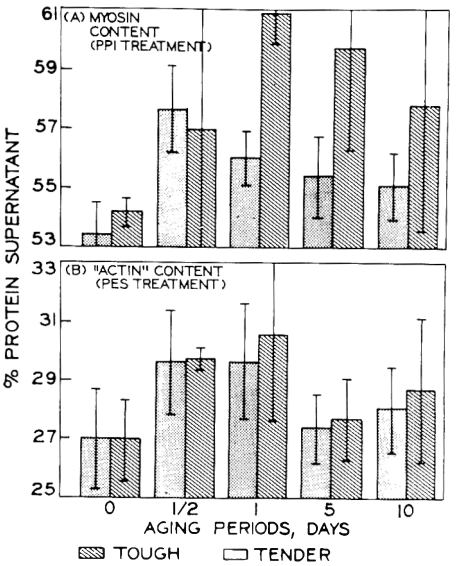

Fig. 7—Dissociation of the natural actomyosin complex of tough and tender muscle. (a) Separation of myosin in 0.6 M KCl, 20 mM Tris-Acetate (pH 6.8), 1 mM Mg^{++}, 5 ± 1 mg/ml of actomyosin and 10 mM PPi, 3°C. (b) Separation of "actin" in 60 mM KCl, 20 mM Tris-Acetate (pH 6.8), 1 mM Mg^{++}, 1 ± 0.1 mg/ml of actomyosin and 0.1 mM PES. Temp. 25°C.

It has been shown that tropomyosin (Laki et al., 1962) and α-actinin (Briskey et al., 1967) bind to actin while troponin binds to actin through tropomyosin (Komiriz et al., 1967). Therefore, these proteins are probably included as part of the "actin" determination. Furthermore, Sayre (1968) has shown that the myosin fraction decreased and the actomyosin fraction increased during the onset of rigor mortis. It has been suggested that the solubility of myosin approaches a minimum at the time of maximum rigor mortis (Fukazawa, personal communication) and increases thereafter, being mainly responsible for the increased solubility of natural actomyosin which occurs with post-mortem aging. All the present experiments considered together have suggested a lower content of myosin in natural actomyosin from muscle 12–24 hr post-mortem than from pre-rigor or aged muscle.

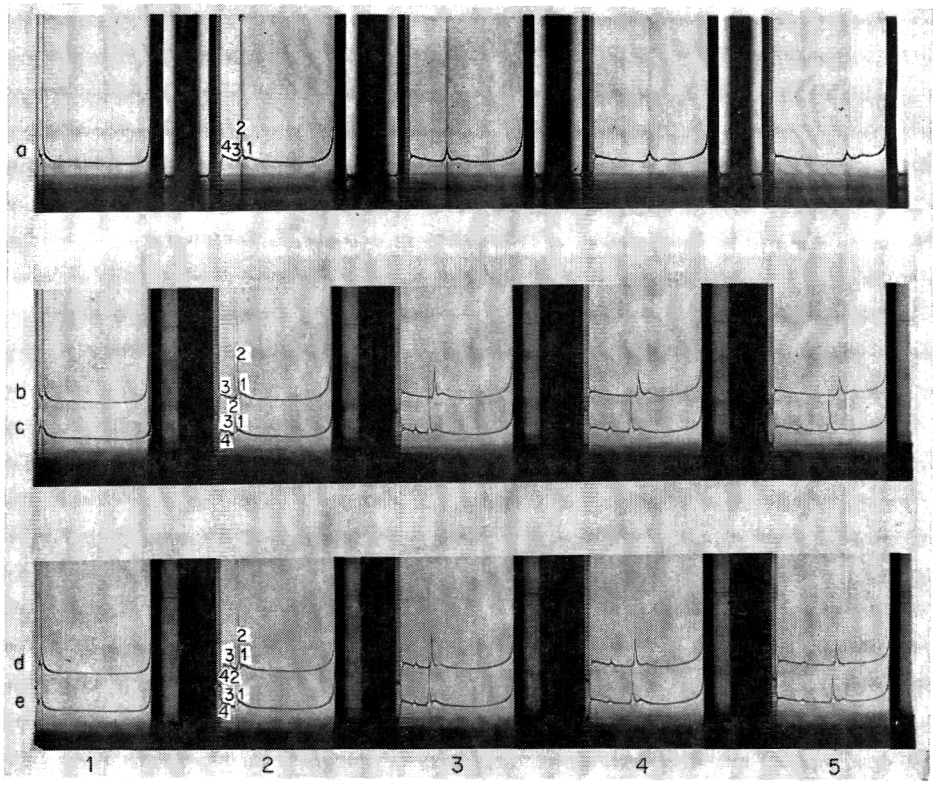

Fig. 8—Sedimentation diagrams of natural actomyosin representing tender muscle. Picture (1) was taken when 44,000 rpm was reached; pictures (2)–(5) were taken thereafter at 8-min intervals. Protein concentration was 2.9 mg/ml in 0.6 M KCl, 20 mM Tris-Acetate (pH 6.8). Temp. as indicated below.

Diagram	Sedimentation coefficients $S_{20,w}$ Peak 1	2	3	4	Relative protein concentration of main component
a. 0-hr natural actomyosin (20.8°C)	40.8	33.5	13.2	3.3	1.0
b. 12-hr natural actomyosin (20.3°C)	35.2	30.5	2.8	—	.95
c. 24-hr natural actomyosin (20°C)	30.4	25.2	12.1	2.9	.86
d. 5-day natural actomyosin (20.1°C)	33.3	26.5	11.7	2.9	1.02
e. 10-day natural actomyosin (20.1°C)	32.1	26.5	12.2	3.3	1.14

Analytical ultracentrifugation

Sedimentation diagrams of natural actomyosin from muscle aged for varying periods post-mortem are shown in Figure 8 for tender muscle and Figure 9 for tough muscle. Aging of muscle had an effect on the sedimentation coefficient of the resulting natural actomyosin. The sedimentation coefficients of the main component (peak 2 in Fig. 8 and 9) were highest (33S to 35S) for natural actomyosin from pre-rigor muscle, lowest (23S to 25S) for natural actomyosin prepared 1 day post-mortem, and intermediate (26S to 28S) for natural actomyosin from post-rigor muscle (5–10 days post-mortem). Similar changes were observed for the polydisperse peak leading the main component (peak 1, Figs. 8 and 9).

The changes in sedimentation rates probably represented fundamental changes in the actomyosin molecule. According to Weber et al. (1952), these molecules are of such a length that the sedimentation rate depends primarily on its thickness, not on length. A slight change in the weight percentage of myosin or actin or some minor protein component could be reflected in a different sedimentation rate. Johnson et al. (1964) have shown that as the weight fraction of myosin was increased, the sedimentation rate of the main component decreased. However, the studies of viscosity and ATP-sensitivity indicated natural actomyosin from 12–24 hr post-mortem muscle had a higher "actin" content than pre-rigor muscle. Results with analytical ultracentrifugation here dispute this however. Therefore, some other factor such as concentration or amount of the main component altered the results.

The concentration of the main component was higher, on a relative basis, in the natural actomyosin from 0-hr muscle than that from muscle 12–24 hr post-mortem in the tender category (Fig. 8). This may have been due to the presence of a higher content of the gel component in natural actomyosin from these latter muscles.

Okitani et al. (1965) reported a higher content of the gel component in natural actomyosin from muscle prepared two days post-mortem than at 0-day. With ultracentrifugal analyses, Scharpf et al. (1966) observed considerably more gel component present in natural actomyosin prepared from pectoralis muscle of turkey at 4 hr than at 48 hr post-mortem and suggested natural actomyosin loses gel-forming ability with "resolution of rigor."

With natural actomyosin from tender and tough muscle in the present experiment, the relative concentration of the main component increased with aging post-mortem of muscle (Figs. 8 and 9), between the 1 and 10-day periods. Whether this increase of the main component was at the expense of the gel component was not determined.

Aging muscle post-mortem also resulted in the appearance of an additional component with a sedimentation coefficient of about 12S to 13S (Fig. 8a,c,d,e). This peak appeared at 24 hr in the tender muscle (Fig. 8c), but did not appear until 5 days aging in tough muscle (Fig. 9e). This component apparently appeared as a result of aging muscle and may have either represented a dissociable substance from the main component of the actomysin molecule itself, which was related with tenderization of muscle post-mortem, or, more simply, it was dimerized myosin which was formed during the storage period at 4°C. That it could have represented an aggregate of myosin was supported by the evidence of the appearance of a peak possibly representing a dimer of myosin with a sedimentation coefficient of 10 to 13S when these samples were centrifuged in the presence of pyrophosphate.

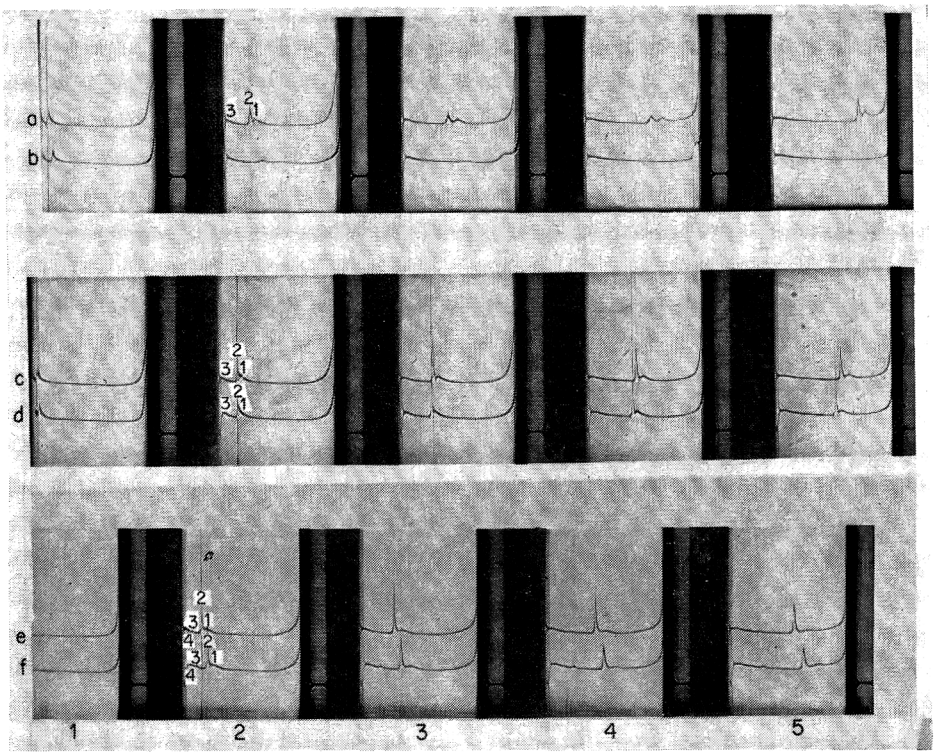

Fig. 9—Sedimentation diagrams of natural actomyosin representing tough muscle. Picture (1) was taken when 44,000 rpm was reached; pictures (2)–(5) were taken thereafter at 8-min intervals. Protein concentration was 2.86 mg/ml (except "b" has 1.67 mg/ml) in 0.6 M KCl, 50 mM Tris-Acetate (pH 6.8). Temp. as indicated below.

Diagram	Sedimentation coefficients $S_{20,w}$ 1	2	3	4	Relative protein concentration of main component
a. 0-hr natural actomyosin (22.3°C)	40.2	35.2	4.0	—	1.0
b. 0-hr natural actomyosin (22.3°C)	—	—	—	—	—
c. 12-hr natural actomyosin (22.8°C)	30.2	26.8	4.1	—	1.23
d. 24-hr natural actomyosin (22.8°C)	25.8	23.1	3.5	—	1.21
e. 5-day natural actomyosin (23.3°C)	31.7	26.5	12.7	3.8	1.25
f. 10-day natural actomyosin (23.4°C)	33.7	28.4	12.6	2.7	1.33

These results with analytical ultracentrifugation lend support to the above studies with viscosity that myofibrillar proteins undergo changes during aging post-mortem in muscle. These changes are of a fundamental nature, however, and further study is needed to ascertain the exact nature of protein changes, especially interactions and solubilization, which occur during the aging process.

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Relation of Chemical Structure to Plant Growth-regulator Activity in the Pineapple Plant: Retarding Senescence of Pineapple Fruit with Applications of 2,4,5-Trichlorophenoxyacetic Acid and 1-Naphthaleneacetic Acid

SUMMARY—The effectiveness of 1-naphthaleneacetic acid (SNA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in markedly retarding further ripening of pineapple fruit and thus extending its marketable life as a fresh fruit has been demonstrated. The effects are evident at different stages of maturity and ripeness, different fruit densities, and from both pre-harvest and post-harvest applications. As little as 1 ppm of 2,4,5-T has noticeable effect, and 100 ppm appears optimum for senescence delay. For SNA, 500 ppm is an optimum level for dipping fruit. A brief wetting of the fruit is adequate. The crowns remain in better condition when not treated with growth regulator. Refrigeration can supplement the effect of the chemical in retarding senescence. Lower temperatures can retard some of the changes which even treated fruit will show, and the chemical can retard ripening changes that otherwise may take place, albeit slowly, under normal refrigeration of pineapple fruit.

INTRODUCTION

A PREVIOUS PAPER (Gortner et al., 1969) reported on the activity of several hundred chemicals with respect to delay of senescence of pineapple fruit when applied as post-harvest dips. Two of the chemicals showing high activity were well known growth regulators, and these were studied in greater detail as being of possible commercial value in treating fruit to extend the marketable life of fresh pineapple. Since these growth regulators have also found use on other crops, it was felt likely that government clearance of one or the other for use on pineapple might be easier to obtain.

The present paper will evaluate some of the conditions for most effective use of the sodium salts of 1-naphthaleneacetic acid (SNA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in retarding senescence of pineapple fruit.

MATERIALS & METHODS

THE SNA and 2,4,5-T were applied as aqueous solutions. When applied post-harvest, 5 lb of captan per 100 gal of solution were usually included as a fungicide to reduce *Thielaviopsis* rot. Crowns were left on the fruit but were not treated except where noted.

Except where otherwise noted, the post-harvest tests were conducted by holding the fruit at room temperature in the shade (70–85°F). Generally, there were 10 fruit per treatment, and the effect of treatment on ripening and senescence (Gortner et al., 1967) was evaluated by noting changes in shell color of the fruit at intervals. Mean values are reported, but the range rarely extended beyond 1 shell color class. In most cases, other subjective observations and chemical analyses were also recorded to confirm effects on senescence.

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RESULTS

Effect of concentration, post-harvest

The effect of concentration of the chemical in post-harvest dips was determined for both 2,4,5-T and SNA. The fruit were immersed only briefly.

The effect of 2,4,5-T solutions ranging from 1 to 1000 parts per million (ppm) is illustrated in Figure 1. As little as 1 ppm of 2,4,5-T delayed shell yellowing of the fruit, and 100 ppm appeared optimal. The data shown in Table 1 demonstrate that the chemical treatments had little effect on the sugar and acid constituents, but had a noticeable effect in minimizing flavor and texture changes associated with senescence.

Table 2 shows the data on fruit treated

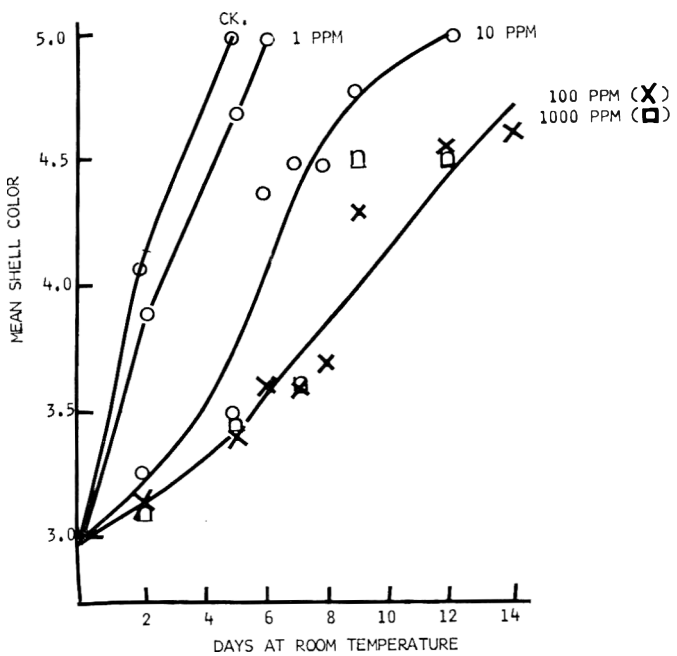

Fig. 1—Effect of dipping the fruit in 1–1000 ppm solutions of 2,4,5-T on shell color changes of half-yellow pineapple. At shell color 3, the fruit is half-yellow, and at shell color 5 it is full yellow. Values are 10-fruit means.

Table 1—Effect of dipping fruit in 1–1000 ppm solutions of 2,4,5-T on subjective and chemical measures of senescence of half-yellow pineapple.

	Shell color[1]	Brix	% acid	Flavor	Fruit texture
At start	3.0	14.4	1.25	Normal	Firm
After 9 days					
Check	Sl. browning	14.2	0.86	Sl. overripe	Spongy
1 ppm	Sl. browning	14.8	0.94	Sl. overripe	Soft
10 ppm	4.8	14.3	0.85	Normal	Firm
100 ppm	4.1	13.4	0.86	Normal	Firm
500 ppm	4.5	13.7	0.98	Normal	Firm
1000 ppm	4.5	13.7	0.90	Normal	Firm
After 13–14 days					
Check	Sev. browning	14.2	0.84	Overripe	Soft, spongy
1 ppm	Sev. browning	14.0	0.77	Sl. overripe	Soft
10 ppm	5.0	13.1	0.70	Normal	Firm
100 ppm	4.6	13.9	0.87	Normal	Firm
500 ppm	4.5	14.2	0.91	Normal	Firm
1000 ppm	4.5	13.7	0.87	Normal	Firm

[1] Shell colors 3, 4, and 5 correspond to one-half, three-fourths, and full yellow shells on the fruit. Values are 10–20 fruit means.

Table 2—Effect of dipping fruit in 100–3000 ppm solutions of SNA on mean shell color changes[1] of one-third yellow pineapple.

	Days at room temperature							
Concentration	0	2	3	5	7	9	11	12
Check	2.3	3.0	—	4.9	5.0	5.0	5.0	5.0
100 ppm SNA	2.2	—	3.0	3.4	3.8	3.8	4.0	—
500 ppm	2.4	2.4	—	2.4	3.0	3.7	—	4.3
1000 ppm	2.4	2.4	—	2.5	3.2	3.5	—	4.4
2000 ppm	2.3	2.7	—	2.7	3.5	3.8	—	4.8
3000 ppm	2.3	2.4	—	2.4	2.7	2.7	—	4.2

[1] Shell colors 2, 3, 4, and 5 correspond to one-fourth, one-half, three-fourths, and full yellow shells on the fruit. Values are 10-fruit means.

Table 3—Effect of dipping time in 10 ppm solution of 2,4,5-T on mean shell color changes[1] of one-fourth yellow pineapple.

Dipping time	Days at room temperature				
	0	2	5	6	7
Check	2.2	3.6	5.0	—	—
4 sec	2.0	2.8	3.6	4.2	4.8
30 sec	2.0	2.8	4.0	4.4	4.8
3 min	2.1	2.6	3.4	3.8	4.8

[1] Shell colors 2, 3, 4, and 5 correspond to one-fourth, one-half, three-fourths, and full yellow shells on the fruit. Values are 5-fruit means.

with 100 to 3000 ppm solutions of SNA. There appeared to be some benefit in retarding shell yellowing in going from 100 to 500 ppm, but little benefit from higher concentrations of SNA.

Effect of dipping time

Data on the effect of dipping time, ranging from 4 sec to 3 min in a solution of 2,4,5-T, on mean shell color are given in Table 3. Because of the need to allow for an improvement in senescence delay from one of the dipping treatments, a suboptimal concentration of 10 ppm of 2,4,5-T was used. Only a brief, 4-sec immersion of the fruit was necessary for the chemical to exert essentially maximum activity. A longer immersion time (Table 3) did not substitute for a higher concentration of the chemical in the solution (Fig. 1).

Effect of dipping crowns as well as fruit

Table 4 compares 2,4,5-T when applied to the fruit only and to the fruit plus crown. At the lower concentrations, there appears to be a minor improvement in delay of shell yellowing when the crown also is treated. However, the growth regulator had a toxic effect on the crown tissue. This caused a loosening of the crown leaves and a weakness in the meristematic tissue that resulted in a snapping off of the heart leaves when a small force was applied. A similar observation from crowns dipped in SNA solution was noted in several tests.

Effect of prior refrigeration

Several tests were run in which the fruit were held for 2 or more weeks under refrigeration and then removed to room temperature. These tests simulated possible commercial practice, wherein the fruit may be refrigerated during ocean and rail shipment and then may be marketed in stores at room temperature.

Table 5 shows the effect of such a treatment with 2,4,5-T treated fruit being held at 50°F for 2 weeks, and includes fruit of two different qualities as determined by density (water flotation) of the fruit. "Floaters" have appreciably higher acidity, lower Brix, and lower esters than pineapple fruit classed as "sinkers" by water flotation. The 2,4,5-T was effective in retarding senescence of the fruit following removal from cold storage. The more dense fruit (sinkers) appeared to have a somewhat greater marketable life than the floaters. Again, the effect of the chemical seems to be on various aspects of senescence and not merely shell yellowing. A number of other observations were also made on these fruit, and little or no difference was observed in the flavor ratings, Brix, acid, Brix: acid ratio, carotenoid pigments, or vitamin C content.

In Table 6, a number of different chemicals are compared for their activity in retarding senescence when the fruit were held for 2 weeks under refrigeration and then removed to room temperature. In this test, the initial storage temperature was 55°F rather than 50°F. Unpublished data indicated that there was less likelihood of "chill injury" at the higher temperature and the greater likelihood of ripening during refrigerated storage would allow a better assessment of effectiveness of the chemical treatments of the fruit. All of the chemicals tested had been rated +++ for senescence delay (Gortner et al., 1969). All of the chemicals markedly delayed shell yellowing under refrigeration, but there were some differences in effectiveness after removal to room temperature. Both 2,4,5-T and 2,3,5,6-tetrachlorobenzoic acid were highly effective. Untreated fruit became fully yellow after 2 weeks at 55°F, in contrast to the moderate yellowing that occurred at 50°F (Table 5).

Data on the effect of SNA when fruit are refrigerated and then removed to room temperature are given in Table 7. In this test, immature fruit as well as fruit of varying degrees of initial shell yellowing were included. Again, considerable yellowing of untreated fruit occurred at 55°F. SNA proved effective in retarding this yellowing and the further browning associated with senescence. This effect of the chemical also was seen regardless of degree of ripeness of the fruit at the start of the test.

All of the fruit in this test had a firm texture after 15 days under refrigeration, but after 30 days of refrigeration some noticeable differences in texture were observed. The immature fruit were mostly firm, even without SNA treatment. The fruit just turning yellow at the start of the test were mostly firm when SNA-treated but were slightly soft without this treatment. The half-yellow fruit treated with SNA were slightly soft, but without SNA treatment many were soft. These texture observations thus parallel observations on shell color changes.

Effect of stage of maturation, pre-harvest

The growth regulators were effective in pre-harvest as well as post-harvest ap-

plications to the fruit. The effects of such pre-harvest application of both SNA and 2,4,5-T are illustrated in Figure 2. In this test, the fruit were allowed to remain on the plant after spray application of the chemical until they reached a one-fourth yellow shell stage of ripeness. They were then picked and held in the shade for further shell yellowing.

Both chemicals were effective in delaying ripening and senescence whether or not the fruit were initially "picking ripe" or quite immature. Again, 2,4,5-T was more effective than SNA. The data indicate that SNA may have had an initial effect which was dissipated before some of the more immature fruit were picked, and thus subsequent ripening proceeded at a rate comparable to the check fruit. A rapid decarboxylation of SNA in the sunlight following application to pineapple plants has previously been demonstrated (Leeper et al., 1962).

Table 8 summarizes the post-harvest ripening times for these fruit sprayed pre-harvest with SNA or 2,4,5-T at varying stages of maturity. The effectiveness of the growth regulator is fairly consistent on fruit ranging from one day to 19 days before they might be picked for the fresh fruit market. The effect of the chemical in delaying further shell yellowing after picking is quite significant, with 2,4,5-T the more potent chemical.

The pre-harvest application of 2,4,5-T is not without hazard. An appreciable number of the fruit sprayed with 2,4,5-T and which were not fully ripe until 3 or more weeks later showed "gassing" or yeasty fermentation during post-harvest ripening. Presumably this growth regulator caused some cracking of the shell that allowed invasion of micro-organisms. Pre-harvest treatment with SNA did not result in gassy fruit.

Residues from SNA dips

To ascertain whether these chemicals pose a problem of residues in the edible portion of the fruit, five half-yellow pineapple fruit were dipped in either 100 or 1000 ppm solutions of SNA, with another five fruit held as untreated checks. Both the crown and the fruit were immersed in order to magnify the possible residue

Table 4—Effect of dipping the fruit or fruit-plus-crown in various solutions of 2,4,5-T on mean shell color changes[1] of half-yellow pineapple.

	Days at room temperature								
	0	2	5	6	7	8	9	12	14
Captan check									
Fruit	3.0	4.1	5.0						
+ Crown	3.0	4.1	5.0						
1 ppm 2,4,5-T									
Fruit	3.0	3.9	4.7	5.0					
+ Crown	3.0	3.9	4.7	4.7	4.9	4.9	5.0		
10 ppm 2,4,5-T									
Fruit	3.0	3.3	3.5	4.4	4.5	4.5	4.8	5.0	
+ Crown	3.0	3.3	3.6	4.2	4.2	4.2	4.2	4.8	5.0
100 ppm 2,4,5-T									
Fruit	3.0	3.3	3.5	3.6	3.6	3.7	4.1	4.6	4.6
+ Crown	3.0	3.3	3.5	3.5	3.6	3.7	4.1	4.6	4.6

[1] Shell colors 3, 4, and 5 correspond to one-half, three-fourths, and full yellow shells on the fruit. Values are 10-fruit means.

Table 5—Effect of fruit density (water flotation) and of 2 weeks' refrigeration at 50°F followed by room temperature on inhibition of senescence of half-yellow pineapple by 2,4,5-T.

	Shell color[1]	Shell appearance[1]	Crown appearance[1]	Fruit texture[1]
At start of treatment				
Floaters	3.1	G	G	G
Sinkers	3.3	G	G	G
2 wks. 50°F, 1 day 75°F				
Check—floaters	3.6	G	G	G
—sinkers	3.7	G	G	G
Treated—floaters	3.6	G	G	G
—sinkers	3.4	G	G	G
2 wks. 50°F, 1 wk. 75°F				
Check—floaters	5.0	F	F	F
—sinkers	5.0	F	F	F
Treated—floaters	4.1	G	G	G
—sinkers	4.2	G	G	G
2 wks. 50°F, 2 wks. 75°F				
Check—floaters	5+	F-P	F	P-F
—sinkers	5+	F-P	F	F-P
Treated—floaters	4.7	F	F	F-G
—sinkers	4.4	F	F-P	G

[1] G = good, F = fair, P = poor; shell colors 3, 4, and 5 correspond to one-half, three-fourths, and full yellow shells on the fruit. Values are 10-fruit means.

Table 6—Effect of dipping fruit in 100 ppm solutions of 2,4,5-T and other acids, followed by 2 weeks' refrigeration at 55°F and then room temperature, on mean shell color changes[1] of one-fourth yellow pineapple.

	At start	After 2 wks., 55°F	Removed to room temperature for				
			2	5	7	9	12 days
Check	2.1	5.0					
2,4,5-T	2.1	2.3	3.0	4.3	4.6	4.7	4.9
2,3,5,6-Tetrachlorobenzoic	2.1	3.3	4.0	4.8	4.8	4.9	5.0
2-Oxobenzothiazolin-3-ylacetic	2.1	3.1	4.0	5.0			
4-Thianaphtheneacetic	2.1	3.1	4.0	5.0			
2-Methylindole-3-acetic	2.1	3.4	4.0	5.0			

[1] Shell colors 2, 3, 4, and 5 correspond to one-fourth, one-half, three-fourths, and full yellow shells on the fruit. Values are 10-fruit means.

Table 7—Effect on shell color changes[1] of pineapple after dipping fruit of different degrees of ripeness in 500 ppm solution of SNA, followed by refrigeration at 55°F, and subsequent storage at room temperature.

At start	Held 15 days at 55°F, then at room temp.		Held 30 days at 55°F
	0	13 days	
Check			
1 week immature	2.2	5.0	Browning
Sl. yellowing	3.6	Browning	Browning
3	4.8	Browning	Browning
4+	Browning	Browning	Browning
SNA-dipped			
1 week immature	1.3	—	3.0
Sl. yellowing	2.7	5.0	4.5
3	4.4	Browning	Browning
4+	5.0	Browning	Browning

[1] Shell colors 2, 3, 4, and 5 correspond to one-fourth, one-half, three-fourths, and full yellow shells on the fruit. Values are 10-fruit means.

Table 8—Post-harvest ripening times for pineapple fruit of varying stages of maturity sprayed with SNA or 2,4,5-T pre-harvest and picked when one-fourth yellow.

Days before normal 1/4 yellow stage when sprayed	Time (days) required after picking 1/4 yellow fruit to attain fully yellow shells		
	Untreated	SNA-sprayed	2,4,5-T sprayed
1	6	14	16
3	8	12	24
5	8	14	26
12	7	12	16
19	8	8	14

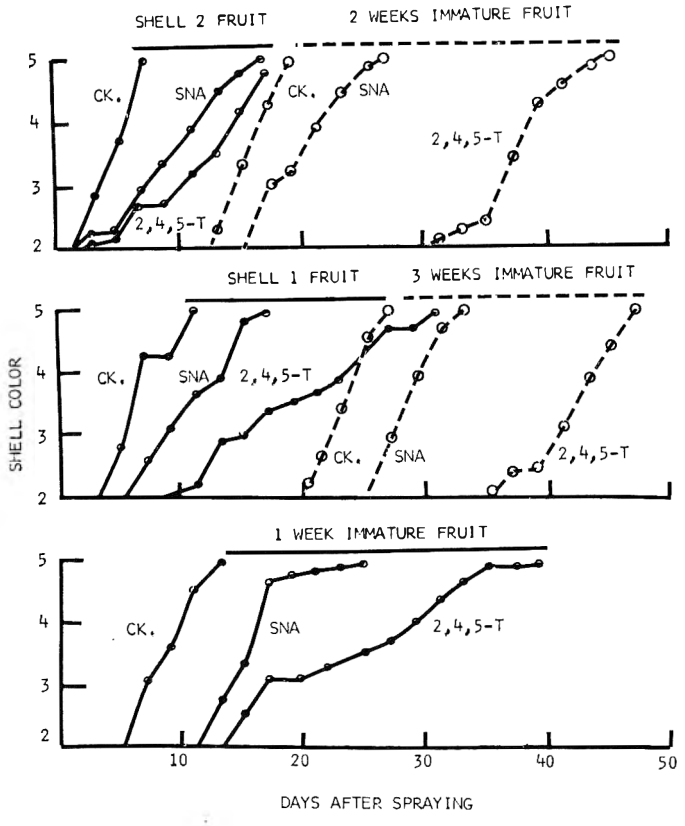

Fig. 2—Effect of pre-harvest sprays of 500 ppm solutions of SNA or 2,4,5-T on maturation to shell color 2 on the plant and on subsequent post-harvest shell color changes of pineapple. Shell colors 2, 3, 4, and 5 correspond to one-fourth, one-half, three-fourths, and full yellow shells on the fruit. Values are 10-fruit means.

problem. Analyses for SNA residues (Young et al., 1963) were made after 3, 7, and 11 days at room temperature. Absolutely no residual SNA was found in the edible portion of any of the fruit, even though the analytical method was able to determine as little as 0.03 ppm of SNA.

DISCUSSION

THE EXTREME potency of a chemical such as 2,4,5-T in delaying senescence of pineapple fruit is evident from these studies. Even a few cc of a 1 ppm solution in contact with the fruit and not applied to the crown gives a noticeable response (Fig. 1). At most, this represents a few micrograms of 2,4,5-T on the surface of the fruit. The effects on texture, flavor, and other qualities indicate that the chemical is not merely affecting the shell metabolism but rather is having an internal effect on changes associated with ripeness and senescence. Yet, there is no evident translocation mechanism. When SNA was the senescence inhibitor, no translocated residues were detectable even from 1000 ppm solutions.

The effect of the chemical is very rapid. This, too, is evident from Figure 1, where the check fruit are undergoing very rapid changes in color that are immediately arrested by the higher concentrations of 2,4,5-T.

It would be interesting to observe the effects of these growth regulators on ethylene production by pineapple fruit (Burg et al., 1962; Lieberman et al., 1962). Dull et al. (1967) have confirmed reports of ethylene production by pineapple fruit and found its concentration to be greater at the base than in the less ripe, upper portions of the fruit.

The effect of either 2,4,5-T or SNA on ripening and senescence does not lead to abnormal metabolism. Good fruit of normal quality are obtained following treatment of partially ripe, or of immature fruit subsequently allowed to ripen. The ripening processes are delayed but apparently not otherwise altered.

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Studies on Compounds and Individual Lipids of Wheat Germ

Summary—A combination of chemical and chromatographic procedures demonstrated that 97% of total wheat germ lipids are nonpolar, while 3% are polar (glycolipids, phosphoipids and proteolipids). The fatty acid composition, in polar and nonpolar lipids, was determined: the former contain a higher percentage of octa-decatrienoic acid than the latter. Nonpolar lipids are tri, mono- and diglycerides and sterol esters. In addition to lecithin, phosphatidyl ethanolamine, phosphatidyl serine and their lyso derivatives, lipids contain unknown compounds tentatively identified as glycolipids or phytoglycolipids. Moreover a high content of compounds identified as phosphatidic acid or polyglycerophosphate derivatives has been observed.

INTRODUCTION

MOST of the knowledge about the composition of wheat germ lipids is from studies on the function of lipids in milling processes (McKillikan et al., 1964; Sullivan, 1940) on the keeping quality of wheat products (Pomeranz et al., 1966a, b), and on baking processes (Sullivan, 1940; Pomeranz et al., 1966a, b).

Germ lipids play an important part in the physiological phenomenon of germination. The chemical composition of wheat germ is of great interest in the technology of wheat products.

The investigations on plant lipids are limited because of the great number of compounds present, and, until recently, there have been few suitable techniques for their separation.

In the present research, an extensive study was made of the composition of the lipids of the germ, which were obtained by industrial processes. No study of this subject has been reported previously, except for some observations of Channon et al. (1934).

EXPERIMENTAL

Preparation of the germ

To obtain a highly pure germ, the procedure described below, as done by Tamburi Mills, was adopted.

By means of separatores (grain washers), cleaners, beaters and brushes, the wheat was cleaned and washed without breaking or removing the germ. It was left for 20–24 hr in a humidified storeroom where a light swelling of the wheat and especially the germ occurred. The grain thus prepared was then ground.

The first steps of grinding (first, second and third breakage) were accomplished by special equipment (Knauff reducers) that smashes the wheat without excessively breaking the bran and leaves the germ whole.

After classification of the products in the shifts (Plansichter), the germ, together with the large bran, passes between pairs of steel rollers, which, by means of sieves in air currents, divide this product into: (1) best flour; (2) bran mixed germ; (3) parts of bran.

The second product (bran mixed with germ) passes onto the smooth cylinders, on which the germs become lightly pressed and broadened. With a successive sifting of the product as it passes through the cylinders one obtains the following fraction: (1) pure germ; (2) germ mixed with parts of bran; (3) best flour; (4) flour.

Of these four fractions only the first (essentially free of endosperm and other material) was used in our research. All the germs so prepared were placed in a tin box, under vacuum, and conserved in a refrigerator at −20°C; from this the necessary amount for immediate research was taken and stored in the laboratory refrigerator at −5°/C until use. The determination of the chemical composition was made according to Am. Assoc. Cereal Chemists Methods (1962), except for lipids.

Extraction of lipids

Different procedures were applied for the extraction of lipids to obtain the best recovery of lipid material together with the lowest alteration of lipid constituents and the least contamination by unbound nitrogenous material. Best results for analysis were obtained with the method described by Brady (1964): 36 g of wheat germ were blended for 2 min with 36 ml cold water; 350 ml of cold methanol were then added, and blending was continued for 60 sec. The homogenate was filtered on a layer of washed Celite, and the residue was washed with three aliquots of 100 ml of 80% methanol (by volume), followed by 250 ml of chloroform-methanol 2:1 (v/v), and then by 200 ml of chloroform, until the residue was free of pigments. The total filtrate was then concentrated to dryness by vacuum distillation of the solvents; the residues were dissolved in chloroform-2:1 and washed according to the method of Folch et al. (1957).

An aliquot of the washed extract was taken to determine the total lipid content by weighing.

Analytical methods on total lipids

The following analytical determinations were carried out: lipid P according to Bartlett (1959) and to the modification of this method described by Marinetti (1962); lipid galactose with the orcinol method of Radin (1964); lipid containing free amino nitrogen according to Lea et al. (1954) with ethanolamine as standard; total amino nitrogen after acid hydrolysis as described by Brady (1964) according to Moore et al. (1948) with L-leucine-HCl as standard; protein with the method of Davenport (1964); alkali labile and alkali stable phospholipids were determined after modified mild alkaline hydrolysis according to Dawson (1960).

Chromatographic methods

Separation of total lipid in polar and nonpolar lipids. To obtain a polar fraction free from nonpolar lipids, a separation was done on a silicic acid column (Bartley et al., 1962). A column (20 mm diameter) containing 18 g of silicic acid (Mallinkrodt, 100 mesh, activated at 110°C overnight) was used. A sample of total lipids (400γ of lipid P) dissolved in chloroform was applied to the column; after removing the nonpolar lipids with chloroform, polar lipids were eluted with 1,000 ml methanol. To complete the elution of polar lipids from the adsorbant, the contents of the whole column were suspended in methanol, shaken and then centrifuged; this operation was repeated three times. The methanolic phases were combined with methanol eluted from the column.

The efficiency of the polar lipids separation with different methods was evaluated by assaying lipids P in total lipids, nonpolar and polar fraction. With the above method the authors obtained a recovery of 81.5% total lipid phosphorus from the methanol-eluted fraction. No lipid phosphorus was present in the nonpolar fraction.

Separation of polar lipids. The eluted polar fraction from the column of silicic acid was chromatographed on alumina (Webster, 1960). The solvent systems were: chloroform-methanol (1:1) 60 ml; chloroform-methanol-H_2O (7:7:1) 30 ml; chloroform-ethanol 0.04 M KOH (10:7:5) 90 ml. Four fractions were separated with this method: (1) choline containing phospholipids; (2) choline and amino groups-free phospholipids; (3) ethanolamine containing phospholipids; (4) serine containing phospholipids. In each fraction phosphorus was determined according to Bartlett (1959).

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Table 1—Chemical composition of wheat germ (hard red winter).[1] (g/100 g).

Water	12.56 ± 0.29
Dry matter	87.04 ± 0.21
Protein[2]	24.44 ± 0.01
Acid soluble nitrogen	0.50 ± 0.21
Total lipids	9.38 ± 0.37
Crude fiber	2.66 ± 0.34
N free extract	45.03 ± 0.21
Ash	4.91 ± 0.22

[1] Average of six determinations.
[2] The protein values have been obtained by multiplying 5.7 × the nitrogen minus soluble acid nitrogen.

Separation of nonpolar lipids eluted from silicic acid column has been conducted according to Barron et al. (1958) on a silicic acid column previously dried at 110°C for 12 hr and prewashed with ethyl ether, 15% benzene in hexane, and hexane. A sample of lipid dissolved in hexane was applied to the column. The loading factor was 10 mg total lipids/g of silicic acid.

Separation of polar and nonpolar lipids on silicic acid impregnated paper. The study of the composition of the four polar fractions, separated as described above, and of nonpolar lipids was conducted by chromatography on silicic acid impregnated paper according to Marinetti (1964). The solvent system was diisobutylketone-acetic acid-water (40:20:3) for the polar fractions, and solvents in sequence (Marinetti, 1964) for nonpolar lipids. The solvent sequence was: chloroform-methanol 2:1 (containing 2% water); heptane-diisobutylketone 1:1; heptane-diisobutylketone-acetic acid 96:6:0.5. Polar and nonpolar lipids of egg were used for a comparison. The methods for the detection of lipids were basically those of Marinetti (1964) for Rhodamine 6-G, ninhydrin and choline test.

Table 2—Lipid constituents of wheat germ.

	mg%/g of germ	Lipids, %
Total lipids	9,380	100.0
Total phospholipids[1]	130	1.38
Glycolipids[2]	71	0.75
Neutral fat[3]	9,024	96.21
Protein[4]	155	1.66
Total amino-nitrogen	13	0.1
Free amino-nitrogen	0.99	0.01

[1] Milligrams of lipid P × 25.
[2] Glycolipids are calculated from values of galactose × 4.55.
[3] Calculated by difference: total lipids − (total phospholipids + glycolipids + protein).
[4] Determined with the method of Davenport (1964).

Gas chromatographic analysis

The fatty acid composition of nonpolar and polar lipids has been determined by means of gas chromatographic analysis. Aliquots of nonpolar and polar lipids were saponified in ethanolic KOH. Methyl esters of fatty acids were prepared by refluxing in dry methanolic HCl (James, 1960). The methyl esters were determined by gas-liquid chromatography on a Beckman GC-2A apparatus equipped with a thermal conductivity detector. A column of 20% diethylene glycol-succinate polyester on Chromosorb W was used at 212°C. Helium was the carrier gas with an inlet pressure of 27 psi. Peaks were identified and quantitatively determined according to James (1960) and Woodford et al. (1960).

RESULTS

THE CHEMICAL composition of germ is reported in Table 1. These results do not present significant differences in respect to the germ of other varieties of wheat (Albritton, 1955). The principal lipid constituents of the washed lipid extracts are given in Table 2; about 97% of the total lipids is made up of neutral or nonpolar lipids, while 3% are polar lipids. The values of total amino nitrogen minus the values of free amino nitrogen remain high and, calculated as protein, are similar to the values obtained with the Davenport method (1964). This indicates that protein or other complex compound, in which the amino acid might be bound or, in any case, containing phenolic rings, is present in the germ lipids. The actual presence of proteolipids in wheat germ has been determined by separation of a fraction at the interface of a system (Folch et al., 1957); this fraction contained 55.6% protein and 44.4% lipid.

Nonpolar lipids, eluted from a silicic acid column, were further separated by column chromatography according to Barron et al. (1958) and three fractions were collected. Preliminary identification of lipids can be made on the basis of their chromatographic properties as studied by Barron et al. (1958). Each of the three fractions (sterol esters, triglycerides, and mono- and diglycerides) was subsequently analyzed by chromatography on silicic acid-impregnated paper.

Figure 1 shows the chromatographic results of the fractionation of nonpolar lipids. Separated lipids were identified by comparing R_f values with values reported in the literature (Marinetti et al., 1964) and with those of pure compounds. The fraction containing sterol esters seems to be a pure fraction and is the only spot corresponding to the R_f value of sterol. The fraction that would contain triglycerides and free fatty acids revealed only the spot of triglycerides; free fatty acids are not present or only in nondetectable quantity.

In the fraction of mono- and diglycerides, a spot identified by pure compounds as tocopherols is present. This spot, Liebermann negative, in the chromatogram shows a blue fluorescence (acidic lipid) after staining with Rhodamine 6G and examination under an ultraviolet lamp. The R_f of standard compounds (triglycerides and sterols esters) was slightly different when they were mixed together than when they were applied separately to the paper and are consistent with the identifications reported in Figure 1.

Table 3 shows the percentage of polar lipids from total phosphorus and galactolipids from total galactose content in the single fraction after separation of polar lipids on an alumina column.

The four fractions of these polar lipids obtained in this way contain phosphatidyl choline, choline- and amino-free phospholipids, phosphatidyl ethanolamine and

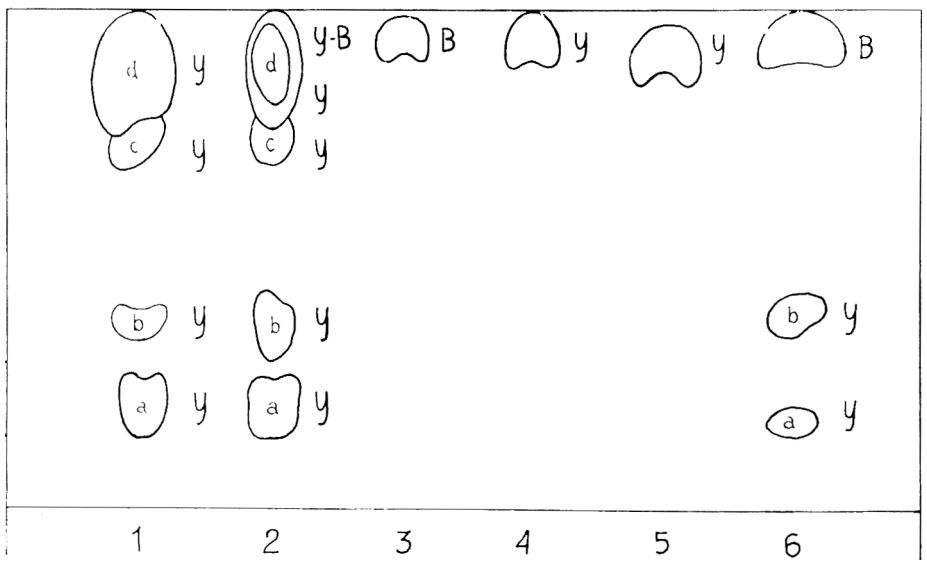

Fig. 1—Chromatography on silicic acid impregnated paper of different lipid fractions from wheat germ. y = yellow; B = Blue fluorescence after staining with Rhodamine 6-G and examination under an ultraviolet lamp; 1 = nonpolar lipids of egg yolk; 1a) monoglycerides; 1b) diglycerides: c) triglycerides; 1d) cholesterol esters; 2 = nonpolar lipids of wheat germ; 2a) monoglycerides; 2b) diglycerides; 2c) triglycerides; 2d) sterol esters + tocopherols; 3 = Tocopherol standard; 4 = Sterol esters fraction of wheat germ; 5 = Triglycerides fraction of wheat germ; 6 = mono- and diglycerides fraction of wheat germ; 6a) monoglycerides; 6b) diglycerides; 6c) tocopherols.

Table 3—Polar lipid fractions of wheat germ lipids.

Solvent[1]	System	Fraction	Bases found	% of the fraction Phospholipids	% of the fraction Galactolipids
C—M	1:1 (v/v) 60 ml	I	Choline	53.32	23.78
C—M—H_2O	7:7:1 (v/v) 30 ml	II	—	29.25	64.73
C—Et—H_2O	2:5:2 (v/v) 60 ml	III	Ethanolamine	13.01	11.49
E—Et—0.4 M KOH	10:7:5 (v/v) 90 ml	IV	Serine	4.40	—

[1] C = Chloroform; M = Methanol; E = Ether; Et = Ethanol.

Table 4—Distribution of lipid P after mild alkaline hydrolysis.

Sample	% of whole lipid P Alkali-labile P	Alkali-stable P
Phospholipids[1]	81.70	18.27
Fraction I[2]	92.00	7.95
Fraction II + III + IV[3]	72.20	27.70

[1] Hydrolysis of a phospholipid sample containing 6.107 μg of lipid P.

[2] Hydrolysis of a sample of the first fraction from the alumina column containing 14.063 μg of lipid P.

[3] Hydrolysis of a sample of the fractions II, III and IV from the alumina column containing 9.175 μg of lipid P.

phosphatidyl serine, respectively. The nitrogen bases composition of the fractions was checked by means of paper chromatography of the acid hydrolysis products, which showed that the fractions did not contaminate one another (Marinetti et al., 1957).

Choline and amino phosphatides are absent in the second fraction, but glycolipids are abundant. The galactose determination indicates their presence in the first and third fractions (23.78 and 11.49%), an abundance of the second (64.73%), and complete absence in the fourth. The phosphorus data show that the fraction containing choline is the most abundant (53.32%).

The distribution of lipid P, after a mild alkaline hydrolysis, is shown in Table 4; the alkali-labile P indicates the phosphatidic compounds, while the alkali-stable P may be an indication of compounds of the sphingolipid type (Dawson, 1960) or other compounds. The data show that alkali-stable phosphorus is present not only in the fraction containing choline but especially in the following fractions; the presence of phosphorus and galactose is suggestive for the presence of, besides sphingolipids, phytoglycolipids (Carter et al., 1958a, b) further study is necessary to rigorously establish the presence of these types of compounds.

The various constituents of the four fractions were studied by means of silicic acid impregnated paper (Table 5). Mobility (R_f values), spot tests (Rhodamine 6G, ninhydrin, choline and phosphate), and quantitative determinations of phosphorus in the spots provided information in the tentative identification of the separated compounds. Fraction I contains lysolecithin, lecithin, phosphatidic acid or polyglycerophosphate (PGP) derivatives and one unknown acidic component, choline-, ninhydrin-, and phosphate-negative.

Fraction II consists of phosphatidic acid or PGP derivatives and two unknown components, which were ninhydrin- and choline-negative, but phosphate and galactose positive. Only phosphatidylserine and phosphatidic acid or PGP derivatives are present in fraction IV. Table 6 reports the amounts (expressed in % of total germ) of all the lipid components examined in this study; for some of the components determined indirectly or by paper chromatography, the quantitation is obviously approximate.

Table 7 reports the methyl esters of the fatty acids of the germ lipids. These data show that the composition of fatty acids in nonpolar lipids is different from that in polar lipids; comparison with the data reported by McKillikan (1964) in free and bound lipid of wheat flour shows that linoleic acid is dominant in the germ as in wheat flour, but the levels of 18:3 are more elevated in lipid germ particularly in the polar fraction.

Table 6—Lipid components of wheat germ.

	content, % of germ
Neutral fat	9.02
Sterols esters	0.56
Triglycerides + free fatty acid	6.10
Mono- and di-glycerides	2.40
Polar lipids containing P	0.130
Phosphatidyl choline (—PA)	0.052
Non-choline non-ethanolamine (—PA)	0.0091
Phosphatidyl ethanolamine (—PA)	0.0129
Phosphatidyl serine (—PA)	0.0032
PA etc.	0.053
Polar lipids containing galactose	0.071
Protein of proteolipids etc.	0.1558

Table 7—Fatty acid spectrum in total lipids, nonpolar and polar lipids of wheat germ.[1]

Fatty acid	Total lipids	Nonpolar lipids	Polar lipids
16:0	20.03	19.77	18.39
16:1	0.30	0.58	1.74
18:0	0.30	—	1.08
18:1	16.68	16.5	19.44
18:2	56.00	58.41	43.49
18:3	5.05	4.61	12.12
20:1	2.06	—	4.09

[1] Percentage of total area of gas-liquid-chromatographic elution diagram.

DISCUSSION

RESULTS for total lipids of wheat germ are similar to those of Cookson et al. (1956).

The present research on lipid wheat germ demonstrates that of the 9.38% total lipids, 97% are nonpolar lipids and 3% polar lipids. Those compounds show the occurrence of common phospholipids: phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl serine and probably of phytoglycolipids, glycolipids, and also proteolipids. The presence of protein obtained after the purification procedure indicates that proteolipids or lipoamino-

Table 5—Properties of phosphatides.

Polar fractions separated on alumina	Phosphatides and other polar lipids[1]	Spot Test R_f	Rh 6-G	Ninhydrin	Phosphate	Choline	Phosphorus, %
Fraction I	Unknown	0.13	Blue	–	–	–	
Choline containing phospholipids	Lysolec	0.18	Yellow	–	+	+	15
	lec	0.38	Yellow	–	+	+	57
	PA	0.83	Blue	–	+	–	28
Fraction II	Unknown	0.23	Yellow	–	+	–	
Choline and amino-free phospholipids	Unknown	0.45	Yellow	–	+	–	24
	PA	0.80	Blue	–	+	–	77
Fraction III	Unknown (MPI?)	0.20	Blue	–	+	–	
Ethanolamine containing phospholipids	Lyso PE	0.30	Yellow	+	+	–	38
	Unknown	0.35	Blue	–	+	–	
	PE	0.52	Yellow	+	+	–	29
	PA	0.89	Blue	–	+	–	33
Fraction IV	PS	0.44	Blue	+	+	–	35
Serine containing phospholipids	PA	0.80	Blue	–	+	–	65

[1] Abbreviations: Lysolec = lysolecithin; Lec = lecithin; Pa = phosphatidic acid; AMP = monophosphoinositides; PE = phosphatidyl ethanolamine; PS = phosphatidyl serine.

acid complexes are present.

Proteolipids have not yet been described in germ, while their occurrence is well known within the vegetable kingdom in the leaf chloroplast (Zill et al., 1961). A report on the proteolipid fractions obtained from wheat germ will be the subject of a forthcoming paper. Also, complexes between lipids and amino acids have been demonstrated in the leaf (Brady, 1964).

Moreover the presence of compounds behaving like phytoglycolipids was detected in germ lipids. These components were demonstrated by Carter et al. (1958a) in soybeans. The relative percentage of the various phospholipids shows a high percentage of phosphatidic acid or PGP derivatives. Although PA and PGP derivatives are present in all the fractions from the alumina column separation, hydrolysis of phosphatides in the column during the separation procedure should be excluded; in fact no such phenomena were ever observed with phosphatide mixtures of animal source. It is however possible that phosphatidic acid or PGP derivatives originate by breakdown of the more complex lipids during the chromatographic separation.

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The Sterols and Triterpenes of Banana Pulp

SUMMARY—Using thin-layer chromatography and gas-liquid chromatography the following substances were identified in banana pulp (Musa sapientum L.): *Cycloartenol, cycloeucalenol, 24-methylene cycloartanol, campesterol, β-sitosterol and stigmasterol. The major triterpene was 24-methylene cycloartanol while β-sitosterol accounted for greater than 72% of the sterol fraction.*

INTRODUCTION

CONTINUED interest in the sterol and triterpene content of the fruits and vegetables that constitute a major portion of man's diet has led us to examine the sterol constituents of banana pulp. In a recent paper (Knapp et al., 1969) the sterols and triterpenes present in banana peel were described. The present study was initiated to compare this with the sterol content of the pulp.

In a study of the dietary constituents of lemurs, Moss (1937) isolated a crude "sitosterol" fraction from banana oil but made no identification. The present study has successfully identified the major sterols and triterpenes of this tissue.

[a]NASA pre-doctoral fellow.

MATERIALS & METHODS

Reagents and chemicals

All solvents were analytical grade, distilled before use, except the ethanol used in the initial extraction. The petroleum ether had a boiling range of 30–60°C. The alumina was Merck, acid-washed.

Thin-layer chromatography

Thin-layer chromatography (TLC) was performed as described previously (Knapp et al., 1968). Preparative TLC plates were spread 500 microns thick with silica gel G (Brinkmann Instrument Co., Westbury, N.Y.) instead of the usual 250 microns. 40 mg of the material to be analyzed were

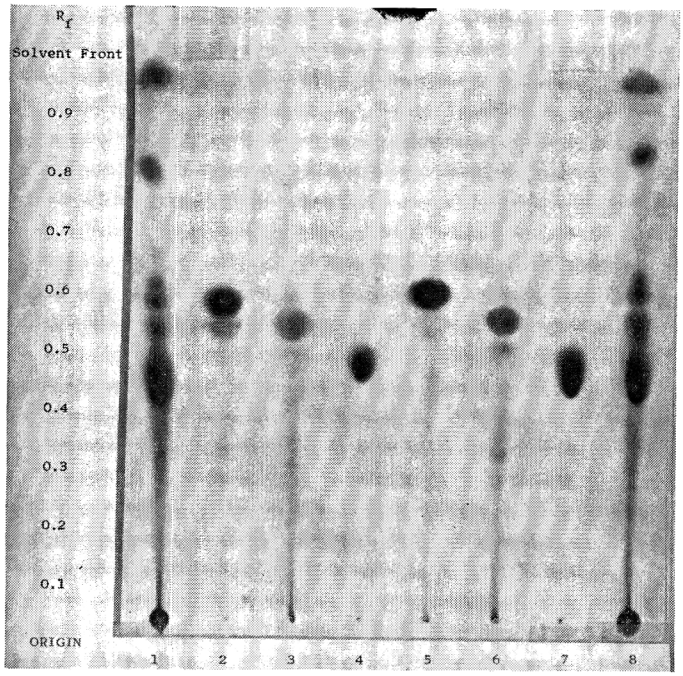
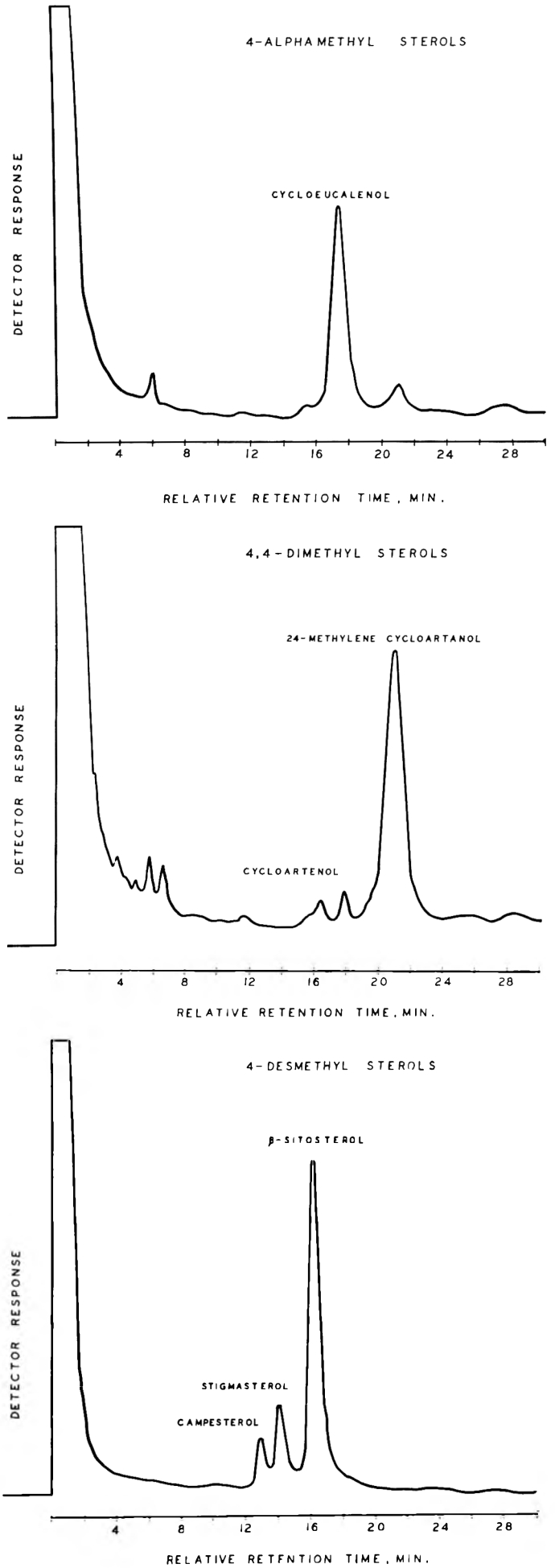


Fig. 1—Photograph of a thin-layer chromatogram. See Experimental Section for TLC details. (1,8) N.S. material from M. sapientum *pulp; (2,3,4) the 4,4-dimethyl, 4α-methyl and 4 desmethyl sterol fractions, respectively, partially purified by preparative TLC of the N.S. material; (5,6,7) standards, 24-methylene cycloartanol, cycloeucalenol and β-sitosterol, respectively.*

Fig. 2—Gas-liquid chromatograms of the three sterol classes from M. sapientum *pulp. Analyses were performed as described in the Experimental Section.*



streaked on the plates, with appropriate markers being applied on the boundaries. After developing, the major portion of the plate was covered with another plate and the markers sprayed with anisaldehyde reagent. After developing, the major portion of the plate was covered with another plate and the markers sprayed with anisaldehyde reagent. After heating to visualize the markers, areas of the unknown were scraped accordingly. Material was eluted from the silica gel with a mixture of chloroform-methanol, 1:1 (v/v), followed by centrifugation.

Gas-liquid chromatography

Gas-liquid chromatography (GLC) was also performed as mentioned previously (Knapp et al., 1968). An SE-30 (3%) column was used for these analyses. Peak areas were integrated by the method of triangulation, with the subsequent calculation of mass ratios.

Preparation of non-saponifiable material

500 lb of banana pulp were sliced and extracted exhaustively with hot, absolute ethanol. The ethanol extract was concentrated to low volume, an equal volume of water added and KOH to a final concentration of 15% (wt/vol). The mixture was refluxed for 1 hr, cooled and extracted thoroughly with ethyl ether. After being washed several times with water the ethereal extract was distilled to yield 8 g of orange wax.

Fractionation of non-saponifiable material

The 8 g of nonsaponifiable material

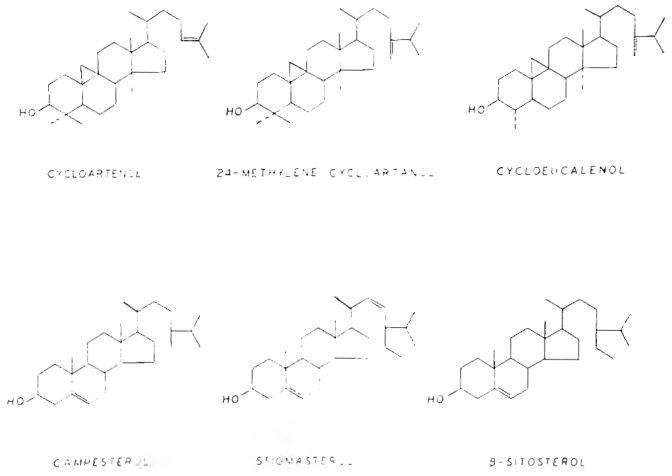


Fig. 3—Structural formulas of the sterols and triterpenes identified in the pulp of M. sapientum.

(N.S.) was chromatographed on a 2 cm diameter glass column containing 300 g of Merck, acid-washed alumina. The column was initially washed with 1 L of petroleum ether. Fractions (100 ml each) were then taken with benzene (1–42), 10% ethyl ether in benzene (43–63), ethyl ether (64–66) and absolute ethanol (67–69). Following examination by TLC appropriate fractions were combined.

RESULTS & DISCUSSION

A REPRESENTATIVE TLC chromatogram of the banana pulp N.S. material is shown in Figure 1. Material at the solvent front presumably represents hydrocarbons and the sterol esters previously described in banana peel (Knapp et al., 1969). This material was not further examined. The material in the R_f 0.80 region co-chromatographs with the unknown triterpene ketone from banana peel. From the alumina column chromatography fractions 21–27 (0.103 g) were found by TLC to contain the 4,4-dimethyl sterols (R_f 0.62), fractions 28 to 43 (0.210 g) the 4α-methyl sterols (R_f 0.54), and fractions 44 to 61 (2.632 g) the 4 des-methyl sterols (R_f 0.47). An aliquot of each of these three sterol fractions was further purified by preparative TLC (Fig. 1) and then examined by GLC.

The GLC chromatograms are illustrated in Figure 2. The 4,4-dimethyl sterol fraction consisted primarily of 24-methylene cycloartanol. A small amount (<2%) of cycloartenol was also indicated in this fraction. Cycloeucalenol was the sole 4α-methyl sterol. The 4 desmethyl sterol fraction consisted of the ubiquitous phytosterols campesterol, stigmasterol and β-sitosterol, in the ratio 1:1.5:6.5. The structures of these compounds are shown in Figure 3.

As would be expected from the high carbohydrate content of banana pulp, the nonsaponifiable material represented only a small fraction of this tissue. The crude pulp extract consisted of a large volume of brown, viscous oil. The amount of nonsaponifiable material present in this large volume of oil was quite small.

Although the sterol constituents of both the peel and pulp are the same, the relative amounts of these sterols in the two tissues differ markedly. In banana peel (Knapp et al., 1969) the major sterol is stigmasterol, while in banana pulp β-sitosterol accounts for greater than 72% of the sterol fraction. In addition, the majority of the triterpenes in banana peel are esterified to long chain fatty acids while the 4 des-methyl sterols are found in the free form (Knapp et al., 1968). In the pulp, however, both the triterpenes and the 4 desmethyl sterols are found in the free form.

A possible hypocholesterolemic action of bananas has been correlated with the high concentration of linoleic, linolenic and arachidonic acid in this tissue (United Fruit Co., 1959). It has been shown that β-sitosterol can interfere with cholesterol absorption in man (Hernandez et al., 1954). This phytosterol may thus aid in controlling hyperchlosterolemia. The presence of β-sitosterol in banana pulp may thus also effect serum cholesterol levels.

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Whiskey Composition: Identification of Additional Components by Gas Chromatography–Mass Spectrometry

SUMMARY—Gas-liquid chromatographic methods are described for the identification of 87 components in distilled alcoholic liquors. Water-free concentrates of ether-pentane or Freon extracts of the distillates were injected into different column systems and the column effluent transported to a mass spectrometer. To our knowledge, 34 of the identified compounds have not been reported previously in whiskey.

INTRODUCTION

IN A PREVIOUS paper (Kahn et al., 1968a) 37 whiskey components were identified by means of a single-pass gas-chromatography-mass sepctrometric technique. It was evident that a number of chromatographic peaks were not completely resolved and in many cases they represented a number of compounds. Some high boiling components were eluted only after several hours. The earlier work has been continued and extended to include columns with different liquid phases and columns operated at higher temperatures.

The identities of 80 compounds have now been confirmed by the comparison of their mass spectra and GLC retention times with those of authentic samples. An additional seven compounds have been tentatively identified on the basis of their GLC retention times only.

During the course of whiskey production, a tails fraction is produced which, depending on the equipment, is recycled in the distillation system or is discarded. This tails fraction was believed to contain compounds which are somewhat less volatile with steam than ethyl alcohol and fusel oils. These higher boiling compounds can be expected to be present in whiskey, since the normal distillation process is designed to allow only some of these compounds to be volatilized. Their concentration in whiskey is so low that their detection is difficult. The tails simply were used as a convenient source of the high boiling components.

EXPERIMENTAL

Equipment

Varian Aerograph Models 1520 and 1200 were used for the development of techniques for the required separations. Equipment used for the identification of compounds by mass spectrometry has been described in the previous publication. Column systems, equipment and operating conditions used are shown in Table 1.

Samples

Sample A was an ether-pentane extract of a typical Bourbon whiskey which had been aged four years in the conventional manner in new charred white-oak barrels.

Sample B was an ether-pentane extract of a 3-year old Bourbon whiskey which initially had an acrolein content of approximately 10 g/100 L at 100° proof. Sample BF was a Freon extract of the same Bourbon as sample B. These samples were discussed in the previous publication.

Sample C was an ether-pentane extract of the tailings from the second distillation in the normal production of Bourbon whiskey. The sample was taken from the low-wine tank containing the compounds distilled between 80° and 0° proof or 90° and 100°C.

Sample D was an ether-pentane extract of a stream sample taken at approximately 40° proof halfway through the distillation of the tails.

Sample preparation and analysis

The techniques used were extensions of those reported previously, but not all components identified were separated on the same column system. Higher temperatures and, therefore, different liquid phases were required to obtain the desired results. In all cases, extracts of the samples were prepared in order to concentrate the trace components and to remove all the water and most of the ethyl alcohol.

Ether-pentane extracts were obtained as reported previously, except that 500 ml, instead of 300 ml, were used for samples C and D because of their lower alcohol content. The Freon 113 (duPont) extract was prepared in a similar manner except that 250 ml of liquor were extracted with two 50-ml portions of Freon.

Table 1—Columns and operating conditions employed.

	Model 1520				Model 1200
	Column 1	Column 2	Column 3	Column 4	Column 5
Length	10 ft + 10 ft each	37 ft	6 ft	10 ft	10 ft + 10 ft each
Liquid phase	10% Tergitol NPX[1] + 10% Surfonic N300[1]	25% Igepal CO880[1]	8.5% DEGS[2] + 1.5% Apiezon M[2]	20% FFAP[2]	10% Hallcomid M-18-OL[2] + 10% Tergitol NPX[1]
Support	Chromosorb W-AW	Chromosorb W	Chromosorb W-HMDS	Chromosorb W-AW-DMCS	Chromosorb W-AW
Mesh size	100/120	60/80	60/80	70/80	100/120
Detector	TC-WX	TC-WX	TC-WX	TC-WX	F.I.D.
Temperatures, °C					
Injector	165	200	225	270	185
Detector	165	200	225	270	185
Column oven and temperature programming rate	From 50° at 2°/min for 10 min, then at 4°/min to 135° and held at that temperature	From 75° at 3°/min for 15 min, then at 5°/min to 170° and held at that temperature	From 100° at 3°/min for 10 min, then at 5°/min to 190° and held at that temperature	At 165° isothermal for 15 min, then temperature-programmed at 5°/min to 240° and held at that temperature	From 40° at 2°/min to 120° and held at that temperature

[1] Applied Science Laboratories, State College, Pa.
[2] Varian Aerograph, Walnut Creek, Calif.
All columns were 1/8 in. O.D. copper for lower temperatures, stainless steel for higher temperatures. Carrier gas was Helium, 100 lb inlet pressure, flow rate approx. 38 ml/min. WX filaments were operated at 175 ma current. A 1 mv 1 sec Westronics recorder was used with a chart speed of 24 in./hr.

Table 2—Compounds identified in ether-pentane extracts of samples A and B and key to Figure 1.

Peak nos. A	Peak nos. B	Component
1	1	Ethyl formate[1]
	1	Acrolein[1]
2	2	Ethyl methyl sulfide[1]
3	3	Ethyl alcohol[1]
4	4	Ethyl acetate[1]
5	5	Acetal[1]
6	6	Ethyl propionate[1]
7	7	*n*-Propyl alcohol[1]
	8	1,1-Diethoxypropane[1]
	9	1-Ethoxy-1-propoxyethane[1]
	10	Allyl alcohol
11	11	Isobutyl alcohol[1]
12	12	Ethyl butyrate[1]
13	13	*n*-Butyl alcohol[1]
14	14	Isopentyl acetate[1]
15	15	Isopentyl alcohol[1] 2-Methyl butanol[1]
	16	3-Ethoxypropionaldehyde[1]
17	17	Triethylorthoformate (tent.)[1]
18	18	Ethyl hexanoate[1]
19	19	3-Methyl pentanol
20	20	Hexyl alcohol
	20	unidentified acetal-type compd.
	21	Ethyl 3-ethoxypropionate[1]
22	22	1,1,3-Triethoxypropane[1]
23	23	2-Furaldehyde[1]
	23	Ethyl heptanoate
	23	unidentified acetal-type cpd. M.W. 132
24	24	Heptyl alcohol
	25	unidentified acetal-type cpd. M.W. 146
	26	unidentified acetal-type cpd. M.W. 160
	27	Benzaldehyde
28	28	Ethyl octanoate[1]
	29	5-Methyl-2-furaldehyde
30	30	Octyl alcohol
	31	unidentified acetal-type cpd.
	32	unidentified acetal-type cpd. M.W. 174
	33	1-Ethoxy-1-isopentoxy-3-hydroxy-propane (tent.)
	34	unidentfiied acetal-type cpd.
	35	Ethyl nonanoate
36	36	Diethyl succinate
37	37	Ethyl decanoate
38	38	Phenethyl formate (tent.)
	39	Phenethyl alcohol

[1] Reported in previous publication.

The Freon extract was analyzed on column 5 using the flame ionization detector of the Model 1200 chromatograph. All other extracts were analyzed with other columns (Table 1) on the Model 1520 using dual WX thermal conductivity (T.C.) detectors. The T.C. detectors were chosen in order to obtain a sensitivity response as similar as possible to that of the equipment employed for subsequent GLC-MS analysis. After satisfactory separations were obtained, the extracts were submitted to GLC-MS analysis, using the same columns and analyzed with the equipment as already described (Kahn et al., 1968a).

Numerous additional compounds, not reported in the previous publications were resolved and identified in the ether-pentane extracts using the Tergitol-Surfonic column system under different operating conditions (Table 2). Essentially the same results were obtained using the Freon extract with the Hallcomid-Tergitol column (column 5).

Table 3—Retention times of eluted whiskey components (min from injection point).

Compound	Column 2 Sample B	Column 5 Sample BF
Acetaldehyde	8.5	3.5
Ethyl formate	13.5	
Acrolein	14.25	6.5
Methanol	15.75	
Ethyl acetate	17	10
Diethoxymethane	16.5	11.75
Ethyl alcohol	17.5	12.5
Acetal	18.5	15.5
Isovaleraldehyde	20	
Ethyl propionate	21.5	16
Benzene	21.5	
Propyl acetate		16.75
Ethyl isobutyrate		18.75
n-Propyl alcohol	23.5	20
Isobutyl acetate		21.25
1,1-Diethoxypropane	22.5	21.75
1,1-Diethoxy-2-propene		22.5
Toluene	27	22.75
Allyl alcohol		23
Ethyl butyrate		23.5
Isobutyl alcohol	26.5	25.75
Ethyl valerate		28
1-Butanol	29.5	29.25
3-Ethoxypropionaldehyde	35.5	30
β-Hydroxypropionaldehyde (tent.)		30
Isopentyl acetate	31.75	31
C_5 alcohols[1]	34.5	35.5
A diethoxy acetal (tent.)		38.5
Ethyl hexanoate (caproate)	41	41.5
2-Furaldehyde	65	43.25
Ethyl 3-ethoxypropionate		43.5
3-Methyl-1-pentanol	45.25	45.25
Hexyl alcohol		47.5
1,1,3-Triethoxypropane	48.5	49.75
Ethyl heptanoate		53.5
Benzaldehyde		57
Ethyl octanoate (caprylate)	71	74.5
Ethyl decanoate (caprate)	100	
A diethoxy acetal (tent.)		85.5

[1] Mixture of 2-methyl butanol and isopentyl alcohol.

Table 4—Compounds identified in ether-pentane extract of sample B and key to Figure 2.

Peak no.	Component
1	1,1,3-Triethoxypropane
2	2-Furaldehyde
3	Ethyl octanoate
4	5-Methyl-2-furaldehyde
4	+ unknown acetal-type compound
5	Ethyl nonanoate
5	+ unknown acetal-type compound
6	Ethyl benzoate
7	Diethyl succinate
8	Ethyl decanoate
9	Phenethyl acetate
10	Phenethyl alcohol
11	(delta) 5-Nonalactone (branched) (tent.)
12	(delta) 5-Nonalactone
13	Ethyl laurate
14	4-Ethyl phenol
15	Ethyl myristate
16	Diethyl phthalate
17	Ethyl pentadecanoate
18	Ethyl hexadecenoate
19	Ethyl palmitate
20	Vanillin
21	possibly a phthalate
22	Ethyl oleate Ethyl linoleate

Table 5—Compounds identified in ether-pentane extracts of samples C and D and key to Figures 3 and 4.

Peak nos. C	Peak nos. D	Compound
1	1	Isobutyl alcohol
2	2	C_5 alcohols[1]
3	3	Ethyl lactate[2]
4		Acetic acid[2]
	5	Ethyl octanoate
6	6	2-Furaldehyde
7	7	Isobutyric acid[2]
8		*n*-Butyric acid[2]
	9	Ethyl decanoate
10	10	Isovaleric acid[2] 2-Methyl butyric acid[2]
	11	*n*-Valeric acid[2]
12	12	Phenethyl acetate
12		Hexanoic acid[2]
	12	Ethyl laurate
13	13	Phenethyl alcohol
14	14	Octanoic acid[2]
15		4-(gamma) Nonalactone[2]
16		Unknown
17	17	4-Ethyl phenol
18	18	Tetramethyl phenol (tent.)[2]
19	19	Ethyl palmitate
20	20	Decanoic acid[2]
21		Aromatic cpd. (MW 120)[2]
22	22	Ethyl oleate
23	23	Ethyl linoleate

[1] Mixture of 2-methyl butanol and isopentyl alcohol.

[2] Identified as such, in tail fractions only; Ethyl lactate, peak 3, was identified in whiskey by several workers and also in this laboratory. (See Kahn et al., 1968b).

The free acids, in general, also were detected in whiskey as methyl esters (Table 6).

To obtain a better separation of low boiling components in these extracts, a 37-ft long "Igepal" column (column 2) was used but the results were below expectations (Table 3).

Since high-boiling compounds were thought to be present and were improperly eluted from the column systems mentioned, a column was employed containing a liquid phase capable of withstanding higher temperatures. Sihto et al. (1963) used a column packed with diethylene glycol succinate (DEGS) and Apiezon M on Celite to separate phenethyl alcohol from beer. Since this alcohol is relatively high boiling, a column using this liquid phase (column 3) was successfully employed for the separation of high boiling components in whiskey extracts (Table 4).

To obtain better separation of high-boiling fatty compounds, which were poorly resolved on the columns mentioned above, the FFAP column (column 4) was used and good results were obtained (Table 5).

By GLC-MS it was determined that some peaks from samples C and D, separated on the FFAP column represent free fatty acids which are not easily identified by their mass spectra. Therefore, these samples were re-extracted, the acidic fraction separated by neutralization and reacidification and the free acids methylated with MeOH-BF_3 reagent.

The resulting methyl esters then were separated by temperature programming the FFAP column from 80° to 240°C at 5°/min and identified by GLC-MS using the same

Table 6—Occurrence and retention times of compounds in methylated acidic fractions from samples A and C on FFAP column.

Compound	R.T. (min)	Samples[1] A	C
Methyl ethyl sulfide	1.25		+
Methyl isobutyrate	2	+	+
Methyl butyrate	2.75		+
Methyl isovalerate	3	+	+
Methyl 2-methyl butyrate	3	+	+
Methyl valerate	4.25		+
Methyl hexanoate	6.25	+	+
Methyl heptanoate	8.75	+	+
Methyl octanoate	11.25	+	+
Methyl nonanoate	14	+	+
Methyl decanoate	16.5	+	+
Phenethyl alcohol	24.75		+

[1] + = present.

column and temperature conditions. Most of the methyl esters found corresponded to the free acids separated by the FFAP column. However, some additional methyl esters were also present (Table 6).

RESULTS & DISCUSSION

THERE WERE 87 components identified in the samples described. The identities of all but six have been confirmed by the comparison of their mass spectra and GLC retention times with those of authentic samples. It is believed, 34 of the confirmed compounds have not been reported previously in whiskey (Table 7).

In addition to the 37 compounds already reported by this laboratory, 50 components have been identified by the techniques here described. Some GLC peaks represented mixtures of compounds but in most cases, these mixtures could be distinguished and identified by their mass spectra. For example, in Table 5, peak 12 represents phenethyl acetate and hexanoic acid. Powell et al. (1966) made the same observation in the analysis of beer.

Several more aromatic compounds were identified such as diethyl phthalate, ethyl benzoate, phenethyl alcohol and its esters, ethyl phenol, a tetramethyl phenol and vanillin.

A number of peaks on the chromatograms represent acetal-type compounds, the identities of which could not be established because of the lack of reference mass spectra and the non-availability of the authentic compounds. It is postulated that many of these unidentified components are mixed acetals, i.e., two different alcohols reacted with one aldehyde. Since many alcohols and several aldehydes are present, numerous combinations for mixed acetals are possible.

Peak 33 from sample B (Table 2) was tentatively identified by GLC-MS as 1-ethoxy-1-isopentoxy-3-hydroxypropane. Its mass spectrum indicated a parent mass of 190 and showed that the compound was an acetal containing a hydroxyl group. The identification and structure could not be confirmed due to lack of reference material. The tentative structure is, however, quite feasible because the compound is a mixed acetal formed by the reaction of ethyl and isopentyl alcohols with beta-hydroxypropionaldehyde.

This hydroxyaldehyde is thought to be an intermediate in the formation of 1,3-propanediol from glycerol (Abeles et al., 1960 and Sobolov et al., 1960) and the product of the reaction of acrolein with water (Hall et al., 1950; Shell Chemical Corp., 1959).

Mass spectral fragments indicate that beta-hydroxypropionaldehyde may be part of the 3-ethoxypropionaldehyde peak from sample BF, Table 2, which actually consists of more than one component. There are also indications that some of the beta-hydroxypropionaldehyde may have lost a molecule of water during the mass spectral analysis thereby forming a small quantity of acrolein. This tentative identification could not be confirmed due to unavailability of reference sample and spectra.

The finding of allyl alcohol was not unexpected, because it can be formed from acrolein. Acrylic acid or ethyl acrylate was expected, since the oxidation-reduction reaction would produce both allyl alcohol and acrylic acid from acrolein. However, attempts to detect acrylic acid or ethyl acrylate were not successful.

Allyl alcohol and other compounds thought to be related to acrolein were not detected in whiskey which did not contain acrolein. However, 1,1,3-triethoxypropane, believed to be the final reaction product of acrolein and ethyl alcohol, was found in trace quantities in acrolein-free whiskey and in large quantities in whiskey which contained acrolein.

A great number of even as well as odd-numbered carbon fatty compounds were identified, including a few unsaturated fatty acid esters. Many of these compounds were reported by Nykanen (1968).

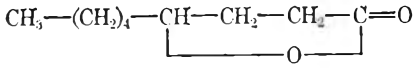
Table 7—Compounds identified in whiskey and/or tail fractions.

No.	Compound
1	Acetal[1]
2	Acetaldehyde[1]
3	Acetic acid
4	Acrolein[1]
5	Allyl alcohol[2]
6	Benzene[1,2]
7	Benzaldehyde[2]
8	1-Butanol[1]
9	2-Butanone[1]
10	sec-Butyl alcohol[1]
11	Butyric acid
12	Carbon bisulfide (tent.)[1]
13	Decanoic acid (capric) (C_{10})
14	Diethoxymethane[2]
15	1,1-Diethoxy-2-methylpropane[1,2]
16	1,1-Diethoxypropane[1,2]
17	1,1-Diethoxy-2-propene[1,2]
18	Diethyl phthalate[2]
19	Diethyl succinate[2]
20	1-Ethoxy-1-isopentoxy-3-hydroxypropane (tent.)[2]
21	1-Ethoxy-1-propoxyethane[1,2]
22	3-Ethoxypropionaldehyde[1,2]
23	Ethyl acetate[1]
24	Ethyl alcohol[1]
25	Ethyl benzoate[2]
26	Ethyl butyrate[1,2]
27	Ethyl decanoate (caprate) (C_{10})
28	Ethyl 3-ethoxypropionate[1,2]
29	Ethyl formate[1]
30	Ethyl heptanoate (enanthate) (C_7)
31	Ethyl 9-hexadecenoate ($C_{16:1}$)
32	Ethyl hexanoate (caproate) (C_6)[1]
33	Ethyl isobutyrate
34	Ethyl lactate
35	Ethyl laurate (C_{12})
36	Ethyl linoleate ($C_{18:2}$)
37	Ethyl methyl sulfide[1,2]
38	Ethyl myristate (C_{14})
39	Ethyl nonanoate (pelargonate) (C_9)
40	Ethyl octanoate (caprylate) (C_8)[1]
41	Ethyl oleate ($C_{18:1}$)
42	Ethyl palmitate (C_{16})
43	Ethyl pentadecanoate (C_{15})[2]
44	4-Ethyl phenol (probably)
45	Ethyl propionate[1]
46	Ethyl valerate[2]
47	2-Furaldehyde[1]
48	Heptane[1,2]
49	Heptanoic acid (C_7)
50	Heptyl alcohol[2]
51	Hexanoic acid (C_6)
52	Hexyl alcohol
53	Isobutyl acetate
54	Isobutyl alcohol[1]
55	Isobutyraldehyde[1]
56	Isobutyric acid
57	Isopentyl acetate[1]
58	Isopentyl alcohol[1]
59	Isovaleraldehyde[2]
60	Isovaleric acid
61	Lauric acid (C_{12})
62	Methanol
63	2-Methyl butanol (C_5)[1]
64	2-Methyl butyric acid (C_5)[2]
65	5-Methyl-2-furaldehyde[2]
66	3-Methyl-1-pentanol (C_6)[2]
67	5-(delta) Nonalactone[2]
68	5-(delta) Nonalactone (branched (tent.)[2]
69	4-(gamma) Nonalactone[2]
70	Nonanoic acid (C_9)
71	Octanoic acid (C_8)
72	Octyl alcohol[2]
73	2-Pentanone[1,2]
74	Phenethyl acetate
75	Phenethyl alcohol
76	Phenethyl formate (tent.)[2]
77	2-Pinene[1,2]
78	Propionaldehyde[1]
79	Propyl acetate[2]
80	n-Propyl alcohol[1]
81	Styrene[1,2]
82	Tetramethyl phenol (tent.)[2]
83	Toluene[1,2]
84	1,1,3-Triethoxypropane[1,2]
85	Triethyl orthoformate (tent.)[1,2]
86	Valeric acid (C_5)
87	Vanillin

[1] Reported in previous publication.
[2] Compound not previously reported in whiskey to our knowledge.

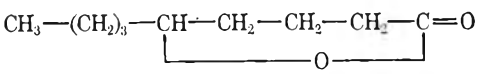
The detection of gamma and delta-lactones in these samples was rather surprising. The presence of gamma-nonalactone (I), which has a strong coconut odor, has been confirmed. Due to the lack of authentic compounds and reference mass spectra, the exact structure determination of one of the delta-lactones (II) was not possible. However, by their mass spectra, it has been established definitely that delta-nonalactones are present.

The structure of the isomeric delta C_9 lactone could not be determined. The gamma and delta-lactones are probably derived from 4-hydroxynonanoic acid and 5-hydroxynonanoic acid, respectively, assuming straight-chain molecular structure:



(I)

(gamma) 4-hydroxynonanoic acid lactone



(II)

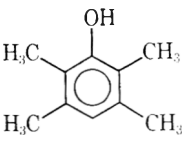
(delta) 5-hydroxynonanoic acid lactone

Knowledge of the exact structures of the lactones detected would be of great interest since they have strong odor characteristics which could contribute to whiskey flavor.

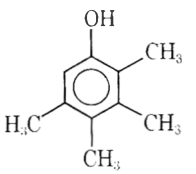
The ethyl phenol compound could be the 3-ethyl or 4-ethyl isomer. No column tested separated the two isomers, and neither is differentiation by their mass spectra possible. The 4-ethyl phenol is believed to be the most likely structure. This isomer has also been reported in distillates from fermented grain. (Steinke et al., 1964).

Although peak 18 listed in Table 5 has not been positively identified, the following reasoning indicates that the material is a tetramethyl phenol. While its mass spectrum is in agreement with a four-carbon substituted phenol, the butyl phenols, methyl propyl phenols, diethyl phenols and dimethyl ethyl phenols produce spectra which differ markedly from that of the compound in question.

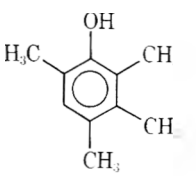
The mass spectrum of recrystallized 2,3.5,6-tetramethyl phenol (I) (Aldrich Chemical Co.) is very similar to the spectrum of the component to be identified, but is not a perfect match. This leads to the assumption that the component is another isomer of a tetramethyl phenol, either the 2,3,4,5 (II) or the 2,3,4,6-isomer (III), neither of which is readily available as reference material.



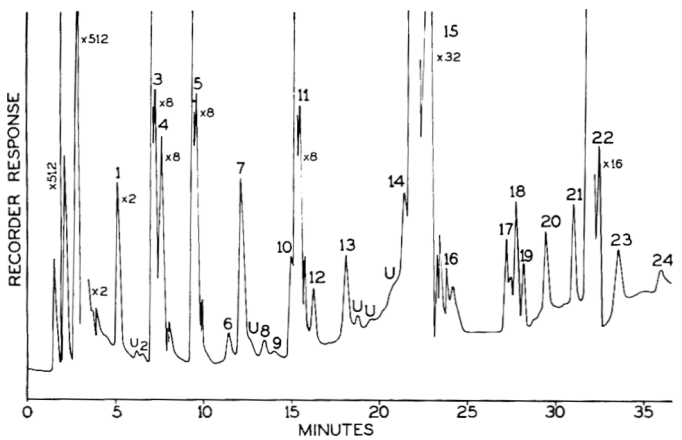
I



II



III



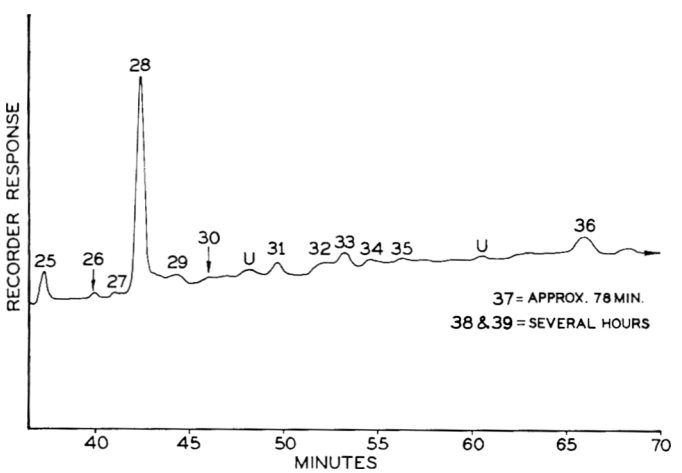


Fig. 1—Chromatogram of ether-pentane extract of Sample A (Tergitol-Surfonic column system).

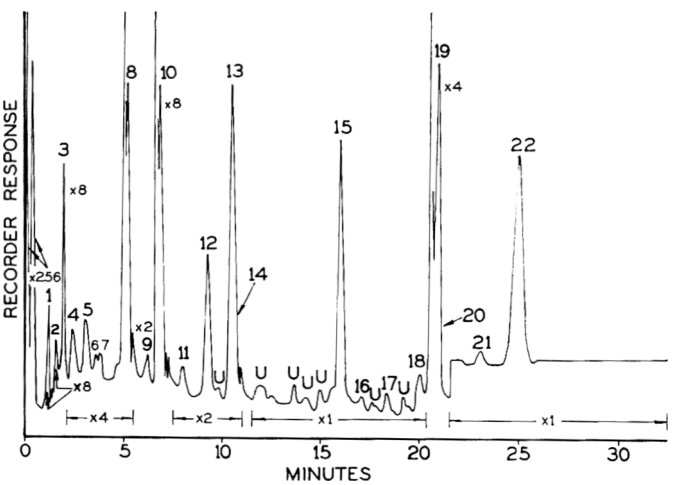


Fig. 2—Chromatogram of ether-pentane extract of Sample B (DEGS-Apiezon column).

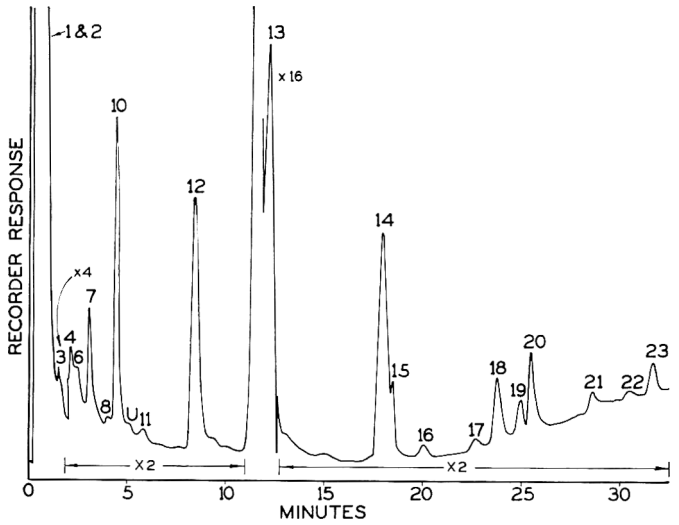


Fig. 3—Chromatogram of ether-pentane extract of Sample C (FFAP column).

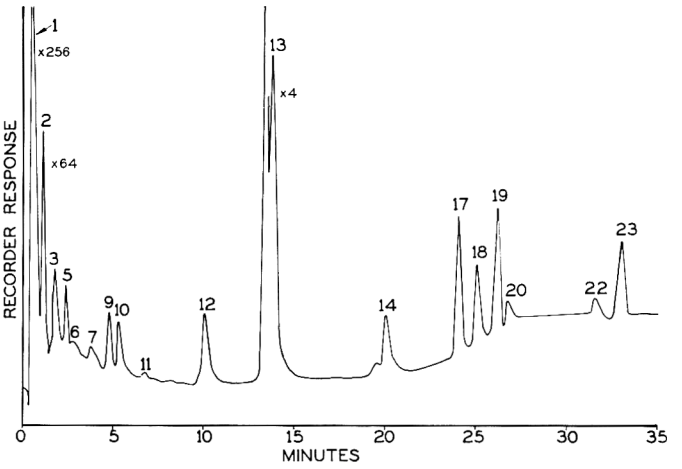


Fig. 4—Chromatogram of ether-pentane extract of Sample D (FFAP column).

Phenethyl formate identification in Table 2 (peak 38) is listed as tentative. This compound has been definitely identified by its mass spectrum, but the formate may be formed on the column and actually may not be present in the whiskey.

In a recent paper, Lam (1967) reported that di-*n*-butyl phthalate and diethyl phthalate contamination may occur in some extracts passed through filter paper. In this laboratory it was found that the diethyl phthalate reported in Table 4 was, in fact, present in the samples and was not due to contamination of extracts by filter paper.

No appreciable differences were noted between ether-pentane and Freon extracts of whiskey (samples B and BF), although five additional components were identified and several more detected in the former extract. The Freon extract, however, was found to contain a higher concentration of aldehydes and esters, including a few esters not detected in the ether-pentane extract of the same sample of whiskey. This agrees with the findings of Schultz et al. (1967).

Several of the unidentified peaks reported in the previous publication from this laboratory now have been identified. Many peaks, however, still remain unidentified because of insufficient concentration of the compounds in the extracts, lack of reference mass spectra or the nature of the molecular structure involved, such as mixed or hydroxyacetals and lactones.

Since the performances of columns 1 and 5 are essentially equal, only the chromatogram from column 1 is shown in Figure 1, and the retention times of the identified peaks from column 5 and those from column 2 are presented in Table 3. Chromatograms from columns 3 and 4 are shown in Figures 2, 3 and 4. Retention times of methyl esters of the free fatty acids separated by column 4, at lower temperatures, are shown in Table 6.

Table 7 lists in alphabetical order all compounds found in whiskey and identified in this laboratory.

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Calcium Chelators Influence Some Physical and Chemical Properties of Rabbit and Pig Muscle

SUMMARY—Intravenous antemortem injections of EDTA, EGTA and CDTA significantly inhibited muscle shortening during development of rigor mortis. Post-mortem micro-injections of $CaCl_2$ increased shortening, but $MGCl_2$ had no measurable effect. Even though average shear values for muscle from EDTA-treated rabbits were lower than those of untreated controls, the differences were not statistically significant. However, muscle from EDTA-treated pigs was significantly more tender than that from untreated controls. The interrelationships between ATP levels and pH values at 0 and 24 hr post-mortem were investigated and their effects upon muscle shortening and tenderness are considered and explained. A highly significant negative relationship was shown to exist between cooking losses and both initial pH and initial ATP values for both rabbit and pig muscle.

INTRODUCTION

THE AMOUNT and state of various protein constituents of meat are generally considered to be of primary importance in regard to its physical characteristics. For many years the amount of stroma proteins, or connective tissue, in meat was believed to be the principal source of variation in tenderness. However, improved understanding of the structure and biochemistry of muscle derived from research on muscle contraction and current knowledge of the biochemical and physical changes occurring during rigor mortis have suggested the importance of fibrillar proteins on the physical properties of meat.

The sliding-filament model of muscle contraction of Hanson et al. (1955) provides an elegant explanation of the great increase in the modulus of elasticity, which occurs as rigor is completed. It is now well accepted that the mechanism of contraction with the onset of rigor mortis is similar to the phenomena occurring in living fibers and in isolated myofibrils. Working with beef, Locker (1960), Herring et al. (1965a, b) and Marsh et al. (1966a, b) indicated that the state of contraction is an important factor contributing to tenderness.

Largely as a result of studies of muscle models and their constituent proteins, changes in the physical state of skeletal muscle have been related to interactions between actin and myosin in the presence of various concentrations of ATP, calcium and magnesium. It is now generally accepted that contraction of skeletal myofibrils, actomyosin and glycerinated fibers requires the presence of calcium in addition to magnesium and ATP (Martonosi et al., 1964).

Martonosi et al. (1964) also pointed out that the relaxing effect of chelating agents and of relaxing factor extract can be explained by their ability to lower the free calcium concentration of skeletal muscle. Weiner et al. (1966) showed that a lethal intravenous injection of EDTA in rabbits inhibited post-mortem shortening of the semitendinosus muscle. Maruyama (1962) suggested that the mechanism of action of chelating agents in an actomyosin system appears to be removal of a trace amount of calcium.

The primary objectives of this study were to determine the effects of intravenous injections of three different chelating agents upon the development of rigor mortis and the subsequent quality factors of meat. Effects of post-mortem, intramuscular injections of calcium and magnesium on the development of rigor mortis were also studied.

EXPERIMENTAL

Experiment I

A total of 20 rabbits weighing between 1.5 and 3.0 kg were used. They were randomly divided into 4 groups (3 treated and 1 control). There were 4, 6, 5 and 5 rabbits in groups I through IV, respectively. Each group was treated as follows: group I—ethylene glycol-bis (B-aminoethyl ether)-N,N-tetraacetic acid (EGTA); group II—1,2-cyclohexanediaminetetraacetic acid (CDTA); group III—ethylenediaminetetraacetic acid (EDTA); and group IV—control.

Treatment consisted of an intravenous injection of a lethal dose (50–150 mg/kg body weight) of the respective chelating agent in isotonic saline solution. Control rabbits received an intravenous injection of 3 ml of isotonic saline solution per kg body weight, so that all animals were injected with approximately the same relative amount of solution.

All rabbits were bled within 5 min of injection. The intact semitendinosus muscle was removed immediately from both rear legs, and shortening was recorded on separate kymographs by means of an isotonic lever loaded with a weight approximately equal to that of the muscle. Muscles were held in a vertical position with two clamps over calcium-free Ringer's solution at 0°C. Nitrogen was bubbled through the solution to prevent the muscles from drying out.

The semitendinosus muscle from the left leg then received several micro-injections of a 0.1 *M* $CaCl_2$ solution to give a total of about 0.4 ml/kg of liveweight. After 6–8 hr, both semitendinosus muscles were removed from the kymograph and checked for extensibility. The muscle from the right leg was frozen in liquid nitrogen, powdered and stored at −30°C for subsequent ATP and pH determinations.

The posterior portion of the longissimus dorsi muscle from the right side was removed immediately after bleeding and frozen in liquid nitrogen. After chilling the carcass for 24 hr at 0°C, the posterior portion of the same muscle from the left side was removed and frozen in the same manner. Frozen muscle samples were powdered and stored at −30°C for subsequent pH and ATP determinations.

The anterior portion of the loin was removed after 24 hr, wrapped in aluminum foil, immediately frozen and stored at −30°C until removed for cooking and tenderness studies. Following thawing, the longissimus dorsi muscle removed from the left side of each loin, trimmed of external fat and weighed. All muscles were then placed on broiling trays and cooked in an electric oven at 175°C to an internal temperature of 75°C.

Samples were then weighed and cooled overnight at room temperature. Three cores 12.5 mm in diameter were removed parallel to the long axis of the muscle fibers and Warner-Bratzler shear values determined.

Experiment II

Eight rabbits weighing between 1.5 and 3.0 kg were used. Four were randomly selected for treatment with EDTA, while the other four were utilized as controls. All rabbits were treated and handled as in Experiment I.

The semitendinosus muscles were removed and shortening was recorded as in Experiment I. The semitendinosus muscles

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from the left legs of the #2 and #4 control rabbits received micro-injections of 0.01 *M* $CaCl_2$. The left semitendinosus muscle of the other six rabbits in this experiment received micro-injections of 0.1 *M* $CaCl_2$. The semitendinosus muscle from the right leg of the #1 and #3 control rabbits received micro-injections of 0.1 *M* $MgCl_2$ equivalent to the amount of 0.1 *M* $CaCl_2$, injected into the paired muscle of the same rabbit.

After 6–8 hr, both semitendinosus muscles were removed from the kymograph and checked for extensibility. The muscles from the #1 control rabbit were kept overnight for use in preparing photographs. The seven remaining semitendinosus muscles from the right legs and three semitendinosus muscles from the left leg of the last three rabbits treated with EDTA were then frozen in liquid nitrogen, powdered and stored at −30°C for pH and ATP determinations.

Experiment III

Twelve crossbred pigs (barrows and gilts) were randomly divided into two groups, one treated and one control with six animals in each group. The animals weighed between 92 and 110 kg. Treatment consisted of an intravenous injection of 250 to 310 ml of the tetrasodium salt of EDTA (0.1 *M*) in isotonic saline solution. Following injection, most of the pigs were in a relaxed state. They were then shackled, hoisted and bled within 5 min. Controls were electrically stunned and bled. In all cases, a treated and control pig were slaughtered simultaneously.

The semitendinosus muscle from the right ham was removed as rapidly as possible, about 10 min post-mortem. A sample was excised and frozen in liquid nitrogen. The remainder was hung in a vertical position at 0–3°C and shortening was recorded over a 24 hr period. After 24 hr, another sample of the same muscle was removed and frozen in liquid nitrogen. The frozen samples were then powdered and stored at −30°C until used for pH and ATP determinations.

After chilling the carcasses at 2–3°C for 24 hr, the hams were removed and trimmed. A 4 cm thick center slice was removed from each ham by cutting perpendicular to the femur, 2.5 cm posterior to the aitchbone toward the shank. Most of the external fat was removed. The weight of the center ham slice was recorded. Following this, the slice from the right ham of a treated (EDTA-injected) and control pig were placed on a broiling rack and cooked as in Experiment I.

After cooking, samples were weighed and cooled overnight at room temperature. Warner-Bratzler shear values were determined on 15 mm diameter cores from the biceps femoris, semimembranosus, rectus femoris and semitendinosus muscles of the left ham and from the biceps femoris, semimembranosus and rectus femoris muscles of the right ham.

Muscle pH was determined by homogenizing approximately 1 g of powdered muscle in 25 ml of 0.005 *M* sodium iodoacetate and values were read with a Corning, Model 12, pH meter. ATP was determined by the bioluminescent method described by Strehler et al. (1952) using an Aminco-Bowman Spectrophotofluorometer.

The data in Experiment I were analyzed for treatment differences by the least squares method. The multiple range test was used for comparing means (Duncan, 1955). Correlation coefficients were also calculated between some of the variables in Experiment I. The data in Experiment III were subjected to analysis of variance. Correlation coefficients were also calculated between all the variables for the treated group and the control group. Levels of significance were calculated as indicated by Snedecor (1956).

Table 1—Means and standard error of the means for shortening[1] of the semitendinosus muscles of the rabbit for the various treatments[2] (Experiment I).

Treatment	Right semitendinosus uninjected	Left semitendinosus injected with $CaCl_2$
Control	14.96[a] ± 1.46	55.71 ± 7.13
CDTA	9.00[b] ± 1.33	32.17 ± 6.53
EGTA	8.25[b] ± 2.31	36.25 ± 7.99
EDTA	5.53[b] ± 1.46	31.88 ± 7.13

[1] Shortening in mm as measured on the kymograph.

[2] Means with different superscripts differ significantly ($P < 0.05$).

Table 2—Effect of micro-injections of magnesium and calcium on shortening[1] of the semitendinosus muscles of the control rabbits (Experiment II).

	Right semitendinosus		Left semitendinosus	
Rabbit number	Uninjected	Injected with 0.1 *M* $MgCl_2$	Injected with 0.01 *M* $CaCl_2$	Injected with 0.1 *M* $CaCl_2$
1		14		100
2	19		25	
3		18		54
4	13		18	
Average shortening	16	16	21.5	77

[1] Shortening in mm as measured on the kymograph.

RESULTS & DISCUSSION

Effects of chelating agents, $CaCl_2$ and $MgCl_2$

Results from Experiment I show that intravenous injection of either EDTA, EGTA or CDTA significantly ($P < 0.05$) inhibited shortening of the semitendinosus muscle (Table 1). No significant difference was observed among the different treatments, but mean values suggest that CDTA (the poorest calcium chelator) decreased shortening the least.

Results are in agreement with an earlier report (Weiner et al., 1966) showing that EDTA inhibited the post-mortem shortening of the semitendinosus muscle of rabbits. Preliminary observations on the semitendinosus muscles from rabbits injected with lethal dosages (approximately 100 mg/kg body weight) of sodium oxalate also showed a marked reduction in shortening as compared to control rabbits (6.00 versus 14.96 mm, respectively).

Davies (1963) suggested that the calcium-actomyosin complex "develops tension during formation and, if the sum-total of the forces developed at any time by these ultramicro-contractions is sufficient to overcome the external load and the resistance of the series elastic elements, the actin filaments will be pulled along the myosin filaments into the A band and the muscle will undergo a quantal contraction." According to Maruyama (1962), the mechanism of chelating agents in an actomyosin system appears to be the removal of trace amounts of calcium.

Hasselbach (1957) showed that the calcium content of undissolved structural proteins from fresh muscle was diminished 50% by washing with EDTA, but that magnesium content was not altered. In accord with these results, addition of chelating agents in the present experiment appeared to form a complex with free calcium and thereby inhibited shortening.

In Experiment III, shortening—measured as the percentage of original length—was significantly less ($P < 0.05$) for pigs treated with EDTA (6.6%) than for control pigs (10.3%). These results support the premise that chelating agents complex enough free calcium to inhibit shortening.

Additional experiments were run to determine the effect of increased levels of calcium and magnesium upon shortening. The data in Table 1 show that post-mortem micro-injections of $CaCl_2$ into the paired semitendinosus muscle (Experiment I) resulted in extensive shortening. Even after injection of $CaCl_2$, the muscles from the treated animals shortened less than comparable muscles from untreated control animals. Results suggest that chelating agents may form a complex with calcium, thereby decreasing the shortening effect of the added calcium.

Values for shortening of the semitendinosus muscles from four control rabbits following micro-injections of $MgCl_2$ and different concentrations of $CaCl_2$ (Experiment II) are given in Table 2. The difference in shortening found on the addition of either 0.1 *M* $MgCl_2$ or 0.1 *M* $CaCl_2$ to the semitendinosus muscles from a rabbit are illustrated in Figure 1. Micro-injections of $MgCl_2$ had no significant effect on shortening during rigor mortis. These results are in agreement with those of Caldwell et al. (1963), who reported that injection of $MgCl_2$ into the muscle fibers did not produce contraction.

The data in Table 2 demonstrate that an increased concentration of $CaCl_2$ within the muscle resulted in a definite

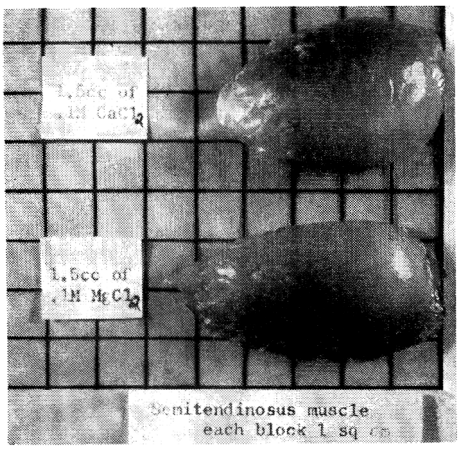

Fig. 1—The difference in shortening found following the addition of $MgCl_2$ and $CaCl_2$ in paired semitendinosus muscles (Experiment II).

Table 3—ATP values[1] for various muscles at different times post-mortem (Experiment I and II).

Muscle	Time post-mortem (hr)	Treatment[2] Control	CDTA	EGTA	EDTA
		Experiment I			
Longissimus dorsi	0	5.970 ± 1.040	6.872 ± 0.950	8.120 ± 1.160	3.690 ± 1.040
	24	0.142 ± 0.015	0.121 ± 0.015	0.105 ± 0.018	0.131 ± 0.015
Right semitendinosus	7	1.129 ± 0.360	1.716 ± 0.330	0.840 ± 0.400	0.753 ± 0.360
		Experiment II			
Longissimus dorsi	0	4.360	—	—	4.340
	24	0.126	—	—	0.108
Right semitendinosus	7	0.748	—	—	1.030
Left semitendinosus[3]	7	—	—	—	0.165

[1] μmoles ATP/g muscle.
[2] Means ± standard error.
[3] Injected with 0.1 *M* $CaCl_2$, 15 min post-mortem.

increase in shortening during the development of rigor mortis. Results agree with those of Niedergerke (1955) and Caldwell et al. (1963), who showed that calcium injected into the sarcoplasm of a muscle fiber caused local contraction at the point of injection. Results of the present experiments show that reduction of free calcium in muscle by chelating agents will reduce the amount of shortening during development of rigor mortis. On the other hand, increased levels of calcium resulted in a greater amount of shortening. However, $MgCl_2$ had no effect on shortening.

ATP

The ATP values at 0 hr post-mortem (Experiment I) ranged from 3.69 μM ATP/g in the longissimus dorsi muscle from the rabbits treated with EDTA to 8.12 μM ATP/g from rabbits treated with EGTA (Table 3). The data from Experiment II showed that there was no appreciable difference in ATP values of the longissimus dorsi muscle from EDTA treated and control rabbits at 0 hr post-mortem.

The variability observed in 0 hr ATP values is probably due to the fact that the rapid phase of ATP decomposition was under way. At 24 hr post-mortem, no consistent difference in ATP values was observed between the different groups in either Experiment I or II.

In Experiment III, no significant difference was obtained between ATP values of the semitendinosus muscle for the treated and control pigs at 0 or 24 hr post-mortem (Table 4). However, pigs treated with EDTA generally had higher ATP values, with 2.42 and 0.451 μM ATP/g at 0 and 24 hr post-mortem, respectively, as compared to 1.95 and 0.308 for the controls.

Trouble was encountered during injection of EDTA in the #2 and #5 pigs. Consequently, they never attained the relaxed state observed in the other treated pigs and were extremely excited at the time of slaughter. ATP levels for these two pigs were the lowest of any of the treated animals at both 0 and 24 hr post-mortem (Table 4). A comparison with other individual ATP values suggests that proper injection of EDTA resulted in higher values at 0 and 24 hr post-mortem (Experiment III).

The difference between the effect of chelators on the ATP values observed for treated rabbits and pigs may be explained by the fact that injection of chelating agents into rabbits resulted in extreme tetany with subsequent death. Treated pigs generally showed slight tetany for a short period of time and then became very relaxed.

On injecting $CaCl_2$ into paired muscles from rabbits treated with EDTA (Experiment II), there was a definite increase in the rate of ATP hydrolysis (Table 3).

Table 4—ATP values and treatment means for the semitendinosus muscles (Experiment III).

Animal number	ATP (μM/gm) 0 hr	24 hr
Pigs treated with EDTA		
1	3.079	0.939
2	2.003	0.146
3	3.268	0.597
4	2.250	0.223
5	0.859	0.118
6	3.081	0.613
Mean	2.42	0.451
Control pigs		
7	2.195	0.747
8	2.106	0.319
9	1.557	0.107
10	2.757	0.481
11	1.443	0.099
12	1.641	0.093
Mean	1.95	0.308

This effect may be explained by the results of Nauss et al. (1966) who demonstrated that presence of free calcium in the sarcoplasm resulted in cyclic formation and breakage of the cross-links between actin and myosin. This was accompanied by enzymatic hydrolysis of ATP due to actomyosin.

The same workers also pointed out that further breakdown of ATP occurs as a result of ATPase activity of the sarcoplasmic reticulum, which is involved in active uptake of free calcium from the sarcoplasm.

Muscle extensibility and its relationship to ATP levels

Rigor has been described as a transformation from the elastic system of actin and myosin to a rigid system consisting of actomyosin, which is joined together by cross bridges between actin and myosin (Nauss et al., 1966). Treatment had no consistent effect upon elasticity or rigidity of the uninjected semitendinosus muscles at 7 hr post-mortem in Experiment I (Table 5). However, paired left semitendinosus muscles from the rabbits in this experiment, which were injected with 0.1 *M* $CaCl_2$, were always rigid and inextensible at 7 hr post-mortem.

Similar results were obtained in Experiment II (Table 5). These results indicate that an increase of free calcium in the sarcoplasm shortens the delay phase during development of rigor mortis, and, thus, is responsible for the increased shortening following $CaCl_2$ injection.

Huxley (1960) pointed out that loss of ATP results in formation of fixed links between actin and myosin filaments as the muscle becomes rigid and inextensible. The rigid system maintains continuous tension in the absence of ATP breakdown (Nauss et al., 1966). In Experiment I, the level of ATP was compared between elastic and inextensible semitendinosus muscles at 7 hr post-mortem. It

Table 5—Effect of treatment on the elasticity or rigidity of muscle at 7 hr post-mortem.

Experiment	Animal treatment	Leg	Treatment of semitendinosus muscle	Condition of muscle[1]	
				Elastic	Rigid
I	Control	Right	Uninjected	2	3
		Left	Injected with 0.1 *M* $CaCl_2$	—	5
	CDTA	Right	Uninjected	3	3
		Left	Injected with 0.1 *M* $CaCl_2$	—	6
	EGTA	Right	Uninjected	2	2
		Left	Injected with 0.1 *M* $CaCl_2$	—	4
	EDTA	Right	Uninjected	3	2
		Left	Injected with 0.1 *M* $CaCl_2$	—	5
II	Control	Right	Uninjected	—	2
		Right	Injected with 0.1 *M* $MgCl_2$	1	1
		Left	Injected with 0.01 *M* $CaCl_2$	—	2
		Left	Injected with 0.1 *M* $CaCl_2$	—	2
	EDTA	Right	Uninjected	3	1
		Left	Injected with 0.1 *M* $CaCl_2$	—	4

[1] Number of semitendinosus muscles from each treatment possessing an elastic and extensible condition or a rigid and inextensible condition.

was found that muscles, which were elastic after 7 hr post-mortem, had significantly higher ($P < 0.01$) levels of ATP (1.78 μM/g) than those which were rigid and inextensible (0.527 μM/g).

Nauss et al. (1966) found that as the muscle began to shorten, the rate of ^{45}Ca efflux increased and ATP values fell. They suggested that the important chemical event determining the onset of physical changes in a muscle passing into rigor seemed to be the internal liberation of a sufficient amount of calcium to initiate interaction between actin and myosin. Results from the present experiments indicate that an intravenous injection of chelating agents will inhibit shortening, but does not always prevent formation of some links between actin and myosin, which will eventually result in an inextensible condition of the muscle following depletion of ATP.

Injection of $CaCl_2$ in these experiments increased shortening of the muscle and lowered the level of ATP at 7 hr post-mortem. Thus, results support the postulation (Marsh, 1966; Newbold, 1966; Nauss et al., 1966) that release of calcium by the sarcoplasmic reticulum is responsible for rapid ATP degradation and subsequent stiffening of muscle during the formation of rigor.

pH

In Experiment I, 0 hr post-mortem pH values for the longissimus dorsi muscle of rabbits treated with EDTA (6.31) were significantly lower ($P < 0.05$) than those recorded for the groups treated with EGTA (6.67) and the control group (6.57). No significant differences in pH values were observed between the other groups at 0 hr post-mortem. The ultimate (24 hr) pH values of the longissimus dorsi muscle did not differ significantly between treatments. In Experiment III, no significant difference in pH was observed in semitendinosus muscle between treated and untreated pigs at either 0 or 24 hr post-mortem.

Shear values

No significant difference in shear values was observed among different treatments in Experiment I (Table 6). However, it should be noted that shear values for longissimus dorsi muscle from rabbits treated with EDTA and CDTA were lower than controls. Data in Table 6 (Experiment III) also show that Warner-Bratzler shear values for hams from pigs treated with EDTA (8.65) were significantly lower ($P < 0.05$) than those for control hams (10.08). Locker (1960), Locker et al. (1963) and Herring et al. (1965b) demonstrated that beef muscles which shorten more during rigor showed a corresponding decrease in sarcomere length and tenderness.

Ramsey et al. (1940) and Gorden et al. (1964) have pointed out that the maximum tension developing in muscles depends on the amount of overlap of the filaments. In accord with these results, inhibition of shortening resulting from removal of some of the free calcium by EDTA in these experiments probably decreased formation of complexes between actin and myosin and seems to be associated with improvement in tenderness. This supports the postulation of Partmann (1963) suggesting that if the interaction between actin and myosin during rigor could be completely or partly impeded, tenderness may be improved.

Table 6—Means and standard error of means for shear values (Experiments I and III).

Treatment	Experiment I[1]	Experiment III[2]
Control	5.64 ± 0.60	10.080 ± 1.22
EDTA	3.43 ± 0.60	8.65* ± 1.22
CDTA	4.76 ± 0.55	
EGTA	5.93 ± 0.68	

[1] Shear force values expressed in pounds—measured by the Warner-Bratzler shear using 12.5 mm cores

[2] Shear force values expressed in pounds—measured by the Warner-Bratzler shear using 15 mm cores.

* $P < 0.05$.

Table 7—Simple correlation coefficients between pH and ATP for the longissimus dorsi muscle of rabbits in Experiment .

Variable	pH		ATP at
	0 hr	24 hr	C hr
24 hr pH	0.50*	—	
0 hr ATP	0.78**	0.45*	
24 hr ATP	0.07	0.22	−0.01

* $P < 0.05$.
** $P < 0.01$.

Interrelationships between some muscle properties

Simple correlation coefficients between pH and ATP levels of the longissimus dorsi muscle of rabbits (Experiment I) are presented in Table 7, whereas, similar relationships for the semitendinosus muscle of pigs (Experiment III) are given in Table 8. In both experiments, data at 0 hr post-mortem show highly significant relationships ($P < 0.01$) exist between ATP levels and pH values. Although this relationship was shown many times before (Bendall et al., 1947, 1949; Bate-Smith, 1948; Briskey, 1964), the present study demonstrates that association between ATP levels and pH values was not altered by injection of either calcium chelators or $CaCl_2$.

The fact that it was possible to inhibit shortening by injecting calcium chelators and to enhance shortening and ATP hydrolysis by micro-injections of $CaCl_2$ further verifies the role of calcium in contraction and relaxation as summarized by

Table 8—Simple correlation coefficients between pH and ATP for the semitendinosus muscle from pigs treated with EDTA and their controls in Experiment III.

Item-Time	pH at 0 hr		pH at 24 hr		ATP at 0 hr	
	EDTA	Control	EDTA	Control	EDTA	Control
pH at 24 hr	0.33	0.76g				
ATP at 0 hr	0.91**	0.92**	−0.04	0.83*		
ATP at 24 hr	0.61	0.47	−0.02	0.70	0.78*	0.76*

* $P < 0.05$.
** $P < 0.01$.

Table 9—Simple correlation coefficients between pH, ATP, muscle shortening, cooking losses and tenderness for rabbit muscle (Experiment I).

	pH		ATP	
	0 hr	24 hr	0 hr	24 hr
Shortening right S.T.[1]	0.24	0.34	0.16	0.06
Shortening left S.T.[2]	0.34	0.21	0.05	−0.05
Cooking losses	−0.72**	−0.45*	−0.64**	0.14
Shear values	0.39	0.16	0.36	−0.19

[1] Right semitendinosus muscle—uninjected.
[2] Left semitendinosus muscle—injected with 0.1 *M* $CaCl_2$.
* $P < 0.05$.
** $P < 0.01$.

Table 10—Simple correlation coefficients between pH, ATP, muscle shortening, cooking losses and tenderness for control pigs and pigs treated with EDTA (Experiment III).

	pH				ATP			
	0 hr		24 hr		0 hr		24 hr	
Treatment	EDTA	Control	EDTA	Control	EDTA	Control	EDTA	Control
Muscle shortening[1]	−0.23	0.12	0.19	−0.36	−0.36	0.34	−0.83*	0.64
Cooking losses	−0.37	−0.97**	−0.10	−0.81*	−0.57	−0.94**	−0.49	−0.60
Shear values right ham	−0.50	0.04	−0.06	0.31	−0.68	0.06	−0.81*	0.14
Shear values left ham	−0.56	−0.06	−0.12	0.07	−0.78*	−0.15	−0.78*	−0.32

[1] Muscle shortening as a percent of original length of the semitendinosus muscle.
* $P < 0.05$.
** $P < 0.01$.

Marsh (1966). On the basis of the effects of injection of calcium chelators and $CaCl_2$, it is postulated that a variable amount of calcium is released from the sarcoplasmic reticulum, depending upon the magnitude of the nervous stimuli occurring as a consequence of stunning and slaughtering. The amount of calcium released may then be the key in determining rate of ATP degradation, and, thus, control glycolysis.

Simple correlation coefficients for ATP and pH values in relationship to muscle shortening, cooking losses and shear values for rabbit (Experiment I) and pig muscles (Experiment III) are presented in Tables 9 and 10, respectively. The cooking losses for rabbit muscle were negatively but significantly ($P < 0.01$) related to pH and ATP values at slaughter, and to pH at 24 hr post-mortem ($P < 0.05$). Similarly, cooking losses for control pigs were also negatively and significantly related to pH and ATP values at 0 hr ($P < 0.01$) and to pH values at 24 hr post-mortem ($P < 0.05$). These results would suggest that muscle having a slower rate of glycolysis and ATP degradation following slaughter retains a greater proportion of moisture during cooking, presumably due to increased hydration of the muscle proteins.

Data in Table 10 also show a negative but significant association between ATP levels and shear values for both the right and left hams from EDTA-treated pigs at 24 hr post-mortem. At 0 hr post-mortem, a similar significant ($P < 0.05$) relationship was also found between ATP values and shear values for the left ham, whereas, the same relationship although not significant for the right ham approached the 5 percent level of probability.

There was also a negative but significant relationship between muscle shortening and 24 hr ATP levels for the EDTA treatment. These results suggest an interrelationship between tenderness (shear values), ATP levels and muscle shortening. Earlier studies on shortening (Locker, 1960; Locker et al., 1963; Herring et al., 1965a, b) would suggest that variation in the state of contracture may be responsible for at least part of the difference in tenderness.

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Fractionation of Muscle Proteins of Fresh Water Fish and Changes during Iced Storage

SUMMARY—Fractionation of muscle proteins of four varieties of fresh water fish after storage in ice was carried out by serial dilution of a buffer extract of ionic strength 0.55. Precipitation of actomyosin was complete at an ionic strength of 0.175 and fibrillar fraction at 0.05. In Ophicephalus sp. *the initial solubility of proteins was 91–93%. The solubility fell to 82% by the 5th day and increased on further storage up to 13 days. Further storage indicated a fall in solubility. The fall in solubility on the 5th day coincided with the highest level of actomyosin and maximum rigor rigidity of the round fish. Actin solubility in buffer showed the same pattern as solubility of total proteins. The quantity of actin not reconvertible into f-actin showed an increase during storage. Viscosity of buffer extracts showed an increase during development of rigor and decrease on further storage as reflected by level of preformed actomyosin.*

INTRODUCTION

DURING our investigations on the possible relationship between solubility of proteins of fresh water fish stored in ice and their texture (Moorjani et al., 1962), certain subtle changes in total solubility and fraction precipitated at ionic strength 0.225 were noticed (Baliga et al., 1962a) particularly in the species *Ophicephalus*. No change in sarcoplasmic and non-protein nitrogen fraction was noticed (Baliga et al., 1962b). Partitioning at ionic strength of 0.225 was based on the work of Weinberg et al. (1960), Greenstein et al. (1940) and Bailey (1954). The above work was reported for fractionation of muscle of chicken and mammalian species. Myofibrillar proteins of fish do not necessarily behave like chicken and mammalian muscle fractions (Connell, 1961).

In view of the above, characteristics of precipitation at different serial dilutions of buffer extract of ionic strength 0.55 have been examined for four species of fresh water fish available in this region during iced storage.

EXPERIMENTAL

THE FRESH WATER fish *Barbus dubious, Labeo sp., Cirrhina fulungee* were caught by gill net in the neighboring Cauvery river. They were iced immediately after catching and transported to the laboratory within 2 hr in the iced condition. They were packed with substantial quantities of crushed ice in insulated boxes and stored in a refrigerated room at 2–3°C.

Ophicephalus, a double breather, was collected from nearby tanks using a cast net, transported to the laboratory in water and rested in fresh water. Active individuals were dispatched by a blow on the head and packed in ice as before. In this fish it is possible to avoid or keep to a minimum struggle before death, whereas in the fish caught using a gill net the duration and extent of struggle in the net is not known.

Extraction in potassium chloride phosphate buffer and estimation of total nitrogen, non-protein nitrogen (NPN) and soluble protein nitrogen extracted were as in a previous paper (Moorjani et al., 1962). The potassium chloride phosphate buffer extract has an ionic strength (r/2) of 0.55 and this was diluted serially at steps of decreasing ionic strength of 0.025 to a low ionic strength of 0.05. During the dilution temperature was maintained at 4°C or less. After each dilution below $r/2 = 0.3$, the precipitate was centrifuged down and protein nitrogen determined.

In the case of *Ophicephalus sp.* the solubility was studied throughout the acceptable storage period of 15 days. The viscosity of the buffer extracts at r/2 of 0.05, 0.175 and 0.55 was measured using an Ostwald type viscometer. The time taken to empty the bulb between the two calibration lines was measured using a stop watch of 0.1 sec accuracy.

The extracts were equilibrated to 30°C in the viscometer which was suspended in water in a bell jar. The temperature of the water was maintained at 30 ± 0.1°C using a centrifugal circulating pump and 400 W immersion heater controlled by a contact thermometer through a relay. The timing of the flow through the viscometer is considerably facilitated by illuminating the bell jar from behind and viewing the viscometer through the side of the bell jar. The water acts as a lense magnifying the calibration marks and maniscus of the extract in the viscometer.

Total actin was determined by drying an aliquot of the muscle using acetone washing repeatedly, drying in a desiccator over concentrated sulfuric acid and extracting the G-actin in water from the dry residue. Similarly soluble actin was determined in the potassium chloride phosphate buffer of $r/2 = 0.55$. To the aqueous solution of G-actin, solid potassium chloride was added to 0.55 M. The solution was stored in the refrigerator overnight and the conversion of G-actin to F-actin was measured by diluting to 0.05 M and estimating protein nitrogen in the precipitate.

The results of four series of iced storage studies are reported here. The first was used entirely for establishing the precipitation characteristics of muscle protein fractions. The other three were used for estimations of the protein fractions and viscosity. At each stage of storage, one fish was withdrawn, both the fillets were separated and composited separately. Identity was maintained throughout the extraction, dilution and other estimations.

Buffer extracts for viscosity measurement were prepared using buffers at $r/2 = 0.05$, 0.175 and 0.55 using 95 ml and 5 ± 0.05 g muscle mince. The protein nitrogen was estimated in solutions obtained by dilution of the extracts at $r/2 = 0.55$ as well as the extracts at $r/2 = 0.175$ and 0.05. All extractions and estimations were carried out in duplicate.

RESULTS & DISCUSSION

THE RESULTS of serial dilution of the potassium chloride phosphate buffer extract of the muscle mince of the four different varieties of fresh water fish after 4 days of storage in ice are shown in Figure 1. Dilution below $r/2 = 0.3$ started slight precipitation up to $r/2 = 0.25$ and sharp precipitation up to 0.2 in the case of *Labeo, Cirrhina fulungee and Barbus dubius* and 0.175 in the case of *Ophicephalus*. Further dilution to $r/2 = 0.15$ produced no precipitation in the case of *Labeo* and *Cirrhina fulungee* and slight precipitation in the case of *Ophicephalus* and *Barbus dubius*. Dilution to $r/2 = 0.05$ produced a sharp precipitation again. Lowering of ionic strength to below 0.05 was achieved by dialysis against buffers of the desired strength. There was practically no precipitate up to $r/2 = 0.01$. Dialysis against distilled water produced a precipitation of 11% of total protein nitrogen in *Ophicephalus sp.*

The slope of the serial dilution curve between 0.25 and 0.20 or 0.175 and between 0.15 and 0.05 varied during storage. The plateau was always obtained between ionic strengths of 0.2 and 0.15 in *Labeo* and *Cirrhina fulungee*, 0.2 and 0.175 in *Barbus dubius* and 0.175 and 0.15 in *Ophicephalus*. The slope of the curves between 0.25 and 0.20 or 0.175 was sharper than the slope between 0.15 and 0.05 during the 4th and 5th days of storage. During the first few days and after the 5th day the slope between 0.15

Table 1—Changes in characteristic muscle fractions during storage of Ophicephalus sp. in ice (as % of total protein nitrogen).

Storage (days)	Salt soluble protein nitrogen	Non-protein nitrogen[1]	Actomyosin nitrogen	Sarcoplasmic protein nitrogen	Total actin nitrogen	Soluble actin nitrogen	Soluble active actin	Soluble inactive actin
1	90.7 ± 2.5	10.2 ± 0.3	1.95 ± 0.1	26.5 ± 4.7	30.8 ± 2.5	19.2 ± 1.2	19.3 ± 0.2	—
4	93.1 ± 2.2	9.6 ± 0.1	1.75 ± 0.1	28.6 ± 4.3	31.8 ± 2.6	21.7 ± 1.2	21.7 ± 1.0	—
5	81.9 ± 3.1	9.8 ± 0.1	26.75 ± 1.0	29.5 ± 4.7	30.8 ± 1.0	15.0 ± 3.1	13.7 ± 0.3	1.25
6	91.1 ± 2.7	10.1 ± 0.1	6.33 ± 1.5	30.0 ± 5.0	34.5 ± 2.5	18.8 ± 1.8	14.2 ± 0.9	4.60
9	91.4 ± 0.8	9.7 ± 0.2	5.70 ± 1.3	29.2 ± 6.6	29.4 ± 2.0	17.2 ± 0.6	12.2 ± 0.3	5.00
11	91.5 ± 2.1	10.1 ± 0.2	3.30 ± 0.6	28.9 ± 5.1	30.2 ± 2.4	19.9 ± 1.6	16.0 ± 0.7	3.90
13	93.8 ± 1.9	9.5 ± 0.3	3.13 ± 1.0	32.0 ± 4.7	30.8 ± 1.7	28.8 ± 1.5	20.8 ± 0.6	8.00
15	88.4 ± 1.6	9.5 ± 0.3	3.05 ± 1.4	31.1 ± 3.8	32.2 ± 1.1	20.9 ± 0.5	16.7 ± 0.2	4.20

[1] Non-protein nitrogen expressed as % of total nitrogen.

and 0.05 was sharper.

The serial dilution of the buffer extract at ionic strength of 0.55 has shown that precipitation of actomyosin of the fresh water fish used here is not complete at r/2 = 0.225 which has been found suitable in the case of mammalian muscle and chicken muscle. Dilution to 0.175 was necessary to complete the precipitation. However, for separation of the myofibrillar fraction from the sarcoplasmic, dilution to r/2 = 0.05 was suitable as in the case of mammalian and chicken muscles.

Table 1 shows the changes in solubility in buffer of r/2 = 0.55, N.P.N., the actomyosin precipitated by dilution to r/2 = 0.175, sarcoplasmic fraction remaining soluble after dilution to r/2 = 0.05, total actin in the muscle mince and soluble total and active actin and soluble inactive actin in the buffer extract. The soluble fraction was 91–93% initially and up to the 4th day of storage. It decreased to 82% by the 5th day. The solubility increased again to the initial value by the 13th day and another fall in solubility was observed on the 15th day. These solubility changes were the same as observed in earlier studies (Baliga et al., 1962a), and showed a very low correlation coefficient (0.15) with length of storage. Storage changes beyond this period were not followed because the concentration of microorganisms by plate count on nutrient agar was more than a million per g of muscle and an increase in protein solubility observed beyond this stage could be a reflection of the microbial action.

N.P.N. showed a negative correlation coefficient (−0.56). Whether this was due to marginal diffusion of water into, or the N.P.N. out of the muscle has not been examined. Such a process has been demonstrated in living fish and is responsible for the elimination of ammonia from protein catabolism in fresh water fish which are ammoniotellic (Baldwin, 1959).

Preformed actomyosin in the extract precipitated on dilution to r/2 = 0.175 was 2% up to the 4th day of storage and showed an increase to 30% on the 5th day. This increase in actomyosin on the 5th day coincided with the 1st fall in solubility in buffer of r/2 = 0.55. At this stage of storage the stiffness due to rigor was marked in the round fish. The quantity of actomyosin decreased on further storage to about 3%. These changes bore a low negative correlation coefficient (−0.22) to storage.

The fraction left in solution at r/2 = 0.05 was taken to represent sarcoplasmic fraction. There was no change in sarcoplasmic fraction during iced storage. During the later stages of storage there appeared a slight increase. On the whole, the values were slightly higher than those found earlier using potassium chloride-citrate buffer (Baliga et al., 1962b). The conditions reported suitable for complete removal of myosin in mullet are an ionic strength of 0.05 and a pH of 6.5 (Hamoir et al., 1960).

Total actin in the muscle ranged from 29 to 34% of the total protein. The amount of actin coming into solution in the buffer of r/2 = 0.55 has a pattern similar to the total soluble protein nitrogen during storage. It was the lowest during the 5th day which was also the period showing the maximum level of preformed actomyosin and the lowest total solubility. Soluble actin nitrogen showed a positive correlation coefficient (0.48) to storage.

Actin exists in fresh muscle as active or fibrous actin. From acetone dried muscle, actin can be extracted in water because it is in the globular form. If to this solution of G-actin, solid KCl is added to give an ionic strength necessary to dissolve fibrillar proteins, the G-actin is converted to F-actin (Connell, 1960).

In a previous study (Baliga et al., 1962a) an increase in solubility in buffer containing pyrophosphate was observed. This was ascribed to possible conversion of F-actin to G-actin, which would dissolve in the buffer even in the presence of pyrophosphates. Such a possibility was tested in these experiments by estimating the proportion of soluble actin that can be converted back into F-actin in the presence of 0.55 *M* KCl.

Almost all the soluble actin was reconvertible into F-actin up to the 4th day of storage. Further storage increased the amount of actin that was not reconvertible into F-actin with a negative correlation coefficient (−0.55). The possibility of this phenomenon vitiating the estimate of sarcoplasmic fraction obtained by diluting the buffer extract of r/2 = 0.55 to 0.05 is indicated by the slight increases in sarcoplasmic fraction during the later stages of the storage period. Sarcoplasmic fraction showed a positive correlation coefficient (0.81) during storage.

A number of reports are available in the literature on the correlation between protein solubility, sarcomere length, hydration, biochemical properties and meat quality factors like tenderness and juiciness (Gothard et al., 1966, Herring et al., 1965a and b, Marsh et al., 1966).

Recently Cook (1967) reported that there was no difference in protein solubility between pre-rigor and post-rigor stretched muscle and the difference was significant when the muscle was allowed to shorten freely by suspending. In the present study the fish were stored as round fish and as such the muscle can be said to exist in a stretched condition. The difference in the behavior of fish muscle reported here and beef muscle (Cook, 1967) may be due to the species characteristics.

Figure 2 shows the correlation between soluble protein nitrogen and the viscosity of the buffer extracts at r/2 = 0.05, 0.175 and 0.55 during storage of *Ophicephalus* in ice. The solubility curves at r/2 = 0.55 and 0.175 very nearly coincide with each other at the initial and final stages and show minimums at 6 day storage, when there is marked difference in amount of protein dissolved due to peak concentration of preformed actomyosin.

The viscosity curves of extracts at r/2 = 0.55 and 0.175 show increased viscosity without increased concentration of protein during the initial few days of storage. This is a reflection of building up of the actomyosin complex. The fall in the viscosity and solubility curves of the extract at r/2 = 0.175 coincide with each other, while the curves of the extract at 0.55 do not indicate such a relationship. This is a manifestation of the capacity of the buffer of the higher ionic strength to hold larger aggregates in solution and also increasing solubility beyond the 6th day of storage.

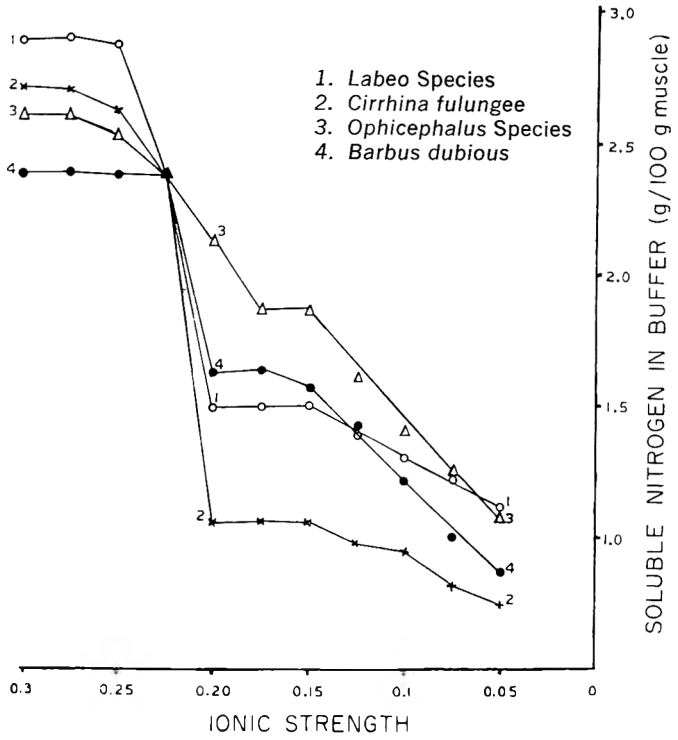

Fig. 1—Serial dilution of potassium chloride-phosphate buffer extract and characteristics of actomyosin precipitation.

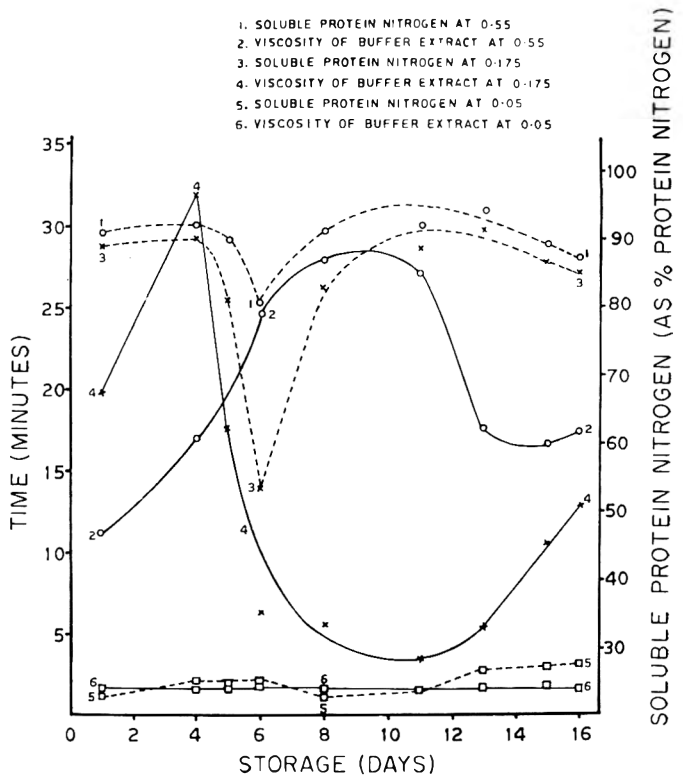

Fig. 2—Correlation between soluble protein nitrogen and viscosity of potassium chloride-phosphate buffer at r/2 = 0.55, 0.175 and 0.05 during storage of Ophicephalus in ice.

The fall in viscosity beyond the 9th day of storage is a manifestation of the resolution of the large aggregates built up during the development of rigor mortis. At r/2 = 0.175 the solubility increases after the 6th day, while viscosity falls up to the 11th day and then increases again. The fall in viscosity is due to resolution of rigor mortis. The increase after the 11th day of the buffer extract probably indicates a tendency for aggregation leading to denaturation and loss of solubility as shown by the solubility curve.

There is good agreement between actomyosin levels at the different storage durations arrived at by diluting a buffer extract of r/2 = 0.55 to 0.175 or by direct extraction of the muscle mince by buffer of ionic strength 0.175. A similar agreement is obtained in the case of sarcoplasmic fraction by dilution of an extract of r/2 = 0.55 to 0.05 or direct extraction at 0.05.

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Environmental Influence on High Energy Phosphate Metabolites in Porcine Muscle

SUMMARY—Adenosine triphosphate, creatine phosphate and glucose-6-phosphate levels in the muscle of Poland China pigs reared under controlled temperature conditions were studied. Longissimus dorsi muscle from animals reared in 32°C environments had higher initial levels of adenosine triphosphate and creatine phosphate ($P < 0.05$) and lower levels of glucose-6-phosphate than that from animals reared at 21°C. Muscle from the latter group of animals underwent more rapid post-mortem glycolysis (measured as muscle pH) and more rapid increase in light reflectance. Results indicate that accelerated post-mortem glycolysis in muscle from pigs grown in specified environments may be a reflection of environmental effects upon the ability of porcine muscle to store and maintain high levels of high energy phosphate compounds.

INTRODUCTION

A NUMBER of recent studies have demonstrated that growing environment, particularly temperature and humidity, affects the ultimate quality attributes of porcine muscle. Thomas et al. (1966) have shown that alternating temperatures at relatively low humidity levels result in paler colored, more exudative longissimus dorsi muscle compared to constant temperature environments. High relative humidity during growth tended to improve muscle color and structure (Thomas et al., 1966; Addis et al., 1967).

It has subsequently been demonstrated that alternating temperature environments induce more rapid post-mortem glycolysis, higher percentage of light reflectance and increased light to dark fiber ratios in the longissimus dorsi muscle of pigs as compared to constant temperature environments, (Howe et al., 1968), but only at moderate relative humidities. Low humidity contributed to more rapid muscle pH decline at either constant or alternating temperatures.

Bendall (1963) has commented on the important relationship between muscle concentrations of high energy phosphate compounds at time of slaughter and the rate at which post-mortem glycolysis and onset of rigor mortis occurs in the muscle. Low levels or depletion of these compounds immediately prior to sacrifice results in accelerated rates of post-mortem reactions.

Lister et al. (1967) have also demonstrated that levels of adenosine triphosphate (ATP) and creatine phosphate (CP) and their interrelation are key determinants of the rate of pH decline in muscle post-mortem. These authors hypothesized that the longissimus dorsi muscle of certain animals is unable to retain large quantities of high energy phosphate compounds and thus is predisposed to rapid pH decline after death while the temperature of the muscle is still high.

In view of the previously cited influences of growing environment on muscle quality and post-mortem pH decline and of the relation of high energy phosphate levels to post-mortem glycolytic rate, this study was conducted to determine the influence of growing environment on phosphate compound levels in porcine skeletal muscle and to evaluate the relation between growing environment, high energy phosphate compounds, muscle pH decline and light reflectance post-mortem.

EXPERIMENTAL

FORTY Poland China barrows which had been reared in environmental chambers were utilized in this study. In Experiment 1, 16 weanling pigs were allotted equally and at random to one of two constant environmental temperatures, either 32°C or 21°C. Temperature was closely controlled (±1°C); however, chamber humidity varied with the moisture content of the outside air.

A constant record was maintained of the relative humidity in the environmental chambers. The first experiment was conducted during the summer months. Therefore the average relative humidity was high, 41% at 32°C and 79% at 21°C (Fig. 1).

Experiment 2 was conducted with 24 weanling Poland China barrows equally and randomly allotted to the same constant temperature environments. Since this experiment was conducted during the fall and winter months, average relative humidities

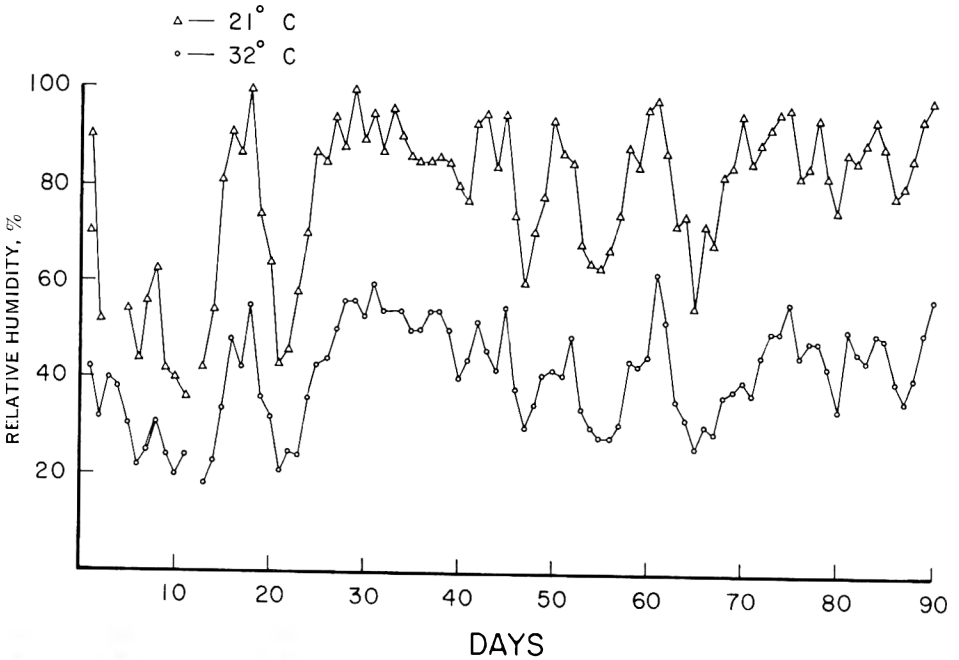

Fig. 1—Daily per cent relative humidity for Experiment 1.

[a] Present address: Wilson and Company, Chicago, Illinois.
[b] Present address: Department of Foods and Nutrition, Purdue University, Lafayette, Indiana.

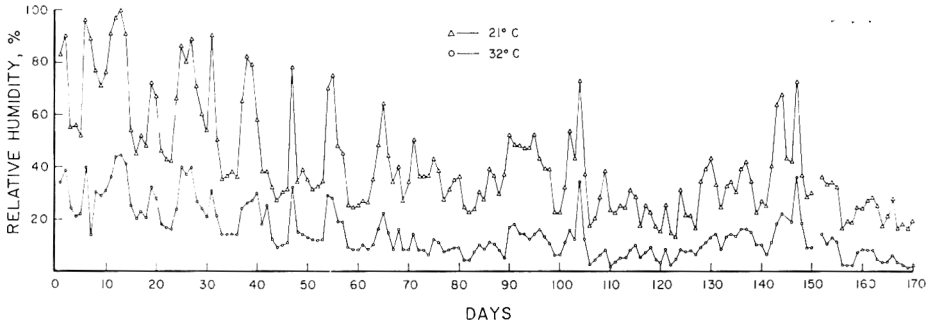

Fig. 2—Daily per cent relative humidity for Experiment 2.

were appreciably lower than in the first experiment and were 15% at 32°C and 41% at 21°C (Fig. 2). It should be noted, however, that the relative humidity during the latter half of the experiment was substantially lower than the average value.

Animals subjected to various environmental conditions grew at different rates. All the pigs in these experiments were slaughtered when the larger animals reached approximately 100 kg body weight. They were stunned with a captive bolt pistol and exsanguinated in their respective chambers.

Samples of the longissimus dorsi muscle were excised and frozen in liquid nitrogen within 2 min of sacrifice. Muscle samples were subsequently pulverized in a Waring blendor in the frozen state and stored at −15°C for future analyses. In no case were the samples stored for more than 48 hr before being analyzed.

Rate of post-mortem pH decline was determined on the cut surface of the longissimus dorsi muscle using a combination probe electrode. Color reflectance changes in the longissimus dorsi during the first 45 min post-exsanguination were measured at 525 mμ with a Spectronic 20 colorimeter equipped with reflectance attachment.

Frozen, powdered muscle was extracted with 6 to 8 volumes of 6% perchloric acid by homogenization in a glass homogenizer. Temperature was maintained at 4°C during extraction. The resulting extract was neutralized with K_2CO_3 and then analyzed for glucose-6-phosphate (G6P), ATP, and CP using the procedures described by Lamprecht et al. (1965a,b). Glucose-6-phosphate dehydrogenase, hexokinase, creatine kinase, triphosphopyridine nucleotide, and adenosine diphosphate were purchased from Sigma Chemical Co. Results are expressed as μmoles/g of fresh muscle.

RESULTS & DISCUSSION

POST-MORTEM muscle pH decline and light reflectance data for both experiments are shown in Figures 3 and 4. There was little difference in initial muscle pH among the various treatment groups. Initial values ranged from 6.31 ± 0.03 for 32°C, 41% relative humidity to 6.20 ± 0.06 for 21°C, 41% relative humidity. However, in both experiments, muscle from animals grown in 21°C environments had more rapid rates of pH fall than muscle from animals grown at 32°C although the differences in rate within each experiment were not statistically significant. Analysis of variance of the combined data indicated significant temperature effects upon rate of post-mortem glycolysis ($P < 0.05$).

Muscle from animals grown in 21°C environments was initially darker at 2 min post-exsanguination (Fig. 4). These muscles underwent a more rapid glycolysis however, and at 30 and 45 min post-mortem, muscle from pigs reared at 21°C had higher light reflectance values.

This relation between rate of pH decline and muscle color agrees with previous reports in the literature (Wismer-Pedersen, 1959; Briskey et al., 1961; Sayre et al., 1963). Howe et al. (1968) have additionally demonstrated that more rapid rates of post-mortem muscle pH decline can be induced in Poland China pigs reared in alternating temperature as compared to constant temperature rearing environments. The more rapid glycolysis was associated with higher light reflectance values of the longissimus dorsi muscle.

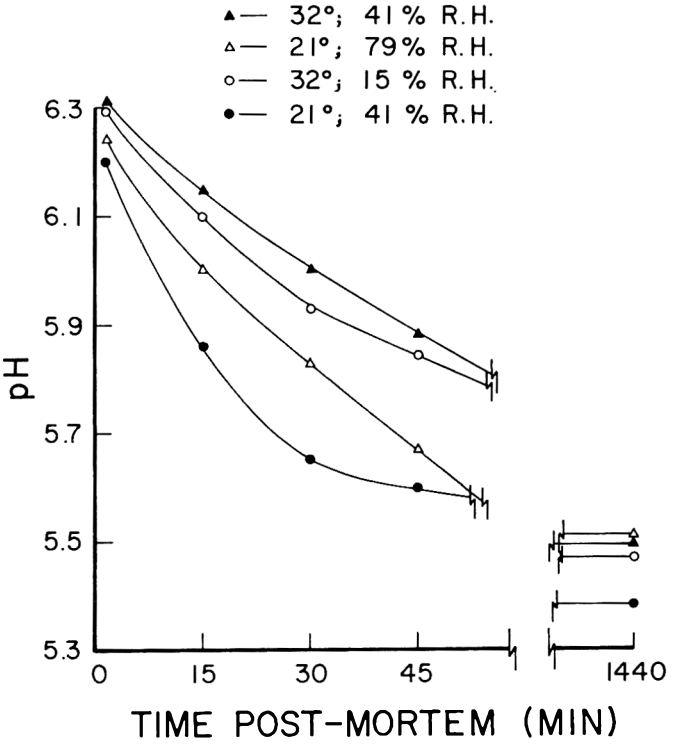

Fig. 3—Post-mortem pH decline for animals grown in 32°C and 21°C constant temperature environments.

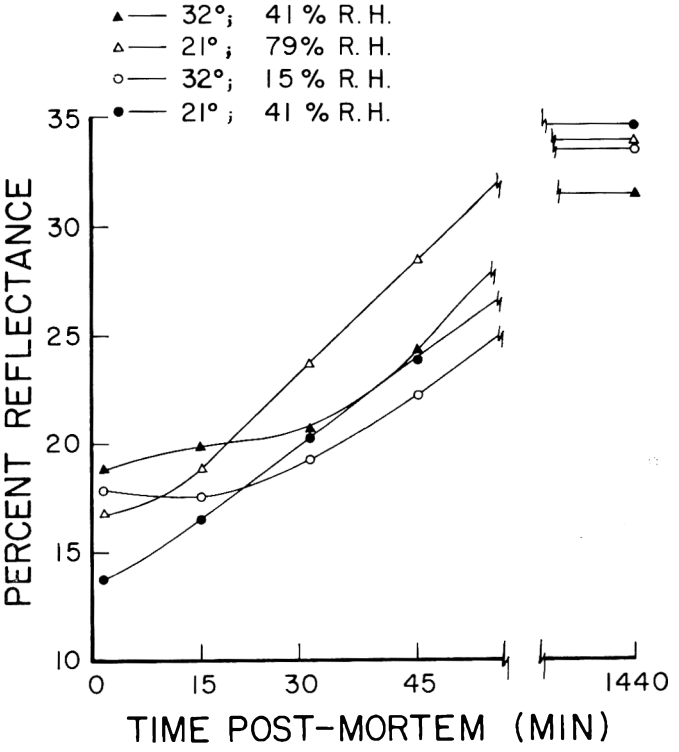

Fig. 4—Post-mortem increase in light reflectance of longissimus dorsi muscle.

Table 1—Initial post-mortem muscle concentrations of glucose-6-phosphate, adenosine triphosphate and creatine phosphate.

Temperature °C	Average relative humidity %	Glucose-6 phosphate[1,2]	Adenosine triphosphate[1,2]	Creatine phosphate[1,2]
		Experiment 1		
32	41	9.71 ± 0.65	7.16 ± 0.31 *	3.88 ± 0.97 *
21	79	10.00 ± 0.49	5.73 ± 0.42	1.67 ± 0.18
		Experiment 2		
32	15	9.39 ± 0.71 *	7.00 ± 0.53	3.56 ± 0.69
21	41	12.15 ± 0.51	6.51 ± 0.42	2.89 ± 0.49

[1] Expressed as μmoles/g of fresh tissue.
[2] Values are given as the mean ± the standard error.
* Means differ significantly ($P < 0.05$).

Table 2—Simple correlation coefficients between glucose-6-phosphate, adenosine triphosphate, creatine phosphate and longissimus Dorsi Muscle pH and Light reflectance.

	G6P[1]	ATP[1]	CP[1]
pH-30 min	−0.34*	0.42**	0.57**
pH-45 min	−0.25	0.46**	0.67**
Ref-30 min[2]	0.07	−0.29*	−0.43**
Ref-45 min	0.07	−0.26	−0.51**
G6P		−0.14	−0.09
ATP			0.68**

[1] G6P—glucose-6-phosphate; ATP—adenosine triphosphate; CP—creatine phosphate.
[2] Ref—Light reflectance.
* $P < 0.05$.
** $P < 0.01$.

Initial longissimus dorsi muscle levels of G6P, ATP and CP are shown in Table 1. Mean G6P concentration ranged from 9.39 to 12.15 μmoles/g of fresh muscle. In general, muscles which had the more rapid rates of anaerobic glycolysis had higher contents of G6P as has been observed by Kastenschmidt et al. (1968) in muscle which differed greatly in glycolytic rate. In both experiments, pigs reared in 21°C environments had higher mean levels of G6P. The effect of temperature was significant ($P < 0.05$) in Experiment 2.

Initial ATP levels ranged from 7.16 μmoles/g for 32°C to 5.73 μmoles/g for pigs reared at 21°C in Experiment 1. In both experiments, muscle from animals reared at constant 32°C temperatures had higher ATP levels immediately after sacrifice than muscle from animals reared at 21°C. The difference was significant ($P < 0.05$) in Experiment 1.

Mean CP concentrations of longissimus dorsi muscle (Table 1) ranged from 3.88 to 1.67 μmoles/g of fresh muscle; however, wider variation was observed within individual treatment groups. As in the case of ATP, muscle from animals grown at 32°C had higher CP levels at death than muscle from animals reared at 21°C. The effect of rearing environment temperature was significant ($P < 0.05$) in Experiment 1.

The concentrations of ATP observed in the muscle at 2 min post-mortem are slightly higher than values reported recently by others. Bodwell et al. (1966) found 5.6 μmoles/g present in porcine longissimus dorsi muscle at 13 min post-mortem.

Kastenschmidt et al. (1968) found approximately 5.6 μmoles/g in muscle which had a slow post-mortem glycolysis (pH > 6.0; 60 min post-mortem) and 3.7 μmoles/g in muscle with rapid glycolysis (pH < 5.5; 30 min post-mortem) at 2 to 3 min after exsanguination. They also reported 6 to 9 μmoles of CP/g for muscle with slow glycolysis and 3 μmoles/g for rapidly glycolyzing muscle.

Mean values for CP (Table 1) compare favorably with the rapid glycolysis muscle, even though rates of pH fall were not as rapid. However, several animals exhibited extremely slow pH decline and had CP levels comparable to those reported by Kastenschmidt et al. (1968).

There may have been some difference in the rate of ATP and CP turnover in the muscle during the period immediately prior to exsanguination and before the muscle sample was excised and frozen which would account for some of the observations reported. The data are not sufficient to determine whether or not turnover rates varied between treatments. However, there is no reason to expect varying turnover rates immediately prior to sacrifice since all animals were well rested and were then stunned under identical conditions in their respective chambers.

Table 2 contains simple correlation coefficients between initial muscle concentrations of G6P, ATP and CP and various measures of the rate of post-mortem glycolysis and muscle quality change. Of the three phosphate compounds assayed, G6P was least associated with the rate of post-mortem reaction as reflected in muscle pH. CP was most highly associated with the rate of pH decline and also with light reflectance at 30 and 45 min post-mortem.

The correlation coefficients emphasize the important relation between the concentration of high energy phosphate compounds in the muscle at sacrifice and the rate at which post-mortem glycolytic reaction proceeds in porcine muscle. The high positive correlation between concentrations of ATP and CP indicates the high interdependence of the two compounds.

It has been adequately demonstrated, as cited by Briskey (1964), that the rate of post-mortem glycolysis and muscle pH decline is a central causative factor in the development of pale, soft, exudative porcine musculature. Lister et al. (1967) have shown that levels of ATP and CP and their interrelation are key determinants of the rate of pH decline. They have also presented evidence that certain animals are unable to maintain large stores of these high energy compounds and are thus more susceptible to rapid pH decline after death while the temperature of the muscle is still high.

Since environment is shown to influence the concentration of ATP and CP initially present in the muscle at death, it can be postulated that environmental effects on rate of muscle pH fall and muscle quality are in turn a reflection of environmental effects on high energy compounds in the muscle.

Other authors have reported that environmental temperature and humidity both influence muscle characteristics (Thomas et al., 1966; Addis et al., 1967; Howe et al., 1968). As noted earlier, it was not possible to regulate humidity in the present experiment therefore no definite conclusions can be drawn about humidity effects upon muscle phosphate compounds. It is correct to state, however, that environment influences the level of high energy phosphate compounds and their rate of post-mortem degradation as shown by pH changes. The exact cause of this effect could be either temperature, or humidity or a combination of both.

Kastenschmidt et al. (1968) have demonstrated that muscles which undergo rapid pH decline are more highly anaerobic at the time of exsanguination. A higher proportion of dark muscle fibers (Howe et al., 1968) and higher myoglobin levels (Thomas et al., 1970) have been reported in muscle from pigs reared in environments favoring slower post-mortem reaction. This indicates greater development of aerobic metabolism. Based on the present results, these animals would appear to possess higher ATP and CP concentrations in their musculature at the time of sacrifice.

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Providing Uniform Meat Cores for Mechanical Shear Force Measurement

SUMMARY—Porcine longissimus dorsi muscle was cut into 5.08 cm sections, heated to an internal temperature of 72°C and chilled to 4°C. Pork muscle cooked to 72°C and chilled to 4°C before boring, was firmer, drier and thus better able to hold its shape during coring than muscle cooked to 60°C and bored warm. Two cores were taken from each pork loin chop. One core was bored by hand, the other by machine. Three different core sizes were used (2.54 cm, 1.90 cm and 1.27 cm). Each core diameter was measured at the same three locations as the core was sheared. A significant ($P < 0.005$) difference in diameter was observed between the hand and machine cut cores when the 1.90 cm and 1.27 cm bores were used. At these core diameters, there was less variation between the machine bored cores than those removed by hand. There was no significant difference between the core diameters of the two different boring methods when the 2.54 cm bore was used. In all cases machine bored cores were larger in diameter and required a greater shear force than cores removed by hand. Mean shear force values indicated that one cannot double the 1.27 cm core shear value and receive comparable results for the 2.54 cm core shear forces.

INTRODUCTION

METHODS for determining relative tenderness of muscle have been accompanied by a large degree of experimental error. Part of this error has been due to the measuring device, animal variation and muscle variation, but a large portion of the experimental error can be attributed to sampling error. It is important to devise a uniform, consistent method for taking cores of meat to be measured by the mechanical shear machine (e.g., Warner-Bratzler).

Several methods for evaluating tenderness have been devised and each method incorporated various sampling techniques. Related work by Kulwich et al. (1963) used the Warner-Bratzler shear apparatus to compare to a slice-tenderness evaluation device. Muscle cores for the Warner-Bratzler test were not removed by machine or if so this was not mentioned.

Proctor et al. (1955) started that samples to be used in a recording strain gage denture tenderometer were to be of the same size. Even though sample size was considered important no special equipment was used to insure uniform samples.

Samples to be measured by the motorized Christel Texturemeter (Miyada et al., 1955) were extracted from semitendinosus muscle of beef by using a cork borer. Each core was bored to a given thickness and remeasured on four sides with a vernier caliper. Miyada et al. (1955) also stated that samples to be used in testing the Hamilton-Beach food grinder were of one approximate size.

Hedrick et al. (1968) used 2.54 cm and 1.27 cm cores for use with the Warner-Bratzler shear machine, however, no specifications were given for the method of removing cores. Previous studies did not reflect that any objective control was exercised to insure uniform, consistent samples to be measured by the various tenderness machines.

More and more research is devoted to the study of muscle and its characteristics, especially in relation to tenderness. Most mechanical methods for measuring tenderness require a uniform section of muscle, and considerable difficulty has been experienced in providing uniform individual cores. This has been particularly true when meat is bored immediately after cooking. The best treatment is to bore cooked meat after it is chilled to 4°C (Henrickson, 1964). Another practice that aids in providing uniform cores is that of heating meat samples to an internal temperature of 72°C. Meat heated to 72°C is well cooked, drier, firmer and thus holds its shape better during boring than muscle cooked at a lower temperature (60°C).

Until now meat cores to be measured by the Warner-Bratzler shear apparatus were obtained manually and there was a tendency for the cores to be of a concave or hourglass shape, which was considered to affect the shear tests (Fig. 1). This was especially true when the hand boring

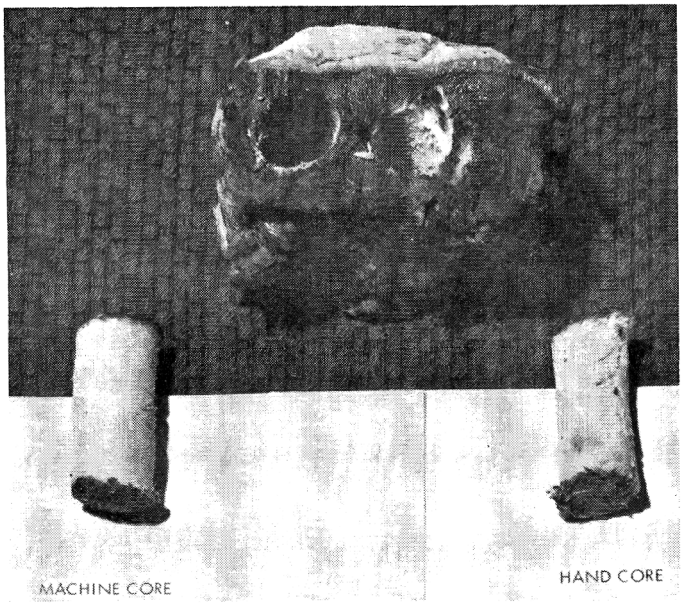

Fig. 1—Machine and hand bored cores showing the variation in diameter.

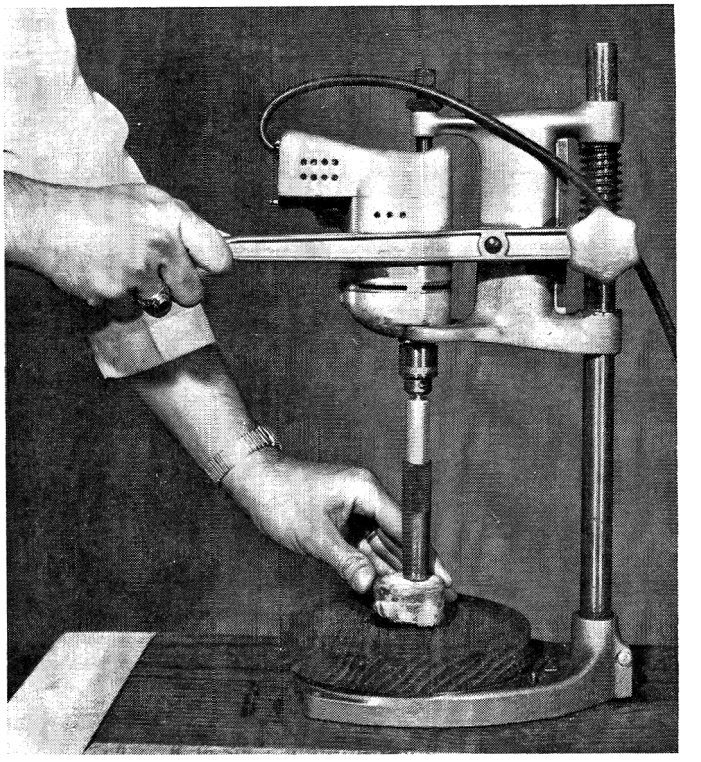

Fig. 2—A mechanical boring device.

method was used on meat cooked medium to rare.

A tool was devised that will assure cores of uniform diameter. This companion tool to the shear machine consists of a Black and Decker type III drill stand, drill and a metal rod adapter machined to fit inside the standard bore (Fig. 2). The borer has one metal projection 2 mm in diameter, extending 2.5 mm perpendicular and 2 cm distant from the terminal end. The male insert was milled with a recess in the thickened end to receive the metal projection. The projection on the bore holds the bore to the modified "chuck" device during operation and allows the bore to be easily detached for core removal and cleaning (Fig. 3).

The purpose of this study was to determine whether there was any significant difference in both core diameter and corresponding shear force required between hand and machine bored cores.

MATERIALS & METHODS

CORING diameters used were 2.54 cm, 1.90 cm and 1.27 cm. The cutting edge of each bore was sharpened and stored with a cork inserted to prevent damage. The metal plunger inserted in the cutting attachment had the dual function of holding the bore and facilitating removal of the core. The assembled attachment was then fitted into the drill which was designed to operate at a slow speed while coring. The drill speed was controlled by a Fisher Powerstat.

The pork chop to be bored was placed on a circular hardwood stand below the drill. This rotating stand had a neoprene center to prevent dulling of the cutting edge of the bore as it passed through the chop. The pork chop was then cored swiftly and uniformly by pressing down manually on the drill's lever. Each core was placed back into the chop after being cut in order to preserve original shape and retain moisture until the core was placed in the shear machine.

The same bore was used to cut both the hand and machine cores. This was done in order to eliminate variation that might result between bores of the same size. The only difference between the hand and machine produced cores was the use of the drill press in the case of the machine bored cores.

Porcine longissimus dorsi muscle was cut into 5.08 cm intervals from the full loin. The resulting slices were then divided into three groups to be cored at one of the three diameters (2.54 cm, 1.90 cm and 1.27 cm). All pork chops were boned and trimmed of excess fat, heated to an internal temperature of 72°C in a 140°C deep fat frylator and chilled in a 4°C cooler for 24 hr. Eighteen chops were randomly assigned to each core diameter. Two cores were taken from each pork chop, one by machine and one by hand. Both cores were removed from the central area of the chop and as close together as possible.

Care was exercised to randomize core removal so that no treatment had any greater

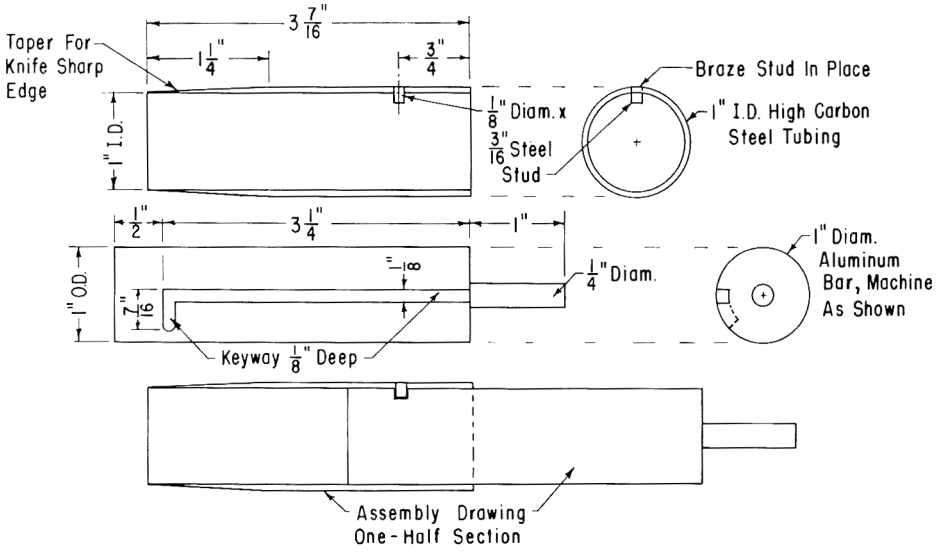

Fig. 3—The modified bore and chuck device for three bore sizes and schematic diagram of the 2.54 cm bore and chuck device.

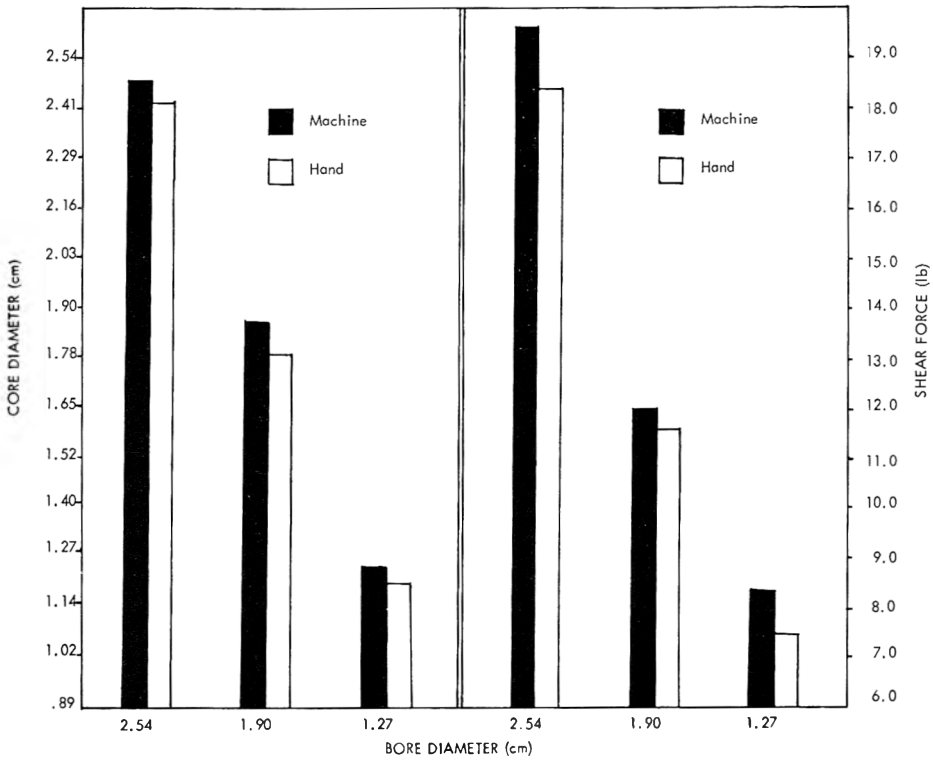

Fig. 4—The relationship of hand and machine cut cores to diameter and shear force.

chance than the other of lying on the medial or lateral side of the center line of the pork chop. A micrometer was used to measure the diameter of each core at each end and the center. An x-ray viewer aided in determining when the micrometer points touched the sides of the core. At each diameter measurement, the core was sheared using the Warner-Bratzler machine, and the required forces recorded.

RESULTS & DISCUSSION

CORES OBTAINED by the machine bored method at each bore diameter were free of the hourglass shape commonly found in hand bored cores. In addition, removing cores by machine was faster and easier than by the hand method. There was a significant ($P < 0.005$) difference between hand and machine core diameters taken at 1.90 cm and 1.27 cm. At these two core diameters there was less variation between machine core diameters than the cores removed by hand.

An analysis of the 1.90 cm core diameters yielded standard errors of the means of .0112 cm ($\bar{x} = 1.7917$ cm) and .0056 cm ($\bar{x} = 1.8753$ cm) respectively for both hand and machine methods. A similiar relationship existed when the diameters of the 1.27 cm cores were analyzed to produce standard errors of the means of both core removal methods.

The standard error of the mean for the hand method (1.27 cm) was 0.0097 cm ($\bar{x} = 1.2085$ cm) and for the machine method 0.0063 cm ($\bar{x} = 1.2515$ cm). Even though there appeared to be a visual difference between the uniformity of hand and machine cores at 2.54 cm, this difference was not statistically significant. This may have been due to the magnitude of error caused while boring a large core as compared to a small one. In every case the machine cut cores were more uniform in diameter from end to end than the hand cores.

Uniform coring is obtained more easily when meat is heated to 72°C and chilled to 4°C for 24 hr. Under these conditions meat was well cooked, firmer, drier and thus better held its shape during coring, than muscle cooked at a lower temperature (60°C) and bored warm.

Both machine and hand cut cores were taken side by side. By doing so both animal variation and some muscle variation were controlled. There was a significant ($P < 0.025$) difference between shear force values of the two treatments when the 1.27 cm bore was used. In contrast, shear values obtained at 2.54 cm and 1.90 cm proved non-significant. The lack of significance can be attributed to great variation in shear force of cores taken from the same chop.

There was a definite trend for shear forces and core diameters taken by machine to be larger than those removed by hand (Fig. 4). Machine cut cores were consistently more uniform and closer to the bore size used to extract the sample. These data indicate that shear force recorded at 1.27 cm cannot be doubled to obtain comparable 2.54 cm core shear values (Fig. 4).

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Treatment and Post-Mortem Aging Effects on the Z-line of Myofibrils from Chicken Pectoral Muscle

SUMMARY—Changes in the morphology of myofibrils prepared from chicken pectoral muscle during post-mortem storage at 5°C were examined by light and electron microscopy. When the 24-hr stored samples were blendorized, electron micrographs showed two types of destruction in the Z-lines of sarcomeres and myofibrillar fragments: (1) The degradation and/or disappearance of Z-lines. (2) The breakdown of the junction of Z-line and I-filaments. A change in the state of the Z-line and the junction of the Z-line and I-filaments appeared to be indispensable for the fragmentation of the myofibrils. It was also shown through phase contrast microscopic observations that sarcoplasmic proteins, participating in the glycolytic cycle, may play a role in the fragmentation of the myofibrils. Evidence has not been obtained, to date, on the participation of proteolytic enzymes in the fragmentation phenomenon.

INTRODUCTION

LARGE QUANTITIES of single sarcomeres can be isolated from the pectoral muscle of the chicken if the muscle is permitted to remain attached to the skeleton for more than 24 hr post-mortem (Fukazawa et al., 1963). Takahashi et al. (1967) have also shown that the tendency for myofibrils, from these pectoral muscles, to break into small fragments consisting of 1–4 sarcomeres, upon treatment with a Waring Blendor, increases progressively with time post-mortem. Fukazawa et al. (1967) conducted an electron microscopic study of this phenomenon and suggested that the increased tendency for fragmentation of the myofibrils might be due to the destruction of the zigzag configuration in the Z-line. This zigzag configuration has been observed by several investigators (Frazini-Armstrong et al., 1964; Knappeis et al. 1962) and is thought to provide continuity between the actin filaments on each side of the Z-line.

Weidemann et al. (1967) investigated the effect of thermal treatment on the histology of ox muscle and concluded that high temperatures caused disruption of the actin filaments and destruction of the cross-bridge linkage between the actin and myosin filaments. Perry (1964) has also shown that extraction of the myofibril with a low ionic strength and high pH solution causes the disappearance of the Z-line. Hanson (1964) noted that Z-lines in skeletal muscle of the pecten and vertebrate animals differ in their resistance to glycerol extraction. Ashley et al. (1952) reported that tryptic digestion, employed in isolation of myofibrils, brought about a partial destruction of the Z-line. More recently, Stromer et al. (1967) demonstrated the disappearance of the Z-line of myofibril from bovine muscle after the myofibrils were treated with trypsin.

The cause of the increased tendency for the myofibril to fragment with time post-mortem is not known. The tendency to fragment may however have a direct relationship to tenderness. Therefore, the present study was conducted to ascertain the effects of several factors on the tendency of the myofibril to fragment and upon certain characteristics of the Z-lines of the myofibrils.

EXPERIMENTAL

8 CHICKENS, approximately 10 weeks of age, were used in each of the following groups: (1) scalded and pectoral muscle retained on skeleton, (2) scalded and pectoral muscle excised from skeleton, (3) dry picked and pectoral muscle retained on skeleton and (4) dry picked pectoral muscle excised from skeleton. Myofibrils were prepared from pectoral muscles as described by Fukazawa et al. (1963). Initial samples were taken within 1 hr after death and 24 hr samples were taken after the carcasses or excised muscles were retained at 5°C for 24 hr post-mortem.

Electron microscopy

The suspensions of myofibrils were centrifuged at 600 × G for 15 min and the sediments were washed twice with 0.1 *M* phosphate buffer (pH 7.0). The myofibrils were prefixed by immersion in cold 2.5% glutaraldehyde buffered in a 0.1 *M* phosphate solution (pH 7.0). After washing in 0.1 *M* phosphate buffer (pH 7.0) the myofibrils were post-fixed in Palade's osmium tetroxide at 0°C (Palade, 1952). Subsequently, the myofibrils were dehydrated in a graded series of alcohol solutions followed by propylene oxide, and embedded in an epon mixture (Luft, 1961). The sections were made with an ultramicrotome (Sorvall-Porter-Blum) using a glass knife, stained in 1% uranyl acetate and lead citrate (Leynolds, 1963) and examined in a Hitachi HU-11B electron microscope operated at 75 KV and 50 KV.

Fragmentation

The technique for studying fragmentation has been described in detail in the report of Takahashi et al. (1967). Approximately 2–3 g of minced muscle were treated in a Waring Blendor with 20 vol of a solution containing 0.1 *M* KCl, 1 mM EDTA, and 39 mM Borate buffer (pH 7.1). Fragmentation was subsequently observed in an Olympus Phase Contrast Microscope. The number of myofibrillar fragments (F) consisting of 1–4 sarcomeres were counted in over 20 microscopic fields of view, representing over 500 fibrils and fragments (Σ). The data are presented as $(F)/(\Sigma)^{*}$ 100.

In the development of this work, it was essential to establish standard conditions for Waring Blendor treatment. Figure 1 shows effect of duration of Waring Blendor treatment on degree of fragmentation. Increasing the duration of treatment with the Waring Blendor increased the number of fragments consisting of 1–4 sarcomeres when the myofibrils were prepared immediately after death. When the myofibrils were prepared from tissue 72 hr post-mortem, the duration of Waring Blendor treatment had no significant effect on fragmentation. To standardize conditions for this experiment, all samples were

Table 1—The extractability of water soluble protein[1] following mechanical disintegration.

Method of extraction	Amount[2] of water-soluble protein extracted, protein mg/g muscle
Sum of 4 times-extraction by blenderizing for 90 sec	114.80
Blenderizing for 90 sec	97.45
Stirring[3] for 180 sec	61.40
Stirring for 60 sec	37.88

[1] Extracted with 0.1 *M* KCl containing 39 mM borate buffer (pH 7.1) and 1 mM EDTA.
[2] Nonprotein nitrogen is included in the values listed in the table.
[3] Continuous stirring by means of magnetic stirrer at moderate speed.

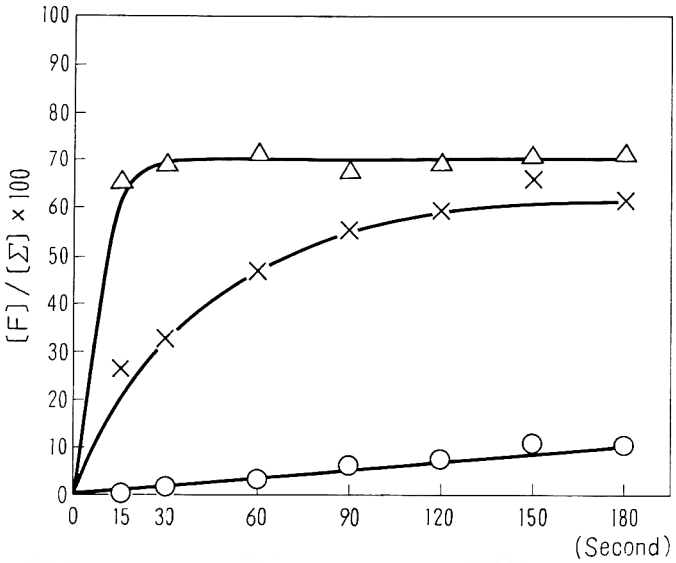

Fig. 1—The effect of time taken to blenderize on the fragmentation of fibrils. O - - - - - O Immediately post-mortem; X - - - - - X 18 hr post-mortem; Δ - - - - - Δ 72 hr post-mortem.

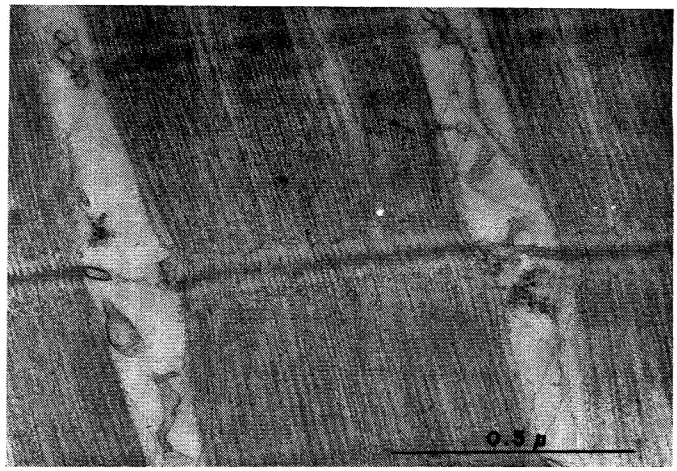

Fig. 2—Electron micrograph of chicken pectoral muscle fixed within 1 hr post-mortem. The zigzag configuration of the Z-line is clearly visible.

removed from the skeleton immediately after death and held at 5°C for the preparation of myofibrils. A Waring Blendor treatment of 90 sec in duration was used for the remainder of this study.

pH determination

The pH values were measured with a combination glass electrode. Maintenance of high initial pH values of muscle was accomplished by injection of insulin as described by Bate-Smith et al. (1956).

Protein determination

The protein concentrations of all samples and preparations were determined by the biuret method of Gornall et al. (1949) after standardization by use of the micro-Kjeldahl procedure.

RESULTS AND DISCUSSION

Electron microscopy of Z-line

Figure 2 shows a typical zigzag configuration of the Z-line in myofibrils which were prepared for electron microscopy within 1 hr after death of the chicken. Figure 3, however, shows the morphological structure of sarcomere and myofibrillar fragments which were prepared from chicken pectoral muscle which had been scalded and the muscles were retained on the skeleton at 5°C for 24 hr. Fukazawa et al. (1967) have shown that, although the zigzag configuration was evident in the Z-line of an intact myofibril isolated immediately after death, such a specific configuration was no longer detectable after the muscle had been stored for 24 hr post-mortem.

In the present experiment the myofibrils from muscle 24 hr post-mortem retained their Z-line constituents after 90 sec of treatment in the Waring Blendor. Nevertheless, these myofibrils were broken at a large number of junctions between Z-line and I-filament giving fragments consisting of 1–4 sarcomeres (Figs. 4, 5 and 6). The breaks of the Z-I junction were not variable in the myofibrillar fragments of the muscles which were excised and those which were retained on the skeleton. The changes in the banding patterns of myofibrillar fragments with time post-mortem will appear elsewhere.

Effect of post-mortem pH value on myofibril fragmentation

The changes in pH values and frag-

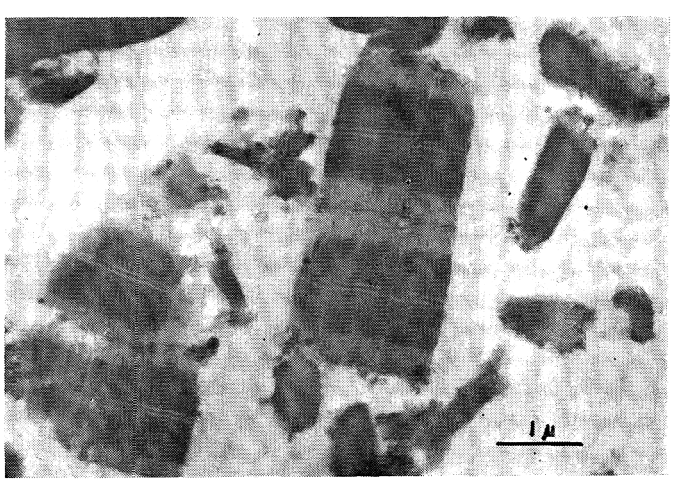

Fig. 3—Low power magnification of sarcomeres and myofibrillar fragments prepared from pectoral muscle of scalded chicken carcass which had been left attached to the skeleton and stored at 5°C for 24 hr. The zigzag configuration of the Z-line is undetectable and/or the Z-line constituent has been broken down.

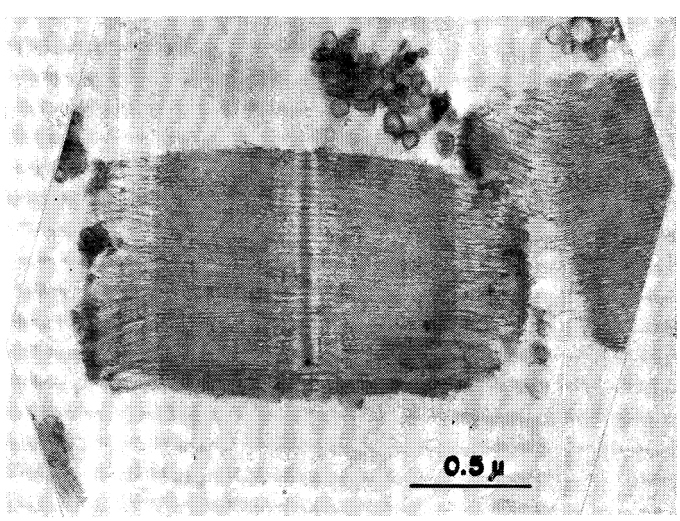

Fig. 4—Electron micrograph of sarcomeres obtained from pectoral muscle of scalded chicken carcass which had been left attached to the skeleton and stored at 5°C for 24 hr. A portion of the Z-line constituents is still maintained, although the zigzag configuration is undetectable.

mentation with time post-mortem are illustrated in Figure 7 (a, b). The injection of insulin was effective in maintaining high pH values for 2 hr post-mortem. The injection of insulin and delay in achieving the ultimate pH in the muscle caused a marked decrease in fragmentation of the myofibrils, in comparison to the myofibrils from control chickens which had a relatively rapid pH decline post-mortem (Fig. 7 a, b). There appeared to be a close relationship between the time required to reach the ultimate pH and the formation of fragments in the myofibrils. During the course of these investigations, it was postulated that some substance involved in the glycolytic cycle might influence the changes in the Z-line and/or the Z-I junction and consequently the degree of fragmentation in the myofibrils.

Effect of sarcoplasmic protein on fragmentation

The water soluble fraction of muscle, which includes the sarcoplasmic proteins, represents a complex mixture of numerous components, many of which are enzymes of the glycolytic cycle. Table 1 shows the quantities of protein extracted from chicken pectoral muscle by several different procedures.

As would be expected, for each extraction procedure, the amount of extractable protein increased with an increase in extraction time. Consequently, it was possible to study the effect of varied amounts of retained sarcoplasmic protein on the fragmentation of the myofibril. The results of this study are shown in Figure 8. It can be clearly seen that the percentage of small fragments increased rapidly with time post-mortem in the nonextracted sample, whereas when sarcoplasmic protein was more thoroughly extracted, $(F)/(\Sigma)$ values did not increase as much with post-mortem time. It seems therefore, that either the sarcoplasmic protein, per se, or the post-mortem change caused by the sarcoplasmic protein contributes to the ratio of fragments to total myofibrils.

Electron micrograph of the intact myofibril isolated free from sarcoplasmic protein immediately after death has shown the intact zigzag configuration in the Z-line even after storage for 24 hr (Fukazawa, unpublished data). The histological integrity and stability of myofibrils prepared immediately and held separate from sarcoplasmic protein implicate either the protein or the reactions carried on by the protein (to be discussed in the next section of this manuscript), to the destruction and breakdown of the Z-line and/or Z-I junction. While the previous experiment (Fig. 8) showed the effect of leaving various amounts of sarcoplasmic protein in the myofibrillar preparations, the present experiment (Fig. 9) shows the effect of the addition of fresh sarcoplasmic protein to fresh myofibrils (weight ratio of fresh muscle extract to myofibril paste is 1:2) previously prepared free of sarcoplasmic protein.

It can be seen that the re-addition of sarcoplasmic protein had no effect on fragmentation and confirms an earlier report by Takahashi et al. (1967). Likewise the use of a borate buffer (pH 7.1) had no effect on fragmentation when compared to distilled water adjusted to pH 7.1. Hence it appears that sarcoplasmic

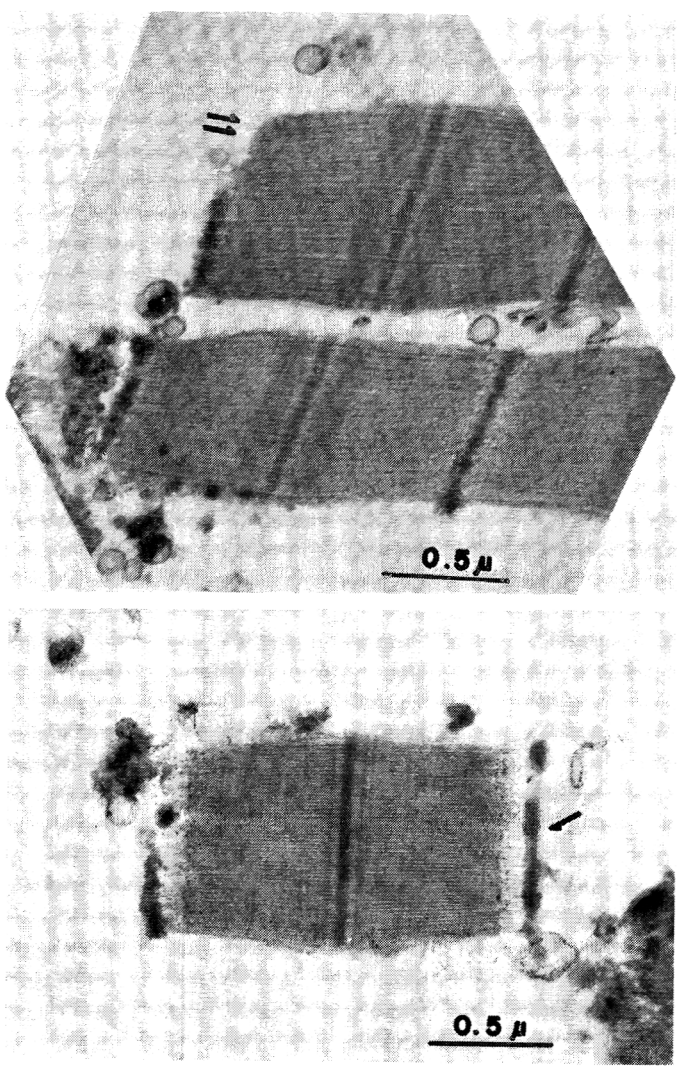

Fig. 5—Electron micrographs of sarcomeres obtained from pectoral muscle of scalded chicken carcass which had been excised immediately and stored at 5°C for 24 hr. Most of the Z-line constituents still remain, and sometimes the zigzag configuration of the Z-line is detectable as shown by ↑ (top). ↗↗ shows the portion where the Z-line has disappeared (bottom).

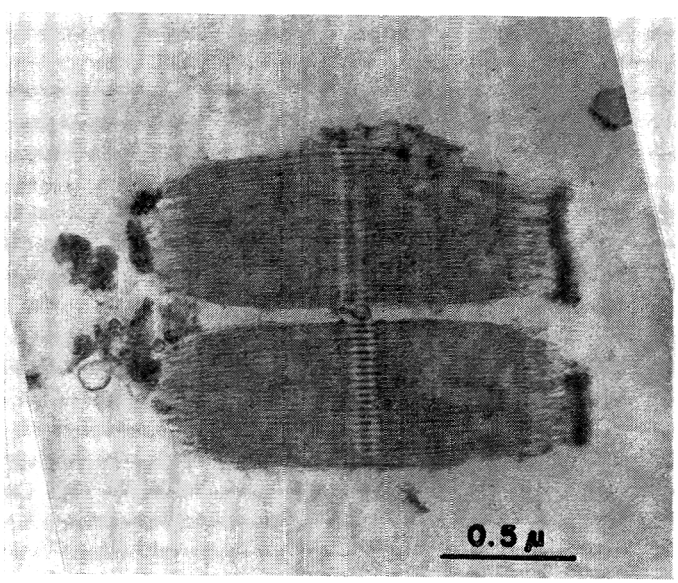

Fig. 6—Electron micrograph of sarcomeres obtained from pectoral muscle of dry picked carcass which had been left attached to the skeleton and stored at 5°C for 24 hr. Although a portion of the Z-line constituents had been removed, the Z-line still remaining shows no zigzag configuration.

protein must be present in its intact state to effect fragmentation. Ashley et al. (1952) observed that tryptic digestion brought about a partial destruction of the myofibrils and experiments on this aspect are presently in progress.

Effect of various pH values on myofibrillar fragmentation

To study the effect of pH on post-mortem fragmentation of myofibrils, 0.1 *M* phosphate buffers with pH values of 6.0, 7.0 and 8.0 were used to prepare the myofibrils from the muscle under storage at 5°C (Fig. 10). These solutions with different pH values had no effect on fragmentation of the myofibrils. Although little is known about the resistance of the Z-line of chicken pectoral muscle to extraction, the data do not support the finding of Perry (1964) that the Z-line of the myofibril disappears upon extraction at low ionic strength and high pH.

However, when the muscle homogenates were washed with 10 vol of 0.1 *M* phosphate buffers adjusted to pH 6.0, 7.0 and 8.0 and then stored at 5°C, no definite effect of pH values on the amount of fragmentation was observable during storage. It should be emphasized, in this case, that the washing procedure removed almost all sarcoplasmic proteins from the preparations.

The present work suggests that the amount of fragmentation in chicken pectoral myofibrils may be associated with the chemical and physical changes which occur in the muscle post-mortem. Changes in nucleotide and glycolytic intermediate levels may be indirectly associated with the activity of various enzymes in the sarcoplasm (Kastenschmidt, et al., 1968). It appears that for fragmentation to occur something must happen to the Z-line and Z-I junction post-mortem. Several papers (Briskey et al., 1967; Masaki et al., 1967; Stromer et al., 1967) have implicated the possible location of tropomyosin and α-actinin in the Z-line of myofibrils (Huxley, 1963; Briskey et al., 1967; and Masaki et al., 1967). Specific changes in Z-line constituents with time post-mortem will be the subject of the forthcoming investigation.

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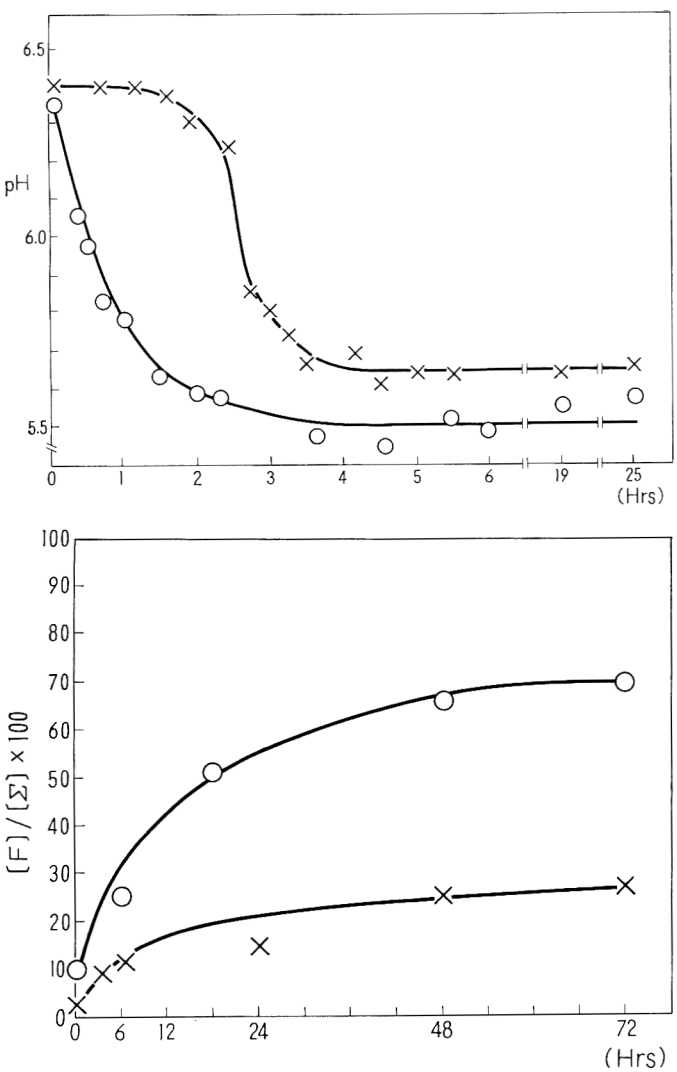

Fig. 7—(top)—The change in pH of chicken pectoral muscle during post-mortem storage. X - - - - - X Insulin injected muscle; O - - - - - O No treatment (control)—(bottom)—The influence of insulin injection on the fragmentation of fibrils during post-mortem storage. X - - - - - X Insulin injected muscle; O - - - - - O No treatment (control).

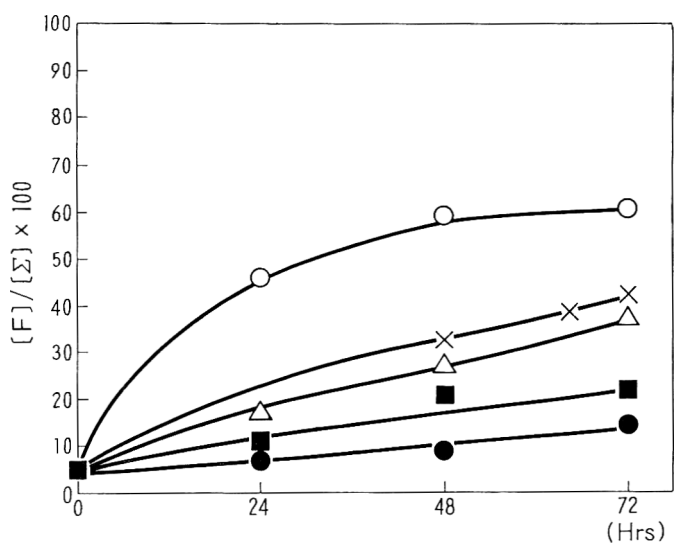

Fig. 8—The effect of varied amount of retained sarcoplasmic protein on the fragmentation of fibrils. O——O Control; X——X Extraction of water soluble protein 1 min; Δ——Δ 2 min; ■——■ 3 min; ●——● 5 min.

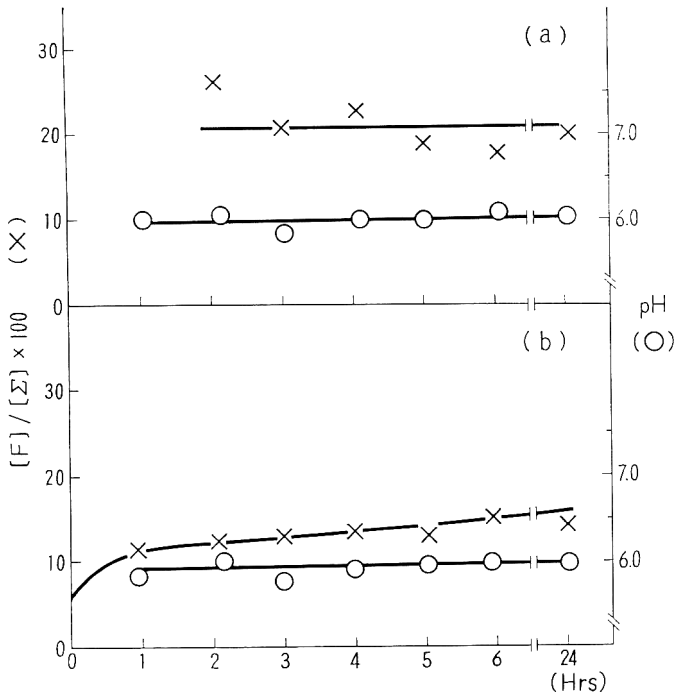

Fig. 9—The changes in the pH values and the formation of myofibrillar fragments in mixtures of freshly prepared myofibril paste and fresh muscle extracts during storage at 5°C. (a) 0.1 M KCl, 1 mM EDTA and 39 mM borate buffer (pH 7.1). (b) distilled water adjusted to pH 7.1 with 0.1 N KOH. (X—X—X myofibril paste); (O—O—O fresh muscle extract).

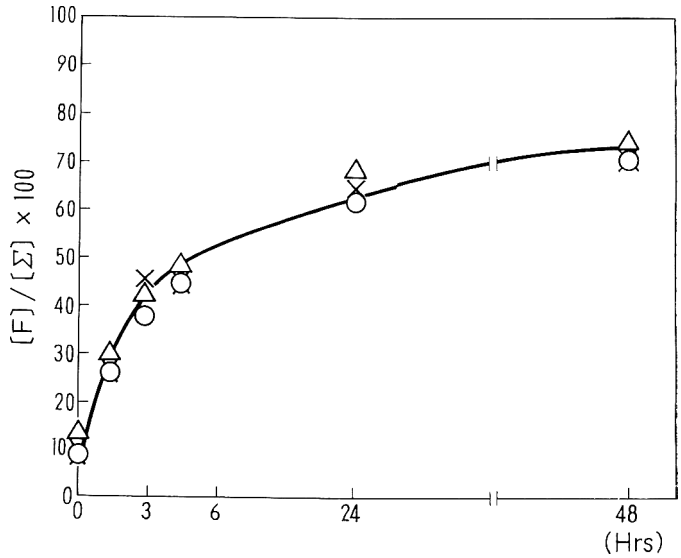

Fig. 10—The effect of pH values of the buffer solution on the fragmentation of fibrils. O——O pH 6.0; X——X pH 7.0; Δ——Δ pH 8.0.

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A Research Note
Composition of Orange Essence Oil

INTRODUCTION

AS A PART of investigations into the composition of essential oils in citrus products (Hunter et al., 1965a, b, c, 1966) by this laboratory, studies on the composition of aroma or essence oils were undertaken. These oils are obtained as a by-product of various types of essence recovery processes.

In the manufacture of orange essence, a portion of the juice is vaporized (up to 25%) and the volatile components concentrated in packed or open columns until the fragrant solution is about one-hundredth in volume in comparison to the original juice. Peel oils in the orange juice are volatilized at the same time, condensed with the essence and float to the top. The essence and the essence oil represent a two phase system in equilibrium to a degree.

It would be expected that the essence oil would lack the non-volatile compounds in peel oil, such as those responsible for the orange color, but would likely be more abundant in some of the more water soluble components. The amount of essence oil available is not known, but it should become substantial as essence recovery becomes more popular. The excellent flavor quality of these oils suggested that the composition differed sufficiently from peel oil to warrant a qualitative study using gas-liquid chromatography.

EXPERIMENTAL

BOTH MIDSEASON (Hamlin and Pineapple) and Valencia essence oils were studied in order to compare these two major types. Each was first separated into a number of fractions by a combination of vacuum dis-

tillation and column chromatography (Hunter et al., 1965a, b). The resulting fractions were subjected to gas-liquid chromatography and were identified either by infrared or mass spectral analysis or by a combination of the two.

Spectroscopic measurements

Mass spectra were obtained with a Bendix Model 3012 (TOF) mass spectrometer, infrared spectra on a Perkin-Elmer Infrared Model 137-A, NMR spectra on a Varian Model A60 and UV data on a Cary Model 14 spectrophotometer.

Separation procedure

Two essence oils were obtained from a commercial plant in Florida producing 100-fold essence by fractionating and condensing the vapors from the first stage of the evaporator. The oil floated to the top of the condensate and was withdrawn periodically. Midseason oil was obtained in March and Valencia oil in June.

The oil samples were fractionated by distillation in a rotary evaporator at 50°C and 4 mm Hg until most of the d-limonene was removed. The residue was fractionally distilled in vacuo at pressures ranging from 3 to 0.3 mm Hg and head temperatures from 40° to 100°C. Four or five fractions were arbitrarily collected in this manner. (The number of fractions make little difference since their composition will eventually be compared to the total essence oil in Table 1.) Each of these fractions was then separated into three portions by chromatography on a column of Fisher's Brockman Activity II neutral alumina and eluted successively with hexane, ethyl ether, and ethanol.

The final analysis was carried out by gas-liquid chromatography on Carbowax 20M, 5% on Chromosorb-P with the temperature programmed from 150° to 230°C at a rate of 2.0°C per min and Carbowax 20M, 20% on Chromosorb-P with the temperature programmed from 150° to 230°C at a rate of 1.5°C per min. Helium was used as the carrier gas.

The volatile oxygenated material which was carried over with the d-limonene was analyzed by the use of adsorption chromatography (Teranishi et al., 1966). Neutral alumina was used rather than silica gel because of the tendency of silica gel to catalyze rearrangements. The eluted polar fractions were analyzed by gas-liquid chromatography under the conditions described above.

Table 1—Approximate percentage composition of two essence oils.

Compound	Midseason	Valencia
Acetone		0.35%
Carvone	0.01%	7.8
Trans-carveol		4.9
Caryophyllene	0.04	0.02
Citronellol	0.0005	
α-Copaene	0.008	0.013
p-Cymene		0.95
n-Decanol	0.11	0.01
n-Dodecanol		0.025
β-Elemene	0.04	0.02
Ethyl butyrate		0.0004
d-Limonene	97.0	84.72
Linalool	0.3	0.06
1,8-p-Menthadiene-9-ol		0.001
1,8-p-menthene-1,2-diol	0.002	
Methylcyclopentane[1]	0.001	
Methylcyclohexane[1]		0.13
Myrcene	0.03	0.04
n-Nonanol	0.004	0.007
n-Octanol	0.5	0.005
Perillyl aldehyde		0.015
α-Pinene		0.006
Piperitenone	0.0005	0.03
Sabinene		0.0004
α-Terpineol	0.5	
n-Undecanol		0.2
Valencene	1.9	0.7

[1] Probably contaminants from the solvents used.

RESULTS & DISCUSSION

THE COMPOUNDS isolated from essence oil are shown in Table 1, along with the approximate weight percentage of the component in the oil, when this information was available. The approximate percentages (Table 1) were calculated from the areas under the peaks of the gas-liquid chromatograph.

Particular care was taken to collect and identify the more volatile compounds contained in the volatile oxygenated fractions. The peaks were collected in traps cooled with liquid nitrogen. This analysis was undertaken in order to determine if significant amounts of the volatile compounds characteristic of orange essence were present in the oil. Ethyl butyrate was the only major component in essence oils which had been commonly observed in the orange essences (Teranishi et al., 1966 and Wolford et al., 1967), but not in cold-pressed orange oils. The presence of this compound in the oil can be explained as the result of the partitioning of volatile essence components between the aqueous essence phase and the oil phase. Two compounds, methylcyclopentane and methylcyclohexane, are probably contaminants from hexane used as a solvent.

The high percentage of valencene represents a notable difference between these oils and cold-pressed orange oils. The percentage of valencene was 10 to 20 times higher than is usual in cold-pressed oils.

The absence of the straight-chain saturated aldehydes, octanal, nonanal and decanal is notable since Moshonas et al. (1968) found them in cold-pressed orange oil and they are common in orange essence.

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Considerations for Beef Tenderness Evaluations

SUMMARY—*Data collected from 690 beef rib steaks were utilized to evaluate the methodology of beef tenderness measurements. The information obtained in this study was critically compared to existing research and evaluated in relation to sample handling procedures. Frozen storage of steaks, especially if they are unprotected from dehydration or are stored for long periods of time, has important implications if such data are to be compared to steaks which were evaluated in the unfrozen state. Cooking frozen steaks with or without thawing apparently has little effect on tenderness or cooking loss. Anatomical location of the sample should be carefully controlled to prevent spurious differences from affecting tenderness measurements. Evidence indicates that a tenderness gradient exists over the cross-section of the l. dorsi and suggests that core samples should be taken from as many and as varied positions as is feasible for existing research conditions. The use of marbling score as an indicator of the tenderness of beef rib steaks resulted in the explanation of 28 percent of the variation in average shear force requirements. The shear force value of the diaphragm muscle was only moderately related ($r = 0.40$) to that of the l. dorsi. Cooking loss percentages were significantly increased as a result of freezing at −34°C and storage of steaks unwrapped at −23°C when compared to unfrozen steaks and by selecting l. dorsi samples from more anterior locations in the wholesale rib.*

INTRODUCTION

TO ACCURATELY assess beef tenderness, careful consideration must be given to the methodology employed. Variations in the following conditions have been reported to influence measurements of beef tenderness: storage period (Ramsbottom, 1947, Law et al., 1967; Dawson et al., 1959), rate of freezing (Pearson et al., 1950), freezer temperature (Hiner et al., 1945; Hankins et al., 1940 and 1941; Hiner et al., 1951), cookery (Cover et al., 1960; Machlik et al., 1963; Hedrick et al., 1968), degree of doneness (Cover et al., 1962; Visser et al., 1960), measurement techniques (Bratzler et al., 1963; Sharrah et al., 1965b), muscle selected (Cover et al., 1962; Sharrah et al., 1965a), core diameter (Paul et al., 1955; Hedrick et al., 1968), orientation of core (Harrison et al., 1959; Hostetler et al., 1964), location within a muscle (Ginger et al., 1958; Taylor et al., 1961), position within a muscle location (Tuma et al., 1962a; Hedrick et al., 1968; Alsmeyer et al., 1965) and temperature of sample core (Hedrick et al., 1968).

Numerous researchers have established precedent for the utilization of the Warner-Bratzler shear device (Burrill et al., 1962; Bratzler et al., 1963; Alsmeyer et al., 1966; Sharrah et al., 1965a; Sharrah et al., 1965b) and the *longissimus dorsi* muscle (Ramsbottom et al., 1948; Cover et al., 1960; Cover et al., 1962; Sharrah et al., 1965b) in beef tenderness measurements when compared to other devices or other muscles, respectively. The errors involved in tenderness evaluations employing the Warner-Bratzler shear machine have been accurately delineated by Hurwicz et al. (1954).

This study was implemented to assess the effects of frozen storage, cooking procedure, anatomical location and core position on the force required to shear core samples from the l. dorsi muscle of beef rib steaks and to compare such findings to previously reported research. Additional data related to cooking loss, marbling, chew counts, and core diameter were analyzed to supplement the main effects studied.

MATERIALS & METHODS

WHOLESALE RIBS, selected from 176 beef carcasses, provided four boneless steaks (3.18 cm in thickness) from the 10th, 11th and 12th rib areas. Carcasses were stored at 2°C for 5 to 9 days post-mortem. The 420 steaks derived from 105 of the ribs were randomly allotted (1 steak/rib/treatment) to one of four treatments.

Treatment groups were as follows: (A) unwrapped while stored at 2°C; (B) wrapped and stored at −23°C; (C) unwrapped and stored at −23°C; and (D) wrapped and stored at −34°C. Storage periods consisted of 12 to 18 hr (A) and 4 to 6 weeks (B, C, and D). Rib area location and carcass identity were preserved for each steak.

Steaks in treatments B and D were double-wrapped in freezer paper, while those in treatments A and C were not wrapped prior to storage. All steaks from the four treatments were carefully spaced and layered in waxed cardboard containers (20 steaks/ container) during storage to assure uniform cooling or freezing rates.

The 284 steaks from the remaining 71 ribs were randomly allotted (1 steak/rib/ treatment) to one of four treatment groups. Treatments consisted of the following: (E) and (F) unwrapped and stored at 2°C; and, (G) and (H) wrapped and stored at −34°C. Wrapping and storage procedures were identical to those described for treatments B and D. Storage periods consisted of 12 to 18 hr (E and F) and 3 to 16 weeks (G and H). Steaks were weighed to the nearest gram at the time of cutting, after storage and following cooking to facilitate weight loss measurements.

After storage for the prescribed time periods, steaks in treatments B, C, D and G were thawed at 2°C to a uniform internal temperature. Steaks in treatments A, E and H were cooked immediately following storage, thus the 71 steaks in treatment H were frozen at the time of cooking. Each steak was cooked to an internal temperature of 75°C using a preheated 176°C gas oven and the "oven-broiling" procedure of Cover et al. (1960). Individual steaks were removed from the oven, allowed to reach room temperature, and hand-cored in parallel orientation with the muscle fibers (Hostetler et al., 1964). The six cores (1.27 cm in diameter) removed from each steak in treatments A, B, C, D, E, G and H were designated P_1 through P_6 (Fig. 1).

The average shear force value for each steak was designated as P-av and represents the mean of positions 1 through 6. In subsequent discussions regarding anatomical location, positions 1 and 2 are termed "dorsal," positions 3 and 4 "medial" and positions 5

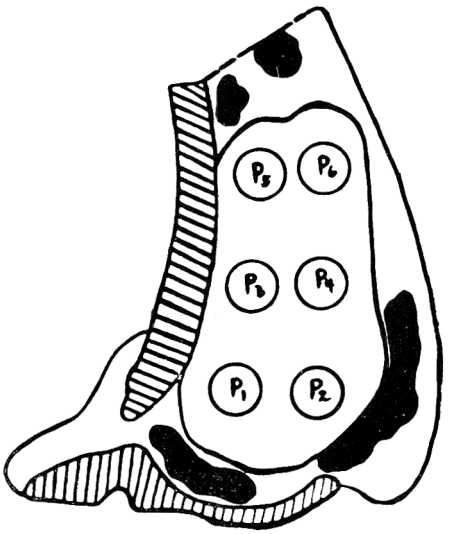

Fig. 1—Core positions for shear determinations.

and 6 "lateral." Six cores, three that were 2.54 cm in diameter (P_2, P_3 and P_0) and three that were 1.27 cm in diameter (P_1, P_4 and P_6) were taken from steaks in treatment F.

A three-member chew-count panel evaluated 30 g slices taken from the anterior end of each 2.54 cm core. All of the core samples were sheared (1 shear/core) using a Warner-Bratzler shear machine. Position identity for cores was preserved for all samples except those in treatment F. Only 57 of the 71 steaks in the latter treatment (F) were used for shear force and chew count measurements.

Marbling and maturity data were available for 95 of the wholesale ribs. All of these ribs were either A- or A in maturity and ranged from devoid to abundant in marbling (USDA standards). The deep digital flexor from the foreshank and crus of the diaphragm were available from 42 and 23 of the carcasses, respectively. Both muscles were stored, cooked, cored and sheared in the same manner as the rib steaks in treatment A. The latter muscles were investigated relative to their accuracy for estimating rib steak tenderness.

Table 1—Multiple range test for frozen versus unfrozen rib steak measurements.

Treatment	n	Shear force (kg) mean	Shear force (kg) S.D.	Thaw drip loss (%) mean	Thaw drip loss (%) S.D.	Cooking loss (%) mean	Cooking loss (%) S.D.
Unfrozen 2°C (A)	105	3.68[a]	0.94	—		28.46[a]	4.80
Frozen wrapped −23°C (B)	105	3.73[a]	1.02	0.86[a]	1.04	28.96[ab]	4.68
Frozen unwrapped −23°C (C)	105	3.81[a]	1.11	1.96[b]	1.06	30.12[bc]	4.96
Frozen wrapped −34°C (D)	105	3.69[a]	0.99	1.18[a]	0.94	30.43[c]	4.22

[abc] Mean values followed by the same superscript are not significantly different at the $P \leq 0.05$ level according to the multiple range test (Duncan, 1955).

Table 2—Comparison of frozen versus unfrozen beef rib steaks and their physical condition at cooking.

Treatment	n	Shear force (kg) mean	Shear force (kg) S.D.	Thaw drip loss (%) mean	Thaw drip loss (%) S.D.	Cooking loss (%) mean	Cooking loss (%) S.D.
Unfrozen 2°C (E)	71	4.29[a]	0.99	—		28.64[a]	4.66
Frozen wrapped −34°C (G) Cooked thawed	71	4.09[b]	0.96	1.15	1.10	30.82[b]	3.67
Frozen wrapped −34°C (H) Cooked frozen	71	4.05[b]	0.84	—		31.94[b]	4.01

[ab] Mean values followed by the same superscript are not significantly different at the $P < 0.05$ level according to the multiple range test (Duncan, 1955).

RESULTS & DISCUSSION

Frozen storage

Under most experimental conditions it is not possible to analyze fresh beef samples using chemical, physical or organoleptic methods within time intervals short enough to prevent the occurrence of influential changes. One of the most convenient and efficient means for sample storage and preservation is freezing. Blumer (1963) reported that while beef is considered to be fairly stable with respect to chemical and physical properties, rather large variations in storage temperatures and times are reported in the literature. Weir (1960) states that various investigators have reported no effect, increased tenderness and decreased tenderness for freezer storage of beef.

The initial comparison of frozen versus unfrozen steaks in this study (Table 1) indicates that nonsignificant differences in shear value resulted from the treatments employed.

This lack of significant difference in tenderness between unfrozen and frozen steaks is in agreement with Dawson et al. (1959) and Ramsbottom (1947) both of whom reported little observable difference in tenderness between frozen and unfrozen steaks even with rather long storage periods.

Results of the second comparison (Table 2) however, support the conclusion that freezing results in a significant decrease in the force required to shear core samples. The latter finding is in agreement with Hiner et al. (1951) who reported 6.83 to 15.20% decreases in shearing resistance for steaks frozen at −18°C in comparison with paired unfrozen steaks; and with Hiner et al. (1947) who found that beef round steaks frozen at −8°, −18°, and −64°C were more tender than paired unfrozen steaks.

The contrasting effects on tenderness resulting from freezing between the two sets of data (Table 1 vs. Table 2) can possibly be explained by consideration of the respective storage periods. In the second comparison (Table 2) 32 pairs of frozen steaks were stored for 4 months prior to cooking, in contrast to the 3 to 6 week storage period for all other pairs or sets of steaks. Smith et al. (1968) suggested that storage could have a significant effect on the tenderness of lamb l. dorsi, if length of time in storage differed between samples. Field et al. (1966) found that long periods of storage at −23°C resulted in significantly lower shear force values for beef, based upon a correlation of −0.27 between days in the freezer and Warner-Bratzler shear values.

Pearson et al. (1950) found that steaks sheared at 0 days were significantly more tender than those stored for 90 and 180 days. Schrewsbury et al. (1945) reported that when beef was frozen and stored for 15 months, there was a definite deterioration in quality. However, Weir (1960) reported that tenderness did not significantly change during storage. Ramsbottom (1947), Dawson et al. (1959) and Law et al. (1967) stored boneless loin steaks at −18°C or −23°C and reported that frozen storage up to 9 months did not influence tenderness.

In the present study the reduction in resistance to shearing of 4.6 to 5.6 percent for steaks frozen at −34°C compared to unfrozen steaks (Table 2) is considerably lower than the 13 to 26 percent reduction previously reported by Hiner et al. (1947).

The observation that steaks frozen at −23°C and −34°C were not appreciably different in tenderness disagrees with the reports of Hankins et al. (1940), Hiner et al. (1945), and Hiner et al. (1947) in which consistent increases in beef tenderness occurred as freezing temperatures were lowered. However, the present study is in agreement with Pearson et al. (1950) who found that rate of freezing did not significantly influence beef tenderness.

Unwrapped steaks, frozen at −23°C, displayed the greatest resistance to shear force among the frozen treatments. The observed difference, although nonsignificant, is of similar direction and magnitude as that observed for lamb chops by Smith et al. (1968) and suggests that physical changes occur under such conditions. Yet, Hiner et al. (1951) placed unwrapped beef cuts in a −18°C freezer, stored them for 24 hr, and upon cooking and shearing reported significant tenderizing effects due to freezing. The large difference in storage times (4 to 6 weeks in the present study versus 24 hr in the study of Hiner et al., 1951) probably explains the contrasting results.

It is generally known that frozen steaks require 10 to 15 min more cooking time than comparable unfrozen cuts. Machlik et al. (1963) state that changes in shear force or tenderness values produced upon heating are undoubtedly related to time-temperature-dependent protein denaturation processes. If such were the case, it is logical to expect a decrease in tenderness for unthawed steaks because of the additional time required to achieve doneness. The results shown in Table 2 fail to support such a conclusion since no significant difference in tenderness resulted when paired steaks were cooked in

Table 3—Relationships between treatment shear values.

Dependent Variable	Independent Variable	Y Intercept	b	b′	Multiple R^2	S.E.E.
Unfrozen (A)	Frozen wrapped −23°C (B)	1.0777	0.6990	0.7554	0.5707	1.3675
Unfrozen (A)	Frozen unwrapped −23°C (C)	1.2756	0.6317	0.7446	0.5545	1.3930
Unfrozen (A)	Frozen wrapped −34°C (D)	1.2580	0.6567	0.6829	0.4664	1.5245
Unfrozen (E)	Frozen cooked thawed −34°C (G)	0.9600	0.8140	0.7873	0.6199	1.3519
Unfrozen (E)	Frozen cooked frozen −34°C (H)	0.6866	0.8897	0.7557	0.5711	1.4359

(A) through (H) refer to treatment comparisons listed in Tables 1 and 2, respectively. For treatments A through D, n = 105. For treatments E through H, n = 71.

Table 4—Anatomical location effects on shear value and cooking loss percentages.

Location[1]	n	Shear value (kg) mean	Shear value (kg) S.D.	Cooking loss (%) mean	Cooking loss (%) S.D.
1	105	3.53[a]	0.94	27.91[a]	4.95
2	105	3.76[b]	1.02	30.12[b]	4.67
3	105	3.83[b]	1.04	30.57[b]	4.72
4	105	3.79[b]	1.05	29.37[b]	4.16

[1] Location 1 denotes the 3.18 cm steak taken opposite the 12th rib; locations 2, 3 and 4 correspond to the next three steaks removed progressing anteriorly toward the cervical region.
[ab] Mean values followed by the same superscript are not significantly different at the $P < 0.05$ level according to the multiple range test (Duncan, 1955).

Table 5—Mean shear force values by position.

Source	No. of cores	Shear Value (kg)						Position order (Smallest to largest)
Unfrozen (A)	630	3.31	3.56	3.60	3.71	3.87	4.04	3-1-4-2-5-6
Frozen wrapped −23°C (B)	630	3.45	3.65	3.66	3.70	3.77	4.15	3-1-2-4-5-6
Frozen unwrapped −23°C (C)	630	3.34	3.73	3.76	3.80	4.03	4.21	3-4-1-2-5-6
Frozen wrapped −34°C (D)	630	3.39	3.56	3.64	3.70	3.79	4.08	3-1-2-4-5-6
Location 1	630	3.27	3.37	3.40	3.58	3.65	3.92	3-4-1-2-5-6
Location 2	630	3.45	3.55	3.67	3.73	3.96	4.21	3-1-2-4-5-6
Location 3	630	3.41	3.77	3.79	3.89	3.92	4.20	3-2-1-5-4-6
Location 4	630	3.36	3.71	3.78	3.79	3.96	4.15	3-4-1-2-5-6
I Subtotal[1]	2520	3.37	3.63	3.68	3.70	3.86	4.12	3-1-4-2-5-6
Unfrozen (E)	426	3.88	4.15	4.28	4.31	4.48	4.65	3-2-4-1-6-5
Frozen cooked thawed −34°C (G)	426	3.82	3.89	4.05	4.18	4.27	4.35	2-3-4-1-5-6
Frozen cooked frozen −34°C (H)	426	3.85	3.85	4.01	4.03	4.23	4.34	3-4-2-1-5-6
II Subtotal[1]	1278	3.87	4.00	4.06	4.17	4.38	4.39	3-2-4-1-5-6
III Total[1]	3798	3.54	3.80	3.81	3.81	4.04	4.21	3-2-4-1-5-6

[1] Mean values underscored by the same line are not significantly different at the $P < 0.05$ level according to the multiple range test (Duncan, 1955).

Table 6—Positional shear force values grouped according to location area within the l. dorsi cross-section.[1]

Location	Mean shear force for treatments A, B, C and D (kg)	Mean shear force for treatments E, G and H (kg)	Mean shear force for all treatments (kg)
Dorsal (Positions 1 and 2)	3.67	4.08	3.81
Medial (Positions 3 and 4)	3.53	3.96	3.67
Lateral (Positions 5 and 6)	3.99	4.38	4.13
n	2520	1278	3798

[1] Mean values were not statistically analyzed.

frozen versus thawed states. It might be postulated that if the additional time is required at the beginning of the heating curve it would have little effect on decreases in tenderness related to denaturation.

In order to achieve accurate comparisons between steaks stored under different conditions, regression equations such as those presented in Table 3 might be utilized.

The equations presented are of little usefulness for other investigations and illustrate the inadequacies of such formulae for correcting and standardizing data obtained from steaks stored under dissimilar conditions. This assessment is based upon consideration of the magnitude of the coefficients of determination (Multiple R^2 value) shown in Table 3.

Differences in drip loss percentages during thawing were significantly greater for steaks frozen at −23°C unwrapped than for other freezing treatments, lending further credence to the hypothesis that significant changes in physical structure occurred when steaks were frozen without the protection of wrapping materials.

Pearson et al. (1950) reported that slow, intermediate and rapid freezing rates resulted in drip loss percentages of 1.04, 1.12, and 1.33, respectively. Moreover, Ramsbottom et al. (1941) found significant increases in drip loss percentage as the period of freezer storage was lengthened. When compared to unfrozen steaks, cooking loss percentages were significantly increased as a result of freezing at −34°C (Tables 1 and 2) and storage of steaks at −23°C while unwrapped (Table 1). Although a difference of 1.12 percent in cooking loss was observed, total weight losses sustained by steaks in treatments G and H were 31.97 and 31.94 percent, respectively.

Thawing steaks prior to cooking had no significant effect on either cooking or total weight losses. Freezing increased cooking losses in relation to that sustained by unfrozen cuts in every comparison except that between treatments A and B in Table 1. Law et al. (1967) reported that storage of boneless beef steaks at −18°C and −23°C for 6 months resulted in significant increases in cooking loss. Pearson et al. (1950) reported significant increases in cooking loss with 90 days of storage but found that rate of freezing had no effect on cooking loss percentages.

In the present study unfrozen rib steaks sustained less evaporative and cooking loss than frozen steaks. This is in agreement with the report of Smith et al. (1968) in which lamb chops were subjected to various storage conditions.

Anatomical location

Numerous researchers have reported

significant variations in Warner-Bratzler shear values at different anatomical locations (proximal to distal) within beef and pork muscles (Weir, 1953; Paul et al., 1955; Kinsman, 1961; Ginger et al., 1958; Doty et al., 1961; Batcher et al., 1960; Taylor et al., 1961 and Mackey et al., 1954). Results of the present study (Table 4) suggest that steaks adjacent to the 12th thoracic vertebra are significantly more tender and undergo significantly less weight loss during cooking than those nearer the 10th or 11th thoracic vertebrae. The differences in cooking loss may result from the greater proportions of fat present in the 10th and 11th rib areas than that present at the 12th thoracic vertebra region.

The observed difference in shear force value (Table 4) is in disagreement with the reports of Romans et al. (1965) and Henrickson et al. (1964) who indicated that l. dorsi steaks from the 9th thoracic vertebra were more tender than those from the 11th thoracic vertebra. Sharrah et al. (1965b) reported nonsignificant differences in mechanical shear or tenderness values between the proximal and distal ends of the l. dorsi.

Position effect

Reports of a tenderness gradient within the l. dorsi muscle have been advanced by numerous researchers (Sharrah et al., 1965b; Cover, 1937; Cover et al., 1962; Hostetler et al., 1964; Tuma et al., 1962a; Alsmeyer, 1960; Alsmeyer et al., 1965; and McBee et al., 1967). The divergence of opinion concerning the direction of this gradient and the need for guidelines in selection of core positions for tenderness assessments prompted this phase of the present study. The relative tenderness of the six cores, 1.27 cm in diameter, taken from steaks in the various treatment groups was of initial concern. Table 5 presents a comparison of the mean shear force values for each sample position.

The multiple range test (Duncan, 1955) for 3798 individual cores indicates that position 3 is most tender, positions 2,4 and 1 similar in tenderness, while positions 5 and 6 were least tender. Cover et al. (1962) reported that the most tender area in the l. dorsi, was the core position farthest from the vertebrae and nearest the fat edge which corresponds to position 6 in this study and thus is in disagreement with these results. Sharrah et al. (1965b) reported that the area nearest the fat cover and in the center of the l. dorsi, which corresponds to position 5 in the present study, required the least shear force to sever.

Since many researchers have studied effects of core sample position when using 2.54 cm cores and thus only three positions (dorsal, medial and lateral), the data listed in Table 5 were regrouped in Table 6 to facilitate comparison with other investigations. The results obtained indicate that the medial position is most tender and the lateral position least tender although the means were not compared statistically. This is in disagreement with McBee et al. (1967) who reported that dorsal cores were most tender and medial cores were least tender. Cover (1937), Alsmeyer (1960), Hedrick et al. (1968) and Tuma et al. (1962a) similarly reported that the dorsal core positions were most tender.

Table 7—Correlation coefficients between individual position and average position (P-av) shear force values by treatments.[1]

Treatment	df	P_1	P_2	P_3	P_4	P_5	P_6
Unfrozen (A)	103	0.83	0.84	0.84	0.76	0.82	0.73
Frozen wrapped −23°C (B)	103	0.79	0.81	0.81	0.86	0.84	0.83
Frozen unwrapped −23°C (C)	103	0.87	0.87	0.82	0.82	0.88	0.80
Frozen wrapped −34°C (D)	103	0.82	0.82	0.84	0.84	0.85	0.82
Unfrozen (E)	69	0.82	0.76	0.80	0.76	0.76	0.71
Frozen wrapped and cooked thawed −34°C (G)	69	0.84	0.65	0.83	0.78	0.85	0.61
Frozen wrapped and cooked frozen −34°C (H)	69	0.83	0.72	0.83	0.75	0.74	0.56
Total unfrozen (A and E)	174	0.84	0.81	0.83	0.78	0.81	0.73
Total frozen −34°C (D, E and H)	245	0.83	0.74	0.84	0.80	0.83	0.70
Overall (A through H)	631	0.83	0.79	0.82	0.81	0.83	0.75

[1] All values are significant at the $P < 0.01$ level.

Alsmeyer et al. (1965) concluded that the dorsal position of beef l. dorsi was most tender based upon S.T.E. shear and puncture data but reported that Warner-Bratzler shear values were lowest (most tender) at the medial position and highest (least tender) at the dorsal position. Both Cover et al. (1962) and Hostetler et al. (1964) reported that the most tender area in the beef l. dorsi was the lateral position. Hedrick et al. (1968) reported that the dorsal core sample location was most tender, however cores in their study were removed perpendicular to the cut surface of the steak.

Little agreement among researchers has been reached concerning the area of the l. dorsi cross-section that is most tender. Two sources of variation among investigations are size of the core sample and method of obtaining the core (Hostetler et al., 1964). Both 1.27 cm and 2.54 cm cores were found suitable by Paul et al. (1955), while Hostetler et al. (1964) reported that when coring was done parallel with the muscle fibers the results were significantly different than if the cores were obtained without regard to the direction of the fibers. One or both of these variables may be responsible for the disagreement among researchers concerning the position effect on Warner-Bratzler shear values. Data reported in Table 11 concerning the correlation between 2.54 and 1.27 cm core shear force values indicates that the relationship, although significant in most instances, is rather low in magnitude (0.14 to 0.56).

Sensory or mechanical measurements of tenderness on one muscle may not apply to other muscles or cuts of meat (Sharrah et al., 1965a; Cover et al., 1957; Cover et al., 1962; and Carroll et al., 1964). To adequately measure l. dorsi tenderness, either to predict its own tenderness or for use as an indicator of carcass tenderness, requires consideration of core position. If 1.27 cm cores are used, it is of interest to know which core or cores are most representative of average shear value from the entire steak. Simple correlation coefficients (Table 7) indicate that little difference in predictive accuracy exists between individual core positions when used to predict average shear value (P-av). Position 6 exhibited the lowest correlation with average shear value (P-av) and thus would be least suitable if only one core was taken (Table 7).

Alsmeyer et al. (1965) suggested that sampling along the l. dorsi cross-section is the preferred practice in assessing tenderness. These workers suggested one core or slice from each of the dorsal, medial and lateral areas. Using all possible combinations of two core positions indicates that combinations of positions 1 plus 4, 5 or 6 and positions 2 plus 5 are most valuable in accounting for the variability in P-av value (Table 8).

When three positions are used (Table 8) the combinations 1, 3 and 6; 1, 4 and 5; 1, 4 and 6; or 1, 5 and 6 have the highest coefficients of determination and smallest standard errors and would be most valuable in accounting for the variability in steak tenderness as measured by average shear force values. These comparisons are based on regression analyses and since most researchers would merely average the position values to

achieve a tenderness rating, Table 9 illustrates the comparative accuracy of regression versus simple correlation assessments. Little difference in predictive capacity is noted thus justifying the use of the more easily determined value.

Marbling

Marbling scores for 95 of the wholesale ribs were studied to determine their relationship to beef rib tenderness. Shear force values for unfrozen steaks, grouped according to marbling scores, were as follows: devoid (5.36 kg), practically devoid (4.86 kg), traces (3.97 kg), slight (4.30 kg), small (4.20 kg), modest (4.03 kg), moderate (3.62 kg), slightly abundant (3.19 kg), moderately abundant (3.49 kg) and abundant (2.15 kg). McBee et al. (1967) reported that the tenderness of beef loin steaks increased with additional degrees of marbling in a linear fashion, but that there was no significant difference with each successive increase in degree of marbling.

In the present study, the relationship between marbling score and shear force values varied considerably when core positions and treatments were compared (Table 10). However, when average shear force values were related to marbling scores, correlation coefficients of from −0.31 to −0.66 resulted. Thus a knowledge of marbling score would be of limited value in predicting tenderness as estimated by shear force values.

Of considerable interest is the fact that marbling score is most closely related to the tenderness of the two least tender core positions in the cross-section of the l. dorsi muscle. Overall correlation coefficients indicate that from 9 to 14% of the variability in shear force value is associated with variation in marbling scores for core positions 1, 2, 3, and 4 while the corresponding coefficients of determination for core positions 5 and 6 are 30

Table 8—Combinations of two or three core positions for predicting average shear force value (P-av) using regression analyses. (Note: All R^2 values significant at the $P < 0.01$ level of significance $n = 633$.)

Variables[1]	Multiple R^2	S.E.E.
P_1 P_2	0.78	1.04
P_1 P_3	0.83	0.91
P_1 P_4	0.85	0.87
P_1 P_5	0.86	0.83
P_1 P_6	0.86	0.84
P_2 P_3	0.84	0.90
P_2 P_4	0.81	0.98
P_2 P_5	0.86	0.84
P_2 P_6	0.81	0.96
P_3 P_4	0.82	0.94
P_3 P_5	0.83	0.92
P_3 P_6	0.83	0.92
P_4 P_5	0.84	0.88
P_4 P_6	0.80	1.00
P_5 P_6	0.80	0.99
P_1 P_2 P_3	0.88	0.77
P_1 P_2 P_4	0.88	0.77
P_1 P_2 P_6	0.90	0.69
P_1 P_2 P_6	0.90	0.70
P_1 P_3 P_4	0.90	0.69
P_1 P_3 P_5	0.91	0.68
P_1 P_3 P_6	0.93	0.61
P_1 P_4 P_5	0.93	0.61
P_1 P_4 P_6	0.93	0.60
P_1 P_5 P_6	0.93	0.61
P_2 P_3 P_4	0.90	0.71
P_2 P_3 P_5	0.92	0.64
P_2 P_3 P_6	0.92	0.62
P_2 P_4 P_5	0.92	0.65
P_2 P_4 P_6	0.89	0.72
P_2 P_5 P_6	0.91	0.65
P_3 P_4 P_5	0.90	0.71
P_3 P_4 P_6	0.90	0.71
P_3 P_5 P_6	0.89	0.73
P_4 P_5 P_6	0.89	0.73

[1] P_1 through P_6 refer to core locations shown in Figure 1.

Table 9—Comparison of regression versus correlation analyses for estimating average position shear force (P-av) using combinations of two or three core positions.[1]

Positions[2]	Regression-R[3]	Correlation-R[4]
P_1 P_4	0.920	0.920
P_1 P_5	0.927	0.927
P_1 P_6	0.926	0.920
P_2 P_5	0.925	0.925
P_4 P_5	0.917	0.917
P_1 P_3 P_6	0.962	0.961
P_1 P_4 P_5	0.962	0.962
P_1 P_4 P_6	0.963	0.960
P_1 P_5 P_6	0.962	0.958
P_2 P_3 P_6	0.960	0.957

[1] $n = 633$.
[2] P_1 through P_6 refer to position locations shown in Figure 1.
[3] Multiple regression for combined variables.
[4] Average values by mean technique rather than weighted combinations used in regression analysis.

Table 10—Simple correlation coefficients between marbling score and shear force values.

Treatment	df	P_1[1]	P_2	P_3	P_4	P_5	P_6	P-av[2]
Unfrozen (A)	52	−0.37**	−0.58**	−0.48**	−0.37**	−0.50**	−0.59**	−0.59**
Frozen wrapped −23°C (B)	52	−0.37**	−0.48**	−0.42**	−0.42**	−0.60**	−0.69**	−0.62**
Frozen unwrapped −23°C (C)	52	−0.47**	−0.57**	−0.50**	−0.40**	−0.62**	−0.69**	−0.66**
Frozen wrapped −34°C (D)	52	−0.36**	−0.43**	−0.40**	−0.40**	−0.69**	−0.60**	−0.59**
Unfrozen (E)	43	−0.39**	−0.11	−0.20	−0.27	−0.53**	−0.56**	−0.47**
Frozen wrapped cooked thawed −34°C (G)	43	−0.25	−0.16	−0.50**	−0.17	−0.47**	−0.37*	−0.46**
Frozen wrapped cooked frozen −34°C (H)	43	−0.22	−0.11	−0.14	−0.03	−0.44**	−0.37*	−0.31*
Total unfrozen (A and E)	97	−0.34**	−0.36**	−0.35**	−0.31**	−0.49**	−0.57**	−0.50**
Total frozen −34°C	142	−0.27**	−0.25**	−0.36**	−0.22**	−0.54**	−0.47**	−0.47**
Overall (A through H)	349	−0.34**	−0.37**	−0.38**	−0.31**	−0.55**	−0.57**	−0.53**

[1] P_1 through P_6 refer to position locations shown in Figure 1.
[2] P-av represents the mean of positions 1, 2, 3, 4, 5 and 6.
* $P < 0.05$.
** $P < 0.01$.

Table 11—Correlation coefficients among various beef rib measurements.

Variables	Cooking loss %	Shear value (P-av)	Shear Value (2.54 cm core)	Shear Value (1.27 cm core)	Chew count	Shear value (deep digital flexor)	Shear value (diaphragm)
Marbling scores	−0.05	−0.53[1]	−0.08	−0.31[2]	−0.35[2]	0.43[1]	0.06
Cooking loss (%)		0.09[2]	0.08	0.27[2]	0.43[1]	−0.15	−0.06
Shear value (P_1)		0.83[1]	0.38[1]	0.59[1]	0.36[1]	−0.03	0.29
Shear value (P_2)		0.79[1]	0.28[2]	0.43[1]	0.28[2]	0.09	0.23
Shear value (P_3)		0.82[1]	0.37[1]	0.51[1]	0.39[1]	0.13	0.43[2]
Shear value (P_4)		0.81[1]	0.26	0.62[1]	0.22	−0.06	0.13
Shear value (P_5)		0.83[1]	0.54[1]	0.49[1]	0.55[1]	−0.02	0.23
Shear value (P_6)		0.75[1]	0.14	0.44[1]	0.22	−0.14	0.44[2]
Shear value (P-av)			0.48[1]	0.74[1]	0.49[1]	−0.01	0.40
Shear value (2.54 cm core)				0.56[1]	0.49[1]	0.38[2]	0.26
Shear value (1.27 cm core)					0.49[1]	0.13	0.17
Chew count						0.18	0.09
Shear value (deep digital flexor)							0.21

[1] $P < 0.01$.
[2] $P < 0.05$.

and 32%, respectively. Furthermore, correlation coefficients of −0.08, −0.31 and −0.35 between marbling score and 2.54 cm core shear force values, 1.27 cm core shear force values and chew counts, respectively, suggests that while marbling score is unrelated to 2.54 cm core tenderness it is significantly ($P < 0.05$) related to both 1.27 cm core tenderness and chew count scores (Table 11).

This further substantiates the premise expressed earlier that differences in core diameter may be responsible for the disagreement concerning gradient differences previously reported in the literature. The magnitude of the overall correlation coefficient (−0.53) between marbling score and shear force value is in partial disagreement with Suess et al. (1966); Walter et al. (1965); Gilpin et al. (1965); Romans et al. (1965); Walter et al. (1963); Wellington et al. (1959); and Blumer (1963) all of whom reported low relationships between marbling score or percent of ether extract with measures of beef tenderness.

The results of the present study are supported by Goll et al. (1965) who reported that the distribution and texture of marbling scores were significantly related to all tenderness measurements made on cooked meat. Tuma et al. (1962b) found that marbling had more influence on tenderness in certain age groups than in others. In the present investigation, marbling score was significantly ($P < 0.01$) related to cooking loss weight and the shear value of the deep digital flexor muscle.

Other relationships

A number of relationships between beef tenderness and cooking loss values are reported in Table 11. Chew count was significantly ($P < 0.01$) related to average shear value, 2.54 cm core shear value, 1.27 cm core shear value, and cooking loss percent. Similar correlations between Warner-Bratzler shear values and chew counts have been reported by Sharrah et al. (1965b) and Burrill et al. (1962).

A correlation coefficient of 0.56 between shear force values for 2.54 cm and 1.27 cm cores although highly significant ($P < 0.01$) was much lower than would have been expected since both sets of cores were taken from the same steak.

The efficacy of using the diaphragm or deep digital flexor muscles for predicting beef rib steak tenderness was investigated and found to be of little value. It is of interest that the shear value of the diaphragm was significantly ($P < 0.05$) related only to core position 3 (the most tender) and core position 6 (the least tender). Tenderness values for these muscles are not sufficiently related to the shear force values of the l. dorsi to serve as effective substitutes for evaluation of beef tenderness.

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Isolation, Identification and Comparison of the Volatiles of Peach Fruit as Related to Harvest Maturity and Artificial Ripening

SUMMARY—Volatiles of peach (Prunus persica L., cultivar, Gleason Early Elberta) fruit were studied by gas-liquid chromatography, thin-layer chromatography and infrared spectrometry. Chromatograms of the volatiles of hard-mature, firm-mature, soft-mature, tree-ripe and artificially ripened, hard-mature fruit were obtained with temperature programing and flame ionization detection. The volatile concentrates of tree-ripe peaches produced 86 peaks. The major peaks were isolated and the infrared spectra determined and compared with authentic compounds.

In general, concentrations of volatile components increased with advancing maturity. The main volatile components were identified as gamma- and delta-lactones, esters, aldehydes, benzyl alcohol and d-limonene.

The highest total lactone concentration occurred in tree-ripe peaches and was more than four times that of firm-mature fruit. Gamma-decalactone predominated among the lactones in tree-ripe peaches. Artificially ripened peaches had very small amounts of gamma-decalactone and lacked gamma- and delta-dodecalactone, with a total lactone concentration about one-fifth that of tree-ripe fruit. Concentrations of esters in artificially ripened fruit reached only one-third to one-half those of tree-ripe peaches. Benzaldehyde was the predominant volatile in tree-ripe peaches and occurred in five times the concentration found in artificially ripened fruit. These may be the determining factors relative to the inferiority of artificially ripened as compared to tree-ripened fruits.

INTRODUCTION

AROMA generally is a factor in evaluating fruit quality. Fruit aroma is believed to involve minute quantities of various volatile substances such as esters, aldehydes, ketones, alcohols, hydrocarbons, etc., in the fruit. Its size, shape, color and composition change as the fruit grows, ripens and deteriorates.

Several investigations with respect to aroma of fruit during ripening are reported (Grevers et al., 1965; Brown et al., 1966; Andersson et al., 1966; Wick et al., 1966; Antoniani et al., 1954; Antoniani et al., 1955; Serini, 1956; and Jennings et al., 1964).

Lim et al. (1964) attempted to rationalize fruit maturity differences in terms of volatile constituents. They reported that presence of detectable volatiles was correlated with the maturity of peaches and nectarines when harvested. To supply distant markets, peaches are generally harvested while on the green side in anticipation of post-harvest ripening during shipping and holding in marketing channels.

A knowledge of the chemical constituents responsible for aroma of peaches at different maturities might facilitate development of a rational and objective method assuring high quality fruits for consumption as well as for processing.

The primary aim of this investigation was to separate, identify and compare the major components of peach volatiles (cultivar, Gleason Early Elberta) as related to harvest maturity and artificial ripening.

EXPERIMENTAL

Fruit selection

Gleason Early Elberta peaches were harvested at four different stages of maturity from the University's Howell experimental orchard in North Ogden, Utah, from 1964 to 1967. A pressure tester (equipped with $^{5}/_{16}$ in. plunger) developed by Magness et al. (1925) was used in classifying the maturities (Deshpande, 1964). The maturity terms suggested by Haller (1952) for describing peaches were followed. Harvested fruits were promptly sorted, pitted, packed in vacuum sealed containers and stored at −18°C. Some hard-mature fruit was artificially ripened at room temperature (21°C) with 35% relative humidity prior to pitting, packing and storage.

Peak volatiles concentrate

Four kg of partly defrosted fruit were blended in a Waring blendor. The homogenates were saturated with ammonium sulfate and extracted with benzene (thiophene free). The extract was decolorized with active carbon, freeze concentrated and steam distilled. The organic layer of the steam distillate was separated from the aqueous layer by a separatory funnel. Ethyl chloride was

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employed in extracting the aqueous layer and this extract was recombined with the benzene portion. The resulting extract was dried over anhydrous magnesium sulfate, with most of the benzene being removed by freeze concentration followed by evaporation. The concentrate, approximately 200 μl, was stored at −18°C.

Gas chromatography

A Micro-Tek GC 2500 R gas chromatograph with dual flame ionization and thermal conductivity detectors was used in the analysis of the aroma concentrate. Two types of stationary liquid phases were selected for the separation and identification procedure. Methyl silicone polymer SE-30 (nonpolar) and Carbowax 20 M (polar) at 10% w/w on Chromosorb P (HMDS treated, 60–80 mesh) were packed in 20 ft × 1/4 in. o.d. and 10 ft 1/4 in. o.d. stainless steel columns respectively. The operating conditions used were:

Initial temperature	70°C for 15 min
Temperature program	3°C/min
Final temperature	SE-30 (230°C) Carbowax 20 M (210°C)
Flow rate	65 ml/min of helium
Injection port temperature 200°C	200°C
Recorder	1 mv full-scale Westronics Model LS11A
Sample size	10–50 μl

The aroma concentrate was co-chromatographed with a purified authentic compound as an internal standard to facilitate the identification of the resolved peaks under the same temperature program as in the original analysis.

To collect the individual fractions separated by gas liquid chromatography for use in identification by infrared spectrometry, a cold trap was constructed of teflon and glass capillaries immersed in a dry ice-acetone mixture. A section of 18 × 1/6 in. i.d. glass capillary tubing was bent to form a U-shape and a 2.5 × 1/8 in. i.d. Teflon tubing was attached to one end of the capillary. The short section of Teflon tubing enabled a tight connection to be made between the outlet of the gas chromatograph which is 1/9 in. o.d. stainless steel tubing, and the U capillary. Flexibility of the Teflon minimized the possibility of breaking the glass capillaries kept in the dry ice-acetone mixture in a Dewar jar. The outlet of the gas chromatograph was heated by a heating tape at 250°C to prevent condensation of individual fractions before they left the gas chromatograph. The thermal conductivity detector was used during trapping operations.

Thin-layer chromatography

Thin-layer chromatographic plates (250 μ adsorbent thickness) were prepared with a Kensco apparatus on 20 × 20 × 0.3 cm glass. Silica gel G and aluminum oxide G were the two adsorbents. The coated plates were activated at 115°C for 30 min before use.

The peach volatile concentrate (prepared as described in the previous section) was treated with 2,4-dinitrophenylhydrazine reagent (Shriner et al., 1964) for derivatization of carbonyl compounds in the concentrate. The reaction mixture was evaporated to a nearly dry state and taken up with 2 ml of chloroform. The 2,4-dinitrophenylhydrazone derivatives of authentic aldehydes and ketones available in our laboratory were prepared according to Shriner et al. (1964).

Samples (unknown and authentic) were spotted 1.5 cm from the bottom edge of the plates and 2 cm apart. All plates were allowed to develop to a height of 15 cm from the bottom edge of the plates. The solvent containing tanks were equilibrated before the plates were introduced. The solvent system consisted of benzene-hexane-chloroform (1:1:1.5, v/v).

Infrared spectrometry

Infrared spectra of the individual peaks were determined in carbon tetrachloride solutions in a NaCl microcavity cell ("D" type, 0.1 mm path, Connecticut Instrument Corp.) with 5 × beam condenser and beam attenuator on a Beckman IR-8 infrared spectrophotometer. The trapped peaks from a 4 kg sample were rinsed from the capillaries with a minimum amount of spectro grade carbon tetrachloride into a 1 ml teflon beaker and carefully evaporated to ca. 5–10 μl. They were then taken up by a Hamilton syringe and transferred to the NaCl cell. A compensating wedge cell, containing spectro grade carbon tetrachloride was placed in the reference beam during spectra determinations.

RESULTS & DISCUSSION

GAS CHROMATOGRAMS of peach volatiles (cultivar, Gleason Early Elberta) were obtained for four different maturities. Figure 1 shows those of tree-ripe and artificially ripened fruit. These chromatograms were obtained from a 10% SE-30 column with temperature program and flame ionization detection. A total of 86 peaks were separated from the volatile concentrate of tree-ripe peaches. Corresponding but lesser peaks in the chromatograms of hard-mature, firm mature, soft-mature and artificially ripened peach volatiles were numbered in the same manner as those of the tree-ripe peach volatiles. Peak S and peaks 1 to 11 indicated the solvent, benzene and accompanying impurities.

Concentrations of identified individual components of peach volatiles at different stages of maturities (Table 1) were calculated from the results of gas chromatography. These figures may not be the exact concentrations of individual components because of the conditions employed in the preparation of the aroma concentrate, such as charcoal decolorization etc. However, it may represent the trend in concentration changes and serve for the purpose of comparison.

Gamma and delta lactones

Peaks 26, 53, 62, 71, 82 and 85 (Fig. 1) were identified as gamma-valerolactone, gamma-octalactone, gamma-decalactone, delta-decalactone, gamma-dodecalactone and delta-dodecalactone respectively (Table 1). The relative retentions of lactones are shown in Table 2. The infrared spectra of lactones isolated from the peach volatiles and of the authentic

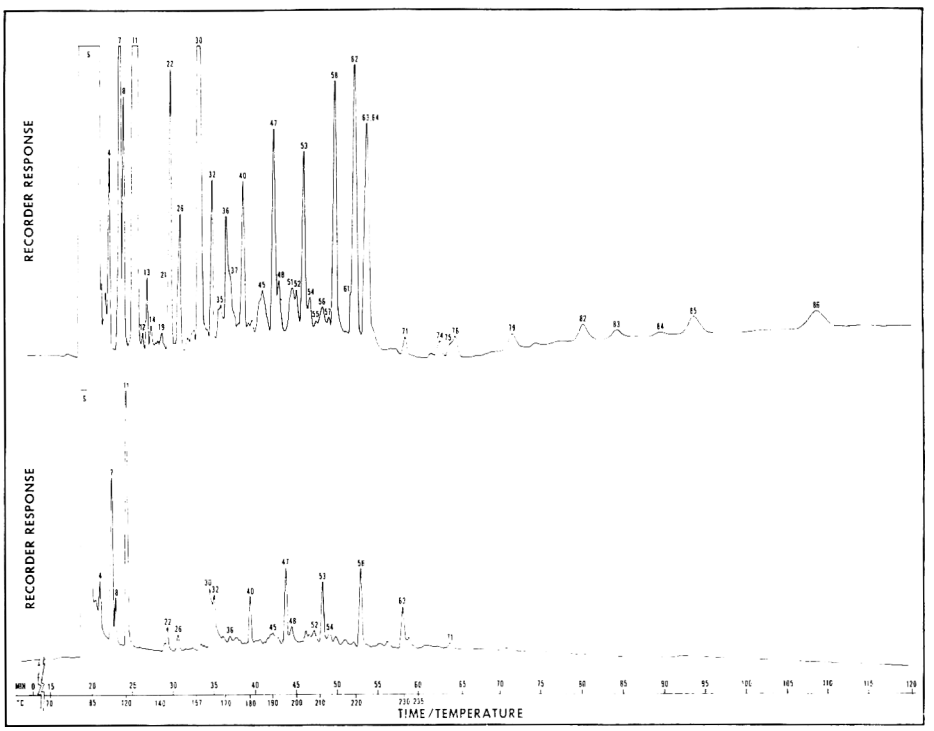

Fig. 1—Chromatograms of Gleason Early Elberta peach volatiles, tree ripe (top) and artificial ripe (bottom).

compounds were in good agreement (Fig. 2). The C=O stretching vibrations of saturated gamma-lactones absorb at shorter wavelengths than delta-lactones (1795–1760 cm^{-1}; 5.57–6.68 μ). Absorption due to C—O stretching in lactones is observed in the 1250–1111 cm^{-1} (8.00 – 9.00 μ) region (Silverstein et al., 1967).

Peak 74 (Fig. 1) was tentatively identified as an unsaturated lactone. The infrared spectrum of peak 74 showed two bands in the region of the spectrum associated with the C=O stretching vibrational mode at 1765 and 1720 cm^{-1}. This is common to most unsaturated five- and six-membered ring lactones in which the double bond is conjugated with the carbonyl group (Silverstein et al., 1967; Jones et al., 1959). The C—O stretching of lactone appeared at 1170 cm^{-1}. Absorptions at 2935, 2860, 1455 and 1395 cm^{-1} were considered CH_2 and CH_3 groups.

Table 2—Relative retentions of peach lactones on SE-30 and Carbowax 20 M.[1]

	SE 30		Carbowax 20 M	
	Knowns	Peach	Knowns	Peach
γ-valerolactone	0.25	0.28	0.29	0.30
γ-octalactone	0.43	0.44	0.46	0.49
γ-decalactone	1.00	1.00	1.00	1.00
δ-decalactone	1.18	1.19	1.29	1.30
γ-dodecalactone	1.86	1.88	1.98	1.99
δ-dodecalactone	2.22	2.25	2.36	2.40

[1] Conditions as indicated in the experiment section except at 190° C.

The presence of several lactones, gamma - caprolactone, gamma - octalactone, gamma-decalatone and deleta-decalactone, Red Globe peach essence have been reported (Jennings et al., 1964a). In this study, gamma-valerolactone, gamma dodeclactone and delta-dodecalactone were identified in the volatiles of Gleason Early Elberta peaches. Gamma-decalactone was highest in concentration among the lactones in the volatiles of tree ripe peaches. By contrast, the volatiles of artificially ripened peaches had a very low concentration of gamma decalactone, and no gamma or delta dodecalactones.

The concentration of total lactones was highest in tree ripe peaches and was more than four times that of firm mature fruit. Artificially ripened peaches had about one-fifth the lactones of the tree ripe peaches. Some lactones possess coconut-like odor and do not resemble peach aroma. Others, however, do have a peachy odor and are important contributors to the aroma of the peaches.

Table 1—Volatile components and their concentrations found in Gleason Early Elberta peach (1967).

Peak	Compound	Identification method	Stages of maturity (Concentration, × 10^{-2} ppm[7]) Hard mature	Firm mature	Soft mature	Tree ripe	Artificially ripe
S, 1–11	Solvent						
12	Isopentanal	R[3], ET[4]	0	0	0	5.0	0
13	Methyl isovalerate	R. ET	0	0	4.0	24.0	0
14–21	U[1]						
22	Isopentyl acetate	R, ET	2.0	8.5	20.5	54.0	12.0
23–25							
26	γ-valerolactone	R, ET	1.0	4.6	13.0	45.0	10.0
27–20	U						
30	Benzaldehyde[2]	R, ET, IR[5], TLC[6]	2.2	9.1	37.6	115.0	25.0
31	U						
32	Hexyl acetate[2]	R, ET	2.0	6.8	16.7	57.0	23.0
33, 34	U						
35	Benzyl alcohol[2]	R, ET	0	3.5	5.0	44.5	9.0
36	d-limonene	R, ET	0	3.0	7.0	26.0	9.0
37–39	U						
40	Benzyl acetate[2]	R, ET	3.5	15.0	27.0	56.0	22.3
41–46	U						
47	Ethyl benzoate[2]	R, ET, IR	5.3	27.5	37.7	73.5	32.3
48	Methyl salicylate	R, ET	1.0	8.3	11.5	23.0	12.5
49–52	U						
53	γ-octalactone[2]	R, ET, IR	4.7	26.5	31.0	66.0	27.2
54–57	U						
58	Hexyl benzoate[2]	R, ET, IR	6.2	37.7	41.5	90.0	31.7
59–61							
62	γ-decalactone[2]	R, ET, IR	0	4.8	27.4	95.2	4.0
63	Ester (?)	IR	3.5	19.3	29.5	75.5	19.0
64–70	U						
71	δ-decalactone[2]	R. ET, IR	0.5	2.5	4.0	7.0	3.0
72–73	U						
74	Unsaturated lactone (?)	IR	0	0	2 5	6.0	1.0
75	U						
76	Ester (?)	IR	0	1.2	4.0	7.0	0
77, 78	U						
79	Ester (?)	IR	0	0	3.0	12.0	0
80 81	U						
82	γ-dodecalactone	R, ET, IR	0	0	7.0	15.0	0
83	U						
84	Ester (?)	IR	0	0	2.0	3.0	0
85	δ-dodecalactone	IR	0	0	4.0	25.0	0
96	Ester (?)	IR	0	0	6.0	30.0	0

[1] U = unidentified.
[2] Compounds identified by Jennings et al. (1964) and Sevenants et al. (1966) in Red Globe peach essence.
[3] R = Relative retention times of volatile components compared with authentic compounds on gas chromatograph using 20 ft × 1/4 in. o.d. SE-30 column and 10 ft × 1/4 in. o.d. Carbowax 20 M column, 150° and 190°C.
[4] ET = Enrichment technique, cochromatography with authentic compound.
[5] IR = Infrared spectrometry.
[6] TLC = Thin-layer chromatography.
[7] Concentrations are mean values of three determinations, repeatability of peak area between determination was ±10%.

Esters

Peaks 13, 22, 32, 40, 47, 48, 58 (Fig. 1) were identified as esters (methyl isovalerate, isopentyl acetate, hexyl acetate, benzyl acetate, ethyl benzoate, methyl salicylate and hexyl benzoate, respectively). The infrared spectra of ethyl benzoate isolated from the peach volatiles and that of the authentic compound matched closely. Absorption bands in the region of 1730–1715 cm^{-1} indicate aromatic ester carbonyl, 3030 cm^{-1} and 1950 cm^{-1} are the characteristics of benzene ring. The absorption bands at 1950 to 1880 cm^{-1} also represent the substituted benzene ring. Aromatic ester C—O stretching appeared to 1445 to 1360 cm^{-1} (Silverstein et al., 1967). There was an excellent match between the infrared spectra of peak 58 and the authentic hexyl benzoate.

In addition to the above identified esters, five more components (peaks 63, 76, 79, 84 and 86) were tentatively identified as esters from their infrared spectra. Peak 63 was separated from peak 64 by rechromatography and retrapping.

Presence of other esters, methyl isovalerate, isopentyl acetate and methyl salicylate, not previously reported as components of peach volatiles, was also observed in this study.

Hexyl benzoate, ethyl benzoate, benzyl acetate and an unidentified saturated aliphatic ester (peak 63) dominate the esters in the volatiles from hard mature peaches. Concentration of esters increased with the on-going ripening process. In artificially ripened fruit, however, it reached only one-third to one-half that of tree-ripe peaches.

Aldehydes and others

Peak 30 was identified as benzaldehyde after rechromatography and infrared spectrum determination. Thin-layer chromatography of 2,4-dinitrophyl-hydrazones of the peach volatile concentrate

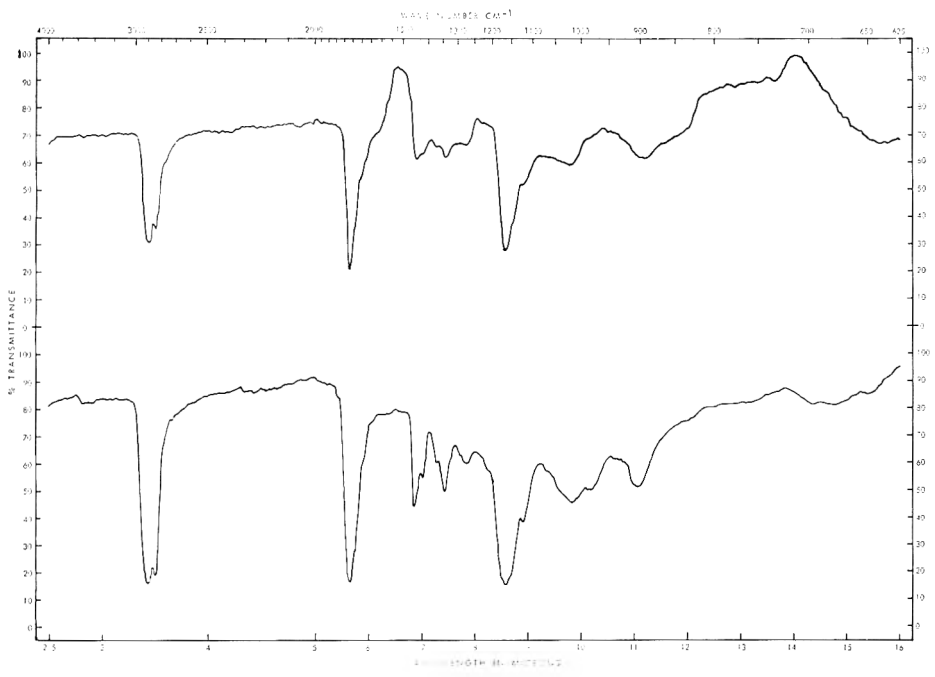

Fig. 2—Infrared spectrum of Gleason Early Elberta peach volatile matched with the authentic compound gamma-dodecalactone.

and of authentic benzaldehyde indicated the presence of this aromatic aldehyde. A pronounced increase in concentration of benzaldehyde was observed at the firm mature stage, and it became the predominating component in the peach volatiles at the tree-ripe stage. The artificially ripened peaches had about one-fifth as much benzaldehyde as occurred in tree-ripe fruits. Isopentanal, benzyl alcohol and d-limonene were also tentatively identified in the peach volatile concentrate.

In general, main constituents of the identified components of the volatiles of Gleason Early Elberta peaches from hard-mature to tree-ripe stages and artificially ripened were gamma and delta lactones, acetate and benzoate esters and benzaldehyde. The lower concentrations of lactones, esters and benzaldehydes might partially account for the low aroma of the artificially ripened peaches. No single compound could be identified as entirely responsible for the characteristic odor of peaches.

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Glycolytic Activity of Chicken Breast Muscle Mitochondria

SUMMARY—A procedure is described for the preparation of chicken breast muscle mitochondria which are capable of aerobic respiration or anaerobic lactate production utilizing any of several glycolytic intermediates including glucose. This indicates that the complete complement of glycolytic enzymes is associated with the preparation. The hexokinase-catalyzed phosphorylation of glucose is the rate-limiting step both aerobically and anaerobically. The aerobic oxidation of glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate is equal to or greater than that with succinate. This high level of glycolytic enzymes associated with the mitochondrial preparation argues against the results being due to an artifact of preparation. This demonstration of the association of the glycolytic complex with the mitochondria of muscle is the first other than with neural tissue and may have important implications in post-mortem carbohydrate catabolism.

INTRODUCTION

THE RELATION between structure and function at the subcellular level is rapidly gaining recognition as important in metabolic control. It is well accepted that mitochondrial energy production and electron transport are closely related to the structural organization of this multi-enzyme system. Green et al. (1966) have suggested that all major energy-producing pathways of the cell may be membraneous bound. Helmreich et al. (1965) and Margreth et al. (1963) discussed the relationship between structural organization and regulation of glycolysis in muscle cells. They suggested that a special localization or organization of the glycolytic enzymes in the muscle cell may regulate enzyme activity and control the on and off contraction-relaxation cycle of muscle.

Rothstein et al. (1959) reached a similar conclusion for yeast cells, viz., the glycolytic system is organized in a manner which is dependent on the integrity of the cell ultrastructure. A further and more general discussion of the importance of subcellular structure to metabolic activity can also be found in a paper by Siekevitz (1962).

Various glycolytic enzymes have been found associated with the particulate fractions of various tissues by both histochemical (Fahimi et al., 1966; Pearce et al., 1959; Pette et al., 1962) and isolation techniques (Hernandez et al., 1966; Amberson et al., 1965; Margreth et al., 1967; Mansour et al., 1966; Rose et al., 1967). Others (Hesselbach et al., 1953; Gallagher et al., 1956; Abood et al., 1959) have shown that the complete glycolytic system is present in a mitochondrial fraction isolated from rat brain.

Johnson (1960) doubts that such a relationship exists in vivo and has presented evidence to show that the glycolytic enzymes, other than hexokinase (HK), are derived from nerve fibers which entrap soluble enzymes and sediment with the mitochrondrial fraction. However, Beattie et al. (1963) prepared mitochondria from beef brain which were shown to be relatively free of contamination by non-mitochondrial particles. These mitochondria were able to metabolize glucose to lactic acid but at a much slower rate than reported by others. Also, Balazs et al. (1959) found 10% of the total glycolytic activity of rat brain associated with their mitochondrial preparation.

Study of the subcellular distribution of lactic dehydrogenase (LDH) in chicken breast muscle (Hultin et al., 1967) showed that there was a relatively high specific activity of LDH in a mitochondrial fraction. The possibility that other glycolytic enzymes may also have sedimented with this fraction was considered. This report presents evidence which shows that a mitochrondrial fraction can be prepared from chicken breast muscle which contains the complete glycolytic system.

EXPERIMENTAL

Materials

The chickens used were obtained from a flock maintained by the Department of Veterinary and Animal Science at the University of Massachusetts. Only female birds were used, and age and feeding habits were not controlled.

The biochemicals used were obtained as indicated: ATP and DPN$^+$(P-L Biochemicals Inc), cytochrome-*c*(Sigma, horse heart Type III), nicotinamide, glucose, glucose-6-phosphate, fructose-6-phosphate, and fructose-1,6-diphosphate (Sigma), o-phenanthroline (Matheson, Coleman and Bell). Other chemicals used were the purest available commercially. The enzymes used were yeast hexokinase (Sigma) and yeast lactic dehydrogenase (Nutritional Biochemicals Corporation).

Preparation of mitochondria

Chickens were sacrificed by the injection of air into the heart. The breast muscle was immediately excised and immersed in ice-cold distilled water. The following procedure was carried out without delay and all steps were conducted at 0–4°C.

Gross connective and adipose tissues were removed and the muscle weighed and sliced into 1 cm cubes. A 6.5% (weight of tissue/volume homogenizing medium) homogenate was made by blending for 30 sec in a solution 0.25 *M* in sucrose and 15 m*M* in histidine-HCl, pH 7.0 (Solution A). The homogenate was filtered through two layers of cheesecloth and the pH determined on a Radiometer pH meter standardized with cold buffer. The pH ranged from 6.7–7.0. The homogenate was centrifuged at 1600 rpm for 15 min in an IEC Model PR-6 centrifuge (rotor 269). The supernatant fraction was decanted and saved. The residue was resuspended in solution A and centrifuged at 2100 rpm for 15 min in the IEC centrifuge.

After decanting the supernatant fraction, the residue was washed a second time in 0.5 *M* sucrose, 15 m*M* histidine-HCl, pH 7.0 solution, and centrifuged at 3000 rpm for 15 min in the same centrifuge. All supernatant fractions were combined and centrifuged in the No. 19 rotor of a Spinco Model L-2 ultracentrifuge at 19,000 rpm for 1 hr. The supernatant fraction was discarded and the residue was resuspended in a volume of solution A equal to about $^1/_8$ of the original homogenizing medium. This resuspended residue

Table 1 Aerobic oxidation of glycolytic intermediates by skeletal muscle mitochondria.

Substrate	Respiration, μl O_2/mg protein/hr
Glucose (10 μmoles)	5.1 (4)
Glucose (20 μmoles)	5.1 (2)
Glucose + 0.4 units HK	36.2 (2)
Glucose-6-phosphate	49.7 (4)
Fructose-6-phosphate	56.8 (2)
Fructose-1,6-diphosphate	20.2 (2)
Succinate	37.5 (2)
Succinate[1]	33

The mitochondria and assay medium were prepared as described in the text. The numbers in parentheses indicate the number of replicate samples assayed. The amount of mitochondrial protein added to each flask was 4–6 mg.

[1] Brain mitochondria, adapted from DuBuy et al. (1956).

was centrifuged for 10 min at 2250 rpm in a Sorvall RC-2 centrifuge (rotor SS-34) and the residue discarded.

The supernatant fraction was centrifuged in the same centrifuge at 11,000 rpm for 30 min. The resulting reddish-brown residue was resuspended in a volume of solution A to yield a final protein concentration of from 8–12 mg per ml. This mitochondrial fraction was immediately frozen and stored at −12°C. Microscopic examination of this fraction using a Zeiss phase contrast microscope at 1000X magnification revealed essentially no nuclear or myofilbrillar contamination. Microsomal contamination was also judged to be negligible as reported in a previous paper (Hultin et al., 1967).

Protein determination

Protein was determined by the procedure of Gornall et al. (1949) using bovine serum albumin as a standard.

Succinoxidase activity

Succinoxidase activity was measured using a Gilson Differential Respirometer in an atmosphere of air at 30°C. The flasks contained 0.2 ml of 20% KOH in the center well. The incubation medium consisted of ATP (3 μmoles), DPN^+ (0.6 μmoles), cytochrome c (0.1 μmole), phosphate buffer, pH 7.0 (6 μmoles), $MgCl_2$ (12 μmoles), succinate (10 μmoles) and 4–6 mg of mitochondrial protein in a total volume of 3.0 ml. The flasks were equilibrated for 10 min prior to addition of the mitochondria from the side well. Oxygen consumption was measured for 1 hr at 10 min intervals. Samples were assayed in duplicate and blanks without mitochondria and without substrate were subtracted from the sample readings.

Glycolysis

Glycolysis was determined anaerobically in an atmosphere of nitrogen at 30°C in a Dubnoff-Metabolic Incubation (Precision Scientific) shaker. The reaction medium consisted of ATP (3 μmoles), DPN^+ (0.2 μmoles), nicotinamide (40 μmoles), $KHCO_3$ (25 μmoles), phosphate buffer pH 7.0 (2.0 μmoles), $MgCl_2$ (40 μmoles) and substrate (10 μmoles except where noted otherwise) in a final volume of 3.0 ml. Mitochondrial protein equivalent to 4–6 mg was added, and at the end of one hour the reaction was stopped by the addition of 0.2 ml of 0.5 M perchloric acid.

Respiration with glycolytic substrates

When the oxidation of glycolytic intermediates was determined aerobically the amount of oxygen consumed was measured as for succinoxidase activity using the glycolytic reaction medium plus cytochrome c (0.1 μmole).

Lactic acid determination

Lactic acid was determined on the perchloric acid extract by the procedure of Schön (1956). All colorimetric determinations were done on a Beckman Model DU-2 spectrophotometer.

RESULTS

MITOCHONDRIA were isolated from chicken breast muscle by the procedure described and glucose was added as the only oxidizable substrate in an aerobic system. As shown in Table 1 a net uptake of oxygen resulted which was not limited by the amount of glucose used but by the rate of its phosphorylation by hexokinase (HK). This was indicated by the addition of yeast HK to the mitochondria in the presence of glucose which stimulated an 8-fold increase in oxygen consumption. Also the rate of glucose-6-phosphate (G-6-P) oxidation by the mitochondria proceeded at a rate always greater than the rate with glucose as substrate. This indicates that HK catalyzes the rate-limiting step in the oxidation of glucose under our assay conditions. This mitochondrial fraction, therefore, contains both the glycolytic and respiratory system with HK the rate-limiting step. The rates with fructose-6-phosphate (F-6-P) and fructose-1,-6-diphosphate (FDP) are also shown, and the rate with succinate is given for comparison.

Table 2 Anaerobic glycolysis by skeletal muscle mitochondria.

Substrate	mμmoles lactate/mg protein/hr
Glucose	83 (2)
Glucose-6-phosphate	1620 (2)
Fructose-6-phosphate	235 (1)
Fructose-1,6-diphosphate	145 (2)
Glucose[1]	890
Glucose[2]	170

The mitochondria and assay media were prepared as described in the text. The numbers in parentheses indicate the number of replicate samples assayed. The amount of mitochondrial protein added to each flask was 4–6 mg.

[1] Brain mitochondria, adapted from Hesselbach et al. (1953).

[2] Brain mitochondria, adapted from Beattie et al. (1963).

Anaerobically HK is also rate-limiting as shown in Table 2. Glucose-6-phosphate is metabolized at 20 times the rate of glucose. The results of only two experiments are shown. Other experiments showed a similar 20-fold increase in G-6-P utilization although the net amount of lactate produced varied considerably from one preparation to another. The rates with F-6-P and FDP were less than with the G-6-P but considerably greater than with glucose.

For comparison, two examples of anaerobic glycolysis by brain mitochondria utilizing glucose as substrate reported by other workers are presented. In both cases the lactate produced is considerably higher than what this study found with the muscle mitochondria with glucose as substrate but of the same order of magnitude comparing their rates with glucose and the authors' with the sugar phosphates.

Finally a series of experiments was carried out for respiration studies. At the terminus of the experiment the lactate formed was measured. The respiratory medium was used in these experiments. Again, the results of other workers with brain mitochondria are given for comparison. The authors' results with glucose were much less, but results with the phosphorylated sugars were comparable to these other systems. The respiratory activity of the muscle mitochondria with glucose was very low, and no detectable lactate was produced.

When calculations are made to compare the moles of O_2 consumed to the moles of lactate produced on the basis that one mole of substrate is equivalent to 6 moles of O_2 and 2 moles of lactate, it is seen that lactate production is the major aerobic pathway with the sugar phosphates in this system; thus, in the case of G-6-P, 69% of the substrate is converted to lactate and 31% is respired to CO_2 and H_2O.

DISCUSSION

A MITOCHONDRIAL fraction isolated from chicken breast muscle was shown to contain the complete glycolytic complex of enzymes. This has not been reported before for any mitochondria other than those isolated from brain tissue. Data on the purity of the mitochondrial fraction were discussed in an earlier report (Hultin et al., 1967). Contamination by other subcellular entities was judged to be very slight; in most cases there was no contamination detectable by biochemical analysis.

The possibility that the association of the glycolytic enzymes with the mitochondria is an artifact of the preparatory precedure must be considered. However, one point casts considerable doubt that it is an artifact. With the phosphorylated sugars the rate of O_2 consumption by the mitochondria is as high or higher than with succinate. Hence, the level of glycolytic enzymes is great enough to not be rate-limiting in the sequence from the glycolytic substrates to termination of oxidation. The one exception found was the hexokinase-catalyzed reaction which was rate-limiting in this system.

As shown by other workers (see Table 3 for example), the phosphorylation of glucose is not rate-limiting in the case of brain mitochondria. This organ difference may be related to differences in carbohydrate metabolism in the two tissues. The brain utilizes glucose as its chief substrate (Brunngraber et al., 1960) while glycogen is the principal energy source of muscle, especially white muscle (Burleigh et al., 1968); hence, there would be less need for hexokinase in carbohydrate catabolism in muscle compared to brain. It is difficult to envision artifactual association of all the glycolytic enzymes with mi-

Table 3 Respiration and lactate production by skeletal muscle mitochondria determined simultaneously under aerobic conditions.

Substrate	Respiration, μl O_2/mg protein/hr	Lactate, mμmoles/mg protein/hr
Glucose (2)	3.2	0
Glucose-6-phosphate (2)	43	1450
Fructose-1,6-diphosphate (1)	20	9150
Glucose[1]	22	430
Glucose[2]	30	1500
Glucose-6-phosphate[2]	19	620
Fructose-1,6-diphosphate[2]	29	1930

The mitochondria and assay media were prepared as described in the text. The numbers in parentheses indicate the number of replicate samples assayed. The amount of mitochondrial protein added to each flask was 4–6 mg.

[1] Brain mitochondria, adapted from Hesselbach et al. (1953).

[2] Brain mitochondria, adapted from DuBuy et al. (1956).

tochondria at a level such that respiration can be fully maintained with the glycolytic substrates. It is more reasonable to propose that these enzymes are associated with the mitochondria in vivo.

Respiratory rates were lowest wih glucose as substrate. In addition, rates of respiratory activities with G-6-P and F-6-P were higher as compared to FDP or succinate. At first this might seem due to using only ATP and no ADP in the reaction medium. Using G-6-P or F-6-P would allow production of ADP from ATP by the action of phosphofructokinase. This ADP would then serve as phosphate acceptor for the respiratory chain enzymes. If this were the explanation, then 0_2 consumption with FDP or succinate should be accelerated by the replacement of part or all of the ATP with ADP. Experiments of this type were tried with both succinate and FDP, but 0_2 consumption was not affected. Either the rate of ADP production from ATP by the system (probably by an ATPase) is faster than required for respiratory activity, or the respiratory system is uncoupled from phosphorylation and does not require a phosphate acceptor. It is not certain which of these is correct as yet since initially the present experiments were only trying to show whether the glycolytic enzymes were present.

Exogenous ADP does not increase the rate of 0_2 consumption with FDP compared with F-6-P, yet the principal contribution of the phosphofructokinase-catalyzed reaction converting F-6-P to FDP is to produce ADP. It is logical to conclude that the ADP produced by phosphofructokinase is more available for respiration than exogenous ADP. This in turn implies that there is a special structural relationship of phosphofructokinase to the apparatus of the respiratory chain. Preliminary results indicate that phosphofructokinase is associated in part with the mitochondria and its relationship to respiration is under investigation.

The significance of the apparent localization of the glycolytic enzymes with mitochondria in post-mortem metabolism of carbohydrate in chicken breast muscle is obscure. However, it is difficult to conceive that such a specific subcellular location would not have a major bearing on the course of glycolysis.

Helmreich et al. (1965) have suggested that "a special localization and organization of the glycolytic enzymes in the structure of muscle . . . would explain why total concentrations of reactants in tissues often are not well correlated to changes in enzymatic rates and why enzymes in vivo irrespective of flow rate seemingly do not establish the equilibrium attained in vitro." These authors then suggest that the glycolytic enzymes might be localized in the sarcoplasmic reticulum. This report has presented experimental evidence that the complete sequence of glycolytic enzymes is associated with a mitochondrial fraction of muscle at a level such that the complete oxidation of the phosphorylated sugars is not limited by the glycolytic sequence.

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Volatile Components of Raw Peanuts: Analysis by Gas-Liquid Chromatography and Mass Spectrometry

SUMMARY—The volatile components associated with the aroma and flavor of raw peanuts have been studied. These components were isolated using low-temperature vacuum distillation and cryogenic trapping. Ten components were identified from the liquid N_2 trap using the combined technique of gas-liquid chromatography and mass spectrometry. The "backbone" compound of the raw peanut aroma appears to be hexanal, with other components adding the proper character.

INTRODUCTION

VOLATILE components associated with typical flavor and aroma of roasted peanuts and with high temperature-cured, off-flavored raw peanuts previously have been investigated. Mason et al. (1967) isolated and identified monocarbonyl compounds as well as nitrogenous components from roasted peanuts. Pattee et al. (1965) cured peanuts at high temperature (55°C) to produce off-flavor, and subsequently isolated and identified certain of the components felt to be responsible for the flavor defect. However, information concerning the volatile components associated with the characteristic normal flavor and aroma of raw peanuts is lacking. To define more accurately chemical defects produced in raw peanuts by improper treatment, the volatile components associated with the good-quality raw product have been studied.

EXPERIMENTAL

Sample treatment

Good-quality peanuts (Variety NC-2) were produced according to the recommended procedures of Beasley et al. (1963). The peanuts were dug, windrowed and field dried to approximately 25% moisture content. They were then combined and placed in forced-air drying bins to a depth of 4 ft. Unheated air was circulated through the peanuts until moisture content was 8%. The samples were stored at 5°C and 50% relative humidity until analyzed.

Preparation of sample for GLC

Volatiles from the peanuts were removed and trapped using low-temperature vacuum distillation and differential cryogenic trapping. Figure 1 shows the apparatus used.

A slurry of 1 kg peanuts blended with 2 L distilled water was placed in a 12-L three-neck distilling flask and attached to the vacuum manifold. The peanut slurry was distilled for 3 hr at 3 mm (±1) without any external heating of the distillation pot. Cryogenic traps used were: (1) wet ice (0°C), (2) dry ice-acetone (−78°C), and (3) liquid N_2 (−196°C).

GLC vapor analysis

Volatile constituents were separated for qualitative retention data with a Micro-Tek model 2500 gas chromatograph equipped with dual-flame ionization detector.

Samples were analyzed using the following columns: 10% Carbowax 20M on 60–80 mesh firebrick super 22 (1/8 in. × 10 ft), operated isothermally at 50°C; 15% polyethylene glycol on 60–80 mesh firebrick super 22 (1/4 in. × 6 ft), 70°C; and a 10% polyethylene glycol column on 60–80 mesh celite (1/8 in. × 19 ft) at 115°C. Helium was used as the carrier gas with flow rates of 15–20 ml/min.

Analysis of the aqueous distillate from the dry ice-acetone trap showed GLC patterns almost identical to those exhibited by the material from the liquid N_2 trap. Since the concentration of vapors was much greater in the liquid N_2 trap, it was subsequently used for all analyses.

After removal of the liquid N_2 trap from the vacuum manifold system the transfer apparatus (Fig. 2) was rapidly inserted and the sample trap reevacuated at liquid N_2 temperature. The sample trap was allowed to warm to room temperature and placed in a water bath maintained at 55°C for not less than 3 min. A 5-ml vapor sample was withdrawn and injected into the GLC. Retention

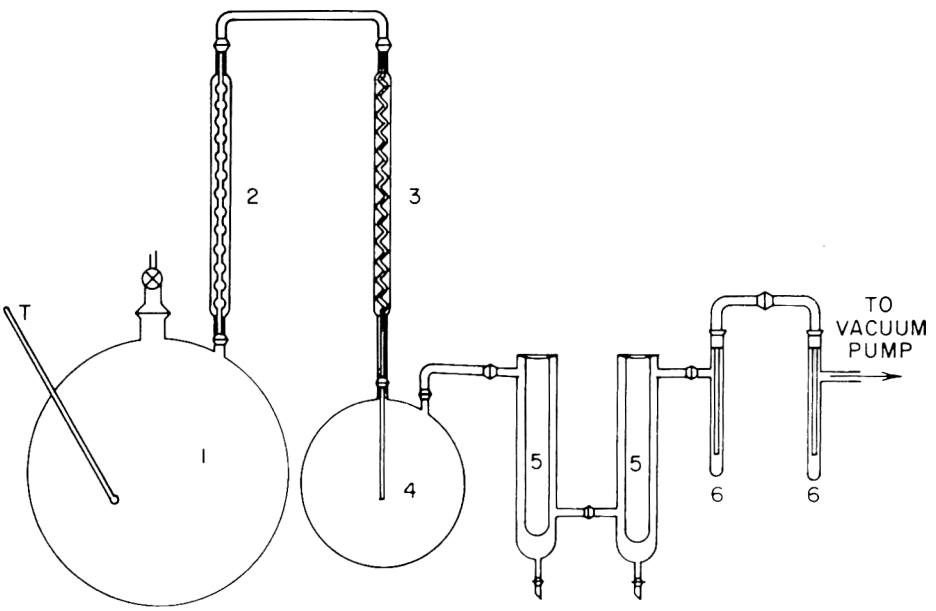

Fig. 1—Drawing of the vacuum distillation and differential cryogenic trapping system used to collect volatile constituents from raw peanuts.

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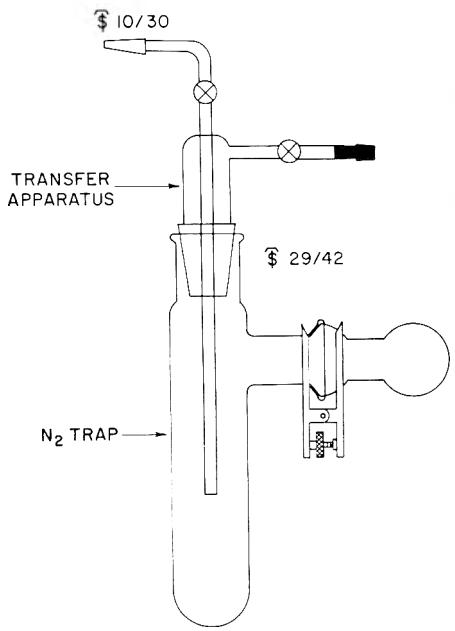

Fig. 2—Drawing of the sample apparatus used to sample the −196°C fraction.

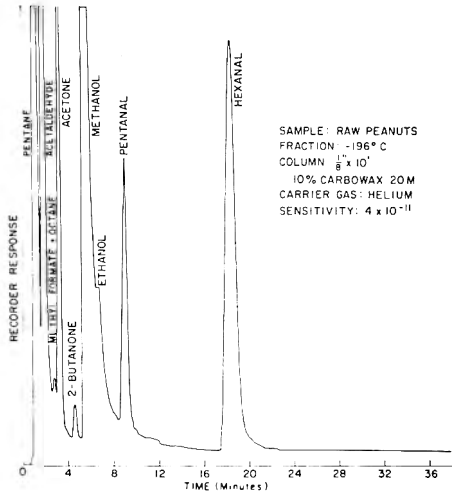

Fig. 3—Typical chromatogram of the volatile constituents of raw peanuts.

times of the emerging components were determined and compared with those of authentic compounds.

Volatiles for concurrent GLC-mass spectral analysis were prepared by sweeping the volatiles from the liquid N_2 sample trap with helium onto a $^1/_4$ in. × 6 in. U-shaped stainless steel column which was packed with 10% carbowax 20M on 60–80 mesh Chromosorb G, and cooled in liquid N_2. The sample trap was allowed to warm slowly to room temperature (10 min) before being immersed in a water bath maintained at 75°C for the balance of the transfer operation. The transfer required approximately 30 min. The U-column was then disconnected from the sample trap and attached to a 20-ft polyethylene glycol column in the GLC oven. Helium was used as the carrier gas at a flow rate of 5 ml/min, and the column programmed from 60°C to 150°C at 3°C/-min.

Mass spectral analysis

Mass spectral analysis of the volatile components was accomplished using a Barber Colman Series 5000 gas chromatograph coupled in tandem to a Bendix Model 12-107 Time-of-flight (TOF) mass spectrometer, similar to the procedure of Teranishi et al. (1963). The GLC was connected to the TOF instrument by leading a 0.02-in ID stainless steel capillary from the exit end of the GLC column (no detector) to a Whitey needle valve, which metered the GLC effluent directly into the ionization source of the mass spectrometer.

The quantity of GLC effluent introduced into the ionization source was determined by bringing the helium peak to its maximum ionization, as observed on a Tektronix Model 545B Oscilloscope, prior to switching on the pre-dynode gate. Total ionization current was monitored according to the method of Gohlke (1962) and directed into a 1-mv recorder for a simulated GLC readout. Following attachment of the U-column in the column oven, the carrier gas was started, the needle valve to the mass spectrometer was opened and mass scanning of the GLC effluent began immediately.

A considerable amount of CO_2 was found to be present, and several scans were made as this component was emerging in order to prevent overlooking other highly volatile components. Subsequent scans were made when the osilloscope pattern changed or by observing the total ionization monitor. The spectra were recorded by a Honeywell Model 1508 Visicorder.

RESULTS AND DISCUSSION

TEN COMPONENTS were identified by analysis of the volatile components in raw peanuts using GLC retention data and mass spectrometry.

Figure 3 shows a typical chromatogram from the liquid N_2 trap containing the volatiles from raw peanuts. This particular chromatogram was obtained with a $^1/_8$ in. × 10 ft carbowax 20M column. The identities of the components are shown on the chromatogram. Methyl formate and octane are eluted as one peak in the GLC analysis, but were easily separated by the conditions used in the GLC–mas spectral analysis. The short $^1/_4$ in. × 6 in. trapping column was packed with carbowax 20M and held at −196°C during the transfer of the sample from the manifold trap and the physical characteristics of the stationary phase were changed resulting in complete separation of these two components.

Mass spectral data also revealed that only a trace of the hydrocarbon octane was present, whereas methyl formate was present in much greater quantities. Ethanol appears as a shoulder on the methanol peak, but the identity of these compounds was established by using a multiple-scanning technique during the emergence of these peaks from the GLC unit. The spectra of the components were compared to reference spectra taken from literature and/or compared to spectra run in this laboratory.

Occurrence of the compounds shown in Figure 3 has been reported from many natural sources. Pentane has been found in oxidized peanut, corn, and soybean oils, and has been suggested as a rancidity indicator for routine analysis of vegetable oil (Scholz et al., 1966). The primary alcohols, ethanol and methanol, have received very little attention in the past as to their contribution to the flavor of a product. However, Day et al. (1965) stated that ethanol and methanol may be of significance as flavor components in blue cheese per se and also as reactants for ester formation. While the metabolic pathway for the production of ethanol is well established, the production of methanol still remains a mystery.

It is generally concluded that the methyl ketones arise from beta oxidation of the fatty acids, and aldehydes arise from certain organic or amino acids and fatty acids. Acetaldehyde is believed to arise via the route of pyruvic acid, whereas pentanal and hexanal most likely arise from the lipid fraction by autoxidation and/or enzymatic reactions. The precursor of methyl formate, according to Kenney (1962) and Merritt et al. (1965), probably also arises from the lipid fraction.

The compounds principally responsible for the raw peanut aroma have not yet been ascertained. However, the aroma of a freshly ground aqueous slurry of raw peanuts is nearly duplicated by the volatiles from the liquid N_2 trap. The nitrogen-trap aroma is highly suggestive of hexanal, but hexanal alone does not establish the subtle aroma of ground raw peanuts. Thus, the characteristic aroma and flavor of raw peanuts probably arise from a physical interaction of the components isolated and identified in this study. The role of certain of these components in the over-all aroma of raw peanuts is currently under investigation.

Since the aroma of raw peanuts is nearly duplicated in the liquid N_2 fraction, this fraction is suggested as a qualitative chemical index of the volatile components associated with the flavor and aroma of good-quality peanuts.

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An Automatic Sampling System for Respiratory Gases and Respiratory Response of Irradiated Citrus Fruits

SUMMARY—An automatic continuous-flow sampling system for monitoring respiratory gas was developed. Continuous airflow at constant pressure and temperature was pumped through 24 chambers. The airflow was regulated by needle valves connected to 3-way solenoids which sequentially switched the sample to a CO_2 infrared analyzer and recorder. A timing cycle was selected so that each sample was analyzed every 3 hr. The recorder was calibrated to read directly in percent CO_2. Results were expressed as ml CO_2/kg product/hr. The system was used to determine the respiratory activity of irradiated and nonirradiated citrus fruits. Irradiated fruits exhibited marked increases in CO_2 production reaching a maximum 1–2 days after treatment then gradually declining. Waxing limited the production of CO_2 especially with irradiated fruits. All fruits were treated with sulfanilamide to prevent post irradiation infection by mold.

INTRODUCTION

RESPIRATION is an index of metabolic activity of fruits and vegetables and is usually determined by the amounts of CO_2 evolved or O_2 absorbed. Continuous airflow systems have been used where evolved CO_2 was determined either titrimetrically (Appleman et al., 1946) or colorimetrically (Claypool et al., 1942). These methods require relatively long periods between sampling, can be insensitive to minute quantities of CO_2 in the respiratory gas or require continuous monitoring by the operator during periods of sampling and analysis.

Infrared (IR) analysis of CO_2 is rapid, sensitive and adaptable to an automated system. Automatic monitoring systems of CO_2 production from plant material by IR analyzers have been utilized for cut roses (Coorts et al., 1965) and citrus fruits (Eaks, 1965; Vines et al., 1965). These continuous flow systems measured CO_2 concentrations in ranges up to 1000 ppm. Respiration chambers varied from one to twelve units and sample switching was initiated by either a cam mechanism or solenoids. Metered calibrated capillaries were used for the control of airflow.

These automatic systems lack one or more desirable features for their successful operation. An improved system will be one which satisfies the following conditions: (a) Simplicity and dependability of the system; (b) Sensitivity to small amounts of CO_2 present in the flowing air; (c) Dependability of the switching mechanism to assure the orderly presentation of different samples to the IR analyzer; (d) Versatility of the system to detect CO_2 produced from a single as well as multiple fruit; (e) Capability to accommodate ample replications of each treatment required for a valid statistical analyses; (f) Portability of the system.

The effects of irradiation upon the respiratory activities of climacteric and nonclimacteric fruits have been studied. Abdel-Kader et al. (1964, 1965) reported that tomatoes, at different maturities, irradiated with dosages of 2.5–700 kilorad (Krad) showed marked increases in CO_2 evolved for a period of 6–7 days. Burns et al. (1957) demonstrated that dosages of 0.5×10^6–1.5×10^6 rep cathode rays increased the respiratory response of mature green tomatoes directly proportional to the dose. Higher respiratory rates were obtained from irradiated Eureka lemons (Maxie et al., 1965a), Washington Navel oranges (Guerrero et al., 1967), nectarines and peaches (Maxie et al., 1967), and pears (Maxie et al., 1965b).

The increased CO_2 evolution from the irradiated Avon lemons, Navel oranges and Temples reached a maximum 24–48 hr following irradiation, then declined gradually as the storage period was prolonged (Ahmed et al., 1966; Dennison et al., 1966). The oxygen uptake by tomato mitochondria was immediately suppressed following irradiation but increased to a maximum during the ensuing 2-day period (Padwal-Desai et al., 1969). A repair mechanism for irradiation damage was suggested during this period of increased activity.

These reports for changes in the respiratory activities of irradiated fruits were conducted where CO_2 production was generally monitored once every 24 hr. Sampling at shorter intervals may determine the approximate times for the onset of the accelerated production of CO_2 and its maximum.

The purpose of the present study was: (a) to develop an improved automatic sampling system for monitoring CO_2 produced at short intervals, and (b) to measure the respiratory responses of irradiated lemons and grapefruit.

EXPERIMENTAL

Apparatus

An automatic sampling system was developed (Fig. 1) in which 24 samples were sequentially monitored. Atmospheric air was pumped (A) through a metal coil (B) to

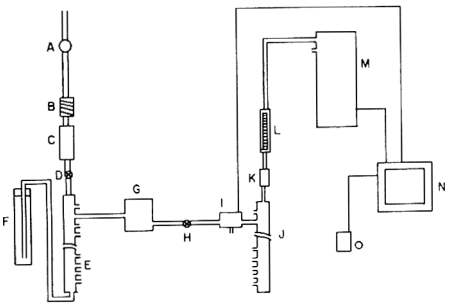

Fig. 1—Schematic diagram for automatic CO_2 sampling system. A—air pump; B—metal coil; C—compressor tank; D—air regulator; E—input manifold; F—Hydrostatic head; G—sample chamber; H—very fine needle valve; I—3-way solenoid; J—output manifold; K—dust and moisture trap; L—flow meter; M—IR analyzer; N—multipoint recorder and; O—timer.

condition the air temperature to that of the respiration room. Air was stored in a compressor tank (C) and allowed to flow through an air regulator (D) to the sample input manifold (E). A hydrostatic head (F) containing ethylene glycol was attached to the input manifold and maintained a constant pressure of approximately 1 psi within the sample chambers (G). This helped to maintain a constant flow rate throughout and permitted the flow rate adjustment of any particular sample while maintaining the others constant.

Air was allowed to flow continuously over the respiring fruits. The flow rate of each chamber was regulated by a very fine needle valve (H). A 3-way solenoid (I) followed each needle valve. The solenoid when energized permitted the sample to flow to the IR analyzer (M) and when de-energized to exit into the atmosphere of the respiration room. Only one solenoid was energized at any sampling period.

The respired air was directed into the sample output manifold (J), through a moisture and dust trap (K), flow meter (L), and into the sample cell of the Leeds and Northrup IR Analyzer Model 7804-A (M). A nichrome element was used as the IR source emitting in the range of 2–14μ(CO_2 absorbs energy at 4.3μ). A filter cell was employed to desensitize the system to other gases which absorb energy within this range. Using a differential thermopile, an electromotive force (EMF) was developed proportional to the quantity of CO_2 in the sample. The EMF was fed to an L&N Speedomax W 24 multipoint recorder (N) which printed directly in the range of 0–2% CO_2.

A switching mechanism, installed in the recorder, sequentially switched the 24 solenoids directing any one sample at a time to the analyzer. An external timer (O) was used to program the switching mechanism and was adjustable within the range of 0–30 min. A timer isolation network was installed in the recorder to allow the manual rapid adjustment of flow rates.

Materials

Field run Bearss lemons and field run and waxed Duncan grapefruit were used for respiratory studies. Grading was limited to sizing and removing damaged fruit. Prior to irradiation, field run fruits were washed while waxed fruits were washed and waxed. Fruits were conditioned at 20°C and 90% RH for 2–3 days prior to irradiation.

Fruits were exposed to ^{60}Co gamma rays ranging from 0 Krad (controls) to 300 Krad. Irradiation was carried out at 13°C and under continuous flow of air. Control and irradiated fruits were treated with sulfanilamide to prevent any post-irradiation infection by decay organisms and placed in respiration chambers.

Respiration measurements

A continuous flow of air at the rate of 100 cc/min was maintained throughout all experiments. A total of 4.5 min were required to purge the previous sample from the lines leading to the analyzer and to permit the IR detector to reach equilibrium. The timer was programmed to switch the solenoids every 7.5 min. The CO_2 content was measured during the last 4 sec of these periods. The respiration room was kept at 21° ± 1°C. The IR detector was checked and calibrated through the use of standard CO_2 gas mixtures at the initiation and at frequent intervals during the experiment. Multiple fruit were used for each replication (12 fruits for lemons and 3 for grapefruit). Unless otherwise stated, each treatment was replicated 5 times, and each replicate was sampled every 3 hr. The amount of CO_2 in the external air was monitored and deducted from the corresponding amounts of CO_2 exhibited by the respiring fruits. Data are expressed as ml CO_2 evolved/kg fruit on fresh weight basis/hr. Data were statistically analyzed by the analysis of variance method.

RESULTS AND DISCUSSION

Effect of sulfanilamide on respiration

Irradiated and nonirradiated fruits and vegetables are often subject to reinfection or infection by decay organisms, which augment their respiratory rates. Decay organisms might be controlled by treatment with sulfanilamide. An experiment was conducted to investigate the effects of this compound on the respiratory rates of Bearss lemons. 7 replicates each of nontreated fruit, fruit dipped in 0.5% aqueous solution for 1 min, and dusted fruit were used. Sulfanilamide did not influence respiratory rates of Bearss lemons (Table 1). Respiration rate varied with time which might be characteristic of the lemon fruit and not due to treatment with sulfanilamide. This was substantiated by the similar trends in respiratory rates of the different treatments.

Table 1—Respiration rate (ml CO_2 evolved/kg fresh weight basis/hr) for Bearss lemons treated with sulfanilamide.

Days after treatment	Treatment			
	None	Dip	Dust	Mean[1]
0	3.57	4.84	4.61	4.35 ab
1	3.80	5.10	5.00	4.64 a
2	3.80	4.06	3.70	3.85 ab
3	3.86	4.33	3.70	3.95 ab
4	3.19	3.74	4.06	3.66 b
5	4.00	4.06	3.80	3.94 ab
6	3.01	3.30	3.00	3.10 b
7	3.01	3.86	3.34	3.40 b
Mean[1]	3.53 a	4.16 a	3.89 a	

[1] Means, within groups, followed by the same letter are not statistically different at the 5% level of probability.

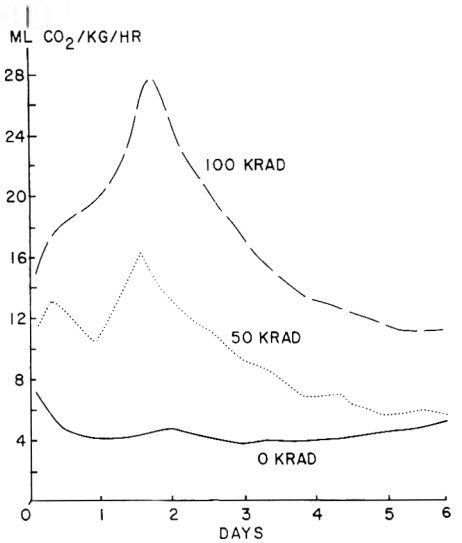

Fig. 2—Respiration rates (ml CO_2 evolved/kg fruit fresh weight basis/hr) for irradiated Bearss lemons.

Respiration rates of irradiated citrus fruits

Increased CO_2 evolution was obtained from irradiated Bearss lemons (Fig. 2). Similar trends were reported by Maxie et al. (1965a) for Eureka lemons. Immediately following irradiation with 50 Krad dose, a rapid increase in respiratory rates occurred for 9 hr, then decreased for a period of 12 hr (Fig. 2). Maximum respiratory response was reached 39 hr after irradiation. This period agrees with those reported by Ahmed et al. (1966) and Dennison et al. (1966) for irradiated Avon lemons, Navel oranges and Temples. A gradual decline occurred for a period of 4 days following the maximum response. However, at the end of this period respiration rates were still higher than for the nonirradiated fruit.

Increased respiration rates were obtained with irradiated Duncan grapefruit (Fig. 3). Similar responses for irradiated citrus fruits have been described by Maxie et al. (1965a) and Ahmed et al. (1966). A slow decline ensued after peak rates were obtained at 21 and 30 hr following irradiation of waxed and field run fruit, respectively. The analysis of var-

Table 2—Analysis of variance for respiration rates one day after irradiation (0-300 Krad) of field run and waxed Duncan grapefruit.

Source	d.f.	M.S.	F-Test
Total	159		
Dose	1	2967.4	115.7**
Treatment	1	903.2	35.2**
Time	7	29.9	1.2
Dose × treatment	1	773.4	30.2**
Dose × time	7	30.4	1.2
Treatment × time	7	11.5	0.5
Error	135	25.6	

** ($P < 0.01$).

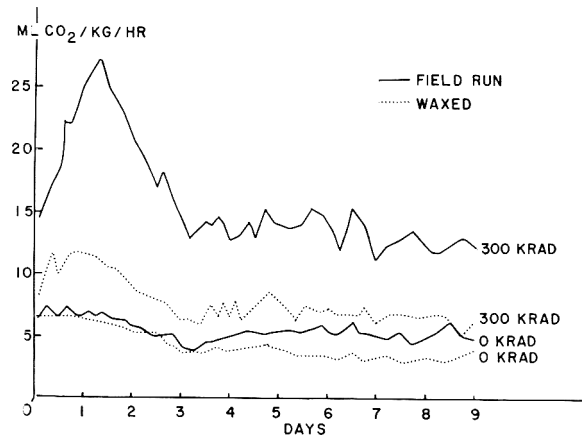

Fig. 3—Respiration rates (ml CO_2 evolved/kg fruit fresh weight basis/hr) for irradiated field run and waxed Duncan grapefruit.

iance for respiration rates for the first 24 hr period following irradiation (Table 2) indicated that a highly significant ($P < 0.01$) difference occurred between dosages (0 vs. 300 Krad) or treatment (field run vs. waxed). The interaction dosage X treatment was significant at the 1% level of probability and indicated varied trends in the respiratory rate of control or irradiated fruit with waxing treatments. Irradiated field run fruit exhibited higher respiratory activity than the irradiated waxed fruit. These trends were typical for each of the analysis conducted over a 9-day period and might be due to restricted gaseous diffusion of the waxed fruit.

The onset of increased evolution of CO_2 occurred within the first 3 hr following irradiation of both lemons and grapefruit (Figs. 2 and 3). Maximum production of CO_2 was found at 39 and 30 hr following irradiation of field run lemons and grapefruit, respectively. Greater fluctuations in the respiratory rates of irradiated grapefruit were observed in comparison to that of Bearss lemons. Sampling at 3-hr intervals made it possible to observe the fluctuations in CO_2 production as well as the timing of maximum gas evolution. These phenomena might not have been noticed if sampling had been carried out at longer periods. This was essentially true with the published reports of Ahmed et al. (1966), Dennison et al. (1966), Guerrero et al. (1967) and Maxie et al. (1965a, 1965b, 1967) where sampling occurred once in every 24 hr.

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Concentration Effect on Odor Addition or Synergism in Mixtures of Methyl Sulfide and Tomato Juice

SUMMARY—The effect of various concentrations of tomato juice and methyl sulfide (Me_2S) in triple distilled water on threshold values of the juice-Me_2S mixtures was studied on juices prepared from five varieties of canning tomatoes. When Me_2S and tomato juice were diluted to less than 10 ppb and 1,000 ppm respectively, before mixing, no significant interaction between juice and Me_2S was observed in the juice-Me_2S mixture. The odor intensities of the two components (as measured by threshold values in distilled water) were additive at subthreshold concentrations. As the concentrations of juice and Me_2S were increased before mixing, an increasing degree of interaction or synergism was observed in the mixture. At concentrations of 10^3 ppm juice and 1 ppm Me_2S, odor intensity of the mixture was approximately double that which would be expected on an additive basis. However, this may not be a case of true synergism if the concentrations which give rise to the effect produce other products of lower threshold values.

INTRODUCTION

PREVIOUS WORK has shown that processing of canned tomato juice produced methyl sulfide (Me_2S) to an extent largely dependent on variety and degree of heat treatment (Miers, 1966). Threshold measurements on many samples of heat processed tomato juice showed that Me_2S was an important contributor to the total odor intensity of the canned product (Guadagni et al., 1968).

One method used to determine the relative contribution of Me_2S to total odor intensity was addition of pure Me_2S to unheated control samples in amounts equivalent to that generated by various process times. The threshold values of these samples were then used to calculate percentage of total odor intensity which was contributed by Me_2S to the heated samples. Results of these calculations showed that Me_2S accounted for about 40 to 100% of the heat-generated odor intensity depending on variety and degree of heat treatment.

In general, as the heat treatment was increased (increasing Me_2S content), percentage of total odor contributed by Me_2S decreased. This was attributed to the likelihood that increased process times produced increased amounts of heat-induced odor other than that contributed by Me_2S. However, analysis of the threshold values of pure Me_2S, the control juice alone and the Me_2S-control juice mixtures, indicated some sort of interaction between juice and Me_2S. The interaction appeared to become greater as the Me_2S concentration was increased.

From the threshold values of pure Me_2S and the unheated juice, it is possible to calculate the fraction of their respective threshold concentrations in the mixture at the threshold value of the mixture. Thus if there is no interaction and the juice components and Me_2S behave in additive fashion at subthreshold concentrations,

$$\left[\frac{C_{Me_2S}}{T_{Me_2S}} + \frac{C_J}{T_J}\right]_{T_M} 100 = 100\%, \qquad [1]$$

where C_{Me_2S} and C_J represent concentrations of Me_2S and juice at threshold concentration of the mixture T_M, and T_{Me_2S} and T_J represent threshold values of pure Me_2S and unheated juice respectively. If values less than 100% are obtained, the result is what is commonly called synergism or more odor than can be accounted for from the individual components of the mixture. If the value is greater than 100% there is either suppression or independence of the individual components.

While there is a great deal of discussion about synergistic effects, very little published experimental data is available. Rosen et al. (1962) and Baker (1963) reported both synergism and additivity in certain mixtures of compounds in water. Guadagni et al. (1963, 1966a and b) reported additivity for a variety of mixtures in water solution. This paper deals with a case of apparent synergism between Me_2S and tomato juice and attempts to explain the phenomenon on the basis of concentration effects.

MATERIALS & METHODS

TOMATOES of five different varieties were obtained from a local cannery over a period of two seasons. Processing of the tomatoes into juice, canning, heat treatment and headspace gas analysis for Me_2S were done as described previously (Miers, 1966). Pasteurized juice (210°F for 30 sec) which did not receive any further heat treatment is considered the unheated control or control. These samples had essentially no Me_2S in the headspace above the canned juice.

Preparation of mixtures for threshold measurements

Stock solutions of 500, 1,000, 5,000, 10,000, 50,000 and 100,000 ppm of tomato juice in triple distilled water were prepared. Methyl sulfide was added to these solutions at the rate of 10^{-5} of the tomato juice concentration. This ratio of juice to Me_2S corresponded to that which occurs in variety VFN8 after 80 min heating. In addition, other juice-Me_2S mixtures were prepared at ratios that correspond to those which occur in natural juice after 5 to 40 min of heating in a boiling water bath. All Me_2S-juice mixtures were prepared from control juices and a 10 ppm solution of GLC pure Me_2S.

All juice-Me_2S mixtures were thoroughly mixed and allowed to stand for 10 min before further dilution. Each of the stock solutions was then diluted to its approximate threshold, and 4 to 6 concentrations were presented to the sensory panel for threshold determination. The range of concentrations was selected so that approximately one half were below and above the threshold concentration. The 500 ppm stock solutions required a 2–4 fold dilution to reach threshold concentration of the mixture, while the 100,000 ppm solution required up to a 1,000-fold dilution to reach threshold.

Threshold measurements

Threshold measurements were made in the same facilities and by the same procedures described previously (Guadagni et al., 1968). Briefly the procedure was as follows: The diluted solutions were placed in 3 oz ruby red glasses with glass plate covers which had been cleaned with an odorless detergent in an ultrasonic cleaner for 20 min. Before using, both containers and lids were rinsed ten times in triple distilled water.

Each of the odor solutions was paired with a blank containing the same volume of triple distilled water. Each of 15 previously trained and screened panelists was presented with 4 to 6 pairs of coded samples. They were asked to remove the glass lids, sniff each sample as much as they liked and check the sample which contained the odorous material. The order of pair presentation was completely randomized with respect to concentrations, and the water and odor solution was first in each pair an equal number of times.

Each series of concentrations was replicated 4 times giving a total of 60 judgments

Table 1—Effect of initial juice-methyl sulfide concentration on threshold values and odor interaction in the mixture.

	Concentrations in initial mixture, ppm				
	Juice—100,000		Juice—500		
	Me_2S	1	Me_2S	0.005	Critical
Variety	A	B	A	B[1]	ratio[2]
145-66	100	53	150	80	17.9
VF14	105	68	195	103	32.3
145-22-8	110	57	165	86	19.7
VFN8	125	55	218	97	40.4
CPC T2	150	71	200	95	21.7

[1] The figures in column A are threshold values in ppm, and those in column B are X values obtained from equation 2.

[2] Critical ratio calculated on basis of threshold values, n = 5-14. Values of 3 and above are considered to represent highly significant differences.

on each series of 4 to 6 pairs. Some of the samples were replicated as many as 14 times over a period of several months. This was especially true of Me_2S whose threshold was determined periodically as a check of sensory panel performance.

The percentage of judgments which correctly identified the odorous sample were plotted against concentration. The threshold was taken at the concentration where detection was significant at $P \leq 0.01$. The mean threshold value of each stock solution, determined on the basis of 60 judgments, did not vary more than ±10%. Individual threshold values of Me_2S determined by this procedure varied from 2.3 to 2.7 ppb over a three-year period.

Calculations

The degree of interaction between Me_2S and juice components was calculated on the basis of the mean thresholds for control or unheated juice (T_J) and pure Me_2S (T_{Me_2S}). These values were checked and verified at the beginning and end of measurements of each series of concentrations and for each variety. From the ratio of Me_2S to juice concentration in the initial mixture, the amounts of juice (C_J) and Me_2S (C_{Me_2S}) at the threshold concentration of the mixture (T_M) were calculated. When these values are introduced in the equation,

$$\left[\frac{C_J}{T_J} + \frac{C_{Me_2S}}{T_{Me_2S}}\right]_{T_M} 100 = X = \quad [2]$$

sum of the percentages of individual thresholds at threshold of the mixture T_M, X is a measure of the degree of interaction between Me_2S and juice.

If the odors from Me_2S and tomato juice behave linearly (simple additivity) at subthreshold concentrations, the value of X should be about 100% and represents no interaction. If the value of X is significantly less than 100%, interaction has occurred (synergism) and the extent of decrease in X value is a measure of the degree of interaction or synergism.

RESULTS & DISCUSSION

THRESHOLD values and degree of interaction for high and low concentrations of juice and Me_2S for five tomato varieties are shown in Table 1. The critical ratios indicate that highly significant differences in threshold values and odor intensity were obtained when the juice and Me_2S were mixed together at relatively high and low concentrations. When mixing was done in relatively concentrated solutions, lower thresholds and apparent synergism were observed. In the dilute solution (500 ppm juice + 5 ppb Me_2S) the threshold values of the mixture indicated an essentially additive relation between the odor of juice and Me_2S.

Since equation 2 yields theoretical X values of 100% when the relation is additive, it is clear that the more concentrated solution produced a perceptible odor when considerably less than the theoretically required amount of juice and Me_2S was present in the mixture. At this particular concentration of juice and Me_2S, 100,000 and 1 ppm respectively, an apparent interaction between Me_2S and juice components yields more odor than is expected on the basis of individual thresholds before mixing. However, when juice and Me_2S were diluted separately to 500 and 0.005 ppm respectively before mixing, the interaction was essentially eliminated as indicated by the increased X values. This phenomenon suggests that at certain concentrations of juice and Me_2S, additional odorous components may be generated or that Me_2S in some way intensifies the odor of existing juice components. Unfortunately, the nature of this "extra" odor generation is such that it defies measurement by even the most sensitive instrumental techniques. Consequently, the only reasonably reliable method of measuring the effect was by threshold measurements.

Since all of our previous work on mixtures of juice and Me_2S was done with a 5,000 ppm stock solution of juice and amounts of Me_2S corresponding to various heat treatments, these same concentrations were studied in detail on the five different varieties of tomatoes.

Figure 1 shows that low concentrations of Me_2S gave essentially additive odor effects. That is, the value of X in equation 2 approached 100%. A rapid decrease in the value of X occurred (synergism) as the concentration of Me_2S was increased to about 10–15 ppb, and thereafter X values remained fairly constant for at least 3 of the 5 varieties tested. Interestingly, X appears to level off at different values for different varieties as Me_2S is increased. Thus, the X value for VF14 drops from 100 to about 70% and then remains relatively constant; the value for 145-22-8 drops from about 95 to less than 50%, and for other varieties the values fall between these two extremes.

Generally there was a 30 to 50% drop in X value from the lowest to the higher Me_2S concentrations. The 50% value represents an approximate doubling of the odor intensity or a 50% reduction in the total amount of odorous components required to give a perceptible odor. As shown in Table 1 these values represent

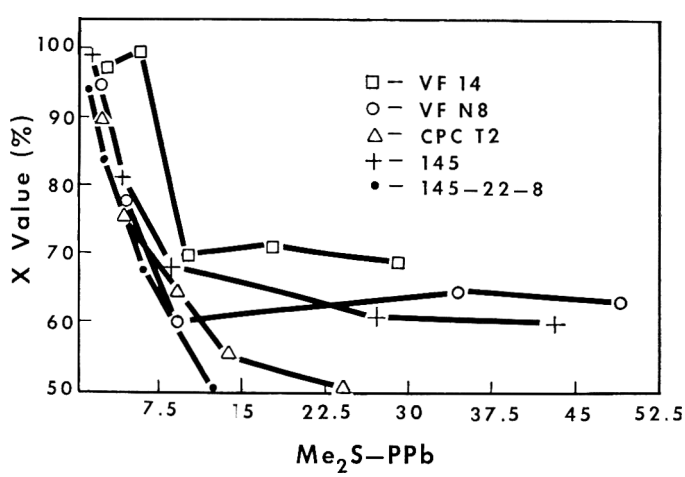

Fig. 1—Effect of Me_2S concentration on odor synergism in 5,000 ppm solutions of tomato juice in triple distilled water. X values are derived from equation 2 in text. Values of 100% represent odor additivity and decreasing values represent increasing interaction or synergism.

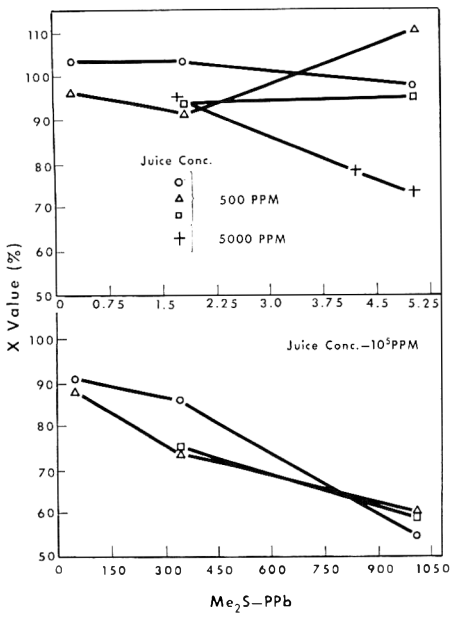

Fig. 2—Effect of Me_2S concentration on odor interaction in solutions of 500, 5,000 and 10^5 ppm of tomato juice in triple distilled water. Derivation and meaning of X values as in Fig. 1. Each line represents different samples of juice from variety VFN8.

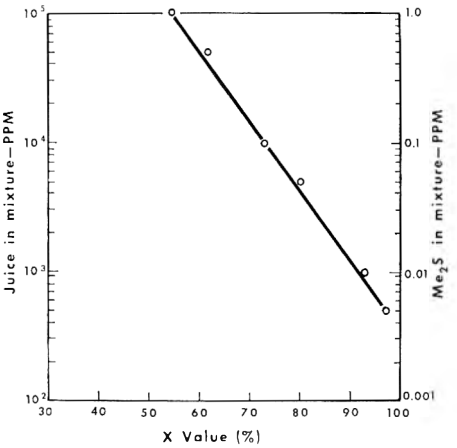

Fig. 3—Effect of Me_2S and tomato juice concentration on odor synergism or interaction in variety VFN8. Derivation and meaning of X values as in Fig. 1.

highly significant differences and cannot be reasonably ascribed to variations in sensory analysis.

This raises a point concerning total contribution of Me_2S to the odor intensity of canned tomato juice. If this degree of interaction can be produced in a relatively dilute juice solution of 5,000 ppm at room temperature in a matter of 10 min, one wonders how extensive the interaction becomes during heat processing of full strength juice and during extended storage. If these conditions produce a greater degree of interaction than occurs under the conditions of this experiment, then Me_2S alone would cause an even greater contribution to the total odor intensity of canned juice than was shown in our previous work (Guadagni et al., 1968).

The effect of Me_2S concentration on intensified odor for three different juice concentrations is shown in Figure 2. Variety VFN8 was selected for this study because it developed the greatest concentration of Me_2S (~10 ppm) during heating for 80 min. For a juice concentration of 500 ppm, the natural Me_2S content after 5 to 80 min of heating would range from about 0.25 to 5 ppb. Over this range of Me_2S concentrations in 500 ppm of control tomato juice, no significant interaction or synergism was observed. The X values in equation 2 remained within ± 10% of 100 which indicates odor additivity of the juice components and Me_2S, but not synergism.

For the same range of Me_2S concentrations and 5,000 ppm of juice, however, there is a definite trend of decreasing X values with increasing Me_2S concentration. For a juice concentration of 10^5 ppm, Me_2S concentrations corresponding to process times of 5 to 80 min range from about 50 to 1,000 ppb. Unlike the 500 ppm juice solution, increasing Me_2S concentrations in the 10^5 ppm solution caused steadily decreasing X values or increased interaction.

Figure 3 shows a plot of X values against juice and Me_2S concentrations for the variety VFN8. The ratio of juice to Me_2S concentration was kept constant and equivalent to that which occurred during 80 min of heating. For juice concentrations between 500 and 10^5 ppm there appears to be a logarithmic relation between juice concentration and interaction with Me_2S as measured by X values from equation 2. Again the interaction between juice and Me_2S increased (lower X values) as the concentrations of the two components were increased before mixing together.

It is evident that the interaction can be essentially avoided by reducing the concentrations of juice and Me_2S to less than 1,000 and 0.01 ppm, respectively, *before* mixing. This suggests that the interaction or so-called synergism in this particular case may be caused by some unknown reaction or association between juice components and Me_2S, and that this reaction or association may be governed by the mixing concentrations of the reactants.

It should be emphasized that there is no evidence whatever for subthreshold synergism between Me_2S and tomato juice. It is clear that the two odors are additive when the postulated reaction or association is prevented by adequate dilution before mixing. Furthermore, if the increased odor arises from the formation of a new compound or compounds, one might reasonably assume that the new compounds are more odorous or have lower thresholds than the original components of the mixture. If such were the case, it is entirely possible that the "extra odor" could be explained on an additive basis considering the thresholds of the new compounds formed in the mixture.

On the other hand, it is important to note that very significant interactions occurred at mixing concentrations of only 0.5% juice and 10 ppb of Me_2S. The problem of detecting the product or products of such interactions in the vapor over solutions of these concentrations is most difficult indeed, but the problem of identification is truly staggering. Yet, identification of the interaction product or products is essential to an understanding of the mechanism by which they are formed. Without this vital information, we can only speculate as to what actually occurs at the concentrations where the effects of these interactions are detected by the human nose.

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The Effect of pH-Temperature Treatments on the Calcium-Accumulating Ability of Purified Sarcoplasmic Reticulum

SUMMARY—Pig sarcoplasmic reticulum fragments obtained from the longissimus dorsi *muscle at 0- and 24-hours post-mortem were purified by salt extraction and density gradient centrifugation. The calcium uptake activity of 0-hour purified preparations was more than 20-fold higher than that from 24-hr old muscles, but there was no significant difference between fractions for calcium activated ATPase activities. When observed electron microscopically after negative staining, the ultrastructures of the 0- and 24-hour membrane fragments were found to be essentially identical. Incubation of isolated sacroplasmic reticulum fragments at pH 7.2 and 37°C or pH 5.6 and 0°C caused negligible inhibitoin of their calcium accumulating ability. However, treatment at pH 5.6 and 37°C for 1 hr almost completely abolished the sarcoplasmic reticulum calcium uptake. Thus it appears that low muscle pH and high temperature may be responsible for the inactivation of the calcium accumulating ability of the sarcoplasmic reticulum that occurs in situ.*

INTRODUCTION

THE SARCOPLASMIC reticulum of skeletal muscle is an extensive membrane network that lies between the myofibrils (Bennett et al., 1953; Porter et al., 1957; Peachey, 1965). This membrane system is believed to control muscle contraction and relaxation by regulating the free calcium concentration in the sarcoplasm (Ebashi, 1960; Weber et al., 1963). This idea was supported by the discovery of an ATP-driven calcium transport mechanism in isolated sarcoplasmic reticulum fragments (Hasselbach et al., 1961; Ebashi et al., 1962) and the establishment of the requirement for traces of calcium to activate maximally the contractile proteins (Weber, 1959; Ebashi, 1960; Weber et al., 1961a; Weber et al., 1961b).

The fact that the sarcoplasmic reticulum plays such an important role in muscle contraction and relaxation and in controlling myofibrillar ATPase activity (Marsh, 1952; Baird et al., 1960; Weber et al., 1963) suggests that the maintenance or loss of its functional integrity may be related to post-mortem changes in muscle. An extremely rapid decline of pH occurs in some pig muscle postmortem. This circumstance results in the condition known as pale, soft and exudative (PSE). PSE muscle (Briskey, 1964) is a problem of practical significance to the meat industry, and in view of recent research (Greaser et al., 1967, 1969a, 1969b) it appears that the function of the sarcoplasmic reticulum post-mortem should be carefully considered in the cause-effect relationship.

It has been demonstrated that the calcium accumulating ability of sarcoplasmic reticulum fragments declines 5- to 10-fold during the first 24 hr post-mortem (Greaser et al., 1967; Greaser et al., 1969a). The rate of loss of activity was much greater in muscles which had a rapid pH decline (Greaser et al., 1969a). Thus the sarcoplasmic reticulum appeared to be relatively labile after death. However, the cause of the loss in calcium accumulating activity remains to be established.

The present study was designed to characterize further the biochemical and ultrastructural changes of the isolated sarcoplasmic reticulum that occur between death and 24 hr post-mortem. Also, the effect of pH and temperature treatments on the calcium accumulating ability of sarcoplasmic reticulum preparations was investigated to determine if these factors might be responsible for the observed post-mortem changes.

EXPERIMENTAL

Preparation and purification of preparations

Muscle samples from the longissimus dorsi of Chester White pigs were obtained within 5 min after exsanguination and at 24 hr postmortem. Following exsanguination and evisceration of the pigs, the carcasses were stored at 4°C beginning about 30 min postmortem. Samples were homogenized in a Waring Blendor in four volumes of ice-cold 0.1 *M* KCl and 5 mM histidine (pH 7.2) in six 15-sec bursts.

The crude heavy sarcoplasmic reticulum fraction from muscle homogenates was isolated by centrifugation of an 8,000 × G (20 min) supernate for 60 min at 30,000 × G (Greaser et al., 1967). The sedimented material was resuspended in 0.6 *M* KCL-5 mM histidine (pH 7.2) to extract contaminating actomyosin (Uchida et al., 1965; Martonosi, 1968a; Greaser et al., 1969c).

After 1 hr at 0°C, the sarcoplasmic reticulum fragments were sedimented at 60,000 × G for 30 min. The precipitates were resuspended in 0.1 *M* KCl-5 mM histidine and part of this suspension was layered on top of a density gradient consisting of 8 ml of 35%, 8 ml of 40%, and 8 ml of 45% sucrose (w/v). The tubes were placed in a Spinco SW25.1 head and centrifuged for 2 hr at 25,000 rpm (Seraydarian et al., 1965). The resulting bands were withdrawn with a Pasteur pipette connected to a plastic springe by means of rubber tubing. The tip of the pipette was bent at a 90° angle to reduce turbulence during removal of the layers. The sucrose suspensions were diluted with 0.1 *M* KCl-5 mM histidine and centrifuged at 60,000 × G for 30 min. The resulting precipitates were suspended in KCl-histidine and stored in ice until use.

Calcium uptake and ATPase determinations

Calcium accumulating ability was measured in a medium consisting of 0.1 *M* KCl, 5 mM histidine (pH 7.2), 5 mM ATP, 5 mM $MgCl_2$, 5 mM $K_2C_2O_4$, and 0.2 mM $CaCl_2$ containing ^{45}Ca. A protein concentration of 0.03 mg/ml was used for all samples. Incubations were conducted at 25°C for 15 min and terminated by Millipore filtration (Martonosi et al., 1964). The amount of bound calcium was determined by liquid scintillation counting of the filtrates and appropriate standards.

Calcium activated ATPase activity was determined in a medium consisting of 0.1 *M* KCl, 5 mM histidine (pH 7.2) 5 mM ATP, 5 mM $MgCl_2$, 5 mM $K_2C_2O_4$ and 0.2 mM $CaCl_2$. EGTA-ATPase (believed to represent ATPases not involved in calcium transport) was measured in a similar medium, except that the $CaCl_2$ was omitted and replaced by 1 mM EGTA [Ethylene bis(oxyethylenenitrilo)-tetraacetic acid]. Incubations were conducted at 25°C, with aliquots withdrawn and added to two volumes of ice-cold 7.5% (w/v) trichloroacetic acid at 1, 2, 3 and 5 min after the initiation of the reaction. The precipitated proteins were removed by centrifugation and the inorganic phosphate concentration in the supernate was determined by the method of Fiske et al. (1925).

Electron microscopy

The negative staining methods of Brenner

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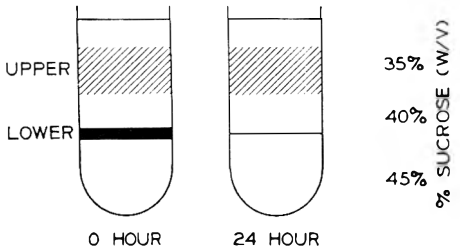

Fig. 1—Density gradient profiles of sarcoplasmic reticulum fragments at 0 and 24 hr post-mortem. Usually two major bands were obtained. The lower band was considerably reduced in size in the 24-hr preparations.

et al. (1959) and Huxley (1963) were used with modifications. A drop of the sarcoplasmic reticulum fragment suspension was placed on a collodion and carbon coated grid which was held with fine-tipped forceps. After approximately 15 sec, the excess liquid was removed by sliding a piece of filter paper between the tips of the forceps. A drop of 1% potassium phosphotungstate (pH 7.0) was immediately placed on the grid, and the excess was removed with filter paper. The grids were allowed to dry and were subsequently examined and photographed in a Siemens Elmskop I electron microscope operating at an accelerating voltage of 80 KV.

pH and temperature treatments

Approximately 2 mg of purified sarcoplasmic reticulum protein in 3 ml of 0.1 *M* KCl-5 mM histidine (pH 7.2) were placed in a series of test tubes kept in ice. Each tube was then equilibrated in a temperature-regulated water bath. After approximately 5 min, the suspensions were poured into a 5-ml beaker and the pH was lowered to the desired value by addition of 0.1 N HCl. The solutions were returned to the water bath and held there for 1 hr. They were then removed and the pH was brought back to 7.2 with 0.1 N KOH. The suspensions were then returned to ice and held there until calcium accumulating ability was assayed.

The pH was monitored by means of a small combination electrode attached to an expandable scale Radiometer pH meter. The meter was standardized for each temperature used, with the standard buffers and the electrode being brought to the temperature of the solutions to be measured. The suspensions were stirred continuously with a micro stirring bar during the pH adjustment to minimize local concentration effects.

Other methods

Protein concentrations were measured using the biuret procedure with bovine serum albumin as a standard (Gornall et al., 1949). ATP was treated with Dowex 50 × 8 in the H^+ form to remove any contaminating calcium (Seidel et al., 1963). Distilled water which had been passed through a mixed bed ion exchange resin was used for all solutions.

Table 1.—Calcium uptake and ATPase activities of sarcoplasmic reticulum preparations obtained at 0 and 24 hrs post-mortem.

		0 hour	24 hours	Significance
Calcium uptake (μM/mg protein)	Salt extraction purified S.R.	4.08 ± 0.30[1]	0.23 ± 0.12	$P < 0.01$
	Salt extraction plus density gradient purified S.R.	5.44 ± 0.23	0.24 ± 0.16	$P < 0.01$
Ca-ATPase (μM Pi/mg protein-min)	Salt extraction purified S.R.	1.35 ± 0.15	0.94 ± 0.25	NS
	Salt extraction plus density gradient purified S.R.	1.62 ± 0.09	1.48 ± 0.14	NS
EGTA-ATPase (μM Pi/mg protein-min)	Salt extraction purified S.R.	0.05 ± 0.01	0.04 ± 0.01	NS
	Salt extraction plus density gradient purified S.R.	0.05 ± 0.01	0.06 ± 0.01	NS

[1] Means ± standard error for 4 preparations.

RESULTS

THE DENSITY gradient profiles of sarcoplasmic reticulum fragments prepared at 0- and 24-hr post-mortem are shown in Figure 1. The 0-hr preparations usually separated into two major bands. The upper band was found to contain the highest Ca uptake specific activity, in agreement with previous work (Seraydarian et al., 1965). The position of the bands was similar in the 24-hr preparations to that in the 0-hr ones. The only difference noted between them was the marked reduction in the size of the 24-hr lower band compared to that found in the 0-hr gradients. The reason for this difference was not apparent.

The calcium accumulating ability and ATPase activities of sarcoplasmic reticulum preparations obtained at 0- and 24-hr post-mortem are shown in Table 1. Salt extraction alone gave a nearly 2-fold purification of the calcium uptake activity in the 0-hr fractions versus the nonpurified ones previously observed (Greaser et al., 1967; Greaser et al., 1969a). However, there was no marked enhancement of activity in the 24-hr preparations.

Density gradient purification increased the calcium uptake specific activity with the 0-hr preparations, but the magnitude of this additional increase was less than that achieved by salt extraction alone. There was no significant increase in calcium accumulating ability of 24-hr sarcoplasmic reticulum fragments purified by density gradient centrifugation. The relative purification achieved by density gradient centrifugation in both 0- and 24-hr samples appeared to be correlated with the amount of less active material that was found in their respective lower bands (Fig. 1). There was no clear explanation of the fate of the majority of the lower band material at 24 hr, although presumably it either precipitated during the low speed centrifugations or broke down to smaller sized fragments so that it would not precipitate at 30,000 × G.

The calcium stimulated ATPase activity (or "extra ATPase") in a medium that contains oxalate is believed to reflect the ATP hydrolysis that supports calcium transport (Hasselbach et al., 1961, 1963). Therefore, this activity was measured as a comparison with the calcium accumulation values. Density gradient purification exhanced the Ca-ATPase activity at both 0 and 24 hr. The activities at 0 hr were higher in both salt extracted and salt extraction plus density gradient purified preparations than at 24 hr, but the differences were not significant. Thus the calcium activiated ATPase activity appeared to be much more stable postmortem than the calcium accumulating ability.

The EGTA-ATPase (or "basic ATPase") is though to be a contaminating ATPase in sarcoplasmic reticulum fractions that is not related to the calcium transporting mechanism (Hasselbach et al., 1961, 1963). Its specific activity was less than 5% of the Ca-ATPase. There was no significant difference in EGTA-ATPase activity between 0- and 24-hr preparations or between different degrees of purification.

Previous electron microscopic investigations using fixing and embedding techniques indicated that there were no observable structural changes in the sacroplasmic reticulum vesicles between 0- and 24-hr post-mortem (Greaser et al., 1967; Greaser et al., 1969b). Additional ultrastructural studies were conducted using the negative staining technique since this method allows greater resolution of fine structure. The appearance of 0-hr purified sarcoplasmic reticulum fragments is shown in Figure 2. Most of the vesicles were spherical in shape, with some possessing tails (T) of various lengths. The surface of the membranes displayed a fine granularity. The appearance of these sarcoplasmic reticulum fragments from pig muscle was similar to that of mouse preparations (Ikemoto et al., 1966; Martonosi, 1968b), with the exception that tailed vesicles were less commonly observed.

The edge of the membranes was lined with a row of spherical subunits whose diameter was approximately 35 to 40 A (see arrows, Fig. 3). These subunits were spaced 50 to 60 Å apart when measured

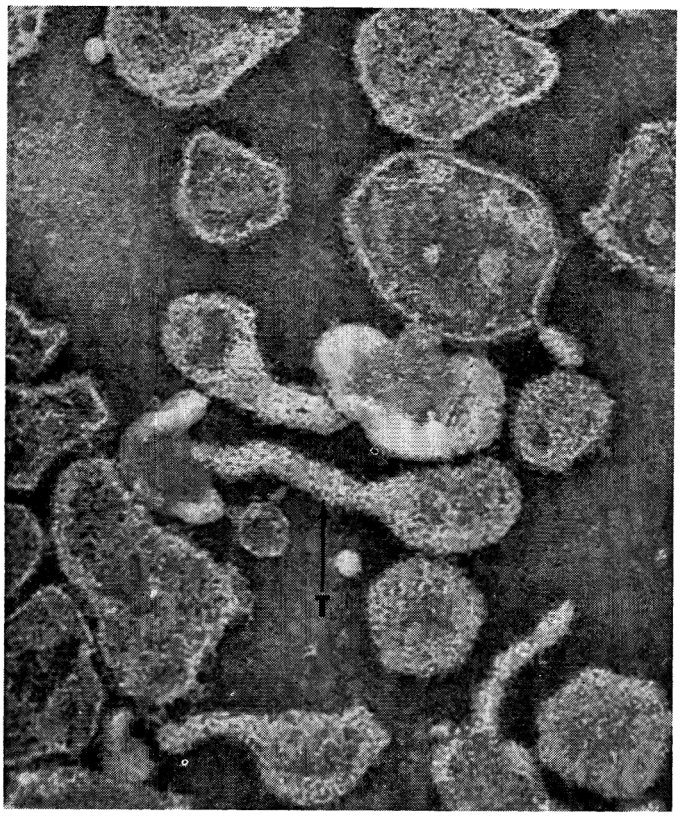

Fig. 2—Purified sarcoplasmic reticulum fragments obtained at 0 hr and negatively stained with 1% PTA. The membranes were usually spherical in shape with some possessing tails (T). ×120,000.

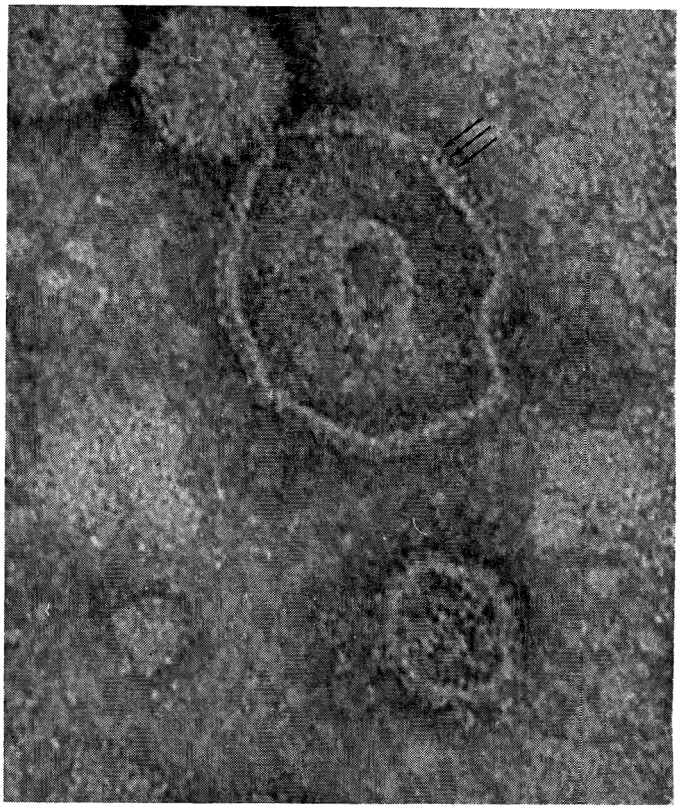

Fig. 3—Purified sarcoplasmic reticulum fragments obtained at 0 hr and negatively stained with 1% PTA. The membrane edges were lined with spherical 35 to 40 Å subunits (see arrows). ×240,000.

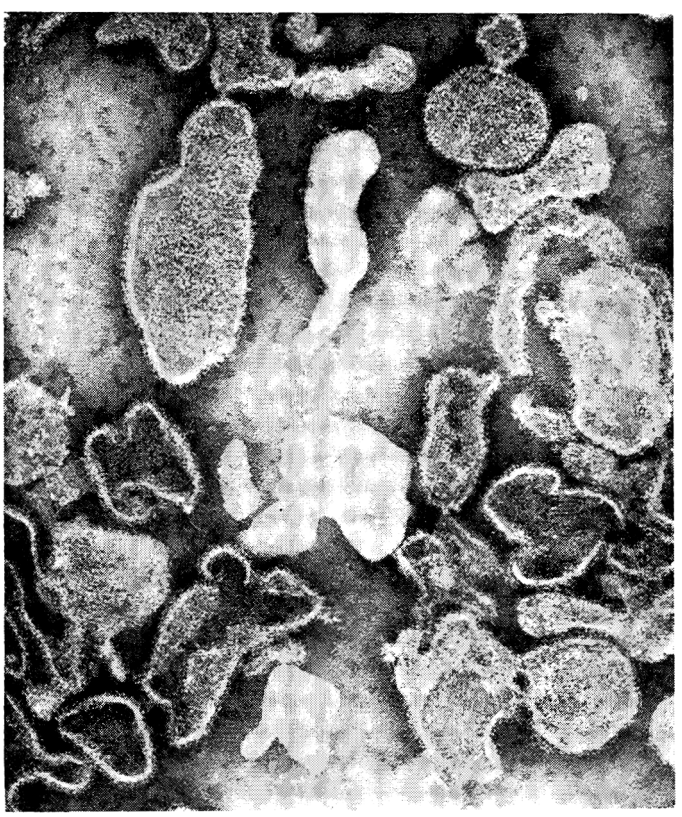

Fig. 4—Purified sarcoplasmic reticulum fragments obtained at 24 hr post-mortem and negatively stained with 1% PTA (phosphotungstic acid). Most of the vesicles were roughly spherical in shape, although some were more irregular. ×120,000.

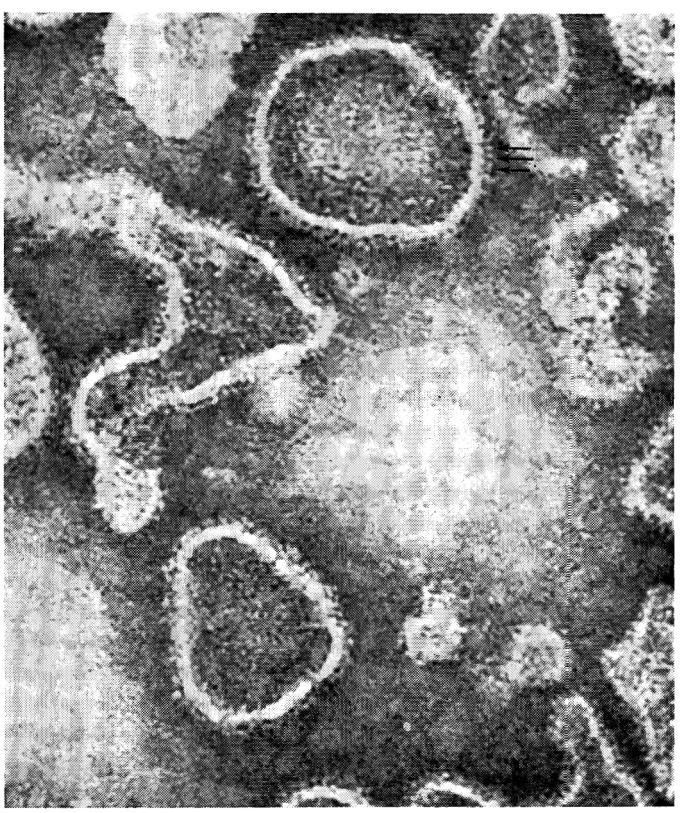

Fig. 5—Purified sarcoplasmic reticulum fragments obtained at 24 hr post-mortem and negatively stained with 1% PTA. The appearance of the subunits (arrows) was identical to that found in 0-hr preparations. ×240,000.

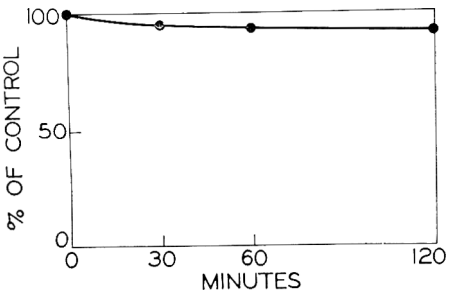

Fig. 6—The effect of 37°C treatment on the calcium accumulating ability of the sarcoplasmic reticulum. Sarcoplasmic reticulum fragments were incubated at pH 7.2 for the specified periods and returned to ice prior to calcium uptake assay. Points represent the means of three preparations.

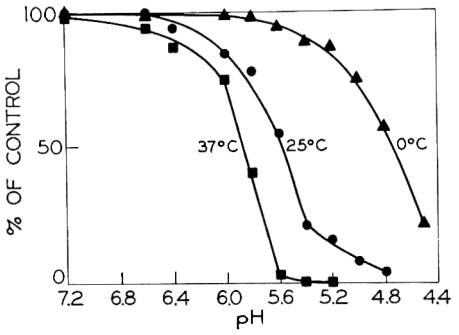

Fig. 7—The effect of pH-temperature treatment on the calcium accumulating ability of the sarcoplasmic reticulum. The pH was adjusted with 0.1 N HCl and the sarcoplasmic reticulum suspensions incubated for 1 hr. The pH was then returned to 7.2 and the preparations placed in ice until calcium uptake assay. Points represent the means of three preparations.

center-to-center. The size and spacing of these subunits were identical to that found in previous studies with mouse, rabbit, and pig sarcoplasmic reticulum fragments (Ikemoto et al., 1966; Martonosi, 1968b; Greaser et al., 1969d).

The appearance of 24-hr purified sarcoplasmic reticulum preparations is shown in Figures 4 and 5. The vesicles were usually roughly spherical in shape, although some had more irregular conformations. However, the proportion of vesicles with irregular shapes was higher in the figure shown than in most preparations. The surface of the membranes was granular and the subunits were still visible (see arrows, Fig. 5). Thus there were no consistent ultrastructural differences observable by negative staining between the 24-hr preparations and those obtained immediately after death even though the calcium accumulating ability of these fractions differed by more than 20-fold.

The inactivation of the calcium accumulating ability of the sarcoplasmic reticulum has been postulated to occur due to the pH changes in the muscle after death (Greaser et al., 1967). This idea was supported by observations that the loss of calcium uptake activity was much more rapid in muscles that had a rapid pH decline (Greaser et al., 1969a). Studies were therefore conducted to determine the effects of different pH and temperature treatment combinations on the calcium accumulating ability of isolated purified sarcoplasmic reticulum fragments.

The effect of incubation at 37°C and pH 7.2 on the calcium accumulating ability of sarcoplasmic reticulum preparations is shown in Figure 6. The rate of inactivation was very slow, with less than a 10% decrease after 2 hr of incubation. Thus 37°C treatment at a neutral pH was relatively ineffective in reducing calcium accumulating ability.

The effects of different temperature-pH combinations on sarcoplasmic reticulum calcium uptake are shown in Figure 7. The activity of preparations incubated at 0°C remained high at pH's normally encountered in post-mortem muscle, with more than 85% of the initial activity left after treatment for 1 hr at pH 5.2 or above. Treatment at pH 4.5 and 0°C abolished the majority of the calcium uptake ability, confirming previous findings (Marsh, 1952; Bendall, 1953; Ebashi, 1958).

The inactivation by pH was greater at higher temperatures. The temperature effect was particularly striking at pH 5.6 where the 0°C, 25°C, and 37°C treatments had 95%, 55%, and 5% of the initial calcium accumulating ability remaining, respectively. With the 37°C treatments, even pH's of 6.0 to 6.4 produced a 15–25% inactivation in 1 hr. Since the muscle temperature in pig carcasses cooled by conventional methods is usually greater than 25°C for most of the first 2- to 3-hr post-mortem (Briskey, 1964), it appears that the combined effects of temperature and pH may explain the inactivation of the sarcoplasmic reticulum calcium accumulating ability that has been observed (Greaser et al., 1967; Greaser et al., 1969a).

DISCUSSION

THE RESULTS of attempts to purify the calcium accumulating ability suggested that there was considerable contamination of the 0-hr crude sarcoplasmic reticulum preparations with salt soluble material. This contaminating material was not found to be present in 24-hr post-mortem preparations, however. These observations, plus those using the negative staining technique, support the concept arrived at previously that the post-mortem loss in calcium accumulating ability of sarcoplasmic reticulum fragments is real and not the result of the reduction in the specific activity of 24-hr preparations by inactive contaminating proteins (Greaser et al., 1967).

The ultrastructure of pig sarcoplasmic reticulum fragments as observed after negative staining was similar to that observed previously with mouse and pig muscle preparations (Ikemoto et al., 1966; Martonosi, 1968b; Greaser et al., 1969d; Greaser et al., 1969e). There was no consistent structural difference between 0- and 24-hr preparations even though the difference in calcium accumulating ability was more than 20-fold. Thus the subunit structure of the membrane does not appear to be the crucial property of sarcoplasmic reticulum fragments for calcium transport.

The studies on pH and temperature treatment of isolated sarcoplasmic reticulum preparation suggested that 37°C incubation at neutral pH was relatively ineffective in reducing calcium accumulating ability. Also, the pH's normally encountered in muscle post-mortem produced little inactivation at 0°C. However, at 25°C or 37°C pH's of 6.4 and 5.4 caused a considerable reduction in calcium uptake. These results are in remarkable agreement with those obtained with post-mortem muscle preparations in which the rate of loss of activity was greatest after the muscle pH was below 6.0 (Greaser et al., 1967; Greaser et al., 1969a). Thus it appears that the post-mortem loss in calcium accumulating ability of sarcoplasmic reticulum fractions may be caused by the pH-temperature conditions that occur in muscle after death. This loss of activity may be related to the rate of ATP breakdown post-mortem and the development of rigor mortis.

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Pacific Cod Muscle 5′-Nucleotidase

SUMMARY—A 5′-nucleotidase, widely distributed in teleost fish muscles, was purified about 20-fold from Pacific cod (Gadus macrocephalus) *by chromatography of a dialyzed aqueous extract of the muscle on DEAE-cellulose. The enzyme was unstable and lost 85% of its activity in 1 hr at 37°C, 53% in 10 min at 42°C and 40% in 1 hr at 30°C. It was stable for 6 days at 0°C, could be dialyzed for up to 3 days at 0°C against 1 mM tris buffer pH 7.5 and quickly frozen and thawed without loss of activity. However, it was inactivated rapidly when held at −30°C. Brief exposure to pH 4.0 or 5.0 effected marked destruction. Attempts at further purification by means of chromatography on hydroxylapatite, adsorption using alumina Cγ and starch gel electrophoresis failed due to instability.*

The enzyme was strongly inhibited by EDTA, pyrophosphate, KF and $ZnCl_2$ (1–10 mM); less markedly inhibited by GSH, 2-mercaptoethanol, carbonate and $CaCl_2$ (10 to 100 mM). It was strongly activated by Mn^{++} and weakly activated by Mg^{++}. The optimum pH was 7.6, and the Km was 5×10^{-4}M with UMP and 8^{-4}M with IMP. It hydrolyzed, in order of effectiveness, UMP, IMP, CMP, d-AMP, GMP, d-IMP, d-GMP, d-UMP and AMP, but not p-nitro phenylphosphate, sugar phosphates or a number of other compounds including 2′,3′-nucleotides.

INTRODUCTION

IT HAS BEEN KNOWN for some time that the inosine monophosphate (IMP), which arises through post-mortem enzymic degradation of ATP in fish muscle, is subsequently either deaminated to AMP or hydrolyzed to inosine and orthophosphate (Saito et al., 1957, Saito, 1961, Kassemsarn et al., 1963, Tarr, 1966). (Abbreviations accepted by the *Journal of Biological Chemistry* are used throughout.) A quantitative measurement of the hypoxanthine arising during enzymic hydrolysis (Tarr, 1955) or phosphorolysis (Tarr, 1958) of the inosine thus formed has been suggested as a criterion of the postmortem age, quality and flavor of flesh foods (Konosu et al., 1960, Jones, 1961, Spinelli et al., 1964, Hashimoto, 1965, Kahn et al., 1968, Fraser et al., 1968).

This investigation concerned a study of the 5′-nucleotidase responsible for postmortem hydrolysis of IMP in fish muscle. A preliminary note has been published (Tarr et al., 1969).

MATERIALS & METHODS

THE NUCLEOTIDES, sugar phosphates and other biochemicals used were the best available from Pabst Laboratories, Calbiochem, Schwarz or Sigma. Diethylaminoethylcellulose (DEAE) was obtained from the Brown Co. It was treated with 0.1 *N* NaOH, washed with H_2O, treated with 0.1 *N* HCl and washed again until the eluate was free from chloride.

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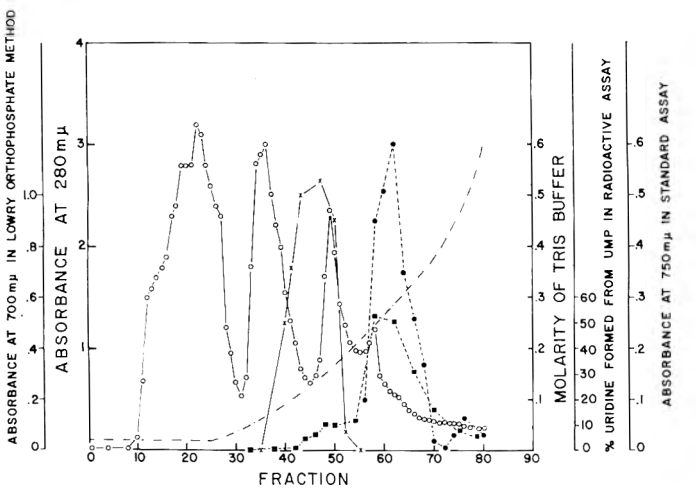

Fig. 1—Elution of fish muscle 5'-nucleotidase from a DEAE cellulose column. Protein by absorbance at 280 mμ, ○——○; molarity of eluting tris buffer, ●—●—●; orthophosphate concentration in fractions by the procedure of Lowry et al., 1946 × —— ×; 5-nucleotidase by standard assay, ●- -●; 5'-nucleotidase by radioactive assay, ■- -■.

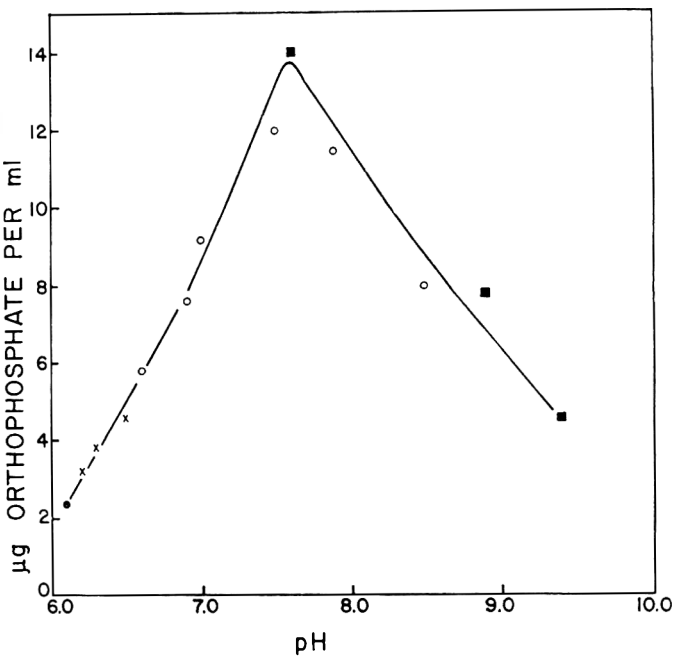

Fig. 2—pH-activity relationships of 5'nucleotidase using 0.2 M buffers. Succinate, ●; maleate, X; tris-HCl, ○; glycine-NaOH, ■. Orthophosphate formed was determined by the standard assay used for purified preparations.

During this procedure fine particles were removed. It was equilibrated with 0.02 *M* tris-HCl buffer pH 7.5 before use.

Nucleotidase activity was determined as follows. In crude extracts or eluates where high orthophosphate concentrations prevented linear determinations of orthophosphate from IMP or UMP under standard assay conditions (see below) the following assay was used. UMP^{14}C, 50 nmoles (0.02 μ curies); tris buffer pH 7.5, 10 μ moles and enzyme in a total volume of 50 μliters in 0.2 ml conical centrifuge tubes were held 20 min at 30°C. The reaction mixture was heated 1 min at 100°C, cooled, 0.5 μmole each of UMP and uridine were added as markers and the mixture was chromatographed on Whatman 3 MM paper 16 hr at 22°C using descending development in isobutyric acid: 0.001 *M* EDTA: 28% ammonia, 66:33:1 (Zetterström et al., 1951).

The paper was dried, the areas corresponding to UMP and uridine were cut out, the paper rolled up and inserted in vials holding 20 ml of a solution containing 6 g of 2,5-diphenyloxazole and 10 mg of 1,4-bis-[2-(5-phenyloxazolyl)]-benzene per liter of toluene, for determination of radioactivity using a Packard Model 3375 Tri-Carb liquid scintillation spectrometer.

For purified enzyme preparations the following standard assay was used. Enzyme and UMP (1 μmole) in a volume of 0.5 ml were held 20 min at 30°C. Since the preparations contained tris buffer pH 7.5 no buffer was normally added. At completion of the reaction 0.375 ml H_2O, 0.2 ml of 5% trichloracetic acid and 0.125 ml of phosphomolybdic acid reagent (Gomori, 1942) were added. The reaction mixtures were centrifuged 5 min at 5000 *g*, the clear supernatant solutions were carefully decanted and 0.05 ml of Elon-bisulfite reagent (Gomori, 1942) was added. The absorption at 750 mμ was determined after 45 min to obtain the amount of orthophosphate found in μg.

Where acid-labile phosphorus compounds (ribose 1-phosphate and deoxyribose 1-phosphate) were used as substrates, or where substances which interfered with normal color development in the Gomori procedure were present (e.g., 2-mercaptoethanol, glutathione and dithiothreitol) orthophosphate was precipitated as magnesium ammonium phosphate (Friedkin et al., 1954) before making the determination.

Protein was determined in DEAE-column eluates by absorption at 280 mμ, and also by the quantitative biuret procedure (Kingsley, 1939). Orthophosphate in DEAE-column eluates was determined by the method of Lowry et al. (1946).

RESULTS

Preparation of enzyme

Muscle of Pacific cod (*Gadus macrocephalus*) frozen at sea prerigor and stored at −30°C for up to 5 months was used, since the muscle of this species had been reported as possessing comparatively high IMP-hydrolyzing activity (Groninger et al., 1968). All procedures were carried out at 0–3°C.

Partly thawed muscle (50 g) was blended with 3 volumes of H_2O and the suspension was centrifuged 10 min at 25,000 G. The supernatant liquid, about 145 ml, after dialyzing several hours against 0.02 *M* tris pH 7.5, was applied to a 38 × 1.8 cm jacketed column of DEAE under a pressure of about 0.5 lb per sq in. The column was washed with 100 ml of 0.02 *M* tris buffer pH 7.5 and was then eluted, under hydrostatic pressure sufficient to maintain a flow rate of about 100 ml per hr, collecting 10 ml fractions.

A concave increasing gradient of tris buffer pH 7.5, formed by passing 230 ml of 0.6 *M* tris in a 4.8 cm diameter straight walled vessel into 320 ml of 0.02 *M* tris in a 6.4 cm diameter vessel, was employed as eluting buffer. The fractions were assayed for protein, orthophosphate and nucleotidase (both by radioactive and standard assay procedures) using UMP as substrate. The results of a typical experiment are shown in Figure 1. Most of the nucleotidase activity emerged as a single peak, but small amounts were found both before and after this peak. These minor peaks may be isoenzymes.

The most active fractions (e.g., fractions 55–69, Fig. 1) were pooled (about 150 ml) and the solution was held at 0°C. This enzyme preparation was quite impure since it contained 5 protein fractions as resolved by starch gel electrophoresis (Tsuyuki et al., 1966), and only one of these possessed feeble nucleotidase activity. The enzyme was quite stable for 5–6 days at 0°C and thereafter lost activity slowly. It was dialyzed at least 5 hr against 1 m*M* tris buffer pH 7.5 before use, and could be dialyzed for 3 days without noticeable loss of activity. The activity of these preparations increased moderately during the first 2 days following preparation, and so far this has not been explained.

The specific activity (S.A.) of 5 different crude muscle extracts varied between 0.35 and 0.54 μmoles of UMP hydrolyzed per mg of protein per hr under the conditions of the standard assay with an

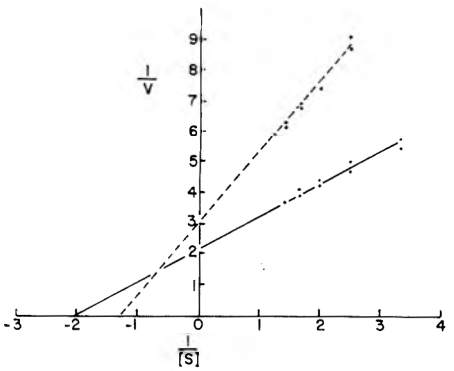

Fig. 3—Lineweaver-Burk plots of fish muscle 5′-nucleotidase with UMP (continuous line) and IMP (broken line) as substrate. V = μmoles orthophosphate formed under conditions of the standard assay for purified preparations. S = substrate concentration (μmoles per ml).

average of 0.45. With the corresponding purified preparations the figures were 7.6–10.6, with an average of 8.7, indicating 19.4-fold purification. The average protein content of the purified preparations was 0.14 mg per ml. The loss of activity during chromatography on DEAE cellulose was high. Thus, with an average of 503 enzyme units applied, only 156 units were recovered. This represented a 69% loss.

All attempts to purify the enzyme further failed. No protection was afforded the enzyme on DEAE columns when 2% mannitol or 0.1 m*M* uridine were incorporated in the eluting tris buffer, since only normal recoveries were obtained. When DEAE-purified preparations were adsorbed on hydroxylapatite columns (Tiselius et al., 1956) at pH 6.8 and elution was carried out with stepwise increasing concentrations of potassium phosphate buffer, nucleotidase activity appeared at about 0.3 *M* phosphate concentration. However, enzyme recovery was very low and all activity disappeared when the active fractions were held overnight at 0°C.

Since the enzyme was quite labile when adjusted for brief intervals to pH 4.0 or 5.5, attempts to effect purification by isoelectric precipitation at pH 5.5 were ineffective. Attempts to effect further purification by adsorbing the enzyme on alumina C$_\gamma$ and eluting it with phosphate buffers of up to 1 *M* concentration failed.

In view of the above, all experiments were carried out with DEAE eluates which were used within 6 days of preparation. Since all enzyme preparations were made from the same lot of frozen muscle and the specific activities were similar, only the volume of enzyme used in different experiments has been recorded.

Table 1—Substrate specificity of cod muscle 5′-nucleotidase.[1]

Substrate	Ratio of activity compared with IMP
IMP	1.00
UMP	1.19
CMP	0.75
d-AMP	0.38
GMP	0.32
d-IMP	0.31
d-GMP	0.22
d-UMP	0.10
AMP[2]	

[1] The standard assay for purified enzyme was employed. The results are an average of two experiments with different enzyme preparations. The following compounds were not hydrolyzed: d-TMP; d-CMP; UDP; IDP; CDP; ADP; GDP; ATP; 2′,3′-UMP; 2′,3′-AMP; 2′,3′-GMP; 3′,5′-AMP; 2′,3′-CMP, glucose 1-phosphate, glucose 6-phosphate, fructose 1-phosphate, fructose 1,6-diphosphate, ribose 5-phosphate, ribose 1-phosphate, deoxyribose 1-phosphate, *p*-nitrophenyl phosphate, and α,β-glycerophosphate.
[2] AMP was largely deaminated before hydrolysis; see text.

Stability

Many enzymes prepared from fish have proved much more unstable than similar enzymes prepared from warm-blooded vertebrates (Tarr, 1970). The present preparation proved to be no exception. Thus, it lost 85% of its activity in 1 hr at 37°C, 53% in 10 min at 42°C and 40% in 1 hr at 30°C. When the pH was adjusted quickly to 4.0 and then promptly re-adjusted to 7.5, enzyme preparations lost about 60% of their activity. Similar adjustment to pH 5.0 occasioned 43% loss in activity. The enzyme could be rapidly frozen and then thawed with no loss in activity, but when stored at −30°C for several days marked losses occurred.

pH activity

The results given in Figure 2 show that the enzyme had a fairly sharp optimum at pH 7.6.

Michaelis constants

Figure 3 shows that the Km was 5×10^{-4} *M* with UMP as substrate and 8×10^{-4} *M* with IMP.

Substrate specificity

Table 1 shows that UMP was utilized most effectively, followed by IMP, CMP, d-AMP, GMP, d-IMP, d-GMP and d-UMP. The results with AMP were complicated by the fact that the preparation contained AMP deaminase (Tarr et al., 1964). Thus, 8 nmoles of AMP-8^{14}C (0.1 μ curie) was held 20 min at 30°C with 40 μl of enzyme and chromatographed on Whatman No. 1 paper using 16 hr descending development in isopropanol: 28% NH_3:H_2O, 7:1:2 (Tener et al., 1955) with 0.5 μmole each of IMP, AMP, adenosine and inosine as carriers. The radioactivities recovered in the various fractions were: AMP plus IMP, 27,000 cpm; adenosine, 1,800 cpm and inosine 8,340 cpm. This showed that while about 18% of the AMP was hydrolyzed to adenosine by 5′-nucleotidase, 82% was first deaminated to IMP prior to hydrolysis.

Under conditions of the standard assay no deamination of CMP as determined spectrophotometrically (Maley, 1967) or of GMP (Roy, 1966) was detected.

Table 1 also shows none of the other

Table 2—Inhibition of cod muscle 5′-nucleotidase.[1]

Compound	Concentration (m*M*)	Inhibition[2] (%)	Compound	Concentration (m *M*)	Inhibition[2] (%)
EDTA	0.1	33	GSH	20	19
EDTA	0.25	44	GSH	40	38
EDTA	0.50	63	GSH	60	58
EDTA	1.0	89	GSH	100	66
EDTA	3.0	92	2-Mercaptoethanol	20	30
Pyrophosphate	0.1	16	2-Mercaptoethanol	50	62
Pyrophosphate	0.25	28	$CaCl_2$	20	13
Pyrophosphate	0.50	35	$CaCl_2$	50	46
Pyrophosphate	1.00	68	Dithiothreitol	20	9.7
Pyrophosphate	2.00	70	Dithiothreitol	40	9.7
Pyrophosphate	3.00	71	Dithiothreitol	60	7.1
$ZnCl_2$	0.05	22	Dithiothreitol	100	0.0
$ZnCl_2$	0.10	55	$MnCl_2$	0.2	+49
$ZnCl_2$	0.20	67	$MnCl_2$	0.5	+64
KF	1.0	29	$MnCl_2$	1.0	+56
KF	2.0	48	$MnCl_2$	5.0	+33
$KHCO_3$	10	6.7	$MgCl_2$	0.2	0
$KHCO_3$	20	8.4	$MgCl_2$	0.5	0
$KHCO_3$	30	17	$MgCl_2$	1.0	+18
$KHCO_3$	50	22	$MgCl_2$	5.0	0
$KHCO_3$	75	32			
$KHCO_3$	100	38			

[1] The following assay was used. UMP (2 μmoles), 0.8 ml enzyme and inhibitor in a volume of 1.0 ml were incubated 20 min at 30°C. The reaction was stopped by addition of 1 ml of ammoniacal magnesium chloride reagent (Friedklin et al., 1954) and orthophosphate was determined in the magnesium ammonium phosphate precipitate.
[2] + indicates % stimulation.

substances studied was hydrolyzed.

Inhibitors

Table 2 shows that the nucleotidase was strongly activated by Mn^{++} (optimum concentration 0.5 m*M*) and feebly activated by Mg^{++}. It was strongly inhibited by EDTA, pyrophosphate, KF and Zn^{++} (1 to 10 m*M*), while 2-mercaptoethanol, GSH, carbonate and Ca^{++} were inhibitive only in higher concentrations (10–100 m*M*).

DISCUSSION

WHILE AMP can be hydrolyzed directly to adenosine and orthophosphate by fish muscle 5′-nucleotidase, most of it is first deaminated to IMP, which is then hydrolyzed to yield inosine and orthophosphate. Similarly, rat heart muscle degraded AMP by two routes (Baer et al., 1966). However, the 5′-nucleotidase from rat heart muscle was much more stable, and the specific activity much greater than that of the fish muscle enzyme. The heart enzyme was about equally distributed between the nuclear and microsomal fractions, with comparatively little in the mitochondrial and soluble fractions.

No serious attempt was made to determine the distribution of the 5′-nucleotidase in muscle cell fractions. Yamamoto et al. (1967) studied the "alkaline phosphatase" of carp muscle. Their results indicated that 50% of the nucleotidase activity was in the soluble fraction when aqueous muscle extracts were centrifuged 60 min at 50,000 G, and 84% when 65,000 G was used.

They purified the particulate fraction by *n*-butanol treatment and DEAE cellulose chromatography and obtained two isoenzymes with very similar properties, but in very poor yields (0.9 and 0.3% of the initial activity). The pH optimum was 7.6 and the Km values of the two fractions were 4.3×10^{-3} *M* and 4.6×10^{-3} *M*. The activity was slightly enhanced by Mn^{++} and depressed or abolished by Zn^{++}, cysteine, EDTA, Ni^{++}, Mg^{++} and Co^{++}. UMP, IMP, AMP, CMP and GMP were substrates, but 2′,3′-nucleoside monophosphates, glucose 1-phosphate, *p*-nitrophenylphosphate and β-glycerophosphate were not utilized. Thus, this enzyme had rather similar properties to that studied in the present investigation.

An attempt was made to obtain a particulate nucleotidase enzyme from Pacific cod muscle. 91% of the activity of aqueous extracts remained in the supernatant liquid after centrifuging 20 min at 9000 G while Yamamoto et al. (1967) reported 50% with carp. Moreover, only 5.2% of the activity of the cod muscle extracts was found in the precipitate after centrifuging 1 hr at 50,000 G.

The results indicate that, even if the 5′-nucleotidase activity does occur in the particulate fractions of fish muscle, it must be quite easily liberated and thus appears largely in the supernatant liquid after high-speed centrifugation. Gubmann et al. (1962), when working with enzymes of the citric cycle in carp liver, observed that these enzymes appeared in both soluble and mitochondrial fractions. Possibly the particulate fractions of fish tissues are more labile than those of warm-blooded vertebrates.

The enzyme is strictly a 5′-nucleotidase. Attempts to inhibit it by structural analogs such as 2′,3′-nucleotides were unsuccessful. The sensitivity of the enzyme to EDTA is of interest in view of the inhibitory effect of this chelating agent in repressing IMP hydrolysis in muscles of carp (Endo et al., 1966) and various species of Pacific ocean fish (Groninger et al., 1968). The sensitivity of the enzyme to carbonate and to various thiols would appear to be rather unusual, especially since the last named compounds are frequently employed to protect enzymes.

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The Maillard Reaction and its Inhibition by Sulfite

SUMMARY—Studies on a sulfited glucose/glycine system showed that rapid color development, due to the Maillard reaction, did not begin until "free" SO_2 (i.e. sulfite/bisulfite ion) had almost disappeared from the system. At this time "free" SO_2 was less than 5% of that added and 28% was present in reversible combination with, e.g., carbonyl compounds. These results are considered in relation to recently published hypotheses on the intermediates involved in the Maillard reaction and on its inhibition by sulfites.

INTRODUCTION

IN A SERIES of papers on the Maillard reaction Song et al. (1966a) and Song et al. (1966b, 1967a, 1967b) reported some studies on kinetics of the reaction and on the mechanism of its inhibition. These authors made what appears to be the first serious attempt at a mathematical treatment of the kinetics of this reaction and its inhibition. This represents a major development in the approach to an understanding of non-enzymic browning reactions (Reynolds, 1963, 1965; Hodge, 1953).

In this paper the authors have not concerned themselves directly with the mathematical approach referred to, but report chemical and physical studies relating to Song and Chichester's conclusions that $\alpha\beta$-unsaturated aldimines are key intermediates in melanoidin production and that sulfite inhibition of the Maillard reaction involves a self-inhibitory conversion of sulfite to a free radical or other equally reactive species. The conclusions reached are, in some respects, different from those reached by Song et al. (1966a, b; 1967a, b). Possible reasons for these differences are discussed and related to other existing work.

EXPERIMENTAL

Materials

Glucose, glycine, sulfuric acid, hydrochloric acid, iodine, sodium hydroxide and soluble starch were of "Analar" quality; sodium metabisulfite was from British Drug Houses Ltd. and had a measurable SO_2 content of 65.1% (theoretical = 67.4%).

Reaction mixture

The glucose + glycine + sodium bisulfite system employed had a composition similar to System III employed by Song et al. (1967b) excepting that ^{35}S radioactive tracer was replaced by an equivalent amount of non-radioactive material, i.e., D-glucose (1.0M), glycine (0.5M), sodium bisulfite (0.043M). The latter component was added as the equivalent amount of sodium metabisulfite and the reaction mixture adjusted to pH 5.5 by addition of 1N sodium hydroxide. The mixture was sealed in bottles and incubated at 55°C.

Measurement of "free" and "combined" SO_2

"Free" and "combined" SO_2 were measured by a method based on that of Sundman (1949) as quoted by Gehman et al. (1954). A 1-ml aliquot of solution was pipetted into a 200-ml Erlenmeyer flask with distilled water (50 ml), 5N sulfuric acid (2 ml) and 1% starch solution (1 ml). The solution was rapidly titrated with 1.0 × $10^{-2}N$ iodine solution to give a measure of the amount of "free" SO_2 (1 ml 1.0 × $10^{-2}N$ iodine = 0.32 mg SO_2). The solution was treated with 1N sodium hydroxide (40 ml) for 10 min to release the "combined" SO_2. To measure this, the mixture was again titrated with 1.0 × $10^{-2}N$ iodine solution after re-acidification with 5N sulfuric acid (15 ml).

Glucose sodium hydroxy sulfonate treated in this way gave "free" SO_2 = 0.24%; "combined" SO_2 = 21.8% (theoretical = 22.8%).

Table 1—Color and measurable SO_2 in glucose/glycine/bisulfite system during incubation at 55°C.

Time, hr	"Free" SO_2, mg/ml	"combined" SO_2 mg/ml	"Total" SO_2, mg/ml	Absorbance, at 490 mμ
1	1.41	1.09	2.50	0
19	1.41	1.16	2.50	0
67	1.42	0.95	2.37	0
115	1.36	1.05	2.41	0
163	1.28	1.1	2.38	0
235	1.24	1.02	2.26	0.01
263	1.19	1.14	2.33	0.0
311	1.14	1.10	2.25	0.0
359	1.05	1.06	2.11	0.01
407	0.98	1.02	2.00	0.01
455	0.89	1.13	2.02	0
507	0.81	0.98	1.79	0.03
555	0.69	1.00	1.69	0.02
603	0.65	1.01	1.66	0.02
651	0.56	0.97	1.55	0.03
723	0.48	0.96	1.44	0.03
819	0.32	0.92	1.24	0.03
892	0.26	0.78	1.04	0.05
940	0.15	0.81	0.96	0.06
988	0.12	0.68	0.80	0.12
1012	0.11	0.60	0.71	0.18
1060	0.10	0.53	0.63	0.26
1087	0.11	0.52	0.63	0.29
1111	0.09	0.48	0.58	0.44
1159	0.085	0.39	0.48	0.55
1184	0.07	0.34	0.41	0.64
1232	0.096	0.23	0.33	0.92

Color measurement

Color intensity was measured using a Unicam SP800 UV/visible spectrophotometer and a distilled water blank. The spectrum was recorded between 400 and 550 mμ. Absorption at 490 mμ was used as a measurement of color intensity to allow direct comparison between the present work and that of Song et al. (1967b).

Paper chromatography

Paper chromatograms (Whatman No. 1; 12.5 cm length) were developed with 4:1 n-propanol:water (descending) for 18 hr before being dried and sprayed with p-anisidine hydrochloride (5% in methanol) or silver nitrate (1% aqueous).

RESULTS

AS ILLUSTRATED with measurements on glucose sodium hydroxysulfonate, the method used for estimating SO_2 is capable of distinguishing between "free" SO_2 (inorganic sulfite/bisulfite) and "combined" SO_2 (*reversibly* combined organic sulfur compounds like carbonyl bisulfite adducts).

Amounts of measurable "free" and "combined" bisulfite in the glucose/glycine/sodium bisulfite system were measured at intervals of 1–3 days during incubation. Results are shown in Table 1 and Figure 1 along with the measurements of absorbance at 490 mμ. Inhibition of color development appears to end at around 985 hr (41 days).

Chromatography of glucose, glucose-bisulfite compound (glucose sodium hydroxysulfonate) and sodium metabisulfite was carried out to investigate the ex-

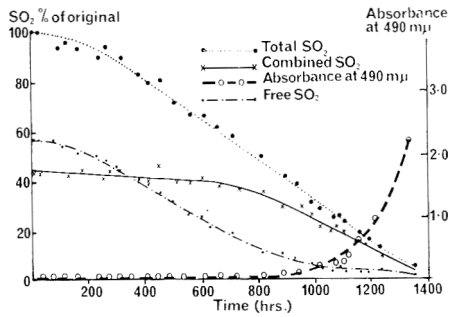


Fig. 1—Color production and loss of SO_2 from sulfited glucose/glycine solution during incubation at 55°C.

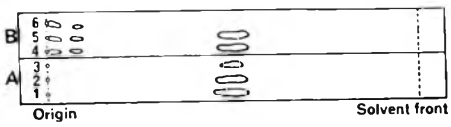


Fig. 2—Behavior of glucose-bisulfite compound during paper chromatography.
A: sprayed with p-anisidine, B: sprayed with silver nitrate.
1 and 4: glucose-bisulfite compound.
2 and 5: gluose + sodium metabisulfite.
3: glucose
6: sodium metabisulfite.

tent to which decomposition of bisulfite compound occurs during chromatographic separation. As shown in Figure 2, observations lead to the conclusion that this compound is completely decomposed during this process and migrates in the same way as a mixture of glucose and sodium bisulfite. *p*-anisidine positive material was found only in the position corresponding to glucose and none was visible at the position of the sulfite spot ($R_F \sim 0.09$) or elsewhere, either in daylight or under U.V. at 254 mμ (at which frequency the *p*-anisidine/glucose pigment fluoresces strongly). The absence of "tailing" in the glucose-bisulfite compound chromatograms indicates that decomposition is both rapid and complete.

After incubating glucose (1.0*M*) and sodium metabisulfite ($SO_2 = 0.045M$) in solution for 1, 5 and 24 hr at 55°C and at pH's corresponding to the beginning of incubation (pH 5.5) and the end of the inhibition period (pH 4.3), amounts of "free" and "combined" SO_2 were measured. The dissociation constants in these systems were calculated; the results (Table 2) show that the equilibrium is

Table 2—K values for the system glucose + sodium bisulfite⇌glucose-bisulfite compound during incubation at 55°C.[1]

Time, hr	pH 5.5	pH 4.3
1	1.28	1.41
5	1.29	1.58
24	1.30	1.58

$$^1K = \frac{[\text{Free Glucose}][\text{Free } SO_2]}{[\text{Combined } SO_2]}$$

established very rapidly. At pH 5.5 and 4.3 the values of K were 1.30 and 1.58 respectively (K = [free glucose] [free SO_2]/[combined SO_2].

DISCUSSION

THE PRESENT studies center on observations of amount of sulfur dioxide chemically determinable during and after the inhibition period in a system similar to that employed by Song et al. (1967b). As seen in Figure 1 and Table 1, onset of rapid color development does not occur until "free" SO_2 has almost completely disappeared from the system. The amount of "free" sulfur dioxide (i.e., sulfite/bisulfite ion) at the end of the inhibition period is only 4.8% of the total amount originally added and only 8% of the "free" SO_2 concentration which existed when the bisulfite + glucose ⇌ glucose hydroxysulfonate equilibrium had first been established (i.e., after a few hr). When expressed as a percentage of original concentration, the total amount of measurable SO_2 (33%) still in solution at the end of the inhibition period (41 days) is markedly different from the 64–73% retention found by Song et al. (1967b).

In comparing relative amounts of "free" and "combined" bisulfite at various times during the incubation, it is noticeable that initially, distribution of measurable bisulfite between the two forms is what might be expected from the glucose + bisulfite ⇌ glucose hydroxy sulfonate equilbrium. As incubation proceeds it is apparent that while some bisulfite is being irreversibly removed, other compounds are being formed which are capable of reversibly combining with bisulfite to give a system in which a much greater proportion of total measurable bisulfite is present in a "combined" form. Presumably this is at least in part due to carbonylic compounds which combine relatively strongly with bisulfite.

The figure for "free" SO_2 as measured, also includes contributions from any other reducing compounds present at the time of measurement and is higher (rather than lower) than the true figure. The shape of the "free" SO_2 curve in Figure 1 suggests that after the end of inhibition these reducing compounds are present at concentrations up to the equivalent of 0.08 mg SO_2/ml.

From their studies of inhibition of color development in the glucose + glycine + sodium bisulfite solution on which the present studies were based, Song et al. (1967b) concluded that sodium bisulfite itself is not an inhibitor of the reaction and that inhibition is due to reactive species (e.g., free radicals) derived from sodium bisulfite. It was also concluded that this conversion of bisulfite to the reactive species is self-inhibiting.

Without comment, at this stage, on the first of these conclusions we draw attention to the fact that the second of these findings is based primarily upon the experimental observation that 64–73% of the bisulfite added originally is still present when inhibition has been completed (Song et al., 1967b). This observation is in conflict with data recorded in the present work (in which the inhibition period is considerably longer than that reported by Song et al., 1967b, for a similar system) and in previous publications by other workers (Stadtman et al., 1945; Hearne et al., 1956).

In attempting to explain the difference between conclusions reached on bisulfite retention (after the end of inhibition) by Song et al. (1967b) and by other workers the bisulfite estimation methods employed have to be considered. It appears that, in contrast to the titrimetric and distillation methods customarily employed for sulfite determination, the estimations reported by Song et al. are based upon measurement of ^{35}S content of incompletely resolved radioactive areas on autoradiograms and electrophoretic radiograms. While these areas are in the general location at which sulfite might appear, no evidence of their identity and purity is reported.

The carbonyl bisulfite compounds in the reaction mixture may suffer at least partial hydrolysis during electrophoretic and chromatographic separation procedures, as in the case with glucose bisulfite compound in the present work. The sulfite so released gives rise to misleadingly high estimates of the amount of free sulfite present in the reaction mixture. Whereas Song et al. (1967b) conclude that 64–73% of the original bisulfite remains unconsumed at the end of inhibition, the present work shows that although considerable amounts of SO_2 are still measurable, the concentration of "free" SO_2 is very low at this time. We conclude that as a result of failure to make a distinction between "free" and "total measurable" SO_2 the concept put forward by Song et al. (1967b), that bisulfite must be converted to a browning inhibiting species by a process which is self-inhibitory, remains to be established satisfactorily. Indeed, it seems likely that the situation which this concept attempted to explain was not a real one.

A simpler explanation of sulfite action (Burton et al., 1963) rests upon the concept that reaction of bisulfite with the olefinic bond of $\alpha\beta$-unsaturated carbonylic compounds leads to formation of relatively stable sulfonated products from which bisulfite cannot readily be regenerated. The highly reactive nature of $\alpha\beta$-unsaturated carbonylic compounds was demonstrated (Burton et al., 1963). It was suggested that they were responsible for the irreversible removal of measurable sulfur dioxide from browning systems with the formation of products which were of low reactivity in browning systems. Stadtman et al. (1946) reported the isolation of a sugar-derived sulfonic acid from sulfited dried fruit which may have arisen by this route. Ingles (1962) reported a similar compound isolated

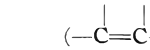
from a sugar/bisulfite reaction system.

The precise role of $\alpha\beta$-unsaturated carbonylic compounds in the Maillard reaction and the nature of the intermediates and products to which they lead in the presence of glycine remains to be established. They may lead, for instance, to formation of $\alpha\beta$-unsaturated aldimines of the type postulated by Song et al. (1966b), arising primarily by a different route. [It should perhaps be pointed out that Song et al. (1966a) are incorrect in attributing the postulation of $\alpha\beta$-unsaturated enolic aldimines as intermediates in the Maillard reaction to McWeeny et al. (1963) and Burton et al. (1963).] The reactions involved in formation of aldimines and the subsequent production of melanoidins in the manner suggested by Song et al. (1966a, b) in their studies of the kinetics of the Maillard reaction represents a new point of departure in hypotheses regarding this reaction and the evidence underlying it merits close examination. Since studies of inhibition of the Maillard reaction must inevitably be linked to theories of the Maillard reaction itself, some observations on the evidence advanced by Song et al. are given below.

Results, grouped under nine headings by Song et al. (1966b), are cited as indirect support for the statement that "unsaturated aldimines have been found to be key compounds during and after the induction period of the Maillard reaction." These results are all non-specific in their significance and existing theories (Hodge, 1953 and Reynolds, 1963 and 1965) of the Maillard reaction can, in common with the aldimine theory of Song and Chichester, explain dehydration of the glucose molecule, formation of reductones, development of unsaturated chromophores, presence of —C=C—, —OH and C=O groups, U.V. absorption at around 280 mμ, formation of enols and decarboxylation of amino acids.

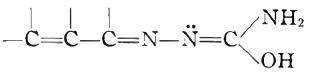
Primary evidence for the aldimine theory of Song et al. (1966a, b) appears to stem from studies made on a compound separated as a relatively major product from a glucose–glycine system before color development had become apparent. Color tests are consistent with it being an unsaturated aldimine, but do not seem to provide specific proof of this. Moreover, it does not seem to be established that this compound is a true intermediate rather than a low reactivity by-product like hydroxymethyl furfural (Kato et al., 1963; Anet, 1959; Yoshihiro et al., 1961 and Burton et al., 1963 and now confirmed by Song et al., 1966b).

Spectral evidence for the importance of aldimines as key intermediates also appears to be open to some question. The statement is made that "$\alpha\beta$-unsaturated aldehydes and their semicarbazone chromophores

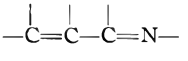


show their respective absorption maxima at 210–245 mμ and 264–274 mμ respectively." Song et al. (1966b) suggest that absorption by a colorless reaction mixture at 273–277 mμ during the induction period indicates "that an intermediate such as an $\alpha\beta$-unsaturated aldimine with a semicarbazone-type chromophore is being accumulated during the induction period."

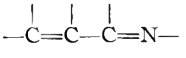
While it is accepted that semicarbazone derivatives of $\alpha\beta$-unsaturated aldehydes absorb at 264–274 mμ, it should be pointed out that these compounds have a chromophoric group which might be typified as



rather than merely



as indicated by Song et al. (1966b). The latter grouping is not a semicarbazone; it is an "azomethine" type with an adsorption maximum at around 220 mμ (Gillam et al., 1957; Scott, 1964). The presence of substituent hydroxyl groups seems unlikely to modify the azomethine-type chromophore sufficiently to bring the absorption maximum to the 273–277 mμ region as observed. Whatever grouping may be responsible for absorption at 273–277 mμ, it is clearly not a simple



chromophore. There may have been some confusion of terms in interpretation of the spectral data quoted and this may have resulted in an apparently erroneous conclusion regarding the chromophore involved.

The new concepts introduced by Song et al. are interesting and open up new avenues of approach to elucidation of the Maillard reaction mechanism. However, it is concluded that acceptance of unsaturated aldimines as key intermediates during and after the induction period must await availability of evidence more specific than presently available. Additionally, evidence for the proposal made by Song et al. regarding bisulfite inhibition of the Maillard reaction may require re-evaluation in light of results now reported.

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